

ANTIOXIDANT ACTIVITIES *IN VITRO* AND HEPATOPROTECTIVE EFFECTS OF *NELUMBO NUCIFERA* LEAVES *IN VIVO*Lin Yuan<sup>1,2</sup>, Xuezhu Gu<sup>3</sup>, Zhenhua Yin<sup>1</sup>, Wenyi Kang<sup>1\*</sup>

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\*E-mail: [kangweny@hotmail.com](mailto:kangweny@hotmail.com)**Abstract**

**Background:** Herbal medicines played a major role in the treatment of hepatic disorders, and a number of medicinal plants and their compounds were widely used for the treatment of these disorders, and oxidant stress injury was one of the mechanism of liver injury.

**Materials and Methods:** Antioxidant activity of *Nelumbo nucifera* leaves (NU) extracts was assayed by the methods of scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzo- thiazoline-6-sulfonicacid) (ABTS) radical and ferric reducing antioxidant power (FRAP) *in vitro*. By intraperitoneal injection carbon tetrachloride (CCl<sub>4</sub>) to establish acute liver injury model in mice, the levels of Glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), superoxide dismutase (SOD) and the content of and maleicdialdehyde (MDA) were detected to evaluate hepatoprotective effect of NU using corresponding test kit.

**Results:** EtOAC (NUEA) and *n*-BuOH extracts (NUBU) of *N. nucifera* leaves had good scavenging DPPH and ABTS radical activity and ferric reducing antioxidant power *in vitro*. DPPH radical scavenging activity and ferric reducing antioxidant power of NUEA (IC<sub>50</sub>= 6.68±0.29 µg/mL, RACT<sub>50</sub>=1749.82±67.03 µmol/g) and NUBU (IC<sub>50</sub>= 4.61±0.01 µg/mL, RACT<sub>50</sub>=1995.27±135.71 µmol/g ) were higher than that of BHT (IC<sub>50</sub>=8.76±0.20 µg/mL, RACT<sub>50</sub>=1581.68±97.41 µmol/g) and Dangfeiliganning (IC<sub>50</sub>=28.06±0.17 µg/mL, RACT<sub>50</sub>=1028.55±3.28 µmol/g). ABTS radical scavenging activity of NUEA (IC<sub>50</sub>= 5.32±0.12 µg/mL) and NUBU (IC<sub>50</sub>= 8.16±0.27 µg/mL) were higher than that of Dangfeiliganning (IC<sub>50</sub>= 9.76±0.16 µg/mL). Thus, hepatoprotective effect of NUEA and NUBU was evaluated on CCl<sub>4</sub>-induced acute liver injury mice. The results showed that the levels of GOT and GPT in each treatment group significantly decreased ( $p<0.001$  and  $p<0.01$ ,  $p<0.05$ , respectively) except for the group of NUEA (130.8 mg/kg) ( $p>0.05$ ). The contents of malondialdehyde (MDA) in liver in groups of NUEA (523 mg/kg), NUBU (840.5 and 420.5 mg/kg, respectively) had significant decrease ( $p<0.001$  and  $p<0.05$ , respectively), and the level of SOD in liver for each treatment group could significantly decrease ( $p<0.001$ ,  $p<0.05$ , respectively).

**Conclusion:** NUEA and NUBU had significantly hepatoprotective effect for Calcium tetrachloride CCl<sub>4</sub>-induced liver injury, which might be attributable to its antioxidant activity.

**Keywords:** Antioxidant activity, Carbon tetrachloride (CCl<sub>4</sub>), hepatoprotective effect, *Nelumbo nucifera* leaves

**Introduction**

Liver injury can be induced by various factors, such as CCl<sub>4</sub>, ethanol and acetaminophen, which are metabolized by Cytochrome P450 2E1 (CYP2E1) to generate unstable free radicals and reactive oxygen species (ROS), these free radicals and ROS can induce liver cell apoptosis and necrosis (Sun et al., 2001; Kuzu et al., 2007), and up-regulation of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-10 (IL-10) and transforming growth factor- $\beta$  (TGF- $\beta$ ) in necrotic hepatocytes that accelerate the progression of liver cell injury.

Carbon tetrachloride (CCl<sub>4</sub>) is one of the oldest and most widely used toxins for experimental induction of liver injury in laboratory animals. At present, the mechanism of CCl<sub>4</sub>-induced liver injury is accepted widely, CCl<sub>4</sub> is metabolized to a highly reactive trichloromethyl free radical (CCl<sub>3</sub>·) and chlorine free radical (Cl·) by cytochrome P450 in liver microsome. These free radicals may be to bind to macromolecular in liver cells covalently, and also attack saturated lipids, which can induce lipid peroxidation and lead to hepatocellular membrane damage (Koch et al., 1974). Herbal medicines play a major role in the treatment of hepatic disorders. A number of medicinal plants and their compounds are widely used for the treatment of these disorders (Park et al., 2000; Gong et al., 2012; Wei et al., 2012; Praveen et al., 2009;). Berberine is an isoquinoline alkaloid of the protoberberine type and silymarin is an antioxidant flavonoid complex, derived from the herb used for treatment of hepatic disorders (Domitrović et al., 2011).

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*Nelumbo nucifera* leaves (NU) is a well known Chinese herbal medicine. It distributed throughout china, and all parts of *N. nucifera* have been used as foodstuffs and Chinese traditional medicines. Moreover, many biological and pharmacological studies have been performed on each part of the plant. Phytochemical researches have shown that flavonoids and alkaloids were the main active components of *N. nucifera* leaves, for example astragalin, quercetin, hyperin, arnepavine and coclaurine (Yang et al., 2007; Xiao et al., 2006).

Pharmacological studies have shown that *N. nucifera* leaves had the effects of antioxidant (Deng et al., 2006), anti-HIV (Kawanishi et al., 2003), antilipemic (Guan et al., 2003), antiobesity (Ono et al., 2006), antibacterium (Li et al., 2003), antigall-stone (Ding et al., 2007), antilipase (Zhu et al., 2007) and antipoliavirus (Boustie et al., 1998).

In this study, antioxidant activity of *N. nucifera* leaves extracts was assayed by methods of DPPH, ABTS and FRAP. By intraperitoneal injection CCl<sub>4</sub> to establish acute liver injury model in mice, the levels of GOT, GPT, SOD and the content of MDA were detected to evaluate hepatoprotective effect of NU.

## Materials and methods

### Chemicals and Materials

Glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), maleicdialdehyde (MDA) and superoxide dismutase (SOD) detection kits were obtained from Jiancheng Institute of Biological Engineering, Nanjing. Dangfeiliganning capsule was obtained from Sichuan Meidakang Pharmaceutical Co. Ltd. (Batch No: 20080302). Coomassie brilliant blue G-250 was obtained from Shanghai Packing Plant of Chemical Reagent Co. (Batch No: 20050115). DPPH was obtained from Tokyo, Japan Chemical Industry Co., Ltd. (Japan). TPTZ was from Acros organics (Belgium). Trolox was obtained from Aldrich (USA). ABTS was obtained from Fluka (USA). Gallic acid propyl (PG), butyl-p-hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and were purchased from Sigma Chemical Co. CCl<sub>4</sub> (AR) were purchased from Kaifeng chemical reagent factory. All the other organic solvents and chemicals used were analytical grade.

### Animals

Male Kunming normal mice weighing  $20 \pm 2$  g were obtained from the Experimental Animal Center of Henan Province. (Zhengzhou, Hennan, China), (12 h light/dark cycle, 25°C and humidity 45 to 65%) and were fed with standard rodent diet and water ad libitum. All animal procedures were approved by the ethical committee in accordance with the 'Institute Ethical Committee Guidelines' for animal experimentation and care (HNPR-2009-05003). Animals were housed in polycarbonate cages.

### Instruments

UV-2000 spectrophotometer (Unico Instrument Co., Ltd, Shanghai). Electronic balance (Mettler-Toledo Instrument Co., Ltd. USA), Multiskan MK<sub>3</sub> microplate reader (Thermo Instrument Co., Ltd. USA), 985370-395-type tissue machine (BIOSREC, Mexico).

### Plant material

*N. nucifera* leaves (Voucher number 20070815) were collected from Henan province in August 2007, and identified by Professor Chang-qin Li (1.Institute of Chinese Meteria Medica, Henan University). The specimen was deposited in Institute of Chinese Meteria Medica, Henan University.

The air dried *Nelumbo nucifera* leaves (3.25 kg) were extracted three times with acetone water solution (acetone:distilled water=7:3) for 7 days at room temperature. After evaporation of solvent *in a vacuum*, the concentrated extract was suspended in water and extract with petroleum ether, EtOAc and *n*-BuOH, respectively. The solution was concentrated under reduced pressure to yield petroleum ether extract (55 g, NUPE), EtOAc extract (81.5 g, NUEA) and *n*-BuOH extract (175 g, NUBU), respectively.

### Antioxidant activity using DPPH assay

DPPH radical scavenging activity was assayed according to the method of Kang (Kang and Wang, 2010). 0.1 mL different extracts of

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NU in methanol had been mixed with 3.5 mL DPPH methanol solution (0.06 mmol/L). The solution was measured at 515 nm after 30 min at room temperature with PG, BHA and BHT as positive control. The antioxidant activity was expressed as an  $IC_{50}$  value, that is the concentration in  $\mu\text{g/mL}$  that scavenging DPPH $\cdot$  absorption by 50%, and was calculated from the concentration-effect linear regression curve.

#### Antioxidant activity using ABTS assay

Scavenging activity on ABTS radical of different extract of NU was evaluated in accordance with the literature (Li et al., 2011; Yue and Kang et al., 2011). The different extracts (0.15 mL) were mixed with ABTS radical stock solution (2.85 mL). The absorbance was observed at 734 nm after incubated 10 min at 37°C with PG, BHA and BHT as positive control. The Scavenging rate of ABTS $\cdot^+$  was calculated using the formula: Scavenging rate (%) =  $[(A_0 - A_1)/A_0] \times 100$ , where  $A_0$  was the absorbance of the control and  $A_1$  was the absorbance of the sample and the standard compound.

#### FRAP reducing activity assay

According to the literature (Li et al., 2011; Yue and Kang et al., 2011), the NU extracts (0.2 mL) and fresh prepared TPTZ stock solution (3.8 mL) were mixed and incubated at 37°C for 30 min. The absorbance was measured at 593 nm. Trolox was used as a reference standard. The standard curve was linear between 25 and 400  $\mu\text{mol/L}$  Trolox. Results were expressed in  $\mu\text{mol}$  Trolox equivalents (TEAC) (TE)/g sample. In this study,  $RACT_{50}$  was used to express Trolox equivalent ( $RACT_{50}$  = the concentration of Trolox cleared 50% free radical/ the concentration of compound or condensate cleared 50% free radical).

#### Hepatoprotective effect *in vivo*

Ninety KM male mice (20 $\pm$ 2 g) were randomly divided into nine groups of ten each. Group 1 (normal control) was treated with distilled water. Group 2 (liver injury model control) was treated with distilled water. Group 3 was fed Dangfeiliganning capsule (1400 mg/kg) as positive control. Group 4, 5 and 6 were given 523, 261.5 and 130.8 mg/kg of NUEA, respectively. Group 7, 8 and 9 were received 840.5, 420.5 and 210 mg/kg of NUBU, respectively. Administration adopted gastric lavage daily for 8 days. On the eighth day, except for the normal group, other group animals were given a single dose of 0.1 mL/10 g b.w.  $\text{CCl}_4$  that was diluted with corn oil by intraperitoneally injection after 2 h of the last administration. All groups were added to the drinking water but not feeding for 16 h. Blood was collected from mice eyeball for the levels of GPT and GOT in serum. The liver was removed to prepare liver homogenate solution of 10% and 1% for determining the content of MDA and the level of SOD by tissue homogenizer, respectively. The content of protein was determined by the method of Comassie brilliant blue G-250. The levels of GPT, GOT and SOD and the content of MDA were determined by the requirements of kits.

#### Statistical analysis

All data were statistically evaluated with SPSS 17.0 software. Statistical comparisons were compared by one-way analysis of variance (ANOVA). The results were considered statistically significant if the  $p$  values were 0.05 or less. All results are expressed as mean  $\pm$  standard deviation (SD) for ten mice in each group.

## Results

#### Assay for antioxidant activity *in vitro*

The antioxidant activity of the extracts with half inhibitory scavenging ( $IC_{50}$ ) and Trolox equivalent ( $RACT_{50}$ ) was shown in Table 1. In DPPH assay, the DPPH radical scavenging activity of NUEA ( $IC_{50}$  = 6.68 $\pm$ 0.29  $\mu\text{g/mL}$ ) and NUBU ( $IC_{50}$  = 4.61 $\pm$ 0.01  $\mu\text{g/mL}$ ) were higher than that of BHT ( $IC_{50}$  = 8.76 $\pm$ 0.20  $\mu\text{g/mL}$ ), and far higher than that of Dangfeiliganning ( $IC_{50}$  = 28.06 $\pm$ 0.17  $\mu\text{g/mL}$ ). The activity of NUBU was a bit higher than that of NUEA. In ABTS assay, the ABTS radical scavenging activity of NUEA ( $IC_{50}$  = 5.32 $\pm$ 0.12  $\mu\text{g/mL}$ ) and NUBU ( $IC_{50}$  = 8.16 $\pm$ 0.27  $\mu\text{g/mL}$ ) were higher than that of Dangfeiliganning ( $IC_{50}$  = 9.76 $\pm$ 0.16  $\mu\text{g/mL}$ ), and far higher than that of NUPE ( $IC_{50}$  = 78.51 $\pm$ 0.20  $\mu\text{g/mL}$ ). The activity of NUEA was a bit higher than that of NUBU. In FARP assay, ferric reducing antioxidant power of NUEA

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( $RACT_{50}=1749.82\pm67.03$   $\mu\text{mol/g}$ ) and NUBU ( $RACT_{50}=1995.27\pm135.71$   $\mu\text{mol/g}$ ) were higher than that of Dangfeiliganning ( $RACT_{50}=1028.55\pm3.28$   $\mu\text{mol/g}$ ) and BHT ( $RACT_{50}=1581.68\pm97.41$   $\mu\text{mol/g}$ ), and far higher than that of NUPE ( $RACT_{50}=405.82\pm17.03$   $\mu\text{mol/g}$ ).

**Table 1:** Antioxidant activity of NU extracts by DPPH, ABTS and FRAP assay

Sample		DPPH IC <sub>50</sub> ( $\mu\text{g/mL}$ )	ABTS IC <sub>50</sub> ( $\mu\text{g/mL}$ )	FRAP RACT <sub>50</sub> ( $\mu\text{mol/g}$ )
Extracts	NUPE	87.30 $\pm$ 0.35	78.51 $\pm$ 0.20	405.82 $\pm$ 17.03
	NUEA	6.68 $\pm$ 0.29	5.32 $\pm$ 0.12	1749.82 $\pm$ 67.03
	NUBU	4.61 $\pm$ 0.01	8.16 $\pm$ 0.27	1995.27 $\pm$ 135.71
Capsule <sup>A</sup>	Dangfeiliganning	28.06 $\pm$ 0.17	9.76 $\pm$ 0.16	1028.55 $\pm$ 3.28
	PG	0.61 $\pm$ 0.01	0.91 $\pm$ 0.02	11554.78 $\pm$ 501.34
Positive Control <sup>B</sup>	BHA	2.16 $\pm$ 0.03	1.78 $\pm$ 0.01	6633.04 $\pm$ 114.04
	BHT	8.76 $\pm$ 0.20	1.54 $\pm$ 0.05	1581.68 $\pm$ 97.41

A: Dangfeiliganning capsule is one of the Chinese patent drugs, not include adjuvant and can be detected in antioxidant capability.

B: Three positive Controls: Propyl gallate (PG), Butylated hydroxyanisole(BHA) and Butylated hydroxytoluene(BHT)are finest antioxidants, have strong toxicity and often use in antioxidant experiments *in vitro*.

### Hepatoprotective activity *in vivo*

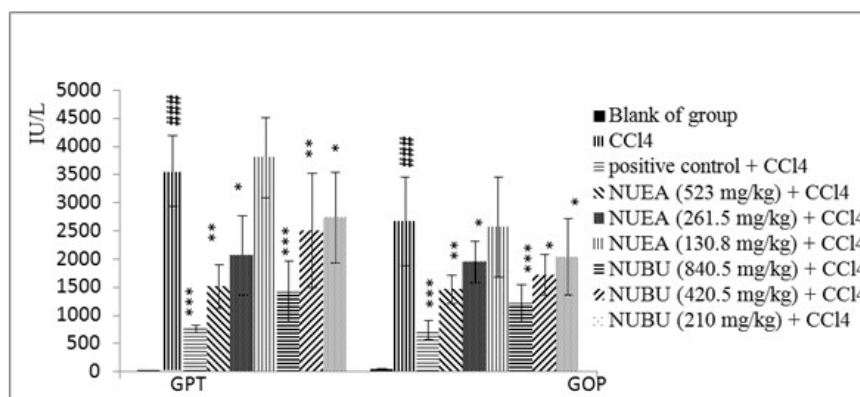
#### Effect of NUEA and NUBU on the levels of GPT and GOT in serum

As shown in Table 2 and Fig.1, compared with blank of group, the levels of GPT and GOT were significantly increased ( $p<0.001$ ) in  $\text{CCl}_4$  group, which indicated  $\text{CCl}_4$ -induced liver injury mice model was established successfully. Compared with  $\text{CCl}_4$  group, the levels of GPT and GOT ( $p<0.01$  and  $p<0.05$ , respectively) in high and middle doses of NUEA groups (523 mg/kg and 261.5 mg/kg) were significantly decreased. The low dose of NUEA group (130.8 mg/kg) had no significantly increased ( $p>0.05$ ). The levels of GPT and GOT ( $p<0.001$  and  $p<0.01$ ,  $p<0.05$ , respectively) in three doses of NUBU groups were significantly decreased.

**Table 2:** Effect of NUEA and NUBU on GPT and GOT in acute liver injury mice

Group	Dose (mg/kg)	GPT (U/mL)	GOT (U/mL)
Blank of group	/	22.1254 $\pm$ 4.4152	40.2149 $\pm$ 7.5030
$\text{CCl}_4$	/	3559.075 $\pm$ 627.1114 <sup>###</sup>	2664.282 $\pm$ 785.5137 <sup>###</sup>
Positive control+ $\text{CCl}_4$	1400	753.0253 $\pm$ 65.7084 <sup>***</sup>	747.3594 $\pm$ 168.2491 <sup>***</sup>
NUEA	523	1507.406 $\pm$ 385.5916 <sup>**</sup>	1466.785 $\pm$ 251.5703 <sup>**</sup>
NUEA	261.5	2064.593 $\pm$ 708.8452 <sup>*</sup>	1953.84 $\pm$ 366.8895 <sup>*</sup>
NUEA	130.8	3802.158 $\pm$ 709.5235	2571.684 $\pm$ 891.8134
NUBU	840.5	1420.032 $\pm$ 547.3112 <sup>***</sup>	1206.779 $\pm$ 338.4166 <sup>***</sup>
NUBU	420.5	2507.503 $\pm$ 1014.0085 <sup>**</sup>	1717.310 $\pm$ 360.2130 <sup>*</sup>
NUBU	210	2736.965 $\pm$ 811.1835 <sup>*</sup>	2036.000 $\pm$ 682.5499 <sup>*</sup>

Data expressed as means  $\pm$  s.d ( $n=10$ ). Dangfeiliganning was as the positive control drug. Blank of group compared to  $\text{CCl}_4$ -induced acute liver injury: <sup>#</sup> $P<0.05$ , <sup>##</sup> $P<0.01$ , <sup>###</sup> $P<0.001$ . Treated group compared to  $\text{CCl}_4$ -induced acute liver injury: <sup>\*</sup> $P<0.05$ , <sup>\*\*</sup> $P<0.01$ , <sup>\*\*\*</sup> $P<0.001$



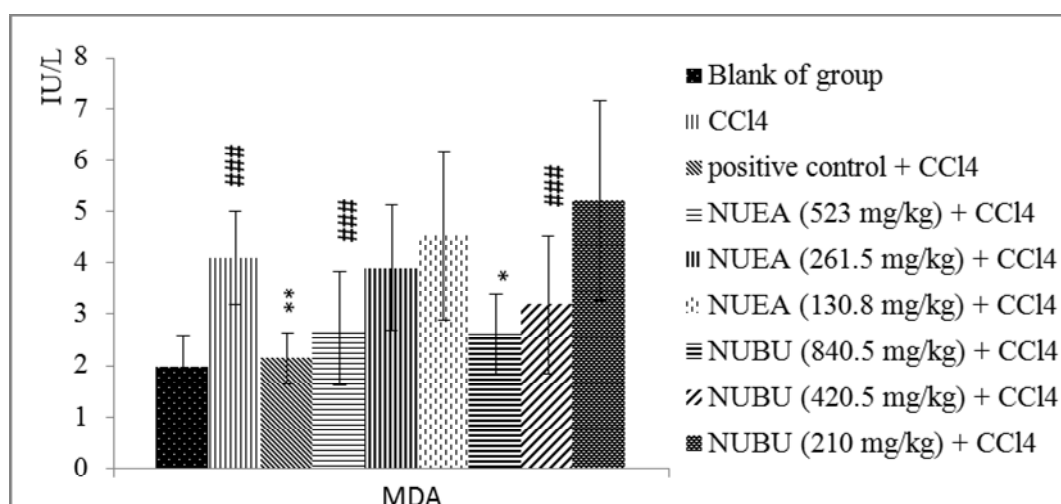
**Figure 1:** Effect of NUEA and NUBU on the level of GPT and GOT in serum.

As shown in Table 3, Fig.2 and Fig.3, compared with blank of group, the content of MDA in liver homogenate solution was significantly increased ( $p < 0.001$ ) and the level of SOD in liver homogenate solution was significantly decreased ( $p < 0.001$ ) liver injury control mice, which indicated that  $\text{CCl}_4$  could cause oxidative stress in mice. Compared with  $\text{CCl}_4$  group, the content of MDA was significantly decreased ( $p < 0.01$  and  $p < 0.05$ , respectively) in high dose of NUEA group (523 mg/kg) and high and middle doses of NUBU groups (840.5 and 420.5 mg/kg, respectively), and the other treatment groups had no significantly increased ( $p > 0.05$ ). The levels of SOD in each treatment group were significantly increased ( $p < 0.001$ ,  $p < 0.01$  and  $p < 0.05$ , respectively).

**Table 3:** Effect of NUEA and NUBU on MDA and SOD in acute liver injury mice

Group	Dose (mg/kg)	MDA (nmol/mgprot)	SOD (U/mgprot)
Blank of group	/	1.9651±0.6129	451.8104±72.7875
$\text{CCl}_4$	/	4.0995±0.9059 <sup>###</sup>	94.4888±72.7875 <sup>###</sup>
Positive control+ $\text{CCl}_4$	1400	2.7324±1.1128 <sup>**</sup>	444.9721±54.7451 <sup>***</sup>
NUEA	523	1507.406±385.5916 <sup>*</sup>	342.4347±79.9700 <sup>***</sup>
NUEA	261.5	3.8997±1.2178	216.6239±97.0241 <sup>**</sup>
NUEA	130.8	4.5278±1.6412	153.8693±47.9484 <sup>*</sup>
NUBU	840.5	2.6186±0.7695 <sup>**</sup>	502.1480±24.1769 <sup>***</sup>
NUBU	420.5	3.1887±1.3394 <sup>*</sup>	437.6063±174.6291 <sup>***</sup>
NUBU	210	5.2201±1.9589	190.3006±41.4079 <sup>**</sup>

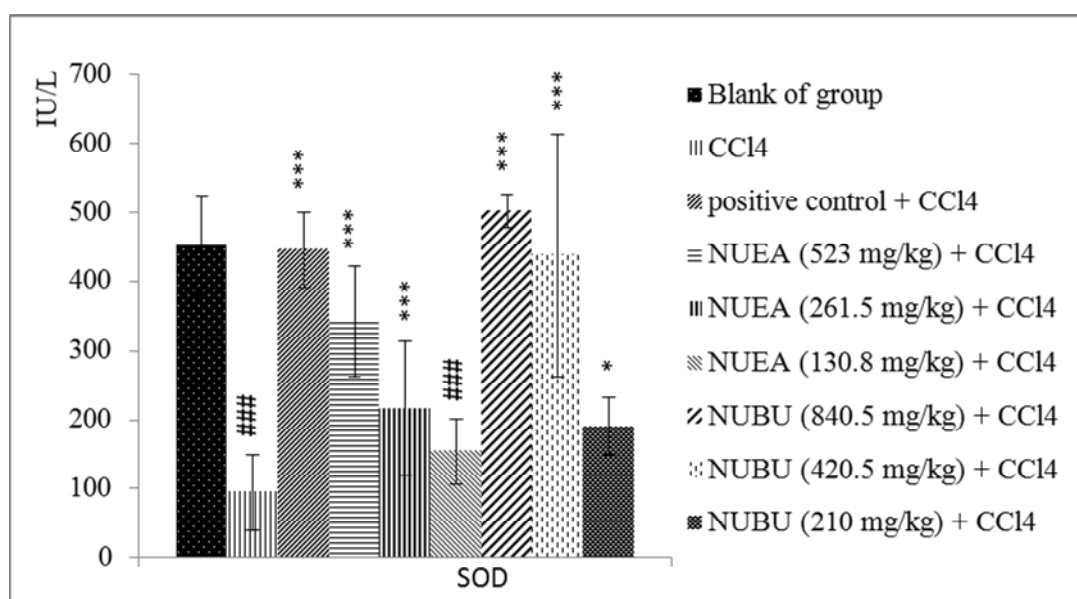
Data expressed as means ± s.d ( $n=10$ ). Dangfeiliganning was as the positive control drug. Blank of group compared to  $\text{CCl}_4$ -induced acute liver injury: <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$ , <sup>###</sup> $P < 0.001$ . Treated group compared to  $\text{CCl}_4$ -induced acute liver injury: <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ , <sup>\*\*\*</sup> $P < 0.001$ .



**Figure 2:** Effect of NUBU on the levels of MDA in liver tissue

## Discussion

It could be seen from the above analysis that NUEA and NUBU had certain scavenging DPPH and ABTS radical activity and ferric reducing antioxidant power *in vitro*. Meanwhile, NUEA and NUBU could significantly decrease the levels of GPT and GOT in serum, the content of MDA in liver homogenate solution and increase the level of SOD in liver homogenate solution in CCl<sub>4</sub>-induced liver injury mice model *in vivo*, which indicated that NUEA and NUBU had hepatoprotective effects against oxidant injury. CCl<sub>4</sub> was metabolized to CCl<sub>3</sub><sup>•</sup> and Cl<sup>•</sup> by microsomal cytochrome P450 system in liver cell, and produce trichloromethyl peroxy radical ( $\cdot\text{OOCCL}_3$ ),  $\cdot\text{OOCCL}_3$  attacked unsaturated fatty acids of phospholipids in the cell membrane, leading to lipid peroxidation in the liver cells and generated amount of free radicals (Halliwell and Gutteridge, 1997). Hepatocellular damage could lead to glutamic-pyruvic transaminase (GPT) and glutamic oxalacetic transaminase (GOT) to penetrate into the blood, which caused the raise of GOT and GPT in the serum (Romero et al., 1998; Berry et al., 1992). At the same time, lipid peroxidation could produce some free radicals including malondialdehyde (MDA).



**Figure 3:** Effect of NUEA and NUBU on the contents of SOD in liver tissue

Superoxide dismutase (SOD) was responsible for the detoxification of deleterious oxygen radicals (Sandesh et al., 2010). In the present study, NUEA and NUBU could decrease the content of MDA, which indicated that lipid peroxidation was weakened and produced less free radicals. Cell membrane was protected and prevented GPT and GOT in the endochylema to plunge into the serum, so the levels of GPT and GOT were decreased in serum. In addition, a significant increase in the level of SOD as a defense against the presence of oxygen free radicals, and reduced the damage of cells. So, antioxidant was one of hepatoprotective mechanisms to decrease lipid peroxidation and oxidant stress for NUBU hepatoprotective effects.

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