Atractylodes lancea rhizome water extract reduces triptolide-induced toxicity and enhances anti-inflammatory effects

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[ABSTRACT] The present study was designed to explore the influence of water extracts of Atractylodes lancea rhizomes on the toxicity and anti-inflammatory effects of triptolide (TP). A water extract was prepared from A. lancea rhizomes and co-administered with TP in C57BL/6 mice. The toxicity was assayed by determining serum biochemical parameters and visceral indexes and by liver histopathological analysis. The hepatic CYP3A expression levels were detected using Western blotting and RT-PCR methods. The data showed that the water extract of A. lancea rhizomes reduced triptolide-induced toxicity, probably by inducing the hepatic expression of CYP3A. The anti-inflammatory effects of TP were evaluated in mice using a xylene-induced ear edema test. By comparing ear edema inhibition rates, we found that the water extract could also increase the anti-inflammatory effects of TP. In conclusion, our results suggested that the water extract of A. lancea rhizomes, used in combination with TP, has a potential in reducing TP-induced toxicity and enhancing its anti-inflammatory effects.

[KEY WORDS] Triptolide; Atractylodes lancea; Licorice; CYP3A; Anti-inflammation effects


Introduction

Triptolide (TP) is one of the major active components in Tripterygium wilfordii Hook F (TWHF). TWHF has been used for centuries in traditional Chinese medicine to treat inflammatory and autoimmune diseases, including rheumatoid arthritis (RA), immune complex nephritis, and systemic lupus erythematosus (SLE), and to prevent rejection following organ and tissue transplantsations [1]. Clinical and experimental studies have demonstrated that TP has multiple pharmacological activities, such as anti-inflammation, anti-tumor, anti-fertility, and anti-rejection [2]. However, many cases of TP-induced toxicity affecting the digestive, urogenital, and blood circulatory systems have been reported [3-4]. The toxicity of TP can be affected by the expression level of hepatic cytochrome P450 (CYP450) enzymes, which are involved in metabolizing toxic compounds [5-6]. A recent study has shown that CYP3A2, one of the genes encoding for CYP450, has an important role in the sex-related metabolism of TP [7]. In vitro studies have also shown that CYP3A plays a major role in the hydroxylation of TP in the liver [8-9]. These observations suggest that induction of hepatic CYP3A may be able to reduce TP-induced toxicity.

In the clinical application of traditional Chinese medicine, TWHF is often used in combination with other herbal medicines (e.g., licorice and paeony extracts) to reduce its toxicity [3, 10]. These herbal medicines usually reduce TP-induced toxicity by inducing hepatic CYP3A, and accelerating the clearance of TP. However, they may also reduce the anti-inflammatory effects of TP. Therefore, alternative combination therapies that do not reduce the efficacy of TP are still needed.

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Atractylodes lancea (Thunb.) DC, belonging to the family Compositae, is well documented in Shen Nong Ben Cao Jing, the first Chinese pharmacopoeia written in the Han dynasty around 200–100 BC [11]. The traditional medicine based on this species is called “Cangzhu” in China, “Khod-Kha-Mao” in Thailand, and “So-jutsu” in Japan [12]. The rhizomes from A. lancea, commonly called Rhizoma atractylodis, are used as an important drug in traditional Chinese medicine to revitalize the spleen and treat dyspepsia, gastroparesis, visceral hypersensitivity, rheumatic diseases, and night blindness [13-14]. These traditional uses are explained by the compound’s ability to eliminate dampness, strengthen the spleen, expel wind-cold from the superficial parts of the body, and clear away the common cold [12]. A. lancea rhizomes also show pharmacological activities such as anti-inflammatory, antimicrobial, and anti-ulcer effects, and hepatoprotection [15-16]. It has been reported that the water extract of A. lancea rhizomes promotes liver protein synthesis, and induces CYP3A expression, resulting in both increased mRNA levels and increased enzyme activity, contributing to its hepatoprotective effect [17]. These findings suggest that when co-administered with TP, A. lancea rhizome extracts may reduce TP-induced toxicity by accelerating TP metabolism in the liver. Furthermore, the combined treatment with TP and A. lancea rhizome extracts may achieve synergistic or additive anti-inflammatory effects, as they both alleviate inflammation. The results from the present study demonstrated that the water extract of A. lancea rhizomes reduced TP-induced toxicity, and enhances the anti-inflammatory effects of TP.

Materials and Methods

Chemicals and reagents

TP (> 99% purity) was purchased from ChromaDex (Irvine, CA, USA). Tween 80 and xylene were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Atractyloside A, vanillic acid, syringic acid, protocatechuic acid, protocatechuic aldehyde, and limonoids were purchased as reference substances from Jingzhu Bio-tech Co. (Nanjing, China). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and l-glutathione (GSH) analysis kits were purchased from Jingzhu Bio-tech Co. (Nanjing, China). Dried rhizomes of A. lancea (100 g) were ground into powder and refluxed with 800 mL of water for 2 h. After filtration, the water extraction procedure was repeated twice. The pooled water extracts were then combined and concentrated under reduced pressure, followed by freeze-drying. The volatile oil extract was prepared according to the Chinese Pharmacopoeia (2015). The yields of the water and volatile oil extracts were approximately 42.9% and 5.21% (W/W), respectively. Licorice was purchased from Haixin Chinese Herbal Pieces Co. (Bozhou, China). The licorice water extract was prepared according to the reported method [5].

Chemical analysis of the water extract of A. lancea rhizomes

Chromatographic experiments were performed on a Waters ACQUITY™ UPLC™ system (Waters Corp., Milford, MA, USA) equipped with a 2998 photodiode array detector (PDA) together with a quaternary pump, an auto-sample injector, an on-line degasser, and an automatic thermostat column oven. The mass spectrometry instrument was consisted of a Waters Synapt™ QTOF/MS (Waters Corp.). Ionization was performed in the negative electrospray (ESI) mode. The mass range was set at m/z 100–1 000 Da with a 0.5 s scan time. UPLC separation was achieved on a Waters ACQUITY™ UPLC™ BEH C18 column (100 mm × 2.1 mm, 1.7 μm) with the column temperature being set at 25 °C. The mobile phase consisted of (A) water containing 0.1% formic acid and (B) acetonitrile. The following gradient elution was used: 95% A (0 min); 80% A (0–1 min); 75% A (1–5 min); 70% A (5–7 min); 40% A (7–10 min); 5% A (10–13 min); and 5% A (13–14 min). The flow rate was set at 0.4 mL·min⁻¹, and the injection volume was 2 μL. Representative chromatograms are shown in Fig. 1.

Fig. 1 The total ion current chromatograms of six constituents as mixed standards (A) and the A. lancea rhizome water extract (B). Peak 1: protocatechuic acid; 2: atractyloside A; 3: protocatechuic aldehyde; 4: syringic acid; 5: vanillic acid; and 6: limonoids

Animals and drug administration

Male C57BL/6 mice (weighing 20 ± 2 g, 8 weeks old) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China, SCXK2010-0001). They were housed for 1 week in an air-conditioned room (25 ± 1 °C, 60% relative humidity, and a 12 h light/12 h dark cycle). The mice had free access to tap water and a regular diet before the experiments. For the CYP3A induction analysis, the mice were treated with the water extract of A. lancea rhizomes or vehicle by oral gavage every day for 2 weeks. For the toxicity
analysis, TP was dissolved in Tween-80, and then diluted to the required concentrations in 0.9% saline (TWEEN-80 was less than 1% V/V in the final solution). Then the mice were administered intraperitoneally (i.p.) with TP on odd-numbered days, and with the water extract of A. lancea rhizomes orally on even-numbered days, for 30 days. For the anti-inflammatory analysis, TP was administered (i.p.) on odd-numbered days, and water extract of A. lancea or licorice was administered by oral gavage on even-numbered days, for 2 weeks. The animal experiments were approved by the Animal Experimentation Ethics Committee of Jiangsu University, and conformed to the guidelines of the “Principles of Laboratory Animal Care” (NIH publication No. 80-23, revised in 1996).

**Real-time polymerase chain reaction (RT-PCR)**

The mice were randomly divided into four experimental groups (n = 3): the vehicle group (0.9% sodium chloride), the water extract of A. lancea group (200 mg·kg⁻¹ normalized to the weight of dry A. lancea rhizomes, similarly hereinafter), the essential oil extract of A. lancea group (200 mg·kg⁻¹) and the phenobarbital group (80 mg·kg⁻¹). After the final drug administration, the mice were fasted overnight. Then, the mice were sacrificed with CO₂ and the total RNA was isolated from the liver tissues with TRIzol Reagent (Solarbio, Beijing, China). The quality of the RNA was assessed using the ratio of absorbance at 260 and 280 nm. Values of A260/A280 from 1.9 to 2.1 were considered to be acceptable. Total RNA was used to synthesize cDNA using a reverse transcription kit (Thermo, Waltham, MA, USA) following the manufacturer’s instructions. The RT-PCR experiments were performed using a TaqMan fast universal PCR master mix kit on a LightCycle 96 Real-Time PCR system (Roche, Basel, Switzerland). The primers were obtained from Suns hine Bio (Shanghai, China) (5'-CAG AGA TCA GAG TAT TCT GT-3' and 5'-TTT GGT AAA GTA CTT GAG GCA GA-3'). The relative expression of Cyp3a11 was normalized to the amount of β-actin in the same cDNA sample.

**Preparation of liver microsomes and Western blotting assay**

Mouse liver microsomes were prepared using differential centrifugation [19]. The protein concentrations of the microsomal suspensions were determined using the bicinchoninic acid method (Beyotime, Nantong, China). Equal amounts of protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF membranes were incubated with primary antibodies at 4 °C overnight, and subsequently with horseradish peroxidase (HRP)-conjugated secondary antibodies at 20-25 °C for 1 h. The protein bands were detected using enhanced chemiluminescence reagents. The chemiluminescent signals were detected and analyzed using a ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA, USA). The following antibodies (and dilutions) were used: anti-CYPOR (SC-55477, 1 : 1 000 dilution; Santa Cruz, Pleasanton, CA, USA), anti-CYP3A4 (SC-53850, 1 : 1 000 dilution; Santa Cruz), and HRP-conjugated goat anti-mouse (A0216, 1 : 1 000 dilution; Beyotime).

**Characterization of general toxicity**

The mice were randomly divided into four experimental groups (n = 8): the vehicle group (treated with 0.9% sodium chloride), the TP group (treated with 300 mg·kg⁻¹ TP), the TP and lower water extract dose group (treated with 300 mg·kg⁻¹ TP and 100 mg·kg⁻¹ of the water extract of A. lancea), and the TP and higher water extract dose group (treated with 300 mg·kg⁻¹ TP and 200 mg·kg⁻¹ of the water extract of A. lancea). After the final drug administration, the mice were fasted overnight. Blood samples were then collected for serum biochemistry detection. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and L-glutathione (GSH) levels were analyzed using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). The organs (liver, spleen, kidney, and testis) were collected and weighted at the time of necropsy of the mice, and the visceral index was calculated. The tissues were fixed in 10% neutral buffered formalin, and processed for paraffin sectioning, followed by hematoxylin and eosin (H&E) staining. The tissue sections were analyzed by Prof. Miao Chen at the Department of Pathology, First People's Hospital, Zhenjiang, China.

**Xylene-induced ear edema assay**

The mice were randomly divided into five experimental groups (n = 8): the vehicle group (treated with 0.9% sodium chloride), the TP group (treated with 300 mg·kg⁻¹ TP), the TP and lower water extract dose group (treated with 300 mg·kg⁻¹ TP and 100 mg·kg⁻¹ of the water extract of A. lancea), the TP and higher water extract dose group (treated with 300 mg·kg⁻¹ TP and 200 mg·kg⁻¹ of the water extract of A. lancea), and the TP and licorice extract group (treated with 300 mg·kg⁻¹ TP and 200 mg·kg⁻¹ of the water extract of licorice). Ear edema was induced in the right ear of each mouse by the topical application of xylene, as previously described [20] with a slight modification. Briefly, 2 h after the last drug administration, 50 µL of xylene was topically applied to the inner and outer surfaces of the right ear of each mouse. After treatment with xylene for 1 h, the mice were sacrificed with CO₂, and both the right and left ears were sampled using a punch (7 mm in diameter). Then the ear sample weights were measured with a precision balance. The edema amount for each mouse was calculated as the difference between the right (xylene-treated) and left (untreated) ear weights. The inhibition rate for each treatment was calculated by comparing the edema amount of the treated group to the average ear weight of the vehicle group.

**Statistical analysis**

All the results are presented as means ± standard deviation (SD). The statistical analysis was carried out using the SPSS 13.0 software package (IBM, Armonk, NY, USA). The statistical significance of the differences between two groups was examined using Student’s t test. The differences were considered statistically significant when P < 0.05.
Results

**Water extract of A. lancea rhizomes induces CYP3A expression**

Liver CYP3A expression levels were detected in mice treated with vehicle or the water extract or volatile oil extract of *A. lancea* rhizomes (200 mg·kg⁻¹), and phenobarbital (80 mg·kg⁻¹). The Western blotting results (Fig. 2A) clearly showed that mice treated with the water extract of *A. lancea* rhizomes or phenobarbital had higher liver CYP3A expression levels than the control group mice did. No protein expression changes were observed in mice treated with the volatile oil extract.

**Fig. 2** Liver CYP3A expression in the mice treated with the *A. lancea* rhizome water extract (*n* = 3). (A) Representative Western blots showing that the water extract increased liver CYP3A protein levels. (B) Graph showing that the water extract resulted in a significant increase in the Cyp3a11 mRNA levels. The data are representative of the results obtained from three separate quantitative PCR analyses. **P < 0.01 (*t* test) as compared with the vehicle group

For quantitative analysis, liver Cyp3a11 mRNA expression was examined (Fig. 2B). The real-time PCR results showed that liver Cyp3a11 mRNA levels were significantly higher in mice treated with the water extract or phenobarbital than in the control mice, whereas no remarkable difference was observed in mice treated with the volatile oil extract. Since the *A. lancea* rhizome volatile oil extract seemed to be less effective in inducing CYP3A expression, the following study focused on the water extract.

**Water extract of A. lancea rhizomes reduces TP-induced toxicity**

The blood biochemical indexes in the mice treated with TP, as well as TP and the water extract of *A. lancea* rhizomes (T&A co-treated) were investigated. In the TP-treated mice, the serum AST, ALT, and BUN levels significantly increased, but the GSH levels significantly decreased (Table 1), in comparison with those in the control group. No significant differences were observed between the T&A co-treated group and the control group (Table 1).

The visceral indexes of the TP-treated and T&A co-treated mice are shown in Table 2. In the TP-treated group, the organ to body weight ratio significantly increased for the spleen, and decreased for the testis compared to that reported for the control and T&A co-treated animals. In the T&A co-treated mice, higher spleen and lower testis weight to body weight ratios were recorded compared to that reported for the control group. It was noteworthy that the weight ratio changes in the group treated with a higher dose of water extract of *A. lancea* rhizomes (200 mg·kg⁻¹) were milder than those in the TP-treated group.

Histopathological analyses of the liver, spleen, kidney, and testis were performed for TP-treated and T&A co-treated mice (Figs. 3A-D). No obvious pathological changes were detected in the liver tissue sections of either group. However, kidney proximal tubular epithelial cell dilation and glomerular lesions were found in the TP-treated mice. In addition, hardly any mature sperm was detected in the testes of the TP-treated mice, and the number of primary and secondary spermatocytes decreased in the seminiferous tubules. However, the symptoms of pathological changes were alleviated in the T&A co-treated mice. The toxicity-reducing effects appeared to be increased when the dosage of water extract of *A. lancea* rhizomes was increased from 100 to 200 mg·kg⁻¹.

| Table 1 The serum biochemical indices for the different experimental groups* |
|-----------------------------|------------|-----------------|-----------------|-----------------|
| Groups                      | ALT (U·L⁻¹) | AST (U·L⁻¹)     | GSH (mmol·L⁻¹)  | BUN (mmol·L⁻¹)  |
| Vehicle                     | 23.3 ± 3.6  | 17.6 ± 2.1      | 18.1 ± 4.5      | 6.8 ± 1.6       |
| TP (300 mg·kg⁻¹)            | 42.1 ± 6.4* | 23.1 ± 3.3*     | 14.3 ± 3.6*     | 11.3 ± 1.3*     |
| TP and the water extract of *A. lancea* rhizomes (300 mg·kg⁻¹, 100 mg·kg⁻¹) | 25.9 ± 4.5  | 16.9 ± 2.5      | 16.6 ± 2.7      | 6.7 ± 3.4       |
| TP and the water extract of *A. lancea* rhizomes (300 mg·kg⁻¹, 200 mg·kg⁻¹) | 24.8 ± 5.9  | 13.8 ± 3.7      | 16.9 ± 2.1      | 7.6 ± 0.9       |

*The values are the mean ± SD for eight mice. *P < 0.05, **P < 0.01 vs vehicle group
Table 2  The visceral indices for the different experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Testis</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>4.6 ± 0.7</td>
<td>0.38 ± 0.04</td>
<td>1.3 ± 0.08</td>
<td>0.72 ± 0.06</td>
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<tr>
<td>TP (300 mg·kg⁻¹)</td>
<td>3.9 ± 0.26</td>
<td>0.65 ± 0.09**</td>
<td>1.4 ± 0.11</td>
<td>0.25 ± 0.08**</td>
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<tr>
<td>TP and the water extract of A. lancea rhizomes (300 mg·kg⁻¹, 100 mg·kg⁻¹)</td>
<td>4.6 ± 0.89</td>
<td>0.55 ± 0.22*</td>
<td>1.2 ± 0.14</td>
<td>0.29 ± 0.05*</td>
</tr>
<tr>
<td>TP and the water extract of A. lancea rhizomes (300 mg·kg⁻¹, 200 mg·kg⁻¹)</td>
<td>4.6 ± 0.42</td>
<td>0.51 ± 0.04*</td>
<td>1.4 ± 0.13</td>
<td>0.38 ± 0.07*</td>
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*The values are the mean ± SD for eight mice. *P < 0.05, **P < 0.01 vs vehicle group. *p < 0.05 vs the TP group.

The water extract of A. lancea rhizome enhances anti-inflammatory effects

The anti-inflammatory effects in the TP-treated and T&A co-treated mice were investigated. In the TP-treated mice, a significantly decreased degree of swelling and a remarkably increased inhibition rate of ear edema were observed compared to that in the vehicle group (Figs. 4A and 4B). In the T&A co-treated mice, an even lower swelling degree and an even higher inhibition rate of ear edema were recorded compared to that in the control mice (Figs. 4A and 4B). Thus, the anti-inflammatory effects in the T&A co-treated mice appeared to be enhanced in comparison with those in the TP-treated mice. For comparison, a water extract of licorice, which has also been shown to reduce TP-induced toxicity when co-administered with TP, was also tested in this study. The anti-inflammatory effects in the TP and licorice water extract co-treated mice were significantly weaker than those in the TP-treated group (Figs. 4A and 4B).

Discussion

In clinical application, TWHF is often used in combination with other herbal medicines to reduce its toxicity. It has been reported that the low activity of CYP3A is a vital factor in the toxicity of TP [7, 9]. Dong et al. [17] have reported that a water extract of A. lancea rhizomes could induce CYP3A mRNA expression levels and raise the enzyme activity. These observations suggest that the water extract of A. lancea rhizomes, as a CYP3A inducer, is a good candidate for reducing
TP-induced toxicity, by accelerating its metabolism. The results from our study showed that a water extract of *A. lancea* rhizomes induced hepatic CYP3A at both the mRNA and protein levels in mice. One might expect that the enhanced metabolism of TP would reduce its efficacy, as the plasma concentration would be lower. To determine whether this was the case, a xylene ear edema assay was performed to test whether the water extract of *A. lancea* rhizomes reduced the anti-inflammatory effects of TP. The results showed that the inflammatory inhibition rates actually improved when the mice were co-treated with TP and the water extracts of *A. lancea* rhizomes, compared to that in the mice treated with TP alone. This improvement was probably due to the synergistic/additive effects of TP and the water extract of *A. lancea* rhizomes, as *A. lancea* also has anti-inflammatory effects.\(^{[16]}\)

Licorice is commonly combined with other herbs to improve the flavor, or to enhance the effectiveness or to reduce the toxicity of other ingredients. As a result, licorice is used in almost half of all Chinese herbal formula.\(^{[21]}\) The toxicity-reducing effects of licorice on TP have been intensively studied in recent years.\(^{[2,22-23]}\) Detailed pharmacokinetic profiling using LC-MS/MS shows that glycyrrhizin (an important active compound in licorice) may accelerate the metabolism of TP, through induction of CYP3A.\(^{[24]}\) However, in those studies, the toxicity-reducing effects of licorice are emphasized, but the efficacy of TP when it is co-administered with licorice (or its active components) seems to be ignored. Our results demonstrated that a water extract of licorice reduced the anti-inflammatory efficacy when co-treated with TP. Thus, licorice water extracts or glycyrrhizin might not be an effective combination partner for TP.

The composition of the water extract of *A. lancea* rhizomes has been reported by a few groups. Li et al.\(^{[25]}\) have prepared a water extract of *A. lancea* rhizomes and isolated a number of compounds, including 2-phenylethyl β-rutinoside, wogonin, wogonin 7-O-glucuronide methyl ester, bis (5-formylfururyl)ether, vanillic acid, syringic acid, protocatechuic aldehyde, protocatechuic acid, limonoids, 5-hydroxymethyl fururaldehyde, and 2-furan carboxylic acid. A few years later, Yahara *et al.*\(^{[26]}\) have separated the water-soluble parts of *A. lancea* rhizomes, and then identified atractylloside A, B, C, D, E, F, G, and H. Six of those components (e.g., atractylloside A, vanillic acid, syringic acid, protocatechuic acid, protocatechuic aldehyde, and limonoids) were detected in our water extract and validated with their reference substances (Fig. 1). However, to the best of our knowledge, none of the aforementioned compounds have been reported to significantly induce CYP3A, or have anti-inflammatory effects in mice. An ongoing study is being performed in our group on six compounds (Fig. 1) in order to determine their roles in liver CYP3A-induction and anti-inflammatory effects of *A. lancea* rhizomes.

**Conclusions**

The present study demonstrated that a water extract of *A. lancea* rhizomes could reduce the toxicity of TP by inducing the expression of hepatic CYP3A in mice. This *A. lancea* rhizome water extract also enhanced the anti-inflammatory effect of TP, whereas the licorice water extract lowered that effect. Our results suggested that water extract of *A. lancea* rhizomes had good potential in reducing TP-induced toxicity, without reducing its anti-inflammatory effects.

**References**
