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Full Length Research Paper

Regulatory effect of *Laggera alata* extract on immune mediated liver injury

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The immune factors were the main reasons of hepatic damage in hepatitis. To validate the beneficial effects of *Laggera alata* on hepatitis in humans, regulatory properties of *L. alata* extract (LAE) was studied using a Bacillus Calmette-Guerin- Lipopolysaccharide (BCG-LPS) induced immune liver injury model in mice. Quantificational analysis of LAE indicated that isochlorogenic acids were the major components in the extract whose content amounted to 51%. LAE reduced the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and nitric oxide (NO) levels of the mice, also decreased the malondialdehyde (MDA) level in mouse liver, and increased the levels of total protein and glutathione peroxidase (GSH-PX) in mouse liver. Meanwhile, resulted in significant recovery of immune-mediated hepatocyte injury in the liver sections. The study suggests that the regulatory properties of LAE may be achieved by ameliorating oxidative stress from BCG-LPS-induced immune liver injury or by preventing tissue damage as a result in inflammation. Isochlorogenic acids may be its substance basis responsible for the regulatory potential. Additional, flavonol components may also play important role in the pharmacological activity of *L. alata*.

Key words: *Laggera alata*, calmette-guerin bacillus, lipopolysaccharide, Isochlorogenic acid, immune mediated liver injury.

INTRODUCTION

Even though hepatitis is a prevalent disease in the world, there is no suitable drug to treat patients with hepatitis. It has been recognized that the immune factors, such as virus/parasite infection, autoimmune stimuli, etc., were the dominant reasons of hepatic damage in hepatitis (Li et al., 2001; Ouyang et al., 2001; You et al., 2001). The genus *Laggera* (*Asteraceae*) is distributed mainly in Tropical Africa and Southeast Asia. *Laggera alata* is one of the only two *Laggera* species found in China. This plant is employed in the traditional chinese medicine for over three hundred years, especially for the treatment of some ailments associated with inflammation including hepatitis. Most studies concerned on *L. alata* focused on folk use and phytochemical analyses of this plant (Bohlmann et al., 1985; Raharivelomanana et al., 1998; Zheng et al., 2003a, b, c). Studies on the role in hepatoprotection and the mechanisms involved in the efficacy for treatment of hepatitis have been limited. Hepatitis implies injury to the liver characterized by the presence of inflammation. In previous investigations, we confirmed the anti-inflammatory activities of *L. alata* extract (LAE) in models of inflammation and its hepatoprotective effect against carbon tetrachloride-induced injury *in vitro* and *in vivo* (Wu et al., 2006a, 2009). The regulating action of LAE in the immune-mediated liver injury remain unknown. To further validate the beneficial effects of *L. alata* on inflammatory liver disorders, protective properties of LAE was studied using a BCG-LPS-induced immune liver injury model in mice.

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Table 1. Effects of LAE on serum AST, ALT and NO levels of the mice with liver damage induced by BCG-LPS.

Group	Dose (mg/kg)	NO (μmol/l)	AST (IU/l)	ALT (IU/l)
Vehicle	-	44.32 ± 4.19 [b]	52.69 ± 5.12 [b]	35.42 ± 2.41 [b]
Model	-	91.27 ± 6.37	134.55 ± 8.49	95.88 ± 6.45
DEX	2.5	61.28 ± 7.32 [a]	81.44 ± 7.27 [b]	50.99 ± 7.28 [b]
LAE	50	85.69 ± 5.97	120.25 ± 14.67	82.11 ± 8.12
	100	80.67 ± 9.28	110.88 ± 12.66	80.67 ± 3.11 [a]
	200	80.64 ± 8.55	111.78 ± 13.91	76.79 ± 4.88 [a]

Values are expressed as mean ± SD, n=10. [a] $P<0.05$ and [b] $P<0.01$ compared with the model control. Student's *t*-test.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (LPS) and dexamethasone (DEX) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Aspartate aminotransferase (AST), and alanine aminotransferase (ALT) diagnostic kits were also purchased from Sigma. Nitric oxide (NO), malondialdehyde (MDA), glutathione peroxidase (GSH-PX), and total protein kits were purchased from Nanjing Jiancheng Bioengineering Institute (China). The Bacillus Calmette-Guerin (BCG) vaccine was from the National Vaccine and Serum Institute (China). All other reagents were of the highest commercial grade available.

Preparation of LAE

L. alata was collected from Tengchong County, Yunnan Province, China, in August 2003, and authentified by Professor Liurong Chen. A voucher specimen (No. ZY982003LA) was deposited in the herbarium of College of Pharmaceutical Sciences, Zhejiang University, China. *L. alata* extract (LAE) was prepared and its main components was quantitatively analyzed according to the method we reported previously (Wu et al., 2006). Quantitative analysis of LAE led to a conclusion that this extract fraction has a high content of phenolic compounds reaching up half of the extract (52.6 g GAE/100 g extract). The high performance liquid chromatography (HPLC) analysis indicated that isochlorogenic acids (4,5-*O*-Dicaffeoylquinic acid, 3,5-*O*-Dicaffeoylquinic acid and 3,4-*O*-Dicaffeoylquinic acid) were the major components in LAE whose content amounted to 51%.

Experimental animals

Male ICR mice weighing 20 to 25 g were kept in a room maintained at 22 ± 2°C and at relative humidity between 40 and 70%. The animal experimental protocol was approved by the Animal Ethics Committee of Zhejiang University, in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology (USA) in July 1989 and revised in March 1999.

BCG-LPS-induced immune hepatic damage in mice

This experiment was followed by the method described by Xu et al. (2005) with some modification. Male ICR mice were divided into six groups comprising ten mice in each group. Group A and B, which served as vehicle and model control, respectively, received 0.5% CMC-Na solution at a dose of 10 ml/kg. Group C served as drug control and received dexamethasone at a dose of 2.5 mg/kg. Groups D, E and F received LAE at doses of 50, 100 and 200 mg/kg, respectively. To induce immune liver damage, the dose of 0.2 ml of BCG (1.0×10^8 CFU/ml, dissolved in normal saline) was injected intravenously into the tail vein of each mouse from Groups B, C, D, E and F. Ten days later, every mouse of Group B-F was again injected intravenously with 0.2 ml (0.05 mg/ml) of LPS in normal saline. Subsequently, the vehicle and drugs were administered orally to the groups of mice at intervals of 0, 4, 8 and 12 h after the intravenous injection of LPS, respectively. Eighteen hours after the administration of LPS, mice were slightly anaesthetized with ether and blood samples were taken from the retro-orbital sinus. The serum was separated for the measurement of AST, ALT and NO. The serum levels of ALT, AST and NO were determined using the ALT, AST and NO detection kits, respectively. Liver samples were rapidly removed, rinsed in cold saline, and homogenized for the examination of MDA, GSH-PX and Total protein levels. The remaining liver samples were rapidly removed and fixed in 10% neutral-buffered formalin, then embedded in paraffin, sliced 5-μm thick, and stained with hematoxylin and eosin (HE). The pathological changes were assessed and photographed under the microscope.

Statistical analysis

Experimental results were reported as mean ± standard deviations of the mean (S.D.). Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and Student' t-test. $P<0.05$ was chosen as the criterion of statistical significance.

RESULTS

The regulatory properties of LAE were evaluated by the BCG-LPS-induced immune liver injury model in mice. The effects of LAE on serum AST, ALT and NO levels of the mice with liver damage are shown in Table 1. The oral treatment of LAE at doses of 50, 100 and 200 mg/kg reduced the mice serum AST, ALT and NO levels. At doses of 100 and 200 mg/kg, LAE showed significant effect on the serum level of ALT. As indicated in Table 2, administration of different doses of LAE reduced the MDA level and enhanced the GSH-PX and total protein levels of liver of the mice. Furthermore, LAE showed significant

Table 2. Effect of LAE on liver MDA, GSH-PX and Total protein levels of the mice with immune liver damage induced by BCG-LPS.

Group	Dose (mg/kg)	MDA (nmol/ml)	GSH-PX (μmol/l)	Total protein (g/l)
Vehicle	-	9.78 ± 0.86 [b]	458.66 ± 22.35 [b]	60.11 ± 5.26 [b]
Model	-	22.64 ± 4.12	311.57 ± 15.79	28.46 ± 2.55
DEX	2.5	13.28 ± 2.11 [b]	435.77 ± 20.22 [b]	57.88 ± 7.28 [b]
LAE	50	20.67 ± 5.07	378.44 ± 17.99 [a]	41.37 ± 9.47
	100	18.55 ± 4.65	405.49 ± 25.88 [b]	52.63 ± 4.64 [a]
	200	15.78 ± 3.11 [a]	421.48 ± 15.12 [b]	52.33 ± 5.27 [a]

Values are expressed as mean ± SD, n=10. [a] P<0.05 and [b] P<0.01 compared with the model control. Student's *t*-test.

effect on the MDA level of the mice liver at a dose of 200 mg/kg. The different doses of LAE remarkably increased the GSH-PX level of the mice liver. At doses of 100 and 200 mg/kg, LAE indicated significant effect on total protein levels of mice liver. Dexamethasone used as the standard drug indicated the remarkable effect on these biomarkers of the mice with liver damage.

Compared with the vehicle control, the serum AST, ALT and NO levels and the liver MDA content of the BCG-LPS-only group were significantly elevated, whereas the total protein and GSH-PX contents of the liver of this group were remarkably reduced, thus indi-cating that immune liver damage was markedly induced. The administration of BCG-LPS caused immune lesions of the liver (granulomatous inflammation exudative lesions etc.), which appeared in all animals of the model control group. No histological abnormalities were observed in vehicle control mice. Administration of different doses of LAE resulted in significant recovery of hepatocytes in different sections of the liver (Figure 1). LAE at doses of 100 and 200 mg/kg showed near normalization of the tissues. The reference drug dexamethasone showed the similar protection. The data suggested that LAE markedly ameliorated BCG-LPS-induced immune hepatic damage.

DISCUSSION

It is known that the immune factors are the main reasons of hepatic damage in hepatitis. The availability of animal models relevant to human hepatitis or hepatocellular immune damage may facilitate discovery of potential therapies. In studies, M*ycobacterium bovis* Calmette-Guerin bacillus infection has been proven to induce immune hepatic injury in rodents (Carpenter et al., 1998; Erb et al., 1998; Ugaz et al., 1999). Moreover, treatment with BCG plus LPS for mice resulted in more severe liver histological changes. Hence, BCG-LPS-induced immune injury model in mice was employed to evaluate the hepatoprotective effect of LAE in the investigation.

Cellular leakage of AST and ALT are conventional indicators of hepatocytes injury. In addition, it has been recognized that NO is produced by cNOS and/or iNOS in mice liver (Moriyama et al., 2000; Vos et al., 1997). The reaction of NO with superoxide anion forms peroxynitrite, a potent cytotoxic oxidative agent eliciting lipid peroxidation and cellular damage. As an indicator of lipid peroxidation, the liver MDA level was measured in the study. Furthermore, the liver GSH-PX level was also measured for evaluating the ability to scavenge radicals. Oxidative stress has been noted to contribute to the pathogenesis of acute hepatitis. Free radicals are toxic to hepatocytes and initiate a reactive oxygen species-mediated cascade causing cell death and leading to acute hepatitis (Bedda et al., 2003; Okuyama et al., 2003). As a consequence, LAE reduced NO production, lowered the liver level of MDA and increased the liver level of GSH-PX, implying that LAE could not only inhibit the lipid peroxidation but also scavenge radicals by enhancing the activities of the antioxidant enzymes. In our previous investigations, the potent anti-inflammatory effects and the hepatoprotective activities against carbon tetrachloride-induced injury of LAE were confirmed and its action mechanisms are probably associated with the inhibition of prostaglandin formation, the influence on the antioxidant systems, and the suppression of lysozyme release (Wu et al., 2006a, 2009). Taken together, the anti-oxidative properties of LAE may contribute to the allevia-tion of immune response and prevent tissue damage as a result in inflammation.

The analysis results of LAE showed that this extract contains plenty of phenolic compounds, especially isochlorogenic acids such as 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid. Isochlorogenic acids exhibit several pharmaco-logical activities such as antioxidative, anti-inflammatory, hepatoprotective and antiviral etc (Kimura et al., 1987; Maruta et al., 1995; Peluso et al., 1995; Mcdougall et al., 1998; Linda et al., 2006). In addition, our previous study found that total flavonoids from the plant also possess hepatoprotective activity against carbon tetrachloride-induced injury (Wu et al., 2006b). Therefore, isochlorogenic acids may be the major active compounds responsible for the biological activity of *L. alata.* Furthermore, flavonol components may also contribute to the curative effect of the plant in some inflammatory ailments including hepatitis. In conclusion, the research

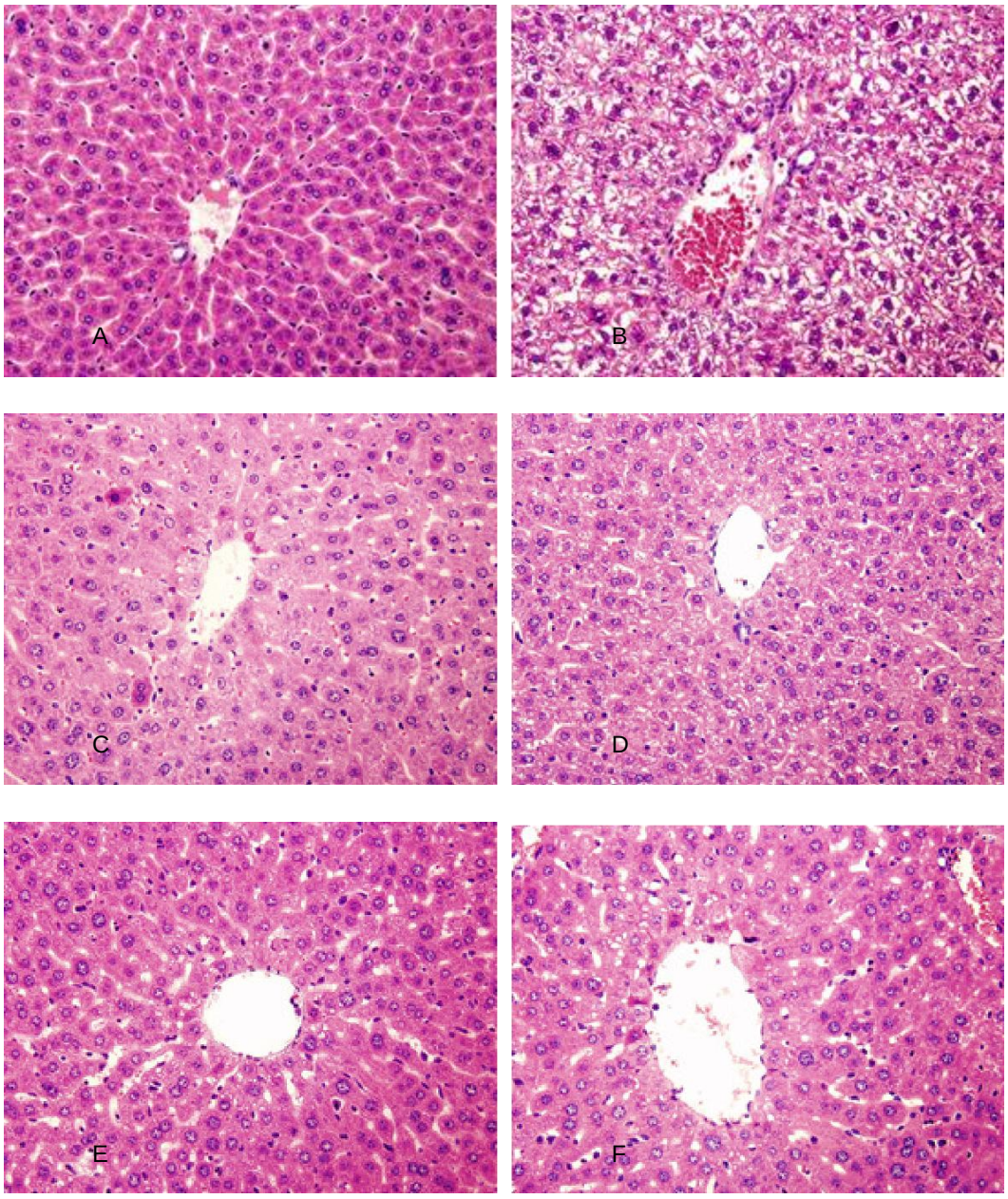

Figure 1. Histopathological effects of LAE on BCG-LPS-induced immune hepatic damage in mice (H&E × 40). (A) a control untreated mouse showing a normal central vein and hepatocytes; (B) a BCG-LPS-treated mouse showing immune lesions of the liver (granulomatous inflammation exudative lesions etc.); (C) a dexamethasone (2.5 mg/kg)–BCG-LPS-treated mouse showing obvious improvement of liver damage; (D) a LAE (50 mg/kg)–BCG-LPS-treated mouse; (E) a LAE (100 mg/kg)–BCG-LPS-treated mouse, and (F) a LAE (200 mg/kg)–BCG-LPS-treated mouse. (D), (E), and (F) show different degrees of improvement of immune liver injury.

suggests that the regulatory properties of LAE may be achieved by ameliorating oxidative stress from BCG-LPS-induced immune liver injury or by preventing tissue damage resulted from inflammation. Isochlorogenic acids may be the active substance responsible for this regulatory potential. Additionally, flavonol components may also play important roles in the pharmacological activity of *L. alata*.

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