Tupistra chinensis extract attenuates murine fulminant hepatitis with multiple targets against activated T lymphocytes

Xuefeng Wu, Jinjin Fan, Zijun Ouyang, Rui Ning, Wenjie Guo, Yan Shen, Xudong Wu, Yang Sun and Qiang Xu

State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, China

Keywords
Con A; experimental hepatitis; inflammation; T cells; Tupistra chinensis

Correspondence
Qiang Xu, or Yang Sun, School of Life Sciences, Nanjing University, 22 Han Kou Road, Nanjing 210093, China.
E-mail: molpharm@163.com; yangsun@nju.edu.cn

Received May 6, 2013
Accepted October 10, 2013
doi: 10.1111/jphp.12176

Introduction
The plant Tupistra chinensis (TCE) Baker, medicinally also known as Kai-Kou-Jian in Chinese, belongs to a species in the genus Campylandra (family Asparagaceae). It mainly distributes in India and China and has been widely used for hundreds of years in southwestern China for the treatment of inflammatory diseases in southwestern China for hundreds of years. The present study was designed to investigate the effects of the extract from T. chinensis against experimental hepatitis and to illustrate its potential mechanisms.

Objectives
The decoction of Tupistra chinensis (TCE) is traditionally used for the treatment of inflammatory diseases in southwestern China for hundreds of years. The present study was designed to investigate the effects of the extract from T. chinensis against experimental hepatitis and to illustrate its potential mechanisms.

Methods
Effects of TCE were investigated on Con A-induced hepatitis. Profiles of multiple cytokines were measured with biometric immuno-sandwich ELISA. Proliferation, activation and apoptosis of T lymphocytes were evaluated using Western blot, MTT analysis and flow cytometry.

Key findings
TCE significantly inhibited levels of serum transaminases and lactic dehydrogenase in mice with Con A-induced hepatitis, accompanied with marked alleviation of the liver microscopic appearances. Moreover, it decreased levels of inflammatory cytokines in a concentration-dependent manner both in vivo and in vitro. It also suppressed mitogen-activated protein kinases and NF-κB-signalling in liver. These effects of TCE are attributed to its inhibition on activated T cells but not to hepatocytes protection. Flow cytometry and immunoblot assay data showed its effects on STAT1/NF-κB-signalling blockade and apoptosis induction of activated T cells.

Conclusion
Our findings illustrate the significant potential of TCE as a novel approach for treatment of T cell-mediated inflammatory diseases.
inflammation, in the present study, we used Con A-induced hepatitis model to examine its effects. We provide first evidence that the ethanol extract of *T. chinensis* exert distinct activities in suppressing hepatic inflammatory reactions evoked by Con A. Our data also revealed the inhibitory effect of TCE on hepatitis was due to its down-regulation of mitogen-activated protein kinase (MAPKs), NF-κB and STAT1 signalling. Moreover, the treatment of TCE led to apoptosis of activated T cells with increase of Bax, suppression of Bcl-2/Bcl-XL and cleavage of caspase 3 and PARP. These results showed multiple effects of TCE against activated T cells for a novel approach to treatment of hepatic inflammation.

**Materials and Methods**

**Plant material and chemicals**

The Chinese herbal drug Kai-Kou-Jian (*T. chinensis*) was purchased from Shennongia Medicinal Material Co. (Hubei, China) and identified by Dr Boyang Yu (Department of Complex Prescription of TCM, China Pharmaceutical University) as rhizomes of *T. chinensis* Baker. A specimen was deposited at State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University with voucher number no. 101018. Powdered root bark of *T. chinensis* was extracted three times with 70% EtOH under reflux. The total filtrate was evaporated under reduced pressure to remove EtOH. The extraction yield (weight of the dried extract/weight of the original sample × 100%) is 60.4%. The extract from rhizomes of *T. chinensis* was named TCE.

**Cells and reagents**

Mouse T cells from spleen or lymph node were purified using magnetic beads (Miltenyi Biotec, Auburn, CA) with more than 95% purity. The cells were incubated in RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100 mg/ml of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO₂ atmosphere at 37°C. Antibodies against STAT1, phospho-STAT1, bcl-2, bcl-xL, bax, GAPDH were from Cell Signaling Technology (Beverly, MA). Anticleaved caspase 3, PARP, T-bet and anti-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ELISA kits for murine IL-1β, IL-2, TNF-α, IL-12 and IFN-γ were purchased from Dakewe Biotech Co. Ltd (Shenzhen, China). Kits for determining serum alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Dexamethasone, Con A, cyclosporin A (Cs A) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Quercetin was purchased from Zhelang Pharmaceutical Technology Co. Ltd (Nanjing, China). Recombinant murine IFN-γ was purchased from Peprotech (Rocky Hill, NJ). Annexin V-FTIC (fluorescein isothiocyanate)/PI (propidium iodide) kit was purchased from BD Biosciences (San Jose, CA), 5, 5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Ethics statement**

All procedures were strictly performed in accordance with the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006). All animal experiments were approved by Nanjing University Animal Care and Use Committee (No. 2012023, March 5th, 2012) and were designed to minimize suffering and the number of animals used.

**Animal treatment**

Female ICR mice (8–10 weeks, 18–22 g) were obtained from the Yangzhou University Animal Center (Yangzhou, China) and group housed at SPF facility under controlled temperature (22 ± 2°C) and photoperiods (12:12-h light-dark cycle). Mice were allowed to acclimate to these conditions for at least 2 days before inclusion in experiments. For each group of experiments, mice were matched by age and body weight.

**Con A-induced T-cell-dependent hepatitis**

Mice received an intravenous Con A injection (15 mg/g body weight) as described before. Mice were given 75, 150 and 300 mg/kg of TCE intragastrically or 5 mg/kg of dexamethasone intramuscularly twice at 1 h and 7 h after Con A administration, respectively. The control animals with Con A-induced hepatitis were given intragastrically the same solvent (normal saline) instead of the drugs. Sera were collected at the indicated time points after Con A administration to measure the serum levels of ALT, AST, LDH and cytokines. Liver tissues were also excised for the histological assay.

**Cytokine assay**

Blood samples were obtained from mice at the indicated time points and centrifuged at 1500 × g for 15 min. Serum samples were stored at −70°C until ready for use. Serum levels of IFN-γ and TNF-α were determined using ELISA kits. For the assay in vitro, T cells purified from lymph nodes were treated with or without various concentrations of TCE in the presence of Con A (5 μg/ml) for 24 h. Then levels of cytokines TNF-α, IFN-γ and IL-2 in supernatants were measured using ELISA kits. All animal experiments were approved by Nanjing University Animal Care and Use Committee (No. 2012023, March 5th, 2012) and were designed to minimize suffering and the number of animals used.
were determined. The threshold of detection was 10 pg/ml and the standard curve’s range was from 0 to 2000 pg/ml.

Measurement of T-cell activation
T cells purified from lymph nodes were treated with or without various concentrations of TCE or Cs A in the presence of Con A (5 μg/ml) for 12 h, the cells were harvested and washed twice with cold phosphate-buffered saline (PBS). Then, the cells were incubated with anti-CD69 or anti-CD25 antibodies (FITC conjugated) for 30 min on the ice before flow cytometric analysis. Data were analysed by Cell Quest software.

Measurement of cell proliferation
T cells isolated from lymph nodes were incubated in 96-Well Plate at a density of 5 × 10⁵/ml cells per well in RPMI 1640 medium (0.2 ml) and stimulated with 5 μg/ml of Con A for 36 h. The cells were cultured with or without various concentrations of TCE. Then cell growth was evaluated with modified MTT assay. The optical density at 570 nm (OD570) was measured by a microplate reader. Then the stimulation index (SI) was calculated as follows: Stimulating index = ODstimulated cells/ODnonstimulated cells.

Cytotoxicity test
Lymph node cells (5 × 10⁵) were incubated for 24 h in the presence of various concentration of TCE, respectively. LDH levels in the supernatants were measured as described by the manufacturer.

Cell apoptosis assay
Cell apoptosis assay was done as described before.[10] The cells were staining with Annexin V-HTC (fluorescein isothiocyanate)/PI (propidium iodide), then cells were measured by flow cytometry as previously reported. Samples were analysed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Annexin V+ cells were considered as apoptotic cells.

Western blot analysis
Western blot was done as described before.[10] Cells or tissue samples were collected and lysed in the lysis buffer containing Triton X-100. The protein lysates were separated by 10% SDS-PAGE and subsequently electrotransferred onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA). Protein bands were visualized using Western blotting detection system according to the manufacturer’s instructions.

Real-time polymerase chain reaction
Real-time polymerase chain reaction (PCR) was performed as described previously.[12] Briefly, tissues were isolated from mice. Total ribonucleic acid (RNA) was extracted with TRIZOL reagent. RNA samples were treated by DNase and subjected to quantitative PCR, which was performed with the Bio-Rad IQ5 multicolor detection system (Bio-Rad, CA) using EvaGreen dye (Soofast, Bio-Rad). Conditions for amplification were 1 cycle of 94°C for 2.5 min followed by 40 cycles of 94°C for 30 s and 58°C for 30 s. The primer sequences used in this study were as follows: Tnf-α forward, 5’-GAGATCCATGCCGTTGGC-3’; Tnf-α reverse, 5’-CAAAATTCGAGTGACAAGCCTG-3’; Ifn-γ forward, 5’-ATGAA CGCTCACACATGCATCT-3’; Ifn-γ reverse, 5’-CCATCTTTTGCCAGTTCCTC-3’; β-actin forward, 5’-TGTCGT CCGTGAATGCGCT-3’; β-actin reverse, 5’-TTTGATGT CAGCCAGAGATT-3’.

Statistical analysis
Results were expressed as mean ± SEM of three independent experiments, and each experiment included triplicate sets. Data were statistically evaluated by one-way analysis of variance (ANOVA) followed by Dunnett’s test between control group and multiple dose groups. Histological differences between groups were evaluated by the Kruskal–Wallis test. The level of significance was set at a P-value of 0.05.

Results
Tupistra chinensis-protected mice from Con A-induced T-cell-mediated acute fulminant hepatitis
The contents of main components in TCE were determined by HPLC (Supporting information Figure S1). According to the references, saponins are believed to form the main constituents of TCE, and are considered responsible for its pharmacological properties.[13,14] So two saponins - β-furost-25(27)-en-1β,2β-D-glucopyranoside (saponin 1) and β-furost-25(27)-en-1β,2β-D-glucopyranoside (saponin 2) were appointed as controls of TCE. The concentration of saponin 1 (retention time 15.34 min) and saponin 2 (retention time 19.09 min) were 1.38% and 1.27%, respectively. To examine the immunomodulatory activity of TCE, we investigated the therapeutic efficacy of TCE in Con A-induced T cell-mediated murine hepatitis. Intravenous administration of Con A in mice induced a severe illness characterized by T cells activation, hepatocytes injury and serum ALT increase which peaked on the eighth hour and resulted in a high mortality rate thereafter. As shown in
Figure 1A, compared with the control group, TCE reduced the serum levels of ALT, AST and LDH in a dose-dependent manner. At the same time, histological analysis 8 h after Con A administration showed massive cell death with cytoplasmic swelling of most hepatocytes, infiltration of inflammatory cells and hyperplasia of Kupffer cells; while a marked reduction in inflammatory response and liver damage was observed in the groups of TCE (300 mg/kg) and dexamethasone (5 mg/kg) treatment (Figure 2B). The results of standard pathological tests in mice from TCE-treated groups (75, 150, 300 mg/kg) had scores of 2.42, 2.17 and 1.58, respectively; while control mice exhibited a score of 3.58 (Table 1, Figure 1C).

T. chinensis-regulated the profiles of cytokines in mice with Con A-induced hepatitis

To analyse some of the inflammatory mediators involved in colonic inflammation during Con A-induced hepatitis, sera were collected at 0 h, 2 h, 6 h, 8 h after Con A administration and further processed for cytokine production by ELISA assay, as described in the Materials and Methods section. Our results demonstrated that the serum level of pro-inflammatory cytokines IFN-γ and TNF-α was significantly increased after Con A injection, suggesting their roles in hepatic inflammation. Compared with the significant increase in serum TNF-α and IFN-γ level due to Con A injection, TCE exhibited notable inhibition in a time and dose-dependent fashion (Figure 2A). The mRNA level of TNF-α and IFN-γ in liver tissues was also reduced by TCE (Figure 2B). All these changes in cytokines correlated with a significant suppression of inflammation and resolution of hepatitis.

T. chinensis suppressed the upregulation of mitogen-activated protein kinases phosphorylation as well as NF-κB-signalling in livers treated with Con A

There is substantial evidence indicating that mitogen-activated protein kinase (MAPK), including extracellular...
signal-regulated protein kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) were involved in Con A induced liver injury, and inhibiting MAPKs activity could suppressing inflammation. In this study, we also found that acute Con A exposure increased phosphorylation levels of ERK1/2, JNK and p38 MAPKs; and TCE could inhibit all three members of MAPK in liver, especially for p38 MAP Kinase (Figure 3A). On the other hand, nuclear

Table 1 Effects of TCE on the liver histopathological changes in mice with Con A-induced liver injury

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Hepatocytes degeneration</th>
<th>Hepatic lobule inflammation</th>
<th>Central veins inflammation</th>
<th>Hepatic sinusoid hyperaemia</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0.33 ± 0.29</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>2 (1–2)**</td>
<td>0.5 (0–0.5)*</td>
<td>0.5 (0–0.5)*</td>
<td>2 (1–2)**</td>
<td>3.58 ± 0.74</td>
</tr>
<tr>
<td>TCE 75</td>
<td>0 (0–0.5)**</td>
<td>0.5 (0–1)</td>
<td>0 (0–0.5)</td>
<td>1 (1–2)</td>
<td>2.17 ± 0.48</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>0 (0–0.5)**</td>
<td>0 (0–0.5)*</td>
<td>0 (0–0.5)*</td>
<td>0.5 (0–1)**</td>
<td>1.58 ± 0.86</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>0 (0–0.5)**</td>
<td>0 (0–0.5)*</td>
<td>0 (0–0.5)*</td>
<td>0.5 (0–1)**</td>
<td>1.50 ± 1.22</td>
<td></td>
</tr>
<tr>
<td>Dex 5</td>
<td>0 (0–1)**</td>
<td>0 (0–0.5)*</td>
<td>0 (0–0.5)*</td>
<td>0.5 (0–1)**</td>
<td>1.50 ± 1.22</td>
<td></td>
</tr>
</tbody>
</table>

The liver tissue sections were stained with haematoxylin-eosin. The histological changes were read on a scale of 0–3 (0, no change; 1, mild; 2, moderate; 3, severe) and expressed as the median and range. Each data indicates median (range) of 6 animals survived. The Kruskal–Wallis test revealed a significant effect at **P < 0.01 vs Normal; *P < 0.05, **P < 0.01 vs Control.
factor κB (NF-κB) plays essential roles in transcriptional induction of those genes involved in inflammation, such as TNF-α and IL-1β. Here, we assessed the effects of TCE on activation of NF-κB signalling in mice livers upon response to Con A. TCE reduced phosphorylation of IKKα, IKKβ and the p65 subunit of NF-κB (Figure 3B), suggesting a decrease of inflammatory reactions in liver. Together, these findings indicated that TCE inhibited activation of MAPKs and NF-κB activity, thus finally reduced inflammation.

Figure 3 Effects of TCE on the Con A-induced activation of MAPKs and NF-κB in murine livers. (a–b) The protein levels of total and phosphorylated JNK, ERK and p38 were determined at least three times, and the representative data are shown. (c–d) The protein levels of total and phosphorylated IKKα, IKKβ, IκBα and p65 were determined at least three times, and the representative data are shown. Expressions of GAPDH were shown as loading controls. Bands from (a) and (c) were analysed by densitometry. Quantitative data are shown. One-way ANOVA revealed a significant difference at *P < 0.01. **P < 0.05, ***P < 0.01, versus TNBS control; ###P < 0.01, versus normal (Dunnet’s test).
**T. chinensis** did not influence the Con A induced T-cell activation and proliferation

Our results demonstrated that TCE could inhibit Con A-induced inflammatory cytokines upregulation, MAPKs phosphorylation and NF-κB activation in vivo, but whether TCE could inhibit T cells stimulated by Con A directly and the particular mechanisms under this phenomenon were still unclear. To this end, T lymphocytes were isolated and characterized by cell activation and proliferation in response to Con A challenges in vitro. As shown in Figure 4A, CD69/CD25, the activation markers of T cells, were not affected by TCE after Con A (5 μg/ml) stimulation for 12 h, while Cs A had a significant inhibition on T cell activation. At the same time, T cells from mice were proliferated in vitro by Con A for 36 h (SI = 2.54 ± 0.17). Con A significantly inhibited this proliferation almost completely at 1 μM with an SI of 1.27 ± 0.01 (P < 0.01), whereas TCE showed no evident inhibition at a concentration up to 100 μg/ml (Figure 4B). Moreover, TCE did not affect unactivated T cells (Figure 4C).

---

**Figure 4** Effects of TCE on T lymphocyte proliferation and activation. (a) T cells purified from lymph nodes were stimulated in the presence of Con A (5 μg/ml) for 12 h with or without TCE of various concentrations or Cs A. Then CD69 and CD25 expression was determined by flow cytometry. (b) Lymph node cells (5 × 10⁵) were incubated for 36 h at 37°C and 5% CO₂ in the presence of Con A with or without TCE of various concentrations or Cs A. Cell proliferation was assessed at 570 nm by MTT uptake. (c) Cytotoxicity of TCE on resting lymphocytes from normal mice. One-way ANOVA revealed a significant difference at P < 0.01. *P < 0.05, **P < 0.01, versus control; ##P < 0.01, versus normal (Dunnet’s test).

**T. chinensis** did not influence the Con A induced T-cell activation and proliferation

Our results demonstrated that TCE could inhibit Con A-induced inflammatory cytokines upregulation, MAPKs phosphorylation and NF-κB activation in vivo, but whether TCE could inhibit T cells stimulated by Con A directly and the particular mechanisms under this phenomenon were still unclear. To this end, T lymphocytes were isolated and characterized by cell activation and proliferation in response to Con A challenges in vitro. As shown in Figure 4A, CD69/CD25, the activation markers of T cells, were not affected by TCE after Con A (5 μg/ml) stimulation for 12 h, while Cs A had a significant inhibition on T cell activation. At the same time, T cells from mice were proliferated in vitro by Con A for 36 h (SI = 2.54 ± 0.17). Con A significantly inhibited this proliferation almost completely at 1 μM with an SI of 1.27 ± 0.01 (P < 0.01), whereas TCE showed no evident inhibition at a concentration up to 100 μg/ml (Figure 4B). Moreover, TCE did not affect unactivated T cells (Figure 4C).
**T. chinensis**-inhibited cytokines production and the activation of NF-κB signalling in T cells

To further determine if TCE affects T cell cytokines in vitro in the same manner as it does in vivo, we examined the T cell-specific cytokine profiles. In this study, T cells were isolated and challenged with Con A (5 μg/ml) for 24 h in vitro. It was shown that in culture supernatants cytokines commonly associated with T cell activation, including IL-2, TNF-α and IFN-γ were significantly down-regulated by TCE (Figure 5A).

According to the well-described role of NF-κB in inflammatory regulation and in cytokines expression, we assessed the effects of TCE on activation of NF-κB in T cells upon response to ConA for 6 h. As shown in Figure 5B, TCE inhibited phosphorylation of IKKα/β induced by ConA in a concentration-dependent manner without notable effect on total IKKα or IKKβ expression. TCE also reduced phosphorylation of IκBα. Moreover, the phosphorylation of p65 subunit of NF-κB was inhibited by TCE. Taken together, these results indicate that TCE inhibits activated T cells via NF-κB suppression, thereby reducing cytokines production.

**T. chinensis**-inhibited IFN-γ/STAT1/T-bet signalling in T cells

IFN-γ (Signal Transducer and Activator of Transcription 1) /T-bet signalling has been described to be essential for T cell activation and cytokines production.

---

**Figure 5** Effects of TCE on cytokines production, STAT-1 phosphorylation, and T-bet expression in T lymphocytes. (a) T cells purified from lymph nodes (5 × 10^5) were incubated in vitro. TNF-α, IFN-γ and IL-2 in supernatants of T cells cultured with TCE of different concentrations in the presence of Con A were determined by ELISA, respectively. (b) The cells were treated with Con A (5 μg/ml) with or without TCE for 6 h. The protein levels of total and phosphorylated IκBα, IκBβ, IkBα and p65 were determined at least three times, and the representative data are shown. Data summary are expressed as a histogram of mean ± SEM of three independent experiments. (c) The cells were treated with or without TCE for 24 h followed by murine IFN-γ (25 μg/ml) incubation for 30 min. Western blot for phospho-SHP2, SHP2, phospho-STAT1, STAT1, T-bet and actin are shown. Data summary are expressed as a histogram of mean ± SEM of three independent experiments. Data are statistically evaluated by one-way ANOVA followed by Dunnett’s test between control group and multiple dose groups, with the level of significance chosen as *p_<0.05, **p_<0.01, versus control, ***p_<0.001, versus normal.

© 2013 Royal Pharmaceutical Society, Journal of Pharmacy and Pharmacology, pp. **--**
resulted in a marked enhancement of STAT1 tyrosine phosphorylation. Co-incubation of IFN-γ-treated CD4+ T cells with TCE (100 μg/ml) completely inhibited the Tyr701 phosphorylation of STAT1 (Figure 5C). As a downstream molecule of STAT1, Th1-specific transcription factor T-bet was also suppressed by TCE in a concentration-dependent manner (Figure 5C). Moreover, we found that SHP2 was activated by TCE in lymph node T cells in a dose-dependent manner with elevated phosphorylation, while the total expression of SHP2 was not affected (Figure 5C). Earlier reports demonstrated that SHP2 is a dual-specificity phosphatase involved in STAT1 dephosphorylation at both tyrosine and serine residues in the nuclei.\(^{20,21}\)

The phosphorylation and binding of SHP2 to non-phosphorylated STAT1, induced by TCE, may lead to a unique regulation of STAT1 signalling. On the other hand, Cs A, the immunosuppressant widely used in clinic, had no significant effects on SHP2 phosphorylation while inhibiting STAT1 activation.

**T. chinensis-induced apoptosis of activated T lymphocytes**

To further delineate the mechanisms of TCE against T-cell-mediated immune response, we incubated T cells with TCE in the presence of Con A for 48 h. Notable cell apoptosis was detected when concentrations higher than 25 μg/ml were used (Figure 6A). The T cells activated with Con A and exposed to TCE underwent apoptosis in a dose-dependent manner (Figure 6A and B). Furthermore, after incubating with various concentrations of TCE (0, 5, 10, 25, 50, 100 μg/ml) at the presence of Con A (5 μg/ml) for 48 h, level of the anti-apoptotic proteins, bcl-2/bcl-xl, was significantly down-regulated while the apoptotic protein, bax, was increased. Caspase 3 and PARP in T cells were found to be cleaved (Figure 6C and D). These results indicated that TCE significantly triggered apoptosis of Con A-activated T cells.

**Discussion**

*Tupistra chinensis* has been used for the treatment of inflammatory diseases for hundreds of years. It has an undoubted role in clinic. In National Herbal Compendium, *Tupistra chinensis* at doses of 2.5 g or 5 g is recommended to be decocted in water for oral administration in the treatment of pharyngolaryngitis, rheumatism, etc. In Chinese Materia Medica, *T. chinensis* at doses of 1.5 or 3 g is recommended for oral use. In our study, *T. chinensis* was extracted with an extraction yield of 60.4%. So a dose of 1.5–5 g of the herbal drug *T. chinensis* provides about 0.9–3 g of TCE. Calculated with formulas for body surface area, we used dosage 75–300 mg/kg in mice.

People have been trying to clarify the ingredient of *T. chinensis* for decades. It has been reported that *T. chinensis* contains many saponins and alkaloids. Total saponin content is about 5.28% of dry weight rhizome. 70% ethanol extract of *Tupistra* contains 13.51% total saponins.\(^{23}\) There are some 20 kinds of ingredients, mainly composed of aliphatic compounds and steroidal saponins, in the chloroform extract of *T. chinensis* detected by gas chromatography-mass spectrometry (GC-MS) spectrometer\(^{26}\) and in the solvent extraction of *Tupistra chinensis*, 29 kinds of ingredients have been detected by GC-MS after screened with computer and referred to nucleotide binding site profiling.\(^{21}\) Shen et al. have isolated two novel polyhydroxylated steroidal sapogeni, wattigenin B and C from the rhizome of *Tupistra wartii* Hook.\(^{26}\) Wang and Yang et al. have reported five new steroidal glycosides named wattosides A–E.\(^{20}\) Pan et al. found a new steroidal saponin, tupichigenin A, from *Tupistra chinensis*.\(^{27}\)

Although some compounds have been isolated from TCE and measured, effects of herbal drug *Tupistra chinensis* itself are far from clearly clarified. Here, we provide the first evidence that TCE can inhibit activated T cells for a novel approach to treatment of T cell-related inflammatory diseases.

Increasing evidence suggests that T cell-mediated immunity is one of the dominant causes in a variety of liver diseases involving autoimmune and viral hepatitis. T cell activation is the critical initial step in the pathogenesis of liver damage.\(^{24,25}\) In mice, T cell-dependent hepatitis can be mimicked with Con A administration in vivo. This mitogen induces polyclonal T-cell activation and causes severe immune-mediated hepatitis characterized by increased serum levels of transaminases and infiltration of peripheral T cells into the liver.\(^{11}\) In this study, TCE significantly alleviated Con A–induced hepatitis with an almost recovery from the elevation of serum transaminase levels at the dose of 300 mg/kg. Histological assay showed that TCE also markedly reduced the extent of liver damage (Figure 1). It should be emphasized that TCE was not effective in carbontetrachloride (CCL₄)-induced liver injury (Supporting information Figure S2). CCL₄, a well-known model compound for producing chemical-induced hepatic injury, is a model substance to elucidate the action of hepatotoxic effects and to screen hepatoprotective activities of drugs.\(^{26}\) This feature of TCE is different from that of bifendate, the clinical used drug for hepatitis, suggesting a distinct effect of TCE only on T cells inhibition but not on hepatocytes protection.

TNF-α and IFN-γ are typical T cell cytokines which play casual roles in the onset of liver damage.\(^{11}\) Compared with the significant increase in serum TNF-α and IFN-γ level due to Con A injection, TCE exhibited obvious inhibition in a time- and dose-dependent fashion (Figure 2).
Figure 6  Effects of TCE on apoptosis in Con A-activated T cells. Cells were seeded in 6-well plate and incubated with TCE or quercetin for 48 h at the presence of Con A (5 μg/ml). (a) The apoptosis of cells was determined by Annexin V/PI staining. (b) Annexin V/PI+ and Annexin V/PI+ cells of three independent experiments were shown in column statistics. Representative Western blot bands (c) and data summary (d) of bax, bcl-2, bcl-xL, cleaved caspase 3 and PARP in activated T cells were shown. Data are expressed as a histogram of mean ± SEM of three independent experiments. Data were statistically evaluated by one-way ANOVA followed by Dunnett’s test between control group and multiple dose groups, with the level of significance chosen as *P < 0.05, **P < 0.01.
Furthermore, we examined the immunosuppressive activity of TCE in allergic contact dermatitis, which had been shown to be entirely dependent on the effects of T cells.[30] Administration with TCE significantly inhibited ear swelling in a dose-dependent manner. Moreover, the mice treated with TCE only showed a mild cellular infiltration and vasodilation without obvious oedema compared with the animals in the control group (Supporting Information Figure S5). Taken together, these results suggested that TCE did play its role through intervention of T lymphocytes.

The next question is how TCE modulates T lymphocytes. In the experiment for Con A induced lymphocyte activation and proliferation, we found that TCE had no influences on the cell number and the expression of cell activation markers (Figure 4), indicating that the activity of TCE against T cells was distinct from Cs A and was not due to limitation for cell amplification.

Then we examined the regulation of cell function by this extraction. From the dynamic changes of cytokines in lymph node cells, we found that T cell-related cytokines expression was remarkably reduced by TCE (Figure 5A–B), which was consistent with the data in vivo. Furthermore, we demonstrated that TCE also increased SHP-2 phosphorylation followed with an inhibition on STAT1/T-bet signalling after IFNγ stimulation of lymph node T cells (Figure 5C–D). It should be noted that Cs A showed no substantial effects on SHP2 phosphorylation, whereas it also inhibited STAT1/T-bet signalling, just like TCE. This feature suggests that compared with TCE, Cs A might influence other negative regulators of STAT1, such as suppressor of cytokine signalling 1 or β-arresting 1, etc.[33,34]

Our findings have pointed to T cells as a potential target of TCE in the treatment of murine fulminant hepatitis. Therefore, effective elimination of pathogenic effector T cells might be a therapeutic strategy. Herein we reported that TCE induced apoptosis of activated T cells rather than inhibiting T cells activation in vitro (Figure 6A–B). Upon examination of apoptotic pathways, we observed the upregulation of bax, truncation of bcl-2/bcl-xL, activation of caspase-3 and the cleavage of PARP induced by TCE (Figure 6C–D), indicating that mitochondria apoptotic pathway is involved in TCE-mediated cell death. These results confirm that the facilitation of apoptosis by TCE in activated T cells could also be responsible in blocking the development of T-cell-dependent fulminant hepatitis.

It is well known that activation of MAPKs and NF-κB play essential roles in transcriptional induction of cytokines genes in T cell-mediated inflammatory diseases, such as TNF-α, IL-1β and IL-2.[35,36] Many studies proved that using inhibitors of MAPKs or NF-κB could ameliorate inflammation.[37,38] Our data showed that Con A injection remarkably activated all three members of MAPK in mice livers; and TCE could inhibit these changes moderately but notably, especially for p38 MAP Kinase (Figure 3A–B). On the other hand, TCE significantly reduced phosphorylation of IKKα/β and IκBα (Figures 3C–D, 5B). The phosphorylation of p65 subunit of NF-κB was also inhibited by TCE, in line with its activity on IKKα/β and IκBα. These results provide evidences that TCE suppress T cell-induced inflammations through regulating MAPKs, NF-κB and STAT1 pathways.

Conclusion

Overall, by using a combination of in vivo and in vitro studies, we have demonstrated that TCE has the potential to attenuate Con A-induced acute fulminant hepatitis, and its effects involves multiple signals against activated T cells: regulation of cytokine profiles, inhibition of MAPKs, NF-κB and STAT1 pathways, as well as apoptosis induction. A possible clinical application due to this unique character could be expected to have benefits for other T cell-mediated inflammatory diseases.

Declarations

Conflict of interest

The authors declared no conflict of interest.

Funding

This work was supported by National Natural Science Foundation of China (Nos. 81173070 and 81001465), Natural Science Foundation of Jiangsu Province (BK2011053, BK20131282) and National Science & Technology Major Project (No. 2012ZX09304-001).

References


© 2013 Royal Pharmaceutical Society, Journal of Pharmacy and Pharmacology, ••: ••••


Supporting Information
Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1 HPLC assay of TCE. Two saponins were controls in TCE: 5β-furost-Δ25(27)-en-1β,3β-β-D-glucopyranoside (saponin 1) and 5β-furost-25(27)-en-1β,3β-26-octaol-6-one-Δ25(27)-β-D-glucopyranoside (saponin 2). HPLC analysis was applied on a Shimadzu 20A series HPLC system consisting of two pumps (LC-20A Solvent Delivery Unit), a column oven (CTO-10ASVP), a SPD detector (SPD-M20AV Photodiode Array Detector) and an LC solution Work Station. TCE was applied to himadzu VP-ODS column (5μm, 150 × 4.6 mm; Shimadzu, Japan) and detected at 203 nm. Column temperature was set up at 25°C and the flow rate was 1 ml/min. The mobile phase was acetonitrile – water two-phase gradient elution.

Figure S2 Effects of TCE on carbontetrachloride (CCl4)-induced hepatitis. Mice were given orally with TCE (75, 150, 300 mg/kg) or Bifendatatum (200 mg/kg) in PBS for five days, respectively. One hour after the final administration, Mice were intraperitoneally injected with CCl4 (0.12%, v/v, dissolved in olive oil, 10 ml/kg bodyweight) to induce acute liver injury, while the mice in the normal group were administered with equal volume of olive oil. The animals were sacrificed 24 h after CCl4 intoxication. Serum was obtained by bleeding for the measurement of alanine transaminase (ALT) and aspartate transaminase (AST) activities respectively. The data indicated the mean ± SEM of experimental animals (n = 10). One-way ANOVA revealed a significant difference at P < 0.01. *P < 0.05, **P < 0.01, vs. Con A control; ##P < 0.01, vs. normal (Dunnett’s test).

Figure S3 Effects of TCE on picryl chloride (PCL)-induced contact hypersensitivity in mice. Mice were sensitized by painting 0.1 ml of 1% PCL in ethanol onto the shaved skin of their abdomen on day 0. Five days after sensitization, they were challenged on right ears with 30 μl 1% PCL in olive oil. At the time of challenge and 5 h later, TCE (75, 150, 300 mg/kg) were given orally twice. (A) Twenty hours after challenge, the ear swelling was evaluated. Each column represents the mean ± s.e.m. of 12 mice. (B) Representative microphotographs showing ear histopathologic changes with hematoxylin–eosin staining (original magnification ×200). (C) Microscopic scores of ears (n = 12). One-way ANOVA revealed a significant difference at P < 0.01. *P < 0.05, **P < 0.01, vs. Con A control; ###P < 0.01, vs. normal (Dunnett’s test).
Dear Author,

During the preparation of your manuscript for publication, the questions listed below have arisen. Please attend to these matters and return this form with your proof.

Many thanks for your assistance.

<table>
<thead>
<tr>
<th>Query References</th>
<th>Query</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AUTHOR: Please reduce to no more than 40 characters for the short title running head.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AUTHOR: Should PARP be written in full? If so, please provide its full form.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AUTHOR: Please provide the full form of RPMI.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AUTHOR: Please give address information for Cell Quest software, e.g. town, city, state (if applicable) and country.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>AUTHOR: If this is a product, please provide manufacturer details for 96-Well Plate, e.g. name of company and address details same as the previous query.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>AUTHOR: Please provide manufacturer/product details for TRIZOL.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>AUTHOR: Journal style requires References with three or more authors/editors, list the first author/editor name followed by ‘et al.’ Please check and confirm that the reference entries comply to this style.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AUTHOR: Please provide the full surnames for the one-character surnames.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>AUTHOR: Please supply the volume number and page range for Reference 33.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>AUTHOR: If this is not a one-page article please supply the first and last pages for Reference 35.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>*AUTHOR: Please provide details for Note $.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>*AUTHOR: The page range ‘373–338’ seems to be incorrect; please check.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>*AUTHOR: AUTHOR: If you wish to have the figures reproduced in colour and have not had previous approval for gratis colour, please fill out the Colour Work Agreement form (CWA) found at <a href="http://www.blackwellpublishing.com/pdfs/SN_Sub2000_F_CoW.pdf">http://www.blackwellpublishing.com/pdfs/SN_Sub2000_F_CoW.pdf</a> and return it to <a href="mailto:jphp@wiley.com">jphp@wiley.com</a>. Figures will automatically be converted to monochrome upon publication if no CWA form is received.</td>
<td></td>
</tr>
</tbody>
</table>

Note: The query which is preceded by * is added by Toppan Best-set.
Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X) The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:

This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We’ve picked out some of these tools below:

1. Replace (Ins) Tool – for replacing text.
   Strikethrough through text and opens up a text box where replacement text can be entered.
   How to use it
   - Highlight a word or sentence.
   - Click on the Replace (Ins) icon in the Annotations section.
   - Type the replacement text into the blue box that appears.

2. Strikethrough (Del) Tool – for deleting text.
   Strikethrough through text that is to be deleted.
   How to use it
   - Highlight a word or sentence.
   - Click on the Strikethrough (Del) icon in the Annotations section.

3. Add note to text Tool – for highlighting a section to be changed to bold or italic.
   Highlights text in yellow and opens up a text box where comments can be entered.
   How to use it
   - Highlight the relevant section of text.
   - Click on the Add note to text icon in the Annotations section.
   - Type instruction on what should be changed regarding the text into the yellow box that appears.

4. Add sticky note Tool – for making notes at specific points in the text.
   Marks a point in the proof where a comment needs to be highlighted.
   How to use it
   - Click on the Add sticky note icon in the Annotations section.
   - Click at the point in the proof where the comment should be inserted.
   - Type the comment into the yellow box that appears.
5. **Attach File Tool** – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

**How to use it**
- Click on the **Attach File** icon in the Annotations section.
- Click on the proof to where you’d like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

6. **Add stamp Tool** – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

**How to use it**
- Click on the **Add stamp** icon in the Annotations section.
- Select the stamp you want to use. (The **Approved** stamp is usually available directly in the menu that appears).
- Click on the proof where you’d like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

7. **Drawing Markups Tools** – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.

**How to use it**
- Click on one of the shapes in the **Drawing Markups** section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the **Help** menu to reveal a list of further options:
Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

<table>
<thead>
<tr>
<th>Instruction to printer</th>
<th>Textual mark</th>
<th>Marginal mark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leave unchanged</td>
<td>⋯ ⋯ under matter to remain</td>
<td>✓</td>
</tr>
<tr>
<td>Insert in text the matter indicated in the margin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delete</td>
<td>/ through single character, rule or underline or / through all characters to be deleted</td>
<td>✓</td>
</tr>
<tr>
<td>Substitute character or substitute part of one or more word(s)</td>
<td>/ through letter or / through characters</td>
<td>✓</td>
</tr>
<tr>
<td>Change to italics</td>
<td>— under matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to capitals</td>
<td>≈ under matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to small capitals</td>
<td>≈ under matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to bold type</td>
<td>≈ under matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to bold italic</td>
<td>Encircle matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to lower case</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Change italic to upright type</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Change bold to non-bold type</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert ‘superior’ character</td>
<td>/ through character or ✓ where required</td>
<td>✓</td>
</tr>
<tr>
<td>Insert ‘inferior’ character</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert full stop</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert comma</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert single quotation marks</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert double quotation marks</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert hyphen</td>
<td>(As above)</td>
<td>✓</td>
</tr>
<tr>
<td>Start new paragraph</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>No new paragraph</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Transpose</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Close up</td>
<td>linking characters</td>
<td></td>
</tr>
<tr>
<td>Insert or substitute space</td>
<td>/ through character or ✓ where required</td>
<td>✓</td>
</tr>
<tr>
<td>between characters or words</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduce space between characters or words</td>
<td>between characters or words affected</td>
<td>✓</td>
</tr>
</tbody>
</table>

E.g. e.g. under character e.g. over character e.g.