

Tropical Journal of Pharmaceutical Research April 2017; 16 (4): 861-866

ISSN: 1596-5996 (print); 1596-9827 (electronic)

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Available online at <http://www.tjpr.org><http://dx.doi.org/10.4314/tjpr.v16i4.16>

Original Research Article

Protective effect of alcohol extract of Yulangsans leaf on chemically-induced liver injury in mice

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Received: 14 January 2017

Revised accepted: 22 March 2017

Abstract

Purpose: To investigate the protective effect of *Millettia pulchra* Kurz var. *Laxior* (Dunn) Z. Wei (Yulangsans) leaf (YLSL) on chemically-induced liver injury in mice.

Methods: Models of carbon tetrachloride (CCl₄) and D-galactosamine (D-GalN)-induced liver injury in Kunming mice were prepared by intraperitoneal injection. Sixty mice were randomly divided into normal saline (NS) group, liver-injury group, low-, medium- and high-dose YLSL groups (7.5, 15 and 30 g/kg dose, respectively), and biphenyldicarboxylate (BPDC) group, with 10 animals per group. Indices for liver, spleen and thymus were assessed. Serum aspartate transaminase (AST) and alanine aminotransferase (ALT) activities, levels of malondialdehyde (MDA) in liver tissues and reduced glutathione (GSH) as well as activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in liver tissue were assayed. Liver tissue damage was assessed histologically.

Results: YLSL could significantly decrease the elevation of AST or ALT in liver injuries induced by CCl₄ or D-GalN in mice, which showed a dose-effect relationship obviously. The high dose YLSL significantly decreased thymus weight relative to CCl₄ and D-GalN (CCl₄ CCL₄+YLSL: 4.4213 ± 1.0544 vs 3.7120 ± 0.8534; D-GalN vs YLSL + D-GalN: 3.7272 ± 1.1655 vs 1.9548 ± 1.2996, p < 0.01). However, SOD activity was significantly increased (p < 0.01, p < 0.05). In treatment groups exposed to CCl₄, GSH-Px activity was significantly increased (p < 0.01) and GSH levels decreased (middle dose group and positive control group). In treatment groups with D-GalN, GSH content was significantly increased (p < 0.01 or p < 0.05), while GSH-Px activity decreased (p < 0.01).

Conclusion: YLSL has protective effect against chemically-induced liver injury in mice. The mechanism may be related to attenuation of free radical-induced lipid peroxidation.

Keywords: *Millettia pulchra*, Liver injury, Biochemical parameters, Thymus, Antioxidant, D-galactosamine, Biphenyldicarboxylate

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INTRODUCTION

Millettia pulchra Kurz var. *Laxior*(Dunn)Z. Wei also known as *Yulangsans* (YLS) or *Longyanshen*, is used as an analgesic, anti-inflammatory, sedative memory-enhancing, immunity-boosting and anti-stress agent. The roots of YLS contain flavonoids [2], saponins [3] and polysaccharides [4]. Studies have shown

that the aqueous extract of YLS roots has hypotensive, free radical-scavenging, myocardial ischemia and reperfusion injury-reducing properties [5]. It also has anti-dementia and anti-inflammatory [6], as well as hepatoprotective effects [7,8]. Studies have so far concentrated mostly on Yulangsans roots, to the exclusion of the leaves.

In the present study, the hepatoprotective effects of alcohol extract of Yulangsans leaves against chemical-induced liver injury were investigated in SPF Kunming mice through a combination of biochemical and histological procedures.

EXPERIMENTAL

Animals

SPF Kunming mice (half male and half female, weighing 20 ± 2 g) were provided by the Guangxi Medical University Experimental Animal Center (Shuangyong Road No. 22, Nanning City, Guangxi Province, China). Experimental animal production and occupation licenses were SCXKG Gui 2009-0002, and SYXKG Gui 2009-0004, respectively. This research was approved by the Animal Ethical Committee of Guangxi Medical University Experimental Animal Center according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [7].

Materials and reagents

The plant was collected from Xinxu in the suburb of Lingshan Guangxi Province in 2014, and was identified as *Millettia pulchra* Kurz var. *Laxior* (Dunn) Z. Wei (*Yulangsans*) leaf by Professor Maoxiang Nai (Guangxi Institute of Chinese Medicine & Pharmaceutical Science, Nanning). A voucher specimen (no. YLS 201406-03) was kept at the herbarium of Experimental Center of The School of Pharmacy, Guangxi Medical University. Yulangsans leaf (YLSL) alcohol extract was prepared by a method utilizing 70 % methanol for extraction for 2 h at reflux; analytical grade carbon tetrachloride (CCl_4) was purchased from Chengdu Kelong Chemical Reagent Factory (Sichuan, China). D-GalN was provided by MP Biomedicals LLC (Shanghai, China). Bifendate dropping pills were purchased from Guangzhou Xingqun Pharmaceutical Co., Ltd (Guangdong Province, China); sodium chloride injection was purchased from Guizhou Tiandi Pharmaceutical Co., Ltd. (Guangdong Province, China), while Coomassie blue detection kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing City, China). Other reagents were of analytical grade.

Instruments and equipment

Visible spectrophotometer was product of Shanghai Precision Scientific Instrument Co., Ltd, Model 722S; electronic balance: ZA1003 (Sartorius (Shanghai) Trading Company Ltd); low-speed large-capacity desktop centrifuge was obtained from Shanghai Anting Scientific Instrument Factory (Shanghai, China); model

TDL-5, trace desktop centrifuge model CL17R, was product of Thermo Company (Shanghai, China); high-speed refrigerated centrifuge was from Shanghai Anting Scientific Instrument Factory (Shanghai, China); model TGL-16G-A and vortex mixer were products of Shanghai Medical Instrument Factory (Shanghai, China). Water ultra-purification System (Model UPT-II-20T) was supplied by Chengdu Chaochun Technology Co., Ltd (Sichuan Province, China); stainless steel electric heated-water bath, model GSY-ii was from Beijing Medical Equipment factory (Beijing, China); while color pathological image analysis system PTPS-2011 was supplied by Chongqing Tianhai Medical Equipment Co., Ltd (Chongqing, China).

Animal models

Healthy Kunming mice of both sexes (mean weight = 20 ± 2 g) were randomly assigned to six groups, with each group having 5 male and 5 female mice. The groups were normal control group, CCl_4 model group, biphenyldicarboxylate (BPDC, (150 mg/kg body weight) positive control group, and three groups given different doses (high, medium and low) of alcohol leaf extract of Yulangsans, i.e., YLSLH, YLSLM, YLSLL groups which received 30, 15 and 7.5 g YLSL/kg body weight, respectively. Mice in positive control group received intraperitoneally (*i.p.*) 0.02 mL ml/g; the CCl_4 model group received the same dose of NS *i.p.* while mice in the treatment groups received their respective doses of YLSL *i.p.*, in addition to NS. All treatments were given once daily for 7 days, and 1 h after the last administration, all the groups were injected with 0.1 % CCl_4 in peanut oil [10] at a dose of 0.1 mL/10 g, except the normal control group which got peanut oil injection in place of 0.1 % CCl_4 (0.1 mL/10 g). The mice fasted for 16 h but had free access to drinking water, and the D-GalN model group was given (*i.p.*) D-GalN solution (800 mg/kg) with the capacity of 0.2 mL/10 g [11], and fasted for 16h with access to drinking water [12].

Determination of serum biochemical indicators

Blood was taken from the retro-orbital plexus, allowed to coagulate, and centrifuged at 3000 rpm at 0 °C for 10 min. The serum was stored at -5 °C, and used for assay of serum AST and ALT with kits according to manufacturer's instructions.

Calculation of visceral indices of liver, spleen and thymus

The mice were sacrificed by decapitation; the fat and mesentery were quickly cut off on an ice tray

in an ice-water bath of physiological saline. The liver, spleen and thymus were excised and rinsed with ice-cold physiological saline to remove accumulated blood. The organs were blotted and weighed; and visceral indices were calculated as follows: liver index = liver weight (g) divided by body weight (g), thymus index = thymus weight (mg) divided by body weight (g) and spleen index = spleen weight (mg) divided by body weight (g).

Preparation of liver homogenates and examination

The right lobe of the liver was selected and rinsed with cold saline to remove blood clots, then weighted after blotting. Ten percent (10 %) liver homogenate was prepared in 0.9 % sodium chloride solution. The homogenate was centrifuged at 3500 rpm at 4 °C for 15 min, and the supernatant was refrigerated at -5 °C prior to use.

Biochemical indices in liver homogenate were assayed with kits were MDA (with thiobarbituric acid); SOD (xanthine oxidase method); GSH and GSH-PX, while protein content was determined by the Coomassie brilliant blue method [13].

Histological examination of liver tissue

Small sections of liver tissue were taken 0.5 cm from the edge of liver leaf edge, and fixed in 10 % formalin fixative at 4 °C overnight. The tissue was sectioned (cut into about 2 mm thickness) and HE stained, then the morphological changes in liver tissue were observed under light microscope.

Statistics

SPSS 18.0 software was applied for statistical analysis. The data are presented as mean \pm standard deviation (SD). The means of multi-groups were compared using analysis of

variance. $P < 0.05$ or 0.01 was taken as statistically significant.

RESULTS

Effect of YLSL on visceral index of mice

For mice with CCl₄-induced acute liver injury, both YLSLH and YLSLM significantly reduced thymus index ($p < 0.01$). YLSLH reduced spleen index ($p < 0.01$). However, the different doses of YLSL had no significant effect on liver index.

For mice with D-GalN-induced acute liver injury, the three doses of YLSL had no significant effect on liver index and spleen index. However YLSLH significantly lowered thymus index ($p < 0.05$; Table 1).

Effect of YLSL on serum biochemical parameters

Experimental results demonstrated that for mice with CCl₄-induced acute liver injury, YLS leaf alcohol extract at low dose significantly reduced the activity of serum AST ($p < 0.01$), while the middle dose significantly reduced the activity of serum ALT ($p < 0.05$). High dose of the extract resulted in significant reduction of MDA ($p < 0.05$), while each of the three doses significantly enhanced the activities of SOD and GSH-PX ($p < 0.01$).

For mice with D-GalN-induced acute liver injury, low dose of YLS leaf alcohol extract reduced the activity of serum AST ($p < 0.01$); each dose significantly decreased serum ALT activity (low- and middle-dose: $p < 0.01$, high dose: $p < 0.05$).

Middle- and high-doses resulted in significantly decreased content of MDA ($p < 0.01$ or < 0.05), while each of the three dose significantly increased GSH content (high- and middle-

Table 1: Effect of YLSL on liver, spleen and thymus indices in CCl₄ or D-GalN-induced acute liver injury in mice

Group	Liver index (%)	Spleen index (mg/g)	Thymus index (mg/g)
NC	0.0460 \pm 0.0037	4.0807 \pm 0.7729	3.7120 \pm 0.8534
CCl ₄ model	0.0472 \pm 0.0033	3.8743 \pm 0.9183	4.4213 \pm 1.0544
BPDC+CCl ₄	0.0506 \pm 0.0047	4.3336 \pm 0.5902	3.8805 \pm 1.2399
YLSLL+CCl ₄	0.0479 \pm 0.0033	4.3535 \pm 0.9405	3.8722 \pm 1.8661
YLSLM+CCl ₄	0.0496 \pm 0.0035	3.4046 \pm 0.6768	2.8778 \pm 0.5853**
YLSLH +CCl ₄	0.0498 \pm 0.0037	3.2068 \pm 0.3859*	2.7682 \pm 0.7235**
NC	0.0474 \pm 0.0097	4.1739 \pm 0.7232	3.7654 \pm 1.0897
D-GalN model	0.0543 \pm 0.0049	4.1427 \pm 1.4056	3.7272 \pm 1.1655
BPDC+D-GalN	0.0631 \pm 0.0061**	4.5190 \pm 0.9977	3.9386 \pm 0.8912
YLSLL+D-GalN	0.0567 \pm 0.0113	4.5056 \pm 0.7446	3.3667 \pm 1.4537
YLSLM+D-GalN	0.0563 \pm 0.0081	3.7721 \pm 1.2645	3.1694 \pm 0.8226
YLSLH+D-GalN	0.0571 \pm 0.0073	3.0471 \pm 0.9327	1.9548 \pm 1.2996*

Values expressed are as mean \pm SD ($n = 10$); * $p < 0.05$, ** $p < 0.01$ vs CCl₄ control and D-GalN control group

dose: $p < 0.01$, low dose: $p < 0.05$). However, each dose significantly increased SOD activity ($p < 0.01$) (Table 2).

Effect of YLSL on liver histopathology CCl₄- and D-GalN-induced acute liver injury

Under the microscope, clear clobular contours, prominent nucleoli, abundant and uniform cytoplasm of hepatocytes, and visible liver sinusoidal cord could be seen in normal mice. After acute liver injury, hepatic lobules were unclear, the cytoplasm was bright and empty with sparse, lightly stained, irregular particles, and the liver cells became enlarged. Besides ballooning degeneration, nuclear condensation and focal necrosis were evident. However, each dose of YLSL relieved the liver cell swelling, as well as focal and zonal necrosis. The results are as shown in Figure 1.

DISCUSSION

There are varieties of enzymes which reflect liver cell injury and the extent of damage. These include ALT, AST, GGT, GLD, and SOD. ALT is the most sensitive indicator of liver cell injury [14]. In general, increased activity of serum ALT is positively correlated with degree of hepatocellular disease. Increased serum AST and ALT activities are of significance in the diagnosis of hepatitis, although ALT is a more reliable index than AST. The activities of ALT and AST in the CCl₄ and D-GalN models were significantly elevated, with variation in ALT greater than that in AST, suggesting that liver tissue damage was successfully induced in these models.

MDA is the end product of lipid peroxidation, which indirectly reflects the severity of free

radical attack on cells [15]. SOD is an important antioxidant enzyme system which indirectly reflects ability to scavenge oxygen free radicals [16,17]. Therefore, MDA levels are often correlated with SOD. In this study, SOD activities in the CCl₄ and D-GalN models were significantly lower than SOD activities in the treatment groups ($p < 0.01$). This indicates that alcohol leaf extract of Yulagsan has hepatoprotective effects, most probably through inhibition of lipid peroxidation. GSH, a tripeptide composed of glutamic acid, cysteine and glycine, is an important non-protein thiol compound which belongs to low-molecular scavengers. GSH can eliminate O₂⁻, H₂O₂ and LOOH, and is necessary for the decomposition of hydrogen peroxide. Besides, GSH can stabilize thiol-containing enzymes and prevent hemoglobin and other cofactors from oxidative damage. Deficiency of GSH or its depletion will lead to toxic effects or aggravated toxic effects by many chemicals or environmental factors. Thus, GSH is an index of antioxidant capacity.

The determination of activities of GSH and GSH-Px are useful for understanding the mechanism of drug action. In the treatment groups exposed to CCl₄, GSH-Px activity was significantly increased ($p < 0.01$) and GSH levels decreased (middle dose group and positive control group). In the treatment groups with D-GalN, GSH content was significantly increased ($p < 0.01$ or $p < 0.05$), while GSH-Px activity decreased ($p < 0.01$).

Thus, an inverse relationship was observed between GSH and GSH-Px, i.e., when GSH reduced, GSH-Px increased, and vice versa. This suggests that the alcohol extract of Yulagsan on the one hand promoted the synthesis of GSH, while on the other hand, it accelerates the conversion of GSH to GSSG.

Table 2: Effect of YLSL on serum and hepatic parameters in CCl₄- and D-GalN-induced acute liver injured mice

Group	AST	ALT	MDA nmol/mgprot	GSH (gGSH)	SOD (U/mgprot)	GSH-PX
NC	72.72±23.69**	24.35±9.05**	2.46±0.43**	0.73±0.19*	160.31±20.25**	133.28±47.15
CCl ₄ model	107.13±21.70	97.71±29.50	4.04±1.10	0.94±0.36	123.33±28.33	172.62±49.86
BPDC+CCl ₄	77.19±17.18**	22.25±10.88**	3.08±0.65*	0.65±0.13*	187.74±42.33**	362.60±42.81**
YLSL _L +CCl ₄	80.50±9.57**	66.58±27.98*	4.42±0.98	0.82±0.11	209.55±25.10**	412.52±54.94**
YLSL _M +CCl ₄	101.56±31.58	66.41±29.55*	3.55±0.66	0.65±0.11*	175.66±32.95**	319.80±56.90**
YLSL _H +CCl ₄	101.61±31.44	77.87±29.62	2.98±0.81*	1.02±0.18	165.36±35.34**	341.00±65.03**
NC	50.47±41.69**	12.34±5.97**	6.70±3.31**	1.77±0.98	169.79±64.97	437.41±125.62
D-GalN model	228.61±70.60	260.58±64.53	12.66±5.51	1.18±0.55	142.57±22.40	479.29±126.48
BPDC+D-GalN	74.88±40.85**	77.36±72.93**	7.08±2.69**	4.72±5.22*	195.64±43.92**	323.74±49.54**
YLSL _L +D-GalN	118.83±93.71**	103.74±86.34**	10.89±5.58	4.01±4.05*	210.74±35.55**	365.62±65.52*
YLSL _M +D-GalN	163.70±155.36	135.22±97.96**	8.08±3.53*	2.38±0.93**	190.60±27.55**	289.77±76.09**
YLSL _H +D-GalN	210.67±109.91	138.19±138.57*	6.37±2.07**	4.19±3.06**	198.90±30.12**	333.56±68.27**

Values expressed are as mean ± SD (n = 10); * $p < 0.05$, ** $p < 0.01$ vs CCl₄ control and D-GalN control groups

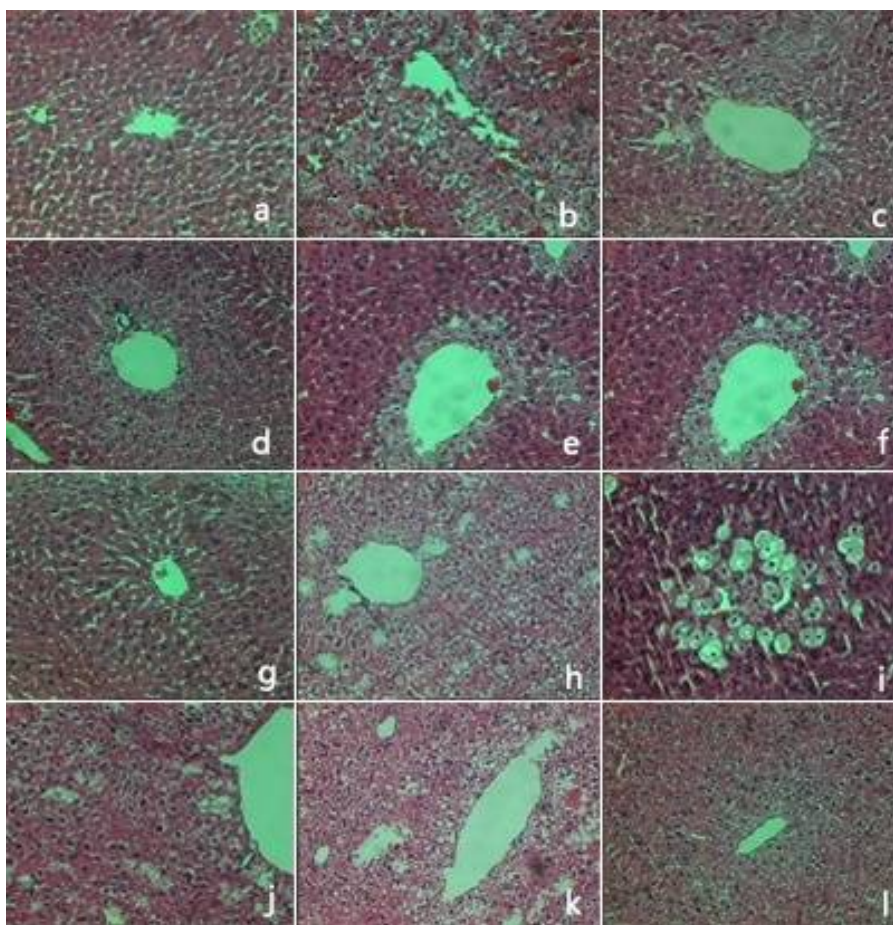


Figure 1: Photomicrograph of liver tissue (H&E $\times 100$). a: oil alone-treated mice; b: CCl_4 alone-treated mice; c: mice pretreated with YLSL ($30\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}\times 7\text{d}$) before treatment with CCl_4 ; d: mice pretreated with YLSL ($15\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}\times 7\text{d}$) before treatment with CCl_4 ; e: pretreated with YLSL ($7.5\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}\times 7\text{d}$) before treatment with CCl_4 ; f: mice pretreated with BPDC ($150\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}\times 7\text{d}$) before treatment with CCl_4 ; g: NC alone-treated mice; h: D-GalN alone-treated mice; i: mice pretreated with YLSL ($30\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}\times 7\text{d}$) before treatment with D-GalN; j: mice pretreated with YLSL ($15\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}\times 7\text{d}$) before treatment with D-GalN; k: mice pretreated with YLSL ($7.5\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}\times 7\text{d}$) before treatment with D-GalN; l: mice pretreated with BPDC ($150\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}\times 7\text{d}$) before treatment with D-GalN

Histological examination is a visual indicator of organ or tissue damage. In this study, CCl_4 -induced liver damage was reflected in cellular necrosis, hemorrhage in the central vein and fatty degeneration. In the D-GalN model group, features seen in histology included diffuse multiple patchy necrosis, cells with a large number of toxic granule with positive PAS staining and more eosinophilic corpuscles. These features are similar to the damage caused by viral hepatitis. The different doses of Yulangsán leaf alcohol extract exhibited varying degrees of relief on the CCl_4 and D-GalN-induced liver cell swelling, necrosis, fatty degeneration, inflammatory cell infiltration and other pathological changes. This shows that the extract has a protective effect against acute liver injury, through a mechanism involving neutralizing

oxygen free radicals and inhibiting lipid peroxidation.

CONCLUSION

YLSL lowers the elevated activities of ALT, AST in acute liver injury mice-induced by CCl_4 and D-GalN, ameliorates liver pathological condition and have protective effects against CCl_4 -induced acute liver injury. The protective effects of wedelolactone against CCl_4 and D-GalN-induced acute liver injury are related to its ability to attenuate oxygen free radicals and inhibit lipid peroxidation. The results of this study provide a further understanding of the pharmacological effects of YLSL as well as the need for further studies of the plant.

DECLARATIONS

Acknowledgement

This work was financially supported by Guangxi Education Department Science Research Foundation (no. KY2015LX191).

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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