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Full Length Research Paper

Antioxidant activities *in vitro* and hepatoprotective effects of *Lysimachia clethroides* Duby on CCl_4-induced acute liver injury in mice

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The antioxidant activity of the extracts of *Lysimachia clethroides* Duby (LC) was assayed by the methods of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzo-thiazoline-6-sulfonicacid) (ABTS) *in vitro*. The ethyl acetate (LCEA) and *n*-butanol extracts (LCBU) of *L. clethroides* were the higher antioxidant activity in DPPH and ABTS assay. LCEA had the highest antioxidant activity (DPPH: IC_{50} = 9.02 µg/ml, ABTS: IC_{50} = 7.43 µg/ml, respectively). Thus, hepatoprotective effect of the extracts of *L. clethroides* was evaluated on CCl_4-induced acute liver injury mice. Intragastric administration of LCPE (500, 250 and 125 mg/kg body weight per day, respectively), LCEA (600, 300 and 150 mg/kg body weight per day, respectively) and LCBU (600, 300 and 150 mg/kg body weight per day, respectively) on CCL_4-induced acute liver injury in mice for 8 days, the level of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in each treatment group significantly decreased ($P < 0.001$ and $P < 0.01$, respectively). The level of malondialdehyde (MDA) in liver for each treatment group could significantly decrease ($P < 0.001$), and the level of superoxide dismutase (SOD) in liver only in group of LCBU (150 mg/kg) had no significant increase ($P > 0.05$), the other treatment groups had significant increase ($P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively). The result showed that LC had a high antioxidant activities *in vitro* and hepatoprotective effect on CCL_4-induced acute liver injury in mice.

Key words: Antioxidant activity, hepatoprotective effect, *Lysimachia clethroides* Duby.

INTRODUCTION

Carbon tetrachloride (CCl_4) is one of the oldest and most widely used toxins induced in laboratory animals. Its metabolic products are responsible for the toxicity. The mechanism of CCl_4-induced acute liver injury is accepted widely, CCl_4 was metabolized to a highly reactive trichloromethyl radical (CCl_3^-) by cytochrome P450 in liver; CCl_3 in liver can induce lipid peroxidation and it leads to hepatocellular membrane damage (Ohta et al., 1998; Drill, 1952). Hepatic damage induced by CCl_4 resulted in an increased level of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in serum (Romero et al., 1998; Berry et al., 1992). Lipid peroxidation can produce some free radicals which include malondialdehyde (MDA). Superoxide dismutase (SOD) was responsible for the detoxification of deleterious oxygen radicals (Sandesh et al., 2010). 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 (Gong et al., 2012; Praveen et al., 2009; Lin et al., 1998; Venkateswaran et al., 1998). 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 (Domitrovi´c et al., 2011).

Lysimachia clethroides Duby belongs to primulaceae

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family. The roots or whole plant of *L. clethroides* (LC) (Zhen-Zhu-Cai in Chinese) are used as a traditional Chinese herbal medicine for the treatment of edema, jaundice, rheumatism, amenorrhea, dysentery and fractures (Hu et al., 1999). Phytochemical research showed flavonoids, phenolic, saponins and organic acids (Yue et al., 2011; Zhou and Tu, 2009; You et al., 2007; Zhou and Tu, 2004; Ding et al., 2001; Ren et al., 2001; Yasukawa and Takido, 1986; Kitagawa et al., 1967). The pharmacological research showed that it has good anti-tumor effect and antibacterial activity (Wu et al., 2011; Wang et al., 2007; Tang et al., 2007; Xu et al., 2003). To the best of our knowledge, there is no research about the antioxidant activities and hepatoprotective effect of LC on CCL_4-induced acute liver injury in mice.

MATERIALS AND METHODS

Plant material and extract preparation

Air-dried plant of LC was collected in Guiyang, China, in June 2011, and identified by Professor Deyuan Chen (Guiyang College of Traditional Chinese Medicine). The specimen was deposited in Institute of Chinese Materia Medica, Henan University (20110622).

The air dried LC (533 g) were extracted three times with methanol for 3 days at room temperature. After evaporation of solvent in a vacuum pump, the concentrated extract was suspended in water and extracted with petroleum ether, EtOAC (ethyl acetate) and *n*-BuOH (*n*-butanol), respectively. The solution was concentrated under reduced pressure to yield petroleum ether extract (LCPE) 5.5431 g, EtOAC extract (LCEA) 15.7458 g and *n*-BuOH extract (LCBU) 13.1471 g, respectively.

Materials in experiments *in vitro*

Gallic acid propyl (PG), butyl-p-hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were obtained from Sigma. 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Tokyo Chemical Industry Co. 6-hydroloxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (trolox) from Aldrich Chemical Co. 2,2´-azino-bis(3-ethylbenzo-thiazoline-6-sulfonicacid) (ABTS) were obtained from Fluka.

Antioxidant activity using DPPH assay

DPPH radical scavenging activity was assayed according to the method of Kang (Kang et al., 2008). 0.1 ml of different extracts of LC in methanol had been mixed with 3.5 ml of DPPH methanol solution (0.06 mmol/L). The solution was measured at 515 nm after 30 min at room temperature with propyl gallate (PG), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as positive control. The antioxidant activity was expressed as an IC_{50} value, that is, the concentration in μg/ml that inhibits DPPH absorption by 50%, and was calculated from the concentration-effect linear regression curve. All reactions were carried out with three replications.

Antioxidant activity using ABTS assay

Scavenging activity on ABTS radical of extracts of LC was evaluated in accordance with the literature (Kang et al., 2010). The different extracts of LC (0.15 ml) were mixed with ABTS radical stock solution (2.85 ml) and incubated at 37°C. The absorbance was observed at 734 nm after 10 min with PG, BHA and BHT as positive control. The percentage inhibition of $ABTS^+$ was calculated using the formula: %Inhibition = $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control and A_1 was the absorbance of the sample and the standard compound. All reactions were carried out with three replications.

Materials and animals in experiments *in vivo*

Materials

UV-2000 spectrophotometer (Unico Instrument Co., Ltd, Shanghai), electronic balance (Mettler-Toledo Instrument Co., Ltd. USA), Multiskan MK3 microplate reader (Thermo Instrument Co., Ltd. USA), 985370-395-type tissue machine (BIOSREC, Mexico).

Glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), maleicdialdehyde (MDA), superoxide dismutase (SOD) and glycogen from the Nanjing Jianchen Bioengineering Institute (Jiangsu, China). Bifendate pills (Zhejiang pharmaceutical Co., Ltd., No: 090205), Coomassie brilliant blue G-250 (packing plant of Chemical Reagent Co. Shanghai, Batch No: 20050115), bovine serum albumin from Beijing AoBoxing research bio-tech co., Ltd (Beijing, China). CCl_4 were purchased from Sigma Chmical Co. All the other organic solvents and chemicals used were of analytical grade.

Animals

Male Kunming normal mice weighing 20 ± 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.

Experimental design and treatment schedule

One hundred and twenty KM male mice (20 ± 2 g) were randomly divided into twelve groups of ten each. Group 1 (normal control) was treated with distilled water. Group 2 (liver injury model control) normal mice were treated with distilled water. Group 3 was given bifendate (70 mg/kg) as positive control. Group 4, 5 and 6 were given 500, 250 and125 mg/kg of LCPE, respectively. Groups 7 to 9 received 150, 300 and 600 mg/kg of LCEA, respectively. Groups 10 to 12 received 150, 300 and 600 mg/kg of LCBU, respectively, and then were treated with drugs 24 h later. The duration of treatment for mice, by intragastric administration, was 8 days. The mice were treated for 8 days and given CCL_4 with 0.05 ml/kg b.w. diluted in olive oil by intraperitoneally injection after 2 h of the last administration except for Group 1.

Sample collection

The animals were sacrificed by cervical dislocation, the liver, kidney and spleen were removed promptly, and weighed. Blood samples were collected after 16 h and centrifuged (3000 rpm for 15 min at 4°C) for separating the serum, blood samples for determining the level of GPT and GOT. The liver homogenate solution of 10 and 1% for determining the level of MDA and SOD was processed at 0 to 3°C by tissue homogenizer, respectively.

Table 1. Antioxidant activity of extracts of *L. clethroides* Duby.

Sample	DPPH radical scavenging capacity IC_{50} (μg/ml)	ABTS radical scavenging capacity IC_{50} (μg/ml)
LCPE	NT	NT
LCEA	11.42 ± 0.07	9.02±0.11
LCBU	9.86 ± 0.13	7.43±0.06
BHA	3.2 ± 0.03	1.88±0.02
BHT	18.71 ± 0.18	7.72±0.04
PG	0.89 ± 0.09	0.81±0.01

Note: NT was indicated not available because of low activity; BHA, BHT and PG were used as positive control.

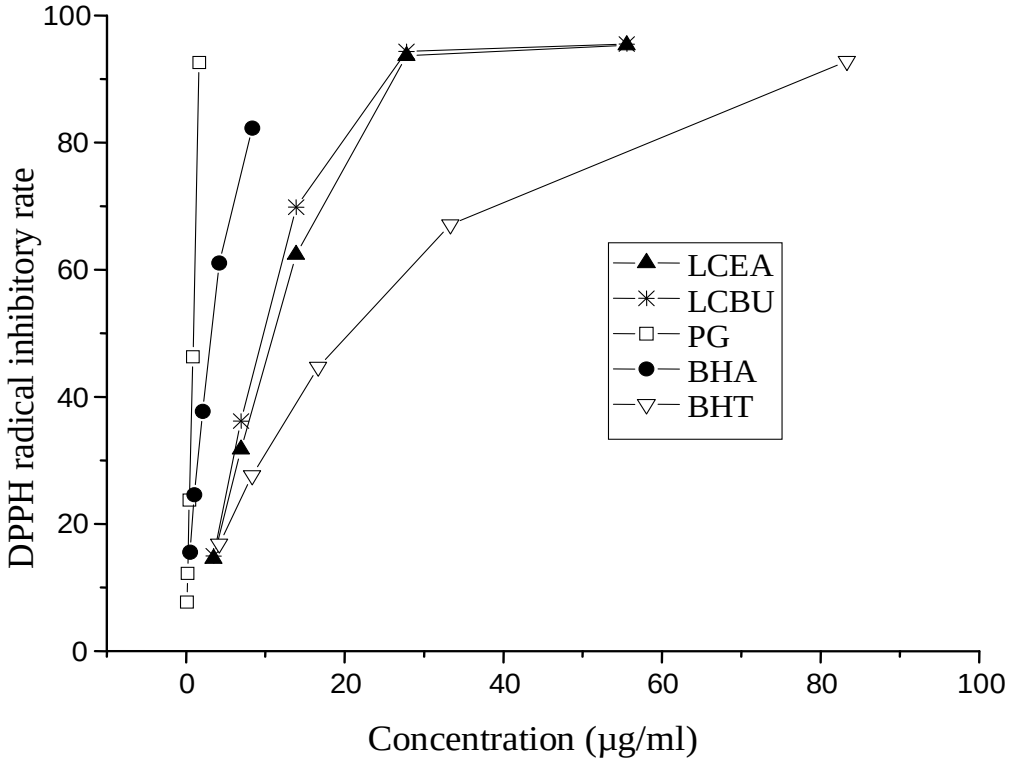

Figure 1. DPPH radical scavenging activity of extracts from *L. clethroides* Duby.

Biochemical analyses

The content of protein in liver samples was determined by Bradford's method (Bradford, 1976). The level of GPT, GOT, SOD and MDA were measured following the commercial kit's instructions.

Statistical analysis

All the grouped 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 ± standard deviation (SD) for ten mice in each group.

RESULTS

In vitro assay for free radical scavenging activity

The antioxidant activity of LC with half inhibitory concentration (IC_{50}) is shown in Table 1, Figures 1 and 2. In DPPH assay, the antioxidant activity of LCBU (IC_{50} =

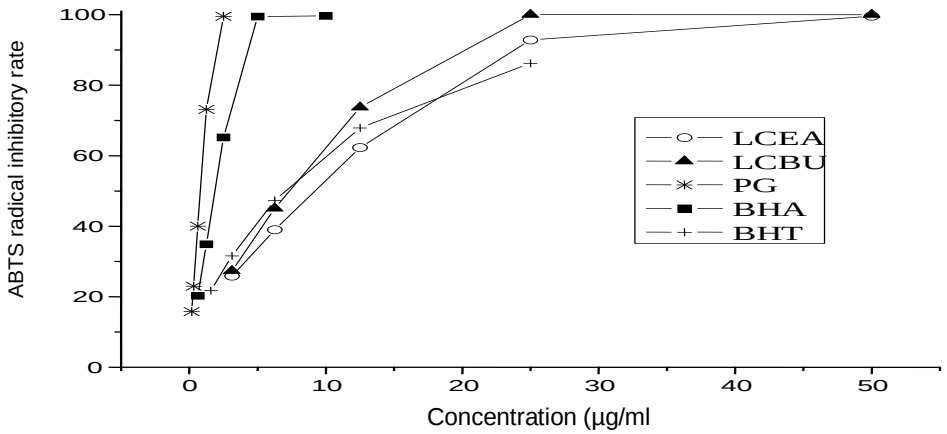

Figure 2. ABTS radical scavenging activity of extracts from *L. clethroides* Duby.

Table 2. Effect of *L. clethroides* Duby on GPT and GOT in acute liver injury mice.

Group	Dose (mg/kg)	GOT (IU/L)	GPT (IU/L)
Normal control	/	95.58 ± 33.68***	87.74 ± 17.20**
liver injury control	/	1486.91 ± 473.04$^{\Delta\Delta\Delta}$	4241.10 ±124.42$^{\Delta\Delta}$
bifendate	70	881.01 ± 203.30**	1213.92 ± 350.65***
LCPE	500	374.03 ± 101.42***	570.83 ± 109.47***
LCPE	250	197.20 ± 78.59***	644.36 ± 137.23***
LCPE	125	258.46 ± 87.42***	453.69 ± 129.64***
LCEA	600	236.08 ± 106.17***	278.51 ± 28.57**
LCEA	300	259.03 ± 81.13***	379.79 ± 85.51***
LCEA	150	541.79 ± 54.33***	1068.27 ± 216.07**
LCBU	600	758.17 ± 174.11***	1555.16 ± 382.92***
LCBU	300	555.64 ± 103.56***	804.88 ± 71.71***
LCBU	150	449.36 ±137.99***	415.23 ± 97.31***

Data expressed as mean ± SD (n = 10). Bifendate was used as the positive control drug. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$: Normal group compared to CCL_4-induced acute liver injury. *P < 0.05, **P < 0.01, ***P < 0.001: Treated group compared with CCl_4- induced acute liver injury.

9.86 μg/ml) was higher than that of BHT (IC_{50} = 18.71 μg/ml). In ABTS assay, the antioxidant activity of LCBU (IC_{50} =7.43 μg/ml) was higher than that of BHT (IC_{50} = 7.72 μg/ml). The LCEA (IC_{50} = 9.02 μg/ml) was slightly lower than that of BHT. The results showed that the antioxidant activity of LCBU was higher than that of LCPE and LCEA, LCBU had the highest antioxidant activity *in vitro*.

Hepatoprotective activity of *L. clethroides* Duby *in vivo*

Effect of LCPE, LCEA and LCBU on GOT and GPT in serum

Table 2 showed the level of GOT and GPT in normal and acute liver injury mice. The level of GOT and GPT was significantly increased in acute liver injury control mice compared with normal mice (p<0.001, p<0.01). Administration of each dose group of LCPE, LCEA and LCBU and bifendate (70 mg/kg) were significantly decreased in the level of GOT and GPT ($P < 0.001$, $p < 0.01$), and LCEA (600, 300 mg/kg) and LCBU (300,150 mg/kg) tend to bring the level to near normal. Compared with positive control of bifendate (70 mg/kg), the result showed that intragastric administration of LCPE, LCEA and LCBU was similar to bifendate as positive control and LCEA showed dose dependence (Table 2, Figure 3).

Effect of *L. clethroides* on MDA and SOD in liver

The effect of different doses of LCPE, LCEA and LCBU

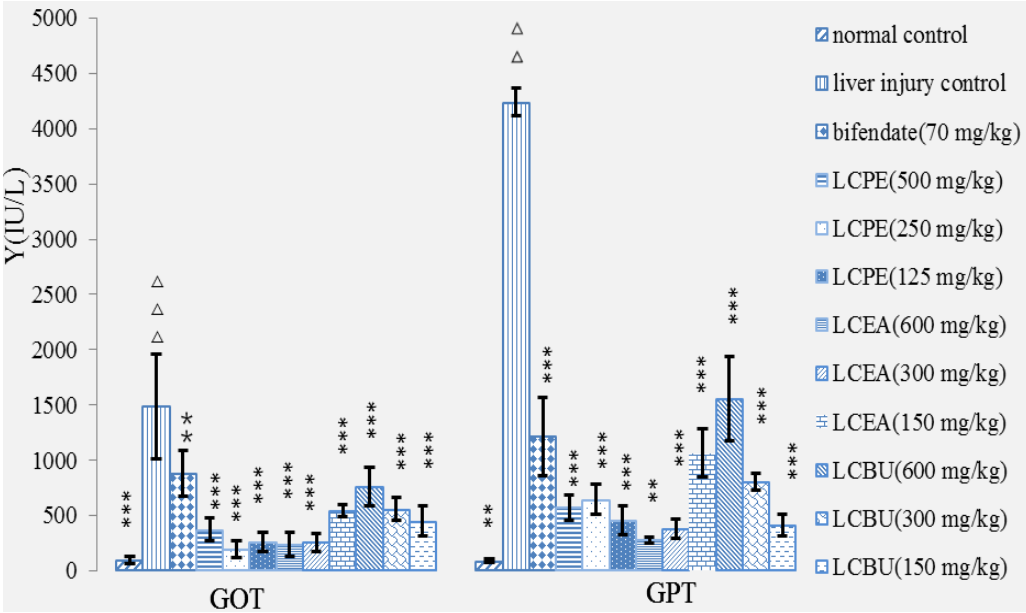

Figure 3. Effect of *L. clethroides* Duby on GPT and GOT in serum.

Table 3. Effect of *L. clethroides* Duby on SOD and MDA in liver.

Group	Dose (mg/kg)	MDA (nmol/ml)	SOD (U/ml)
Normal control	/	27.22 ± 2.43***	505.22 ± 72.61**
liver injury control	/	39.40 ± 2.99$^{\Delta\Delta\Delta}$	273.46 ± 22.81$^{\Delta\Delta}$
bifendate	70	23.17 ± 2.80***	415.14 ± 44.27**
LCPE	500	23.74 ± 2.15***	445.55 ± 41.21**
LCPE	250	16.74 ± 2.37***	431.00 ± 38.37**
LCPE	125	17.76 ± 2.70***	349.98 ± 16.75**
LCEA	600	15.98 ± 3.79***	518.82 ± 75.47*
LCEA	300	18.98 ± 2.50***	349.45 ± 23.74*
LCEA	150	26.08 ± 0.77***	509.38 ± 74.85*
LCBU	600	17.14 ± 3.31***	509.38 ± 31.70***
LCBU	300	12.55 ± 1.87***	572.03 ± 31.93***
LCBU	150	13.71 ± 2.67***	234.03 ± 82.03

Data expressed as mean ± SD (n = 10). Bifendate was used as the positive control drug. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p<0.01$, $^{\Delta\Delta\Delta}p<0.001$ Normal group compared to CCl_4-induced acute liver injury. $^{*}P < 0.05$, $^{**}P<0.01$, $^{***}P<0.001$ Treated group compared with CCl_4-induced acute liver injury.

on the level of SOD and MDA in normal and CCl_4-induced liver injury mice was given in Table 3, Figures 4 and 5. Compared with normal mice, the level of MDA in liver was significantly increased in liver injury control mice ($p < 0.001$), and the level of SOD in liver was significantly decreased ($p < 0.01$), it indicated that the model of CCl_4-induced liver injury control mice was established. Intragastric administration of LCPE, LCEA, LCBU and bifendate to mice for 8 days, and the level of MDA was decreased significantly ($p < 0.001$). The level of SOD in liver only in administration of LCBU (150 mg/kg) had no significant increase ($p > 0.05$); the other treatment groups had significant increase ($p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively).

DISCUSSION

Oxygen species are common mechanisms of injury in most liver diseases, hence an antioxidant may be a

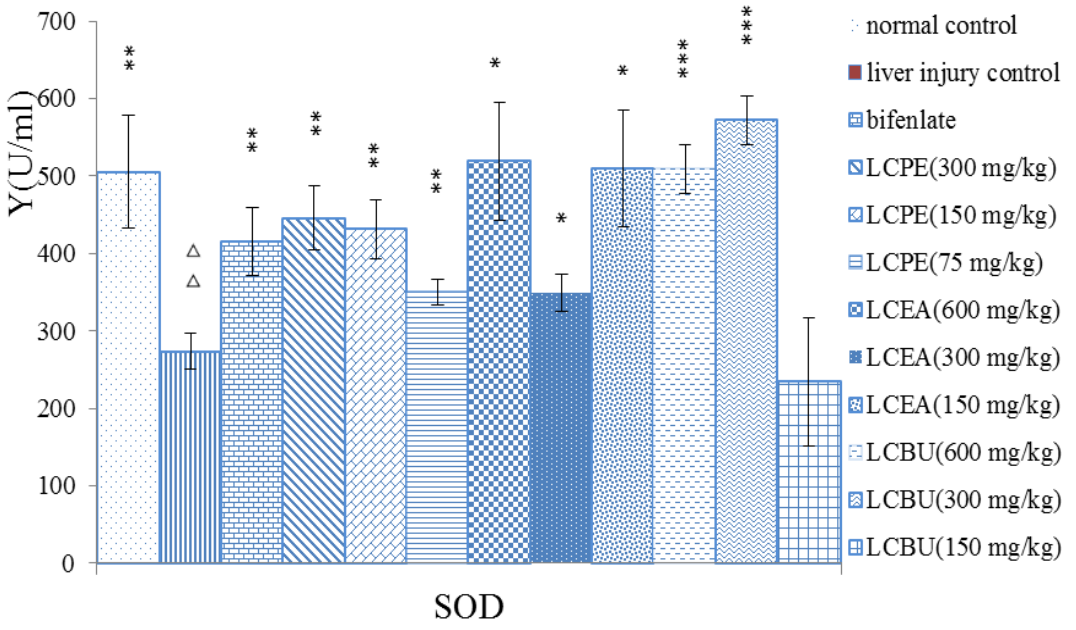

Figure 4. Effect of *L. clethroides* Duby on SOD in liver.

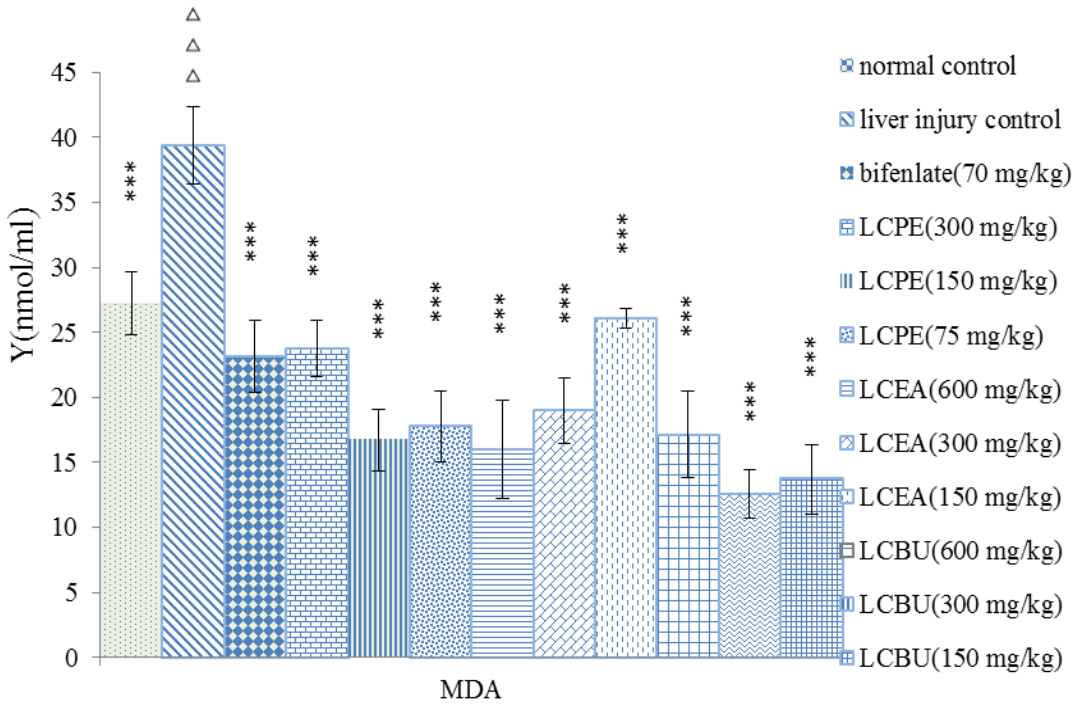

Figure 5. Effect of *L. clethroides* Duby on MDA in liver.

useful tool for protecting the liver cells against superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), and the singlet oxygen ($^1O_2$) induced damage (Jain et al., 2008). CCl_4, a hepatotoxin for evaluating hepatoprotective agents, is commonly used to induce liver damage by producing free radical intermediates (malondialdehyde and 4-hydroxy-2-nonenal). Hepatoprotective studies showed that plants have active ingredients that are capable of free radical scavenging in living systems (Mitra et al., 1998). A major

defense mechanism involves the antioxidant enzymes, which includes SOD, catalase and glutathione peroxidase (GPx), which converts active oxygen molecules into non-toxic compounds. Scavenging of free radicals is one of the major mechanisms that inhibit the chain reaction of lipid peroxidation (Merlin and Parthasarathy, 2011). In this study, the free radical scavenging activity of extracts of *L. clethroides* was evaluated by DPPH and ABTS assay *in vitro* and antioxidant activity was assayed by estimation of MDA and SOD levels *in vivo*. Results showed that LCEA and LCBU were the higher antioxidant activities in DPPH and ABTS assay. LCBU had the highest antioxidant activity (DPPH: IC_{50} = 9.86 ± 0.07; ABTS: IC_{50} = 7.43 ± 0.06). Low and high molecular weight phenolics, including flavonoids, phenolic acids and tannins, have been shown to be good quenchers of free radicals. The free radical scavenging activity of LCEA and LCBU may also be attributed to the presence of phenolics and flavonoids (Yasmin et al., 2010).

The level of MDA in liver for each treatment group could be significantly decreased ($p < 0.001$), and the level of SOD in liver only in group of LCBU (150 mg/kg) had no significant increase ($p > 0.05$), the other treatment groups had significant increase ($p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively). Reducing the level of MDA, and increasing the level of SOD to decreased lipid peroxidation and/or decreased utilization, enhanced antioxidant capability and protected the body from further oxidative damage by free radicals (Sandesh et al., 2010; Quan et al., 2008; Hu et al., 2008).

The elevation of concentrations of serum enzymes such as GOT and GPT is generally regarded as one of the sensitive markers of hepatic damage (Amat et al., 2010). The use of CCl_4 to induce liver injury is well-documented as is the use of GOT and GPT as marker enzymes (Venkateswaran et al., 1998). The results showed that administration of each dose group of LCPE, LCEA, LCBU and bifendate (70 mg/kg) significantly decreased the level of GOT and GPT, ($p < 0.001$, $p < 0.01$) and LCEA (600, 300 mg/kg) and LCBU (300, 150 mg/kg) tend to bring the level to near normal. Compared with positive control of bifendate (70 mg/kg), the result showed that intragastric administration of LCPE, LCEA and LCBU was similar to bifendate, and LCEA showed dose dependence.

Based on the results, it can be concluded that it is apparent that *L. clethroides* has components that have hepatoprotective properties. It can be concluded that possible mechanism of hepatoprotective activity may be due to its free radical scavenging and antioxidant activity. However, further studies are in progress for better understanding of the mechanism of action and its hepatoprotective activity.

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