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ANTIOXIDANT ACTIVITY *IN VITRO* AND HEPATOPROTECTIVE EFFECTS *IN VIVO* OF COMPOUND *LOBELIA*

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Abstract

Background: Chinese medicine has its own uniqueness, advantageous in the treatment of hepatic diseases, and they were widely used in the oxidation. At the same time, oxidation is one of the mechanism of protect liver.

Materials and Methods: In the present study, the antioxidant activity *in vitro* of different extracts from Compound Lobelia were estimated respectively by the methods of measuring the [2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid] diamonium salt (ABTS) radical scavenging, ferric reducing antioxidant power (FRAP). The protective effects on carbon tetrachloride (CCl4)-induced acute liver injury in mice which was investigated by analyzing the result of biochemical parameters such as glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in serum, superoxide dismutase (SOD) and malondialdehyde (MDA) in liver tissue homogenate.

Result: the result showed that ABTS free radical scavenging activity of ethanol extract (IC_{50}=29.26±0.49 μg/mL) was stronger than that of water extract (IC50=42.09±2.44 μg/mL), but they were lower than that of BHT as positive control (IC50=2.47±0.09 μg/mL). The ferric reducing antioxidant power of ethanol extract (FRAP=329.03±46.30 μmol/g) was higher than that of water extract (FRAP=206.03±54.30 μmol/g); they were lower than that of BHT (FRAP=1541.87±9.70 μmol/g). Water extract and ethanol extract could significantly reduce GOT activity (P<0.001) and GPT activity (P<0.001) in serum, reduce significantly the content of MDA (P<0.001) and significantly increase SOD activity (P<0.001) in the mice liver tissue homogenate.

Conclusion: Compound lobelia had a certain antioxidant and satisfied hepatoprotective effects. Moreover, the liver-protective effect was concerned with antioxidant activity and enhances the body immunity. Compound lobelia is expected to be a new medicine for protecting liver.

Key words: Compound Lobelia, antioxidant, hepatoprotective

Introduction

Chinese medicine has its own uniqueness which are safer, effective and lower toxicity than synthetic drug efficacy in the treatment of certain diseases. From the medical economic considerations, China is rich in medical herb resources, with the advantages of easily obtain materials, the people is willing to accept them due to low prices and their faith in traditional Chinese medicine. Traditional Chinese medicine is obviously well marketed and has technological advantages in the development of emergency medicine. Liver disease is significantly threatening people's health problem in china with its high incidence. However, the herbal preparations occupy a certain advantage in the number of drugs used in treating hepatic disease at home and abroad. It is necessary to find safe and effective hepatoprotective drugs from natural products to improve liver damage. Therefore, Chinese medicine has a broad perspective.

Compound *Lobelia* was composed of *Oldenlandia diffusa* Willd (*O. Diffusa*), *Lobelia chinensis* Lour (*L. Chinensis*) and *Scutellaria barbara* D.Don (*S. barbara*) (Wang et al, 2007), which had the main effects of heat-clearing and detoxicating,

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antibacterial diminish inflammation, diuresis detumescence. In addition, some studies also showed that they may have effect on α-glucosidase inhibition, antioxidant, antibacterial, mutagenesis resistance active and enhanced immunity (Shi et al, 2010), which was widely used in sore throat, bronchitis (He et al, 2004), infantile bronchitis (Yang et al, 2005), neonatal pneumonia (Yu, 2002), infantile upper respiratory tract infection (Li, 2004), infantile autumn diarrhea (Wang et al, 2007; Li et al, 2006), pathogenic heat (Xue, 2002), multiple furuncle swollen, tonsillitis, gastric cancer, breast cancer, kidney and other diseases. Adverse reactions were mainly manifest for big sweat, shivers, pale complexion, pulse micro, bosom frowsty, difficult breathing and also on anaphylactic shock symptoms, face and opisthenar urticaria, blurred vision, eyes conjunctival congestion and abdominal pain (Yan, 2005; Tan and Ci, 2005; Chen, 1995; Li et al, 2005). Furthermore, we found that there was strong antioxidant activity and protect liver function of *O. diffusa*, *L. chinensis* and *S. barbara* by collecting literature (Jiang et al, 2012; Shi et al 2009; Liao et al, 2012; Li et al,210; Zhao et al,2012; Mei, 2008). However, the researches of Compound *Lobelia* were focused on clinical applications, adverse reactions and biological activity in the domestic and foreign (Shi et al, 2010). There was no research on hepatoprotective effects which were assayed using the CCl_4-induced liver injury mice model of different extracts from Compound *Lobelia in vivo*. In order to make better use of Compound *Lobelia*, it was necessary to investigate the hepatoprotective effects of different extracts from Compound *Lobelia in vivo*, which at the same time have provided a scientific basis for the rational use of Compound *Lobelia*.

Materials and Methods

Plant Materials

Oldenlandia diffusa Willd, *Lobelia chinensis* Lour. and *Scutellaria barbara* D. Don were all purchased from TongLe large pharmacy of Kaifeng, in Henan province, China and were identified by Associate Professor Changqin Li (Institute of Chinese Materia Medica, Henan University). The specimen was deposited in the Institute of Chinese Materia Medica, Henan University, Kaifeng, China.

Preparation of Extracts

Dried *O. diffusa*, L. *chinensis* and *S. barbara* (each 0.5 kg) were decocted two times with water for 2 and 1 h. The total water decoction was merged, filtered, concentrated and dried to give compound lobelia water extract (Figure **1**). Dried *O. diffusa*, *L. chinensis* and *S. barbara* (each 0.5 kg) were decocted two times with 70% ethanol for 2 and1 h. The total water decoction was merged, filtered, concentrated and

dried to give compound lobelia ethanol extract (Figure **1)**.

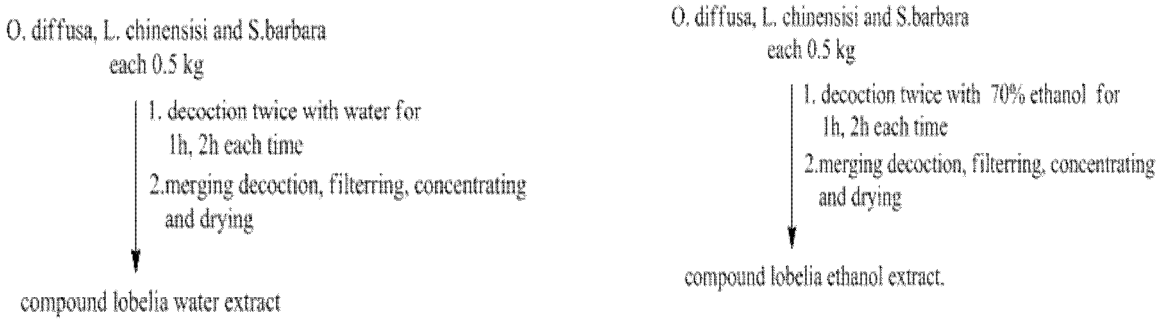

Figure 1: The flowchart of the process of extracts from Compound *Lobelia*

Animals

Male Kunming normal mice that weighted 20 ± 2 g were provided by the Experimental Animal Center of Henan province

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(Zhengzhou, Hennan, China) and were maintained in a temperature (25 ± 2°C) and humidity (45 to 65%) controlled room, and were housed in plastic cages with free access to food and water.

Reagents

[2, 2′-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid] diamonium salt (ABTS), butylated hydroxytoluene (BHT) from Acros organics, 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) from Acros organics, 6-hydroloxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) from Aldrich.

Glutamic Oxaloacetic Transaminase (GOT, NO: 20120720), Glutamic-pyruvic transaminase (GPT, NO: 20120717), Superoxide Dismutase (SOD, NO: 20120721) and Malondialdehyde (MDA, NO: 20120724) were purchased from the Nanjing Jianchen Bioengineering Institute (Jiangsu, China). CCl_4 were purchased from Tianjin Hongyan Chemical Reagent Company. Bifendate pills (Zhejiang pharmaceutical Co., Ltd., No: 100311). Coomassie brilliant blue G-250 (packing plant of Chemical Reagent Co. Shanghai, Batch), bovine serum albumin from Beijing AoBoxing research biotech co., Ltd (Beijing, China).

Two Methods of Micro-method Analysed Antioxidant Activity of the Extracts *In Vitro*

The initial concentration of screening by the two methods of micro-method were 1 mg/mL methanol solution, if radical scavenging rates of ABTS were greater than 50%, measures need to be taken to further dilute the sample, so that the rate of free radical scavenging is 20%~80%, according to the mapping of the concentration-inhibition rate to obtain the IC_{50} value. For FRAP method, if the measured absorbance A was not in the standard curve range, there is need to further dilute the sample, and then measured. Each method was parallel operation three times with BHT as the positive control.

ABTS Scavenging Activity

The reducing power was estimated by scavenging activity on ABTS radical of different extract from compound *lobelia*, and according to the literature (Kang et al, 2010 and Wei et al. 2012). The sample solution (10 µL) were mixed with a prepared ABTS working solution (200 µL) for three parallel, and was measured by at 405 nm after shaking and placing at room temperature for 20 min in the dark. Another blank control group (10 µL sample and 200 µL methanol) with BHT as positive control. ABTS radical scavenging ability which was calculated using the following formula: ABTS radical scavenging rate (%) = $[(A_{control}-A_{sample})/A_{control}]\times100\%$, Where, $A_{control}$ is the absorbance of ABTS itself and A_{sample} is the absorbance of the samples and the standard compound.

FRAP Reducing Activity

According to the literatures (Thaipong, et al. 2006; and Benzie, et al. 1996), the samples solution (10 µL) were mixed with a fresh prepared TPTZ stock solution (200 µL), and the absorbance of mixed solution was measured at 595 nm after mixing and incubating at 37°C for 40 min. At the same time, with BHT as the positive control, and Trolox as reference standard, results were expressed in µmol Trolox equivalents (TEAC) (TE)/g sample. The standard curve was a good linear when Trolox concentration is in between 50 and 1600 µmol/L.

***In Vivo* Experiment Methods and Biochemical Analyses**

Mice were arbitrarily divided into nine groups with 10 mice per group. Group 1, 2 and 3 were given 600, 300 and 150 mg/kg of water extract per day, respectively. Group 4 to 6 received 600, 300, and 150 mg/kg of ethanol extract, respectively. Group 7 (liver injury model control) were treated with distilled water. Group 8 was given bifendate (75 mg/kg) as positive control. Group 9 (normal

control) was treated with distilled water. The duration of treatment was 8 days for mice by intragastric administration. After 8 days, at 2 h after the final administration except for the Group 9, the mice were intraperitoneally injected with 0.4% olive oil solution of carbon tetrachloride at the dose of 0.1 mL/10g bodyweight and were not given their food. Mice blood were taken by picking the eyeball after fasting for 12 h, and serum was separated (3000 rpm for 15 min) and stored at 0 to 3°C. Determination of serum GPT and GOT levels (results were listed in Table 1) according to the instructions of kits which were produced from Nanjing Jiancheng Bioengineering Institute. The liver was removed quickly and weighed. The levels (results were listed in Table 2) of MDA and SOD were estimated using the 10% and 1% solution of liver homogenate that processed at 0 to 3°C by tissue homogenizer respectively.

Statistical Analysis

The antioxidant activity was expressed as an IC_{50} value, and was calculated by origin 6.0 software. The data of GPT, GOT, MDA and SOD were carried out with SPSS 17.0 software, and which were expressed as $\bar{x}\pm s$ (mean values ± standard deviation). Statistical comparisons were analyzed using one-way of variance (ANOVA) followed by Tukey's multiple comparisons test. The results were considered statistically significant if the *P* values were 0.05 or less. Otherwise, the results were not statistically significant.

Results

Two Methods of Micro-Method Analysed Antioxidant Activity of the Extracts *In Vitro*

Table **1:** Antioxidant activity of different extracts of Compound *Lobelia*

Samples	ABTS radical scavenging		FRAP reducing
	I%	IC_{50} (μg/mL)	TEAC (μmol/g)
Water extract	57.58	42.09±2.44	206.03±54.30
Ethanol extract	89.06	29.26±0.49	329.03±46.30
BHT	99.30	2.27	1541.87±9.70

Note: BHT was used as positive control. NT showed not enter the complex screen because of low activity.

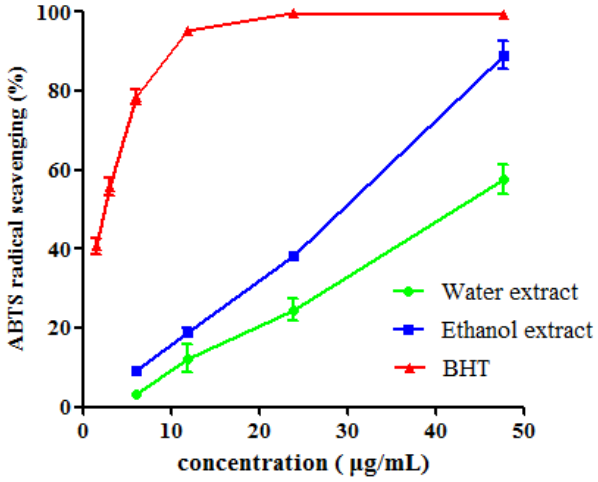

Figure 2: ABTS radical scavenging activity of extracts from Compound *Lobelia*

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In ABTS assay, as shown the Table **1** and Figure **2**, the antioxidant activity of ethanol extract (IC_{50}=29.26±0.49μg/mL) was lower than that of the positive control BHT (IC_{50}=29.26±0.49μg/mL), but was higher than water extract (IC_{50}=42.09±2.44 μg/mL). In addition, their clearance rates were increased with increasing concentration in a certain range.

In FARP assay, the Table 1 showed that antioxidant activity of ethanol extract (TEAC=329.03±46.30 μmol/g) and water extract (TEAC= 206.03±54.30 μmol/g) were lower than that of BHT. Results indicated that the antioxidant activity of ethanol extract was higher than water extract, but their antioxidant activity were lower than BHT.

Hepatoprotective Effects of Different Extracts *In Vivo*

Table 2: Effect of Compound *Lobelia* extracts on GPT and GOT in serum of liver injury mice ($\bar{x} \pm s$, n=10)

groups	Dose (mg/kg)	GPT (U/L)	GOT (U/L)
Water extract	600	487.08±114.99	368.06±62.09***
	300	302.88±54.13***	244.70±20.98***
	150	860.95±86.16	336.26±59.40***
Ethanol extract	600	506.15±39.47*	501.34±99.04**
	300	523.20±23.38**	394.68±72.78***
	150	516.682±31.65***	227.69±54.81***
Model bank	/	1075.95±5.40$^{\Delta\Delta\Delta}$	635.89±51.91$^{\Delta\Delta\Delta}$
Bifendate	75	577.31±80.36***	283.51±60.99***
Normal control	/	43.40±6.62***	130.74±6.55***

Note: Bifendate was used as positive control. $^{\Delta}P$<0.05$^{\Delta\Delta}P$<0.01$^{\Delta\Delta\Delta}P$<0.001, treated group compared with normal control group. $^{*}P$<0.05$^{**}P$<0.01$^{***}P$<0.001, treated group compared with model bank.

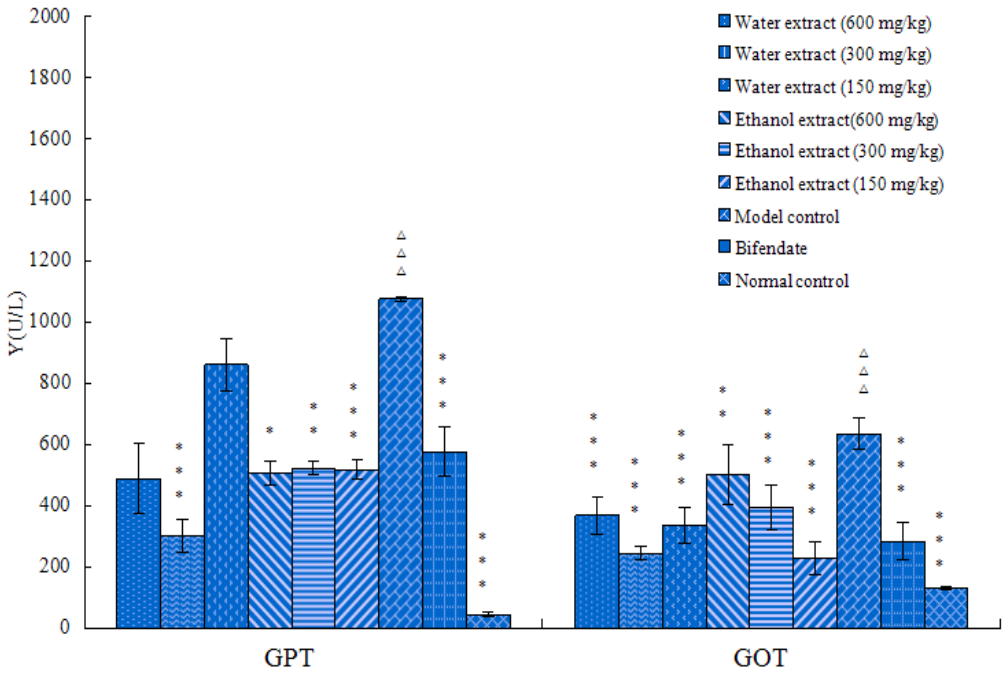

Figure 3: Effect of Compound *Lobelia* extracts on GPT and GOT in serum of liver injury mice ($x \pm s$, n=10)

As shown in the Table 2 and Figure 3 , the levels of GOT and GPT of model control were increased significantly compared with

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normal control (P<0.001), which showed that the mice were caused serious liver damage after interfering with carbon tetrachloride, that is, acute liver injury model of mice was successful in the experiment. The levels of GOT and GPT of Bifendate mice were reduced significantly compared with model control (P<0.001). The levels of GPT of medium dose group of water extract was decreased (P<0.001) significantly except for high and low dose groups (P>0.05 and P>0.05, respectively) compared with model control, and GOT were all reduced significantly compared with model control (P<0.001). The levels of GPT of high, medium and low dose groups of ethanol extract were decreased significantly (P<0.05, P<0.01 and P<0.001, respectively) compared with model control, and the three groups of GOT were reduced extremely significantly (P<0.01, P<0.001 and P<0.001, respectively). The results revealed that water extract and alcohol extract of Compound *Lobelia* had some protective effect on acute liver damage mice. And the water extract of medium dose group and alcohol extract low dose group were best treatment effect.

Table 3: Effect of extract of Compound *Lobelia* extract on MDA and SOD in liver homogenate of acute liver injury mice ($\bar{x}\pm s$, *n=10*)

	groups	Dose (mg/kg)	SOD (U/mgprot)	MDA (nmol/mgprot)
Note:	Water extract	600	$627.43\pm15.21^{***}$	$8.75\pm1.89^{***}$
		300	$625.22\pm44.64^{***}$	$8.99\pm0.89^{***}$
		150	$660.01\pm38.45^{***}$	$9.96\pm0.41^{***}$
	Ethanol extract	600	$639.60\pm32.21^{***}$	$8.65\pm1.82^{***}$
		300	$580.533\pm18.28^{***}$	$8.91\pm0.82^{***}$
		150	$736.75\pm23.91^{***}$	$8.57\pm0.79^{***}$
	Model bank	/	$436.81\pm29.72^{\Delta\Delta\Delta}$	$14.73\pm2.10^{\Delta\Delta\Delta}$
	Bifendate	75	$741.03\pm38.00^{***}$	$9.60\pm1.95^{***}$
	Normal control	/	$732.27\pm25.16^{***}$	$9.88\pm1.44^{***}$

Bifendate was used as positive control. Treated group compared with normal control group: $^{\Delta}P$<0.05$^{\Delta\Delta}P$<0.01$^{\Delta\Delta\Delta}P$<0.001. Treated group compared with model bank:$^{*}P$<0.05$^{**}P$<0.01$^{***}P$<0.001.

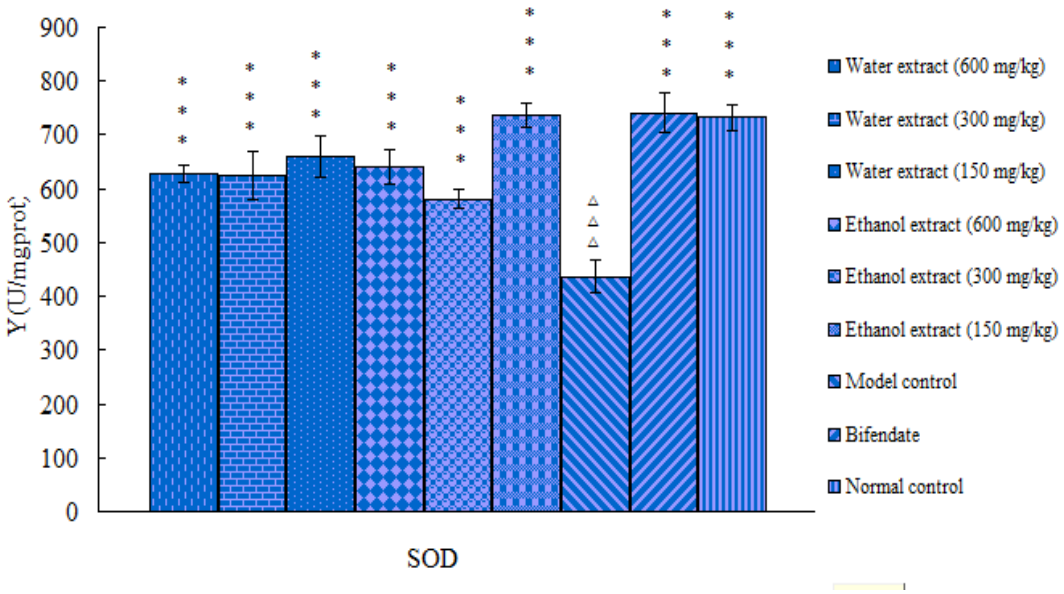

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Figure 4: Effect of extract of Compound *Lobelia* on SOD in liver homogenate of acute liver injury mice (*x*±*s*, *n=10*)

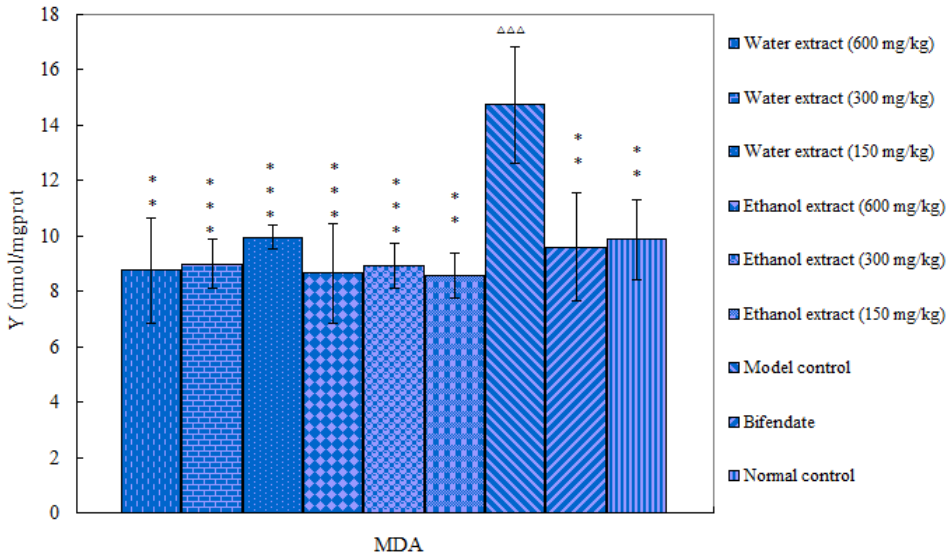

Figure 5: Effect of extracts of Compound *Lobelia* on MDA in liver homogenate of acute liver injury mice ($\bar{x}$±*s*, *n=10*)

In Table 3 and Figure 4 and 5, the level of SOD was reduced (P<0.001) significantly in model control, whereas MDA were increased (P<0.001) significantly compared with normal control, to indicate that acute liver injury model of mice was established successfully. The levels of SOD of Bifendate and extract of Compound *Lobelia*, were both increased significantly (P<0.001) and the contents of MDA were both reduced significantly (P<0.001) compared with model control. The results showed that the different concentrations water extract and alcohol extract of Compound *Lobelia* played a protective role on SOD and MDA in acute liver damage mice that were induced by carbon tetrachloride.

Discussion

In this experiment, the antioxidant activity of extracts of Compound *Lobelia* was tested by two trace methods *in vitro*. The results showed that the method was much faster and more convenient than that of the article (Shi et al, 2010). The experiment results were described that the antioxidant activity of ethanol extract were higher than that of water extract, which showed that antioxidant activity was different due to different reaction mechanism and reaction conditions. In other words, the antioxidant activity of extracts was associated with the type and polarity of extraction solvent. At the same time, there were many antioxidant functional factors that could eliminate reactive oxygen species in the plant, for example, flavonoids and tannins had the effect of antioxidant and scavenging oxygen free radical (Wang et al., 2011). In addition, some studies have shown that polysaccharides of *O. diffusa* (Jiang, et al, 2012), polyphenols, flavonoids, hydroxy anthraquinone, organic acids, polysaccharides and flavonoids of *S. Barbata* (Wang, et al, 2008; Dong, et al, 2008) and flavonoids of *L. chinensis* (Lu et al, 2007) all inhibited significant lipid peroxidation, and cleared free radicals, meanwhile, increased SOD enzyme activity, decreased MDA levels, and had strong antioxidation. Because SOD was the main antioxidant enzyme of clearing free radicals intracellular; this could effectively inhibit lipid peroxidation by clearing free radical. Consequently, SOD played an important role of protecting the cell membrane, and further protected the liver cells. In summary, antioxidant *in vitro* was thought to be related to hepatoprotective effect *in vivo*.

Liver injury was mostly caused by a variety of factors, such as viruses, drugs, reagents, lack of oxygen and immune factors and so on, leading to inflammation, liver fibrosis, cirrhosis, liver failure and other diseases, which was a common disease that threatens human health, because the highness of the morbidity and mortality. Liver injury was induced by intraperitoneal injection of CCl_4,

which was the most commonly used typical model in the study of acute liver damage. It was well-known that CCl_4 was a typical kill liver poison; there were a number of CCl_3• and Cl• radical that were metabolized by the cytochrome P_{450} in the sliding surface endoplasmic reticulum when the toxicants were excessive. And these free radicals could activate phospholipase to cause lipid peroxidation oxidation, damage of liver cell and increase permeability, at the same time, the levels of the GOT and GPT were increased in serum, because these enzymes were cytoplasmatic and are released into the blood after cellular damage (Recknagel et al., 1989; Berry et al., 1992; Romero et al., 1998). There was consistent evidence of reducing the content of MDA and increasing the level of SOD to decreased lipid peroxidation and enhances the antioxidant defense mechanisms and protects the body from further oxidative damage from free radicals (Hoek et al, 2002; Sandesh et al., 2010). Many drugs have been used to treat liver damage. However, the curative effect of acute and chronic liver diseases was often not satisfactory, but many studies have reported that herbal medicines play an important role in the treatment of hepatic disorders for their strong antioxidants (Park et al., 2000; Tang et al., 2006; Yue et al, 2011; Gong et al., 2012). The experimental results showed that the extracts of Compound *Lobelia* could decrease significantly the levels of GOT and GPT and the content of MDA, increase the activity of SOD by the mechanisms, which accelerated free radical scavenging, reduce the occurrence of lipid peroxidation and stabled cell membrane structure to protect the liver of acute liver injury in mice. In order to make better use of Compound *Lobelia*, further studies are required for tracking the active site, investigating the pharmacological of the constituents and going to clinical trials of liver injury.

Acknowledgments

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