Toxicological evaluation of the petroleum ether extract of Leonurus japonicus on liver, kidney and testis in rats

Yi Luo¹, Ben-Hong Zhou¹, Gang Liu¹, Hong-Yan Liu¹ and Xian-Ming Hu²

¹Department of Pharmacy, Renmin Hospital of Wuhan University, Wuhan 430060, PR China.
²College of Pharmacy, Wuhan University, Wuhan 430072, PR China.

Accepted 1 February, 2012

The sub-acute toxicity of the petroleum ether extract of Leonurus japonicus (PELJ) in rats was investigated, and as a comparison, that of the boiled decoction of L. japonicus (DLJ) was also investigated. In rats which received a once-daily administration of PELJ and DLJ at the dose of 60 g/kg by intragastric gavage for 15 days, there was a significant decrease in the body weight gain and an increase in the relative weight of testis and levels of serum blood urea nitrogen (BUN) as well as in the amount of creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), lactic dehydrogenase (LDH) and γ-glutamyltransferase (GGT), urinary protein concentration and urinary NAG. Also, histopathological changes of liver, kidney and testis were observed. The altered levels of body weight, relative organ weight and the biochemical parameters except for urinary protein concentration tended to be restored, and the histopathological changes of liver, kidney and testis were ameliorated in rats that had been withdrawn from the treatment for another 15 days recovery period. PELJ and DLJ exhibited similar toxic effects and the toxic effects of DLJ were lower than those of PELJ. The results demonstrated that PELJ and DLJ at the given dose produced apparent toxicity in rats and that permitting rats to recover for 15 days resulted in partial recovery. Besides, the compositions of PELJ were analyzed by GC–MS. The major components identified in PELJ were 17b-hydroxy-1a, 17-dimethyl-5a-androstan-3-one (21.47%), stigmast-5-en-3-ol (11.86%), E-phytol (9.94%), and caryophyllene oxide (8.69%).

Key words: Leonurus japonicus, petroleum ether extract, GC-MS, toxicity, liver, kidney, testis.

INTRODUCTION

Aerial part of Leonurus japonicus Houtt (syn L. heterophyllus Sweet), a traditional Chinese herbal medicine known as “Yi-Mu-Cao”, has been used widely for several thousand years in China. By invigorating blood circulation, regulating menstrual disturbance and relieving edema, L. japonicus is usually used for blood stasis syndrome with menoxenia, dysmenorrhea, metrorrhagia, postpartum lochiorrhrea, coronary heart disease, cerebral infarction, blood hyperviscosity, myocardial ischemia etc. and for the treatment of various kinds of nephritis with edema (Pharmacopoeia Committee, 2005; Yan and Chen, 2000; Xu et al., 2002; Zou et al., 1989; Wang et al., 1988; Tao et al., 2003; Yin et al., 2010). Besides, in vitro anticancer activities of L. japonicus have also been shown (Chinwala et al., 2003). In practice, some herbalists often use L. japonicus at doses 30 to 60 g up to 120 g per day for hypertension, acute nephrotic edema and central retinocchoroiditis (Bian and Lv, 1998). In recent years, a limited number of studies have provided evidence for the nephrotoxicity of L. japonicus at high dose (Cai et al., 2000; Sun et al., 2005), and some traditional Chinese physicians recommend that administration of L. japonicus should be contraindicated for those patients once suffered kidney injury. So the clinical application of L. japonicus is restricted unavoidably. Nevertheless, to the best of our knowledge, the toxicity of L. japonicus has not been studied intensively and systematically so far, and little research on the toxic effects of various fractions of ethanol extract of L. japonicus, prepared by using solvent...
of varying polarity, has been reported. Therefore, the present study was designed to evaluate the subacute toxicity of petroleum ether extract fraction of *L. japonicus* (PELJ) on Sprague Dawley rats and the reversibility of its toxic effects, and to identify the target organs. Meanwhile, the toxicity of the boiled decoction of *L. japonicus* (DLJ) was also been investigated as a comparison, and the chemical composition of PELJ was analyzed by GC-MS.

**MATERIALS AND METHODS**

**Plant material**

Aerial part of *L. japonicus* was collected from Enshi, Hubei Province, in late May 2005. The plant was identified to conform with the Chinese Pharmacopoeia criterion by Prof. H. Zhang, Wuhan University, China. A voucher specimen was deposited in the herbarium of the College of Pharmacy, Wuhan University, China. The plant material was then dried in open air and kept for use.

**Preparation of the extract**

**PELJ**

The dried plant material was ground to pass 20 mesh sieves before extraction. The powder was refluxed with 95% ethanol twice, each for 1.5 h. The filtrate was evaporated at low pressure to obtain nearly dried extract. The extract was suspended in distilled water and then was partitioned with petroleum ether (60 to 90°C). PELJ, a brown yellowish viscous paste, was obtained after removal of the solvent in vacuo (yield: 1.21%, w/w). PELJ was dispersed in distilled water and diluted to make up the concentration equivalent to original herb 4 g/ml for testing, and stored at 4°C until used. The mixture was subjected to ultrasonic waves for 15 min in order to disperse uniformly before administration. Based on the yield of PELJ, 12.1 mg PELJ was equal to 1 g original herb.

**DLJ**

The dried plant material was soaked in water for 30 min, and then extracted by water boiling method for 2 times, each for 1.5 h. The filtrate was combined and then concentrated to make up the concentration equivalent to original herb 4 g/ml, and was stored at 4°C until used.

**GC-MS analysis of PELJ**

The GC-MS analysis of PELJ was performed using a Thermo-Finnigan trace GC-MS equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm i.d.; coating thickness 0.25 μm). The GC oven temperature was programmed to increase from 60°C, held 1 min, to 290°C at a rate of 6°C/min, and finally held at 290°C for 10 min. Total run time was 63 min. The injector temperature was set at 250°C; and the carrier gas was helium at a flow rate of 1.0 ml/min; 1:40 split ratio. The MS operating parameters were as follows: Ionization energy, 70 eV; scan mode, EI; ion source temperature, 250°C; scanning mass range, 40 to 650 atomic mass units.

Qualitative identification of the components was performed by comparison of their mass spectra with those stored in the database of NIST/WILEY of GC-MS system and those reported previously.

Percentages of the components were calculated from the GC peak areas using the normalization method.

**Animals**

Male adult Sprague Dawley (SD) rats weighing 120 to 150 g were obtained from the Laboratory Animal Center, Tongji Medical College of Huazhong University of Science and Technology after the approval for the study. All animals were housed in the animal facility of the experimental center of the Renmin Hospital of Wuhan University with a 12 h dark/light cycle; the temperature was kept at 22±1°C with 60±5% relative humidity. The animals were allowed to have free access to food and clean water and were acclimatized for a week prior to experimentation. This study was performed based on the guidelines for the care and use of animals established by Wuhan University. The experimental protocol of the study was approved by the Ethics Committee, Wuhan University.

**Subacute toxicity study**

The animals were randomly divided into three groups of 12 animals respectively. Two served as experimental group, namely PELJ group and DLJ group, in which rats received PELJ, DLJ by intragastric gavage daily at a dose of 60 g/kg body weight for a period of consecutive 15 days, respectively. The other served as normal control group in which rats received water. All of the test solutions were administered in a volume of 15 ml/kg body weight per day.

Body weights of the rats were recorded before the beginning of treatment, every day and at the end of treatment. The rats were observed for signs of abnormalities throughout the study. On day 15 (at the end of administration period), six rats randomly selected from each group were housed individually in stainless-steel metabolic cages that allowed for separate collection of urine and faeces and for the next day, 24-h collections of urine were done. On day 16, these rats were anesthetized with ether after the collection of urine samples, and then were sacrificed by dislocating cervical past after blood samples were obtained by cardiac puncture technique. These rats' main organs were observed, collected, and weighted individually. The specimens of liver, kidney and testis of them were also collected for pathological examination. The remaining rats in the control and experimental groups, served as recovery animals observed for reversibility, persistence and delayed occurrence of toxic effects, were further cared without treatment for another 15 days and their urine, blood and organ samples were similarly collected and examined.

The collected urine sample was centrifuged at 3000 rpm for 5 min, and the supernatant was stored at -20°C until the level of urinary protein content (U_protein) and the activity of urinary N-acetyl-β-D-glucosaminidase (NAG) were measured. Determination of the level of U_protein by the Bradford coomassie brilliant blue method (Bradford, 1976) was carried out by using commercial kit (Nanjing Jiancheng Bioengineering Institute, P.R. China). Urinary NAG activity was determined with commercial kit (Shanghai Taiyang Biology Technology Co. Ltd., P.R. China) by using p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate (Pedraza-Chaverri et al., 2000).

Blood sample was allowed to clot at room temperature for 30 min, and then was centrifuged at 4500 rpm for 15 min to separate the serum. Serum sample was stored at -20°C until analyzed. The contents of blood urea nitrogen (BUN), creatinine and the activities of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), lactic dehydrogenase (LDH) and γ-glutamyltransferase (GGT) in the serum were determined using commercial spectrophotometric diagnostic kits (Nanjing Jiancheng Bioengineering Institute, P.R. China) according to the manufacturer's directions. BUN was
assayed by the Fearon method and serum creatinine by the Jaffe method (Tietz et al., 1994). Estimation of GOT activities and GPT activities were done using Reitman-Frankel method (Reitman and Frankel, 1957). The ALP activity was determined by Kind and King’s method (Kind and King, 1954). The activity of GGT was measured using the colorimetric method based on diazo reaction (Liu et al., 2009). The activity of LDH was detected with a colorimetric assay based on the conversion of pyruvic acid to phenylhydrazone (Wang et al., 2005).

The shapes, sizes and colors of internal organs, namely, heart, liver, spleen, lungs, kidneys, and testes in rats were visually observed for any signs of gross lesions. The relative organ weight of each animal was then calculated using the following formula: Relative organ weight = (absolute organ weight (g)/body weight of rat on day of sacrifice (g)) ×100. Small pieces of tissue samples (liver, kidney and testis) were fixed immediately in 10% phosphate-buffered formalin. The fixed tissues were processed routinely and tissue slides were stained with hematoxylin and eosin (H & E) using standard techniques for histological examination by optical microscope.

**RESULTS**

**Chemical composition of PELJ**

The results of GC–MS analysis on PELJ were shown by Figure 1 and Table 1. A total of forty-two compounds, accounting for 89.87%, were identified in PELJ. The major components were 17b-hydroxy-1a, 17-dimethyl-5a-androstan-3-one (21.47%), stigmast-5-en-3-ol (11.86%), E-phytol (9.94%), caryophyllene oxide (8.69%), spathulenol (6.55%), hexadecadienoic acid methyl ester (6.50%) and cis-pinane (5.37%).

**Effects on the general conditions**

All animals survived to scheduled sacrifice and showed normal behavior and activity. After 15 days treatment, PELJ and DLJ decreased the body weight gains of the rats significantly when compared to the control. The shapes, sizes and colors of internal organs (heart, liver, spleen, lungs, kidneys, testes) in experimental rats were normal. For the relative organ weight, only the relative weights of testes of the rats in PELJ and DLJ groups were significantly higher than that of control rats (Table 2). After 15 days recovery period, decreased body weight gains of rats in PELJ and DLJ groups were reversed to normal levels, the relative organ weights of heart, liver, spleen, lung and kidney remained normal whereas significant increases were still observed in the relative weight of testes of rats in PELJ and DLJ groups (Table 2).

**Effects on biochemical parameters**

After 15 days treatment period, as shown in Table 3, the levels of GPT, GOT, ALP, LDH, GGT, BUN, creatinine, U_protein and NAG in PELJ group and DLJ group were increased significantly when compared with those of the control group (P<0.05, P<0.01). The levels of all biochemical parameters tested in PELJ group were significantly higher than those of DLJ group (P<0.01).

After being kept for recovery period of 15 days, as shown in Table 3, the levels of GPT, GOT, ALP, LDH, GGT, BUN, creatinine, and NAG were decreased in PELJ
The identified compounds are listed in the order of their elution on the column. *Only the percentages over 1% are indicated in this table.

The identified compounds are listed in the order of their elution on the column. *Only the percentages over 1% are indicated in this table.

Table 2. Body weight gains and relative organ weights of rats treated with PELJ, DLJ after 15 days treatment and 15 days recovery period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>After treatment period</th>
<th>After recovery period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>PELJ group</td>
</tr>
<tr>
<td>Body weights gains (g)</td>
<td>116.08±12.82</td>
<td>78.58±7.24**</td>
</tr>
<tr>
<td>Relative heart weight</td>
<td>0.55±0.09</td>
<td>0.50±0.06</td>
</tr>
<tr>
<td>Relative liver weight</td>
<td>4.47±0.57</td>
<td>4.11±0.23</td>
</tr>
<tr>
<td>Relative spleen weight</td>
<td>0.33±0.03</td>
<td>0.31±0.03</td>
</tr>
<tr>
<td>Relative lung weight</td>
<td>0.62±0.05</td>
<td>0.60±0.03</td>
</tr>
<tr>
<td>Relative kidney weight</td>
<td>0.49±0.02</td>
<td>0.46±0.02</td>
</tr>
<tr>
<td>Relative testis weight</td>
<td>0.57±0.03</td>
<td>0.65±0.02*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. (n=6). Significantly different from control group, *P<0.01, **P<0.05.

group and DLJ group, whereas, the level of U_{protein} did not demonstrate a tendency to decrease. Significant increases in the levels of all biochemical parameters tested in PELJ group were still observed as compared with the corresponding control group (P<0.05, P<0.01). When compared to the control group, there were significant increases in the levels of GOT, ALP, LDH, GGT and U_{protein} (P<0.05, P<0.01) in DLJ group, but no significant differences in the levels of GPT, BUN, creatinine and NAG were found. The levels of all biochemical parameters tested in PELJ group were significantly higher than those of DLJ group (P<0.01), with the exception of GGT.

Histopathological examination of liver, kidney and testis

The liver sections of control rats showed normal histopathological feature (Figures 2A). Histopathological examination of rats treated with PELJ for 15 days showed granular degeneration and fatty degeneration of hepatocytes and mild inflammatory cell infiltrations. Individual cell necrosis was also present (Figure 2B).

In the kidney, histopathological examination of PELJ treated rats revealed slight tissue damage (Figure 3B) when compared with the control (Figure 3A). The changes included granular degeneration of tubule
Table 3. Mean values of biochemical parameters in rats treated with PELJ, DLJ after 15 days treatment and 15 days recovery period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>PELJ group</th>
<th>DLJ group</th>
<th>After treatment period</th>
<th>Control group</th>
<th>PELJ group</th>
<th>DLJ group</th>
<th>After recovery period</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPT (IU/L)</td>
<td>7.78±1.16</td>
<td>56.25±2.43**</td>
<td>32.13±2.99**</td>
<td>7.29±1.16</td>
<td>16.54±2.47**</td>
<td>9.07±2.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GOT (IU/L)</td>
<td>9.37±1.87</td>
<td>118.89±2.76**</td>
<td>85.48±4.94**</td>
<td>9.46±1.78</td>
<td>56.31±2.99**</td>
<td>34.18±2.93**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>51.18±3.18</td>
<td>392.30±40.64***</td>
<td>153.94±7.66**</td>
<td>53.35±4.88</td>
<td>201.62±2.32***</td>
<td>92.94±4.69**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>8774.69±489.17</td>
<td>21719.14±792.58***</td>
<td>13222.22±127.23**</td>
<td>8378.21±489.17</td>
<td>13503.21±195.90***</td>
<td>9548.08±145.32**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>25.60±6.90</td>
<td>262.50±24.09***</td>
<td>123.21±12.73**</td>
<td>22.22±10.47</td>
<td>80.00±14.14**</td>
<td>68.33±10.27**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>4.29±0.53</td>
<td>14.54±0.39***</td>
<td>9.29±0.66**</td>
<td>5.17±0.74</td>
<td>6.47±1.04***</td>
<td>4.48±0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>91.67±24.64</td>
<td>381.20±27.40***</td>
<td>200.63±8.06**</td>
<td>81.30±5.66</td>
<td>128.73±15.96***</td>
<td>79.71±6.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U protein (mg/L)</td>
<td>50.53±6.19</td>
<td>163.30±16.94***</td>
<td>66.49±9.56*</td>
<td>47.57±8.57</td>
<td>237.28±16.76***</td>
<td>66.92±6.77*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAG (IU/L)</td>
<td>16.83±1.72</td>
<td>38.86±2.67***</td>
<td>32.56±2.00**</td>
<td>17.53±1.01</td>
<td>22.91±0.84***</td>
<td>18.22±0.83</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.D. (n=6). *, P<0.05, **, P<0.01, significantly different from control group; ##, P<0.01 significantly different compared with DLJ group.

Figure 2. The histological morphology of rat livers from the control group (A) and PELJ group (B) treated for 15 days (H&E×400). Individual hepatocyte necrosis (arrow) was present in PELJ group.

epithelial cells and mild interstitial inflammatory cell infiltrations.

On histopathological examination of testis, control rats exhibited well-developed cytoarchitecture, indicated by the presence of normal seminiferous tubules, undergoing normal progression of spermatogenesis, from spermatogonia to primary spermatocytes and secondary spermatocytes to spermatids (Figure...
4A). In PELJ treated rats, reduction in the number of primary spermatocytes, secondary spermatocytes and spermatids was observed in some seminiferous tubules as compared with the control (Figure 4B).

Histopathological changes of the liver, kidney and testis sections of DLJ treated rats were similar to that of PELJ, whereas the alternations occurred in the lower incidence and severity.

At the end of 15 days recovery period, the histopathological changes induced by PELJ and DLJ were ameliorated. However, histopathological alterations were minor in DLJ treated rats and the recovery rats; these rats are not included in the figure for sake of simplicity.

DISCUSSION

PELJ was tried to be disperse in a known volume of 0.05% CMC (Féres et al., 2006), 10% DMSO (Nevin and Vijayammal, 2005) or 0.5% Tween 80 (Costa et al., 2006), but the homogeneity of the aforementioned suspension did not have more advantage than that in distilled water, so distilled water was selected to be used as vehicle for administration, and the mixture for administration was subjected to an ultrasonic wave treatment.

In the present study, the results of the changes in the body weight gains of experimental rats indicate that the adverse effects of PELJ and DLJ on the body weight are reversible. Regarding the toxic effects on liver and kidney in rats, the activities of GPT, GOT, ALP, LDH, GGT in serum were determined as markers of liver damage, as well as the levels of creatinine, BUN in serum and $U_{\text{protein}}$ and NAG in urine were tested as markers of kidney damage (Novelli et al., 1998; Nevin and Vijayammal, 2005; Trevor, et al., 2003).

The significant increases in the levels of all determined parameters in experimental rats treated for 15 days indicate the hepatic and renal toxicity of PELJ and DLJ, which were further proved by the histopathological observations of liver and kidney sections in rats. After 15 days recovery period, the increased levels of most of parameters decreased except for $U_{\text{protein}}$ in experimental rats.

The increased level of $U_{\text{protein}}$ did not display a tendency...
to reverse, thus suggesting a lack of reversibility in this parameter. The recovery period of 15 days in this study may not have been long enough, and it is possible that the altered levels of all parameters including Uproteinc may return to the control value and the histopathological changes be normalized after longer recovery period. However, these assertions need to be clarified. In addition, it was reported that high dose of DLJ given orally may cause renal interstitial fibrosis in rats (Cai et al., 2000; Sun et al., 2005).

But in our study, only slight renal pathologic changes without interstitial fibrosis were observed in DLJ treated rats. The discrepancy may be explained by the fact that variation may sometimes occur in toxic components in the same herb collected in different habitats. Differences in the experimental protocol could account for the discrepancy as well.

To investigate the toxic effects of PELJ and DLJ on testis, serum testosterone concentrations were measured by radioimmunoassay in the study. Because the used of commercially available kit is for human serum, the absolute level of testosterone in rats could not be qualified and the data were not listed in this paper. However, the comparative level of testosterone could be ascertained as follows: the levels of testosterone in experimental rats significantly descended when compared with the control and did not return to the control value after recovery.

As for the testes, testicular damages characterized by reduction of the number of spermatocytes and spermatids were observed in experimental rats, and the changes were not normalized after recovery. So it is evident that administration of PELJ and DLJ can induce testicular toxicity. Although it is difficult to extrapolate result from animal experiments to humans due to interspecies differences, and there have not been a clinical report on the adverse effect of L. japonicus on reproductive system till now; our results suggest that the high attention on the reproductive toxicity of L. japonicus at high dose should be aroused.

In conclusion, the results obtained in the present study demonstrated that PELJ has obvious toxic effects on the liver, kidney and testis in rats under the present experimental conditions, and showed partial recovery of the toxic effects in short period. Meanwhile, the present study revealed that PELJ exhibited a higher toxicity in rats than DLJ at the same dose. Further studies will focus on isolating the toxic compounds responsible for the toxicity of L. japonica on the basis of the results obtained herein and elucidating the mechanism of the toxic effects of L. japonica.

REFERENCES


