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Theacrine, a Purine Alkaloid Obtained from *Camellia assamica* var. *kucha*, Attenuates Restraint Stress-Provoked Liver Damage in Mice

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ABSTRACT: Theacrine (1,3,7,9-tetramethyluric acid), a purine alkaloid, has proven to be beneficial in maintaining several brain functions and is being studied for potential medicinal uses in recent years. In this study, we isolated theacrine from *Camellia assamica* var. *kucha* and investigated its protective effects on liver damage induced by restraint stress in mice. Results showed that 18 h of restraint stress could induce liver damage, with an obvious increase in levels of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST). This finding was further confirmed by hepatic pathological examination, which showed inflammatory cell infiltration and focal necrosis of hepatocytes. However, oral administration of theacrine (10, 20, 30 mg/kg for 7 consecutive days) was found to decrease plasma ALT and AST levels, reduce hepatic mRNA levels of inflammatory mediators (IL-1β, TNF-α, IL-6, and IFN-γ), and reverse the histologic damages in stressed mice. Simultaneously, theacrine also significantly decreased the content of malondialdehyde and increased oxygen radical absorbance capacity (ORAC) level in the plasma and liver of stressed mice. These results suggested that the protective effects of theacrine on stress-induced liver damage might be correlated with its antioxidative activity. The antioxidative capacity of theacrine was further evaluated by *in vitro* ORAC and cellular antioxidant activity assay. The results suggested that the antioxidative capacity of theacrine was not due to the direct action on free radical clearance. Moreover, the elevated activities and gene expressions of superoxide dismutase, catalase, and glutathione peroxidase, as well as the reduced activity of xanthine oxidase by theacrine treatment in stressed mice suggested that the antioxidative activity might be due to the strengthening of the antioxidant system *in vivo*. On the basis of the above results, theacrine is possibly a good candidate for protecting against or treating lifestyle diseases and might contribute to the study of natural products.

KEYWORDS: *theacrine, restraint stress, oxidative stress, liver damage, inflammation*

INTRODUCTION

It has been widely reported that stress could trigger many mental and physical health problems and lifestyle diseases.[1−3] Our previous studies have proved that restraint stress increases the susceptibility to viral or bacterial pathogens,[4,5] inhibits lipid and glucose metabolism,[6,7] diminishes the strength of host immune defense,[8,9] and provokes liver damage.[10,11] Until now, many studies have demonstrated that oxidative stress and diminished antioxidant protection are the main reasons for stress-provoked diseases.[8,11] Therefore, it is necessary to develop potential therapies for stress-associated diseases to stay healthy and improve the quality of life.

Purine compounds, such as caffeine, have many health-promoting properties and have proven to be beneficial in treating various health conditions.[12,13] Theacrine (1,3,7,9-tetramethyluric acid), a purine alkaloid structurally similar to caffeine and abundantly present in *Camellia assamica* var. *kucha*, has in recent years come under investigation for potential medicinal uses. *C. assamica* var. *kucha* is a tea plant grown in wild woodland at 1370 m altitude in Yunnan Province of China and has been consumed there for a long time. Theacrine, a food-derived compound, has been proved to exhibit anti-inflammatory and analgesic properties in mice at doses of 8, 16, and 32 mg/kg body weight.[14] Besides, our previous studies found that theacrine (10 and 30 mg/kg) possessed potent sedative, hypnotic, and antidepressive properties.[15,16] Moreover, theacrine (24 and 48 mg/kg) was also reported to affect the central nervous system via adenosine and dopamine receptors in rats.[17] However, little is known concerning the pharmacological effects of theacrine on liver damage caused by stress. In the present study, the potential effect of theacrine obtained from the commonly consumed *C. assamica* var. *kucha* was studied on restraint stress-induced liver damage in mice. The related mechanism was further explored by examining the improvement of oxidative damage and inflammation. The data collected in this study would hopefully introduce new uses of theacrine as an herbal medicine and promote the prevalence of *C. assamica* var. *kucha* as a functional food.

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MATERIALS AND METHODS

Materials. *Camellia assamica* var. *kucha* was obtained from Jinpin County of Yunnan Province in China. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), glutathione peroxidase (GPx), xanthine oxidase (XOD), catalase (CAT), glutathione-S-transferase (GST), malondialdehyde (MDA), and Coomassie brilliant blue kit were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Sodium 1-octanesulfonate was purchased from Kasei Kogyo Co., Ltd. (Tokyo, Japan). 2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH), sodium fluorescein, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carcoxylic acid (Trolox, a water-soluble vitamin E analogue) were purchased from Wako Pure Chemical Industries., Ltd. (Osaka, Japan). TRIzol, oligo$(dT)_{20,}$ dNTPs (dATP, dGTP, dCTP, dTTP), RNase inhibitor, 5× first strand buffer, dithiothreitol, superScriptTMIII RT, 10× PCR buffer, Taq DNA polymerase, 6× DNA loading buffer, DNA marker I, RNase-free ddH_2O, and Goldview solution were obtained from Tiangen Biotech Co., Ltd. (Beijing, China). All other reagents were analytical grade.

Animals and Treatment. Male Kunming mice (18.0−20.0 g) were purchased from Center of Laboratory Animal Science Research (Guangdong, China). All mice were housed in a pathogen-free room under controlled temperature (23 ± 1 °C) and humidity (60 ± 5%) with a 12 h day−night cycle (lights on at 06:00). Procedures for animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. Fifty mice were randomly divided into five groups, which were normal control group, model (restraint stress) group, and three theacrine treatment (10, 20, and 30 mg/kg body weight) groups. Experimental groups received oral administration of theacrine for 7 consecutive days, while other groups received the same volume of distilled water. All mice except the normal control group were physically restrained in a 50 mL polypropylene tube with holes for 18 h before being sacrificed for sample collection.[11]

Isolation of Theacrine. Theacrine was isolated from *C. assamica* var. *kucha* as previous described.[15,16] Briefly, fresh tea leaves were steamed for 3 min and then dried in an oven at 80 °C. Dried leaves were accurately weighed (100 g) and extracted twice with 2000 mL of boiling water for 30 min. After filtration, the filtrate was concentrated by evaporation under vacuum to 500 mL. After the pH of the filtrate was adjusted to 7.8, a 20% $Pb(OAc)_2{\cdot}Pb(OH)_2$ solution (w/v) was mixed with the filtrate until no further precipitate was formed. After discarding the precipitate, the filtrate was extracted with an equal volume of chloroform three times to yield 1.74 g of chloroform extract. The extract was subjected to column chromatography on octadecylsilanized (ODS) silica gel and then eluted with H_2O, MeOH/H_2O (1:9), and MeOH, respectively. The fraction eluted with MeOH/H_2O (1:9) was further separated by preparative HPLC to yield 890 mg of theacrine. The structure was confirmed by spectral data (MS, NMR, elemental analysis) and illustrated (Figure 1).

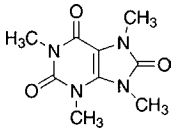

Figure 1. Chemical structure of theacrine.

Analysis of Theacrine in *Camellia assamica* var. *kucha* by RP-HPLC. The aqueous extract of *C. assamica* var. *kucha*, which was prepared as mentioned in the above section, was centrifuged for 15 min at 13000*g*. The supernatant was filtered through a 0.45 μm nylon 6-6 filter and analyzed. The analysis was performed by HPLC at 231 nm (Hitachi, Tokyo, Japan) with a Develosil ODS-HG-5 column (4.6 × 150 mm; Nakalai Tesuque, Kyoto, Japan) at a flow rate of 1 mL/min. The mobile phase consisted of two solvent (solvent A = 95:5:0.05 v/v/v H_2O/CH_3CN/85% H_3PO_4; solvent B = 50:50:0.05 v/v/v H_2O/CH_3CN/85% H_3PO_4). All solvents used were HPLC grade except for H_3PO_4, which was analytical grade. The gradient elution program was as follows: 0 min, 10% B; 5 min, 10% B; 8 min, 30% B; 10 min, 30% B; 15 min, 80% B; 20 min, 80% B. Quantification of theacrine was calculated by standard calibration curves. Data were obtained as average values in triplicate experiments.

Plasma and Liver Sample Collection and Protein Level Measurement. Under ether anesthesia, blood was collected in test tubes with heparin sodium (50 U/mL blood) following cardiac puncture, and the liver was quickly removed. Blood samples were centrifuged at 5000*g* for 10 min at 4 °C by refrigerated centrifuge (Sigma, Germany) to obtain plasma. Liver samples were homogenized in chilled 0.01 M PBS (pH 7.4) using an Ultra-Turrax T8 homogenizer (GmbH, Germany) and centrifuged at 10000*g* for 10 min at 4 °C. A 2% liver homogenate was used to determine protein concentration using a Coomassie brilliant blue kit with bovine serum albumin as standard.

Histological Analysis. For histologic analysis, small sections of fresh liver tissues were immediately fixed in 10% buffered formalin and embedded in paraffin wax. Paraffin-embedded tissues were sectioned at 4 μm and mounted for histological staining. Fixative was removed using a graduated series of xylene, rehydrated, and then flushed for 15 min in deionized water to remove any remaining fixative. Tissue sections were randomly selected for hematoxylin and eosin (H&E) staining and examined in a blinded fashion by two experienced observers under optical microscopy (Olympus, Melville, NY, USA). Representative images from each treatment group were taken. To determine the severity of liver pathology, six randomly selected 200× fields were assessed per animal. Liver inflammation was semiquantified by enumerating inflammatory cells under the same microscopic area. Necrosis was semiquantified as the ratio of area of spotty necrosis to the same microscopic area.[18]

Measurement of ALT and AST Activities in Plasma. ALT and AST levels in the plasma were measured by the Reitman−Frankel method using commercial kits.[19] In the presence of ALT or AST, alanine reacts with an α-ketoglutaric acid to produce pyroracemic acid. Carbonyl addition reaction between pyroracemic acid and dinitrophenylhydrazine produces phenylhydrazone, which is brown in color under basylous medium. Phenylhydrazone absorbance was measured at 492 nm using an MK3 microplate reader (Labsystems, Finland).

Measurement of XOD, GPx, SOD, CAT, and GST Activities in Plasma or the Liver. The activities of XOD, GPx, SOD, CAT, and GST in the liver were determined using commercial kits.[5,20,21] In brief, XOD catalyzes the oxidation of hypoxanthine to xanthine to produce superoxide radical, which eventually results in a pink adduct detected at 530 nm with an MK3 microplate reader (Labsystems, Finland). The activity of GPx can be calculated by determining the optical density of the enzyme tube and the nonenzyme tube at 412 nm after reduced glutathione (GSH) had reacted with 5,5′-dithiobis(2-nitrobenzoic acid). SOD in samples can scavenge superoxide radical and reduce the level of nitrite. When it reacted with a color-developing reagent, nitrite turned purple-red and could be measured by an MK3 microplate reader (Labsystems, Finland) at 550 nm. CAT activity was determined based upon the decomposition of H_2O_2, which has an optical density at 415 nm. GST activity was determined by spectrophotometric kinetic measurements of the conjugation of 1-chloro-2,4-dinitrobenzene with GSH according to the method of Habig et al.[22] The conjugation is accompanied by a decrease of GSH content, which can be determined by the absorbance at 412 nm. All the results of XOD, SOD, GPx, CAT, and GST activities in the liver were standardized with liver protein concentrations and were expressed in units per gram or microgram of protein. The activity of SOD in the plasma was also determined and expressed as units per milliliter.

Measurement of MDA Content in Plasma and the Liver. MDA contents in plasma and the liver were measured with a commercial MDA kit. In acidic medium, MDA reacted with thiobarbituric acid (TBA) upon boiling, and the resultant pink MDA−TBA adducts was measured by an MK3 microplate reader (Labsystems, Finland) at 532 nm.

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Table 1. Sequence of Primers Used for RT-PCR

gene	forward (5′−3′)	reverse (5′−3′)	size
Cu/ZnSOD	ATGGCGATGAAAGCGGTGTG	TTACTGCGCAATCCCAATCAC	456 bp
MnSOD	AAGCACAGCCTCCCAGACCT	TCACTTCTTGCAAGCTGTGTATCTT	597 bp
GPx	GAAGTGCGAAGTGAATGG	TGGGACAGCAGGGTTT	255 bp
CAT	CCAGCGACCAGATGA	CCTTTGCCTTGGAGTA	273 bp
IL-1β	ATGGCAACTGTTCCTGAACTCAACT	CAGGACAGGTATAGATTCTTTCCCTTT	563 bp
IL-6	CCAACAGACCTGTCTATACCAC	GTGACTCCAGCTTATC	425 bp
GR	TGGTGTGCTCCGATGA	AGGGTAGGGGTAAGC	294 bp
TNF-$\alpha\alpha$	GGCGGTGCCTATGTCTC	GCAGCCTTGTCCCTTGA	362 bp
IFN-$\gamma\gamma$	TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC	TGGACCTGTGGGTTGTTGACCTCAAACTTGGC	385bp
18s	GGGAGAGCGGGTAAGAGA	ACAGGACTAGGCGGAACA	241 bp

ORAC (Oxygen Radical Absorbance Capacity) Assay. The procedures of the ORAC assay for theacrine *in vitro* and *in vivo* (plasma and liver) were carried out according to the previously described method.[8] The ORAC level *in vitro* represents the direct free radical clearance ability of theacrine, while the *in vivo* level reflects the effect of theacrine on the total antioxidative capacity in plasma and the liver of stressed mice. An automated ORAC assay was carried out on a GENios luciferase-based microplate reader (TECAN, Switzerland) with an excitation/emission filter pair of 485/527 nm as previously described. Fluorescein was used as a fluorescence probe, and the reaction was initiated with the addition of AAPH. Trolox was used as a standard. The final results were calculated on the basis of the difference in the area under the fluorescence decay curve between the AAPH control and each sample.

CAA (Cellular Antioxidant Activity) Assay. The CAA assay is a biologically representative method for quantifying the antioxidant capacity of phytochemicals and food extracts in cell culture. In this study, the CAA of theacrine and ascorbic acid was determined according to Wolfe and Liu with slight modification.[23] Stock solutions of 10 mM dichlorofluorescin diacetate (DCFH-DA) (dissolved in ethanol) and 10 mM AAPH (dissolved in PBS) were prepared and stored at −20 °C. RAW 264.7 macrophage cells were grown in DMEM high-glucose medium with 10% FBS. Cells were cultured and seeded at a density of 6×10^4/well on a 96-well microplate at 37 °C with 5% CO_2. The outside wells of the plate were not used, as there was much more variation from them than from the inner wells. Twenty-four hours after seeding, the growth medium was removed and triplicate wells were treated for 1 h with antioxidants (dissolved in growth medium) plus 10 μM DCFH-DA dissolved in treatment medium. After 1 h of treatment, wells were then washed twice with PBS and then 200 μM AAPH was applied to the cells. The microplate was immediately placed into a GENios luciferase-based microplate reader (TECAN, Switzerland) at 37 °C with an excitation/emission filter pair of 485/538 nm. Fluorescence was detected every 5 min for 1 h and plotted into a curve. Each plate included triplicate control and blank wells, where control wells contained cells treated with DCFH-DA and AAPH and blank wells contained cells treated with DCFH-DA without AAPH. Antioxidant ability was expressed as CAA units by calculating the difference in area under the curve between tested samples and control wells.

Measurement of Cu/ZnSOD, MnSOD, GPx, CAT, IL-1β, IL-6, TNF-α, and IFN-γ mRNA Levels in the Liver. The gene expression was semiquantitatively assessed utilizing reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the liver using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Three micrograms of total RNA was reversely transcribed into cDNA at 42 °C for 1 h in 20 μL of reaction mixture system containing mouse moloney leukemia virus reverse transcriptase (Tiangen, Beijing, China) with oligo (dT) 15 primer (Tiangen, Beijing, China), followed by PCR amplification. PCR was carried out with 1 μL of cDNA, 1 μM forward primer, 1 μM reverse primer, and 12.5 μL of 2× Taq PCR MasterMix (Tiangen, Beijing, China), in a total volume of 25 μL. The cDNA was amplified using specific primers (Table 1, Invitrogen, Carlsbad, CA, USA) for 30 cycles at 94 °C for 30 s, an annealing temperature of 58 °C for 40 s, and then 72 °C for 50 s, with a final incubation at 72 °C for 7 min. The PCR products were fractionated on a 1% agarose gel and visualized by ethidium bromide staining. The band intensity of ethidium bromide was measured by an image analysis system (Bio-Rad, Hercules, CA, USA), then quantified with Quantity One analysis software (Bio-Rad, Hercules, CA, USA) and expressed as the ratios to 18s.

Statistical Analysis. The data obtained were represented as mean ± SD and analyzed by the SPSS 13.0 statistical software. One-way analysis of variance (ANOVA) was applied to analyze for differences in data of biochemical parameters among the different groups, followed by Dunnett's significant posthoc test for pairwise multiple comparisons. Levels of significance at $p < 0.05$ were considered statistically significant.

RESULTS

Analysis of Theacrine in *Camellia assamica* var. *kucha* by RP-HPLC. The tea sample was analyzed by HPLC as illustrated in Figure 2. The content of theacrine in *C. assamica* var. *kucha* was 1.8 ± 0.05%.

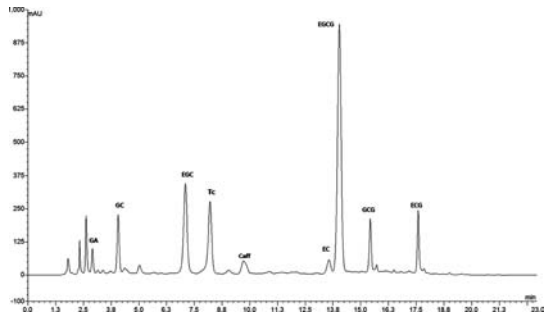

Figure 2. Chromatogram of sample of *Camellia assamica* var. *kucha*. Theacrine (Tc), caffeine (Caff), gallic acid (GA), (+)-catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-gallocatechin gallate (GCG), (−)-epigallocatechin gallate (EGCG), (−)-catechin gallate (CG), (−)-epicatechin gallate (ECG).

Effects of Theacrine on ALT and AST Activities in Plasma of Stressed Mice. As shown in Table 2, plasma ALT and AST levels in normal control group were 28.24 ± 4.61 and 32.82 ± 5.70 U/L, respectively. However, 18 h of restraint stress significantly increased ALT (79.32 ± 15.42 U/L) and AST (84.11 ± 25.15 U/L) levels. Theacrine treatment (10, 20, 30 mg/kg) could significantly decrease plasma ALT and AST levels by different extents in stressed mice ($p < 0.01$).

Effects of Theacrine on Pathological Damage of the Liver in Stressed Mice. As shown in Figure 3, there was no obvious pathological abnormality in the normal group. Liver parenchyma had good morphology, and the hepatocytes were

Table 2. Effects of Theacrine on the Activities of ALT, AST, and SOD, the Content of MDA, and the Level of ORAC in Plasma of Restraint-Stressed Mice[a]

	plasma				
group	ALT (U/L)	AST (U/L)	SOD (U/mL)	MDA (nmol/mL)	ORAC (μM Trolox equiv)
normal control	28.24 ± 4.61	32.82 ± 5.70	261.61 ± 32.50	6.16 ± 1.44	6.97 ± 0.66
model (restraint)	79.32 ± 15.42$^{\#\#}$	84.11 ± 25.15$^{\#\#}$	193.10 ± 34.66$^{\#\#}$	10.56 ± 1.74$^{\#\#}$	5.38 ± 1.10$^{\#\#}$
restraint + Tc (L)	45.06 ± 11.78**	70.83 ± 6.83	229.84 ± 42.01	6.59 ± 0.74**	8.41 ± 0.68**
restraint + Tc (M)	34.46 ± 13.79**	62.65 ± 10.82**	254.89 ± 22.86**	6.24 ± 1.10**	9.18 ± 0.65**
restraint + Tc (H)	30.59 ± 11.96**	48.29 ± 12.72**	282.66 ± 25.03**	7.02 ± 1.89**	8.55 ± 1.02**

[a]The results are represented as the mean ± SD of values in each group (n = 10). Significance of differences from the normal group was at $^{\#\#}p < 0.01$; that from the model group, at $^{**}p < 0.01$.

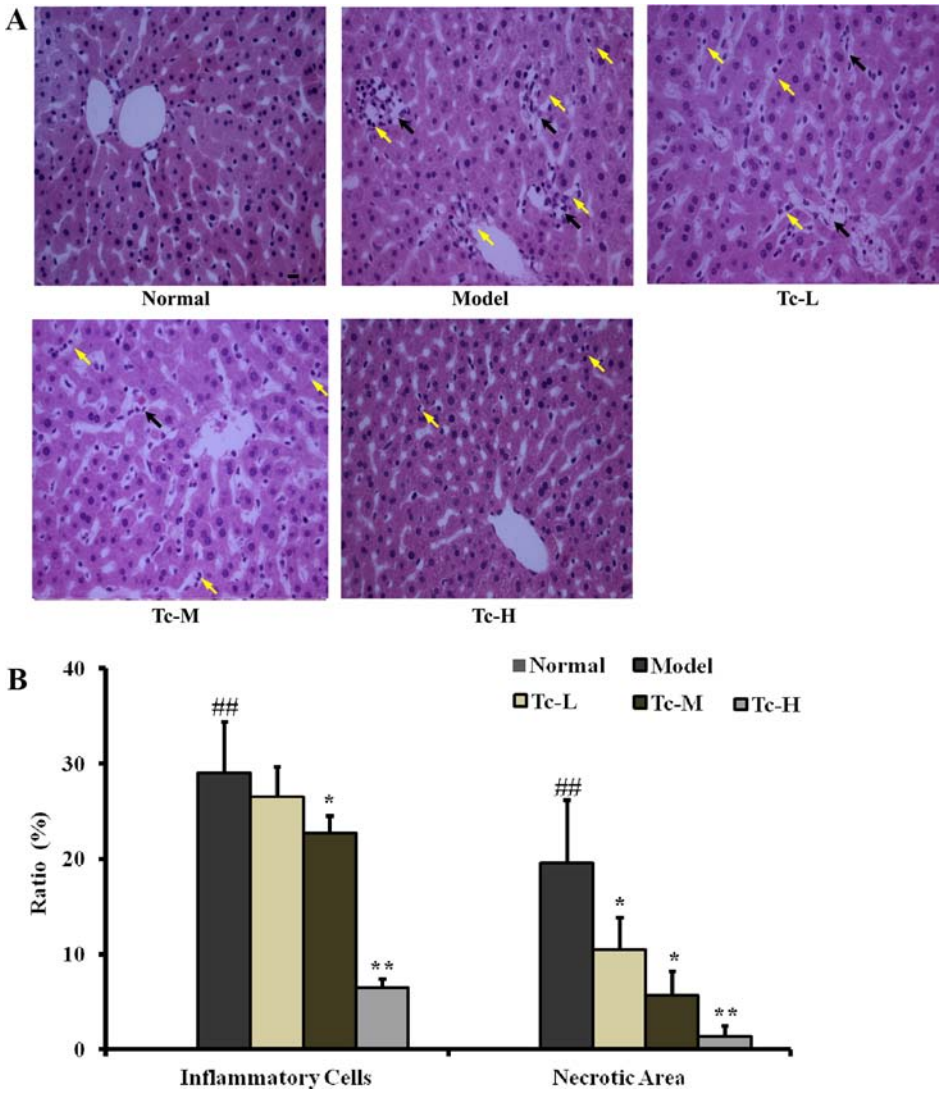

Figure 3. Effects of theacrine on pathological damage of the liver in stressed mice. (A) Hematoxylin and eosin (H&E) staining images of representative liver sections shown at the same magnification (200×), bar = 30 μm. Black arrow represents spotty necrosis; yellow arrow represents inflammatory cell infiltration. (B) Semiquantification of the inflammatory cells (%) and spotty necrosis area (%). The number of inflammatory cells and normal cells were enumerated and expressed as the ratio of inflammatory cells to total hepatic cells. The ratio of spotty necrosis area was calculated by comparing the necrotic area with the same microscopic area. Data represent the mean ± SD of values obtained from 6 mice in each group. The significance of differences from the normal control group was at $^{\#\#}p < 0.01$; that from the model group at $^{*}p < 0.05$, $^{**}p < 0.01$.

arranged around the central vein. There was no sign of inflammation infiltration or necrosis. Compared with normal mice, liver tissue sections from stressed mice showed severe pathological alterations. Spotty, focal necrosis and inflammatory cell infiltration were notably found. By semiquantification, it was discovered that the ratios of inflammatory cell infiltration and necrotic area were significantly elevated. However, in theacrine (10, 20, and 30 mg/kg)-treated groups, the area and extent of necrosis were attenuated and the immigration of inflammatory cells was reduced.

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Antioxidative Capacity of Theacrine Evaluated by ORAC and CAA. The antioxidative capacity of theacrine *in vitro* was evaluated by the ORAC method. The ORAC values of different concentrations of theacrine were calculated as the relative value of the area under the fluorescence decay curve (Figure 4A) using 1 μM Trolox as a standard, and the results

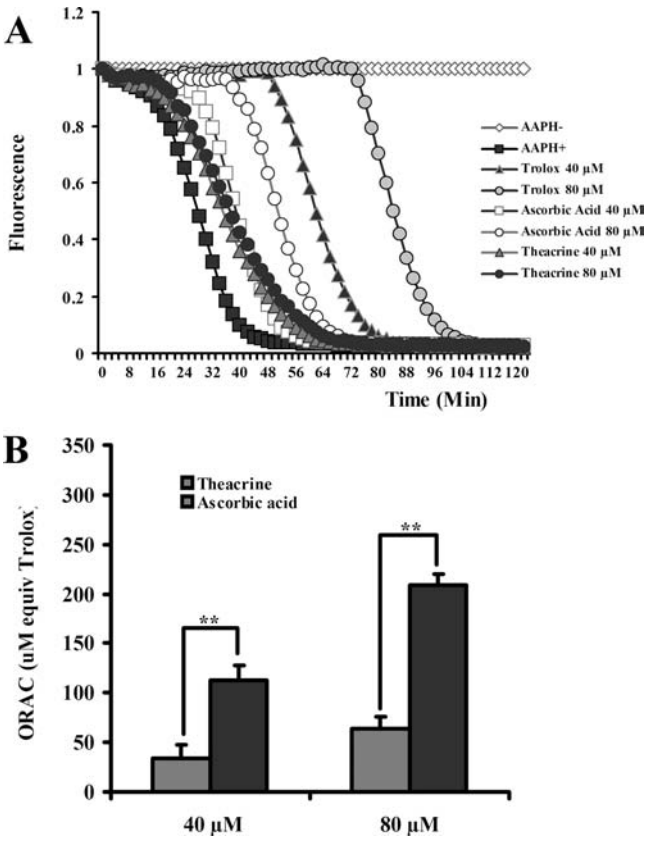

Figure 4. Antioxidant capacity of theacrine *in vitro* evaluated by the ORAC method. (A) Curves of fluorescence decay induced by AAPH in the presence of theacrine, ascorbic acid, and Trolox at different concentrations. (B) ORAC values of theacrine and ascorbic acid. The ORAC value was calculated as the relative value of the area under the fluorescence decay curve using 1 μM Trolox as a standard. Data represent the mean ± SD of values obtained from 5 wells in each group. The significance of differences was from ascorbic acid, $^{**}p < 0.01$.

showed that the antioxidative capacity of theacrine was much lower than ascorbic acid *in vitro* at the same concentration (Figure 4B). The antioxidative capacity of theacrine was also evaluated by the CAA method. The fluorescence changes are shown in Figure 5A. CAA units were calculated based on the area under the curve of fluorescence versus time. The smaller the CAA unit, the higher the antioxidant capability of the sample. As shown in Figure 5B, theacrine showed a comparable antioxidant activity to ascorbic acid at the same dose within the cells. All these results suggested that theacrine possessed strong antioxidative capacity, while this capacity was not due to the direct action of free radical clearance.

Effects of Theacrine on ORAC Level in the Liver and Plasma of Stressed Mice. The results showed that ORAC levels in both the plasma (Table 2) and liver (Table 3) of the model group were comparatively lower than those of the normal group ($p < 0.01$). Compared with the model group,

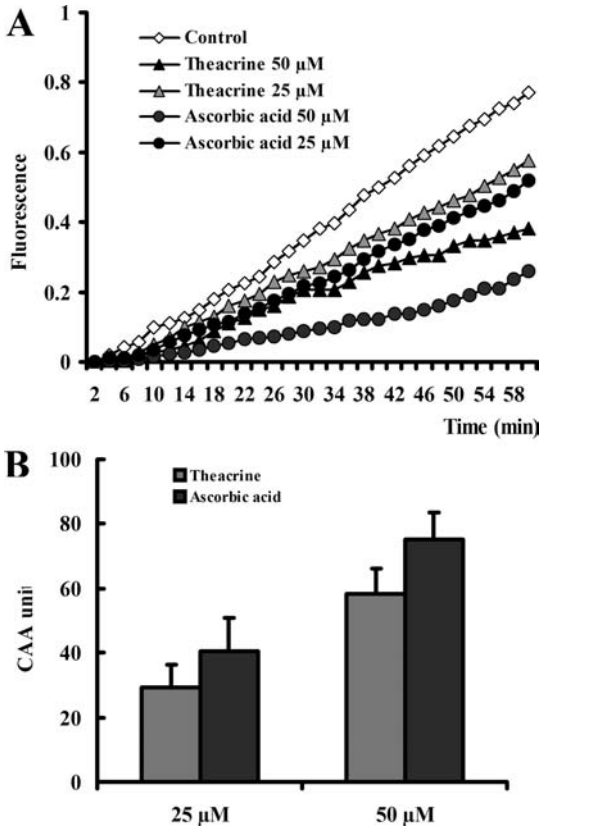

Figure 5. Antioxidant activity of theacrine evaluated by the CAA method. (A) Peroxyl radical-induced oxidation of DCFH to DCF in RAW264.7 cells and the inhibition of oxidation by theacrine and ascorbic acid at different concentrations. (B) CAA units of theacrine and ascorbic acid. The CAA unit was calculated based on the difference in area under the curve between tested samples and control wells. Data represent the mean ± SD of values obtained from 5 wells in each group.

Table 3. Effects of Theacrine on the Content of MDA and the Level of ORAC in the Liver of Restraint-Stressed Mice[a]

	liver	
group	MDA (nmol/mg pro)	ORAC (μM Troloxequiv)
normal control	1.62 ± 0.45	20.53 ± 1.55
model (restraint)	2.71 ± 0.43$^{\#\#}$	13.30 ± 2.26$^{\#\#}$
restraint + Tc (L)	2.08 ± 0.56*	16.77 ± 1.98*
restraint + Tc (M)	1.71 ± 0.55**	18.96 ± 3.04**
restraint + Tc (H)	1.68 ± 0.31**	19.53 ± 2.88**

[a]The results represent the mean ± SD of values in each group (n = 10). The significance of differences from the normal group was at $^{\#\#}p < 0.01$; that from the model group, at $^{*}p < 0.05$, $^{**}p < 0.01$.

theacrine treatment (10, 20, 30 mg/kg) remarkably elevated the ORAC level in both the liver and plasma ($p < 0.01$).

Effects of Theacrine on SOD Activity and MDA Content in Plasma of Stressed Mice. As shown in Table 2, SOD activity in the plasma was significantly decreased in the model group when compared with the normal control group (193.10 ± 34.66 vs 261.61 ± 32.50 U/mL), while the MDA level in the model group was significantly higher than that in the normal control group (10.56 ± 1.74 vs 6.16 ± 1.44 nmol/mL). Theacrine (10, 20, 30 mg/kg) was found to significantly increase SOD activity and decrease MDA content in the plasma ($p < 0.01$).

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Table 4. Effects of Theacrine on the Activities of SOD, XOD, GPx, CAT, and GST in the Liver of Restraint-Stressed Mice[a]

	liver				
group	SOD (U/mg pro)	GPx (U/mg pro)	XOD (U/g pro)	CAT (U/mg pro)	GST (U/mg pro)
normal control	417.56 ± 69.71	83.17 ± 12.54	4.34 ± 1.54	87.21 ± 8.66	129.43 ± 16.65
model (restraint)	220.70 ± 55.01##	42.31 ± 15.63##	6.91 ± 1.21##	55.71 ± 11.23##	89.29 ± 8.56##
restraint + Tc (L)	272.52 ± 40.91*	58.60 ± 14.48	5.24 ± 1.48*	59.34 ± 9.98	81.32 ± 11.28
restraint + Tc (M)	308.64 ± 94.48*	68.67 ± 13.44**	4.54 ± 0.79**	76.54 ± 12.25*	93.26 ± 13.94
restraint + Tc (H)	376.09 ± 69.81**	76.33 ± 17.60**	4.02 ± 0.81**	79.74 ± 18.02*	101.32 ± 14.68

[a]The results represent the mean ± SD of values in each group (n = 10). The significance of differences from the normal group was at ##p < 0.01; that from the model group, at *p < 0.05, **p < 0.01.

Effects of Theacrine on SOD, GPx, CAT, GST, and XOD Activities and MDA Content in the Liver of Stressed Mice. As shown in Table 4, restraint stress significantly suppressed SOD, GPx, CAT, and GST activities and increased XOD activity in the liver when compared with the normal control group (p < 0.01). Meanwhile, a significant increase of MDA content in the liver was caused by restraint stress (p < 0.01). Theacrine administration (10, 20, 30 mg/kg) could increase the activity of SOD and GPx while decreasing that of XOD in the restraint stress mice in a dose-dependent manner (p < 0.01). CAT activity was also significantly elevated by theacrine treatment (20, 30 mg/kg, p < 0.05). Nevertheless, theacrine had no significant influence on GST activity. Moreover, as shown in Table 3, MDA content in the liver was restored by theacrine treatment (10, 20, 30 mg/kg, p < 0.01).

Effects of Theacrine on Gene Expressions of Cu/ZnSOD, MnSOD, GPx, CAT, IL-1β, IL-6, TNF-α, and IFN-γ in the Liver of Stressed Mice. The mRNA levels of hepatic Cu/ZnSOD, MnSOD, GPx, and CAT in the model group were all significantly decreased when compared with the normal control group (p < 0.01 or p < 0.05) (Figure 6). On the other hand, the expressions of IL-1, IL-6, TNF-α, and IFN-γ were increased by restraint stress in mice (p < 0.01) (Figure 7). However, theacrine could obviously reverse restraint stress-induced changes of these gene expressions in the liver (10, 20, 30 mg/kg for Cu/ZnSOD, MnSOD, and GPx; 20, 30 mg/kg for CAT; p < 0.05 or p < 0.01) (Figure 6 and Figure 7).

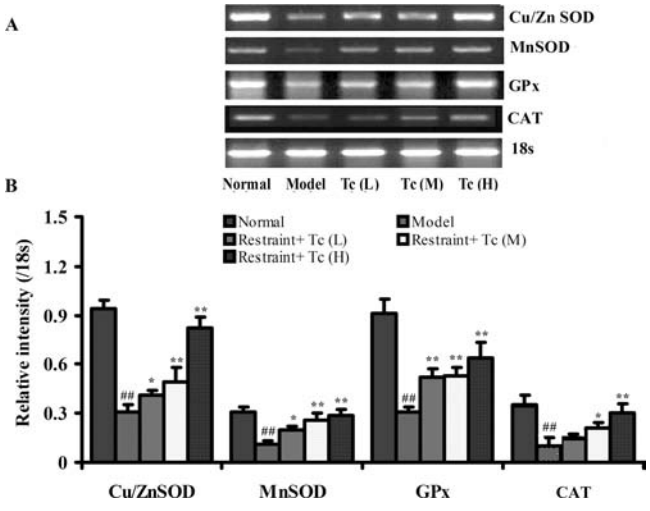

Figure 6. Effects of theacrine on Cu/ZnSOD, MnSOD, CAT, and GPx mRNA expressions in the liver of stressed mice. (A) Agarose gel electrophoresis of RT-PCR amplication of CuZnSOD, MnSOD, CAT, GPx, and 18s mRNAs. (B) Densiometric analysis of PCR products of CuZnSOD, MnSOD, CAT, and GPx mRNAs. Results were generated as relative intensity units by densitometry and expressed as the ratio to 18s. Data represent the mean ± SD of values obtained from 10 mice in each group. The significance of differences from the normal control group was at ##p < 0.01; that from the model group, at *p < 0.05, **p < 0.01.

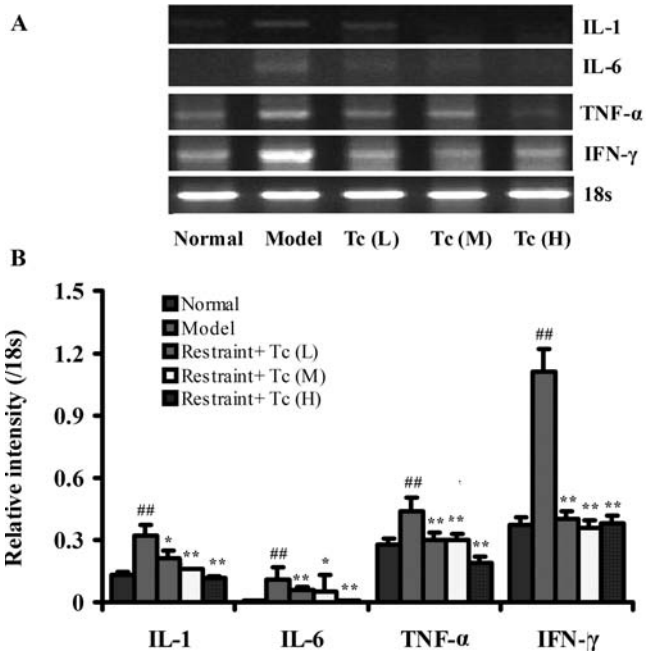

Figure 7. Effects of theacrine on IL-1β, IL-6, TNF-α, and IFN-γ mRNA expressions in the liver of stressed mice. (A) Agarose gel electrophoresis of RT-PCR amplication of IL-1β, IL-6, TNF-α, IFN-γ, and 18s mRNAs. (B) Densiometric analysis of PCR products of IL-1β, IL-6, TNF-α, and IFN-γ mRNAs. Results were generated as relative intensity units by densitometry and expressed as the ratio to 18s. Data represent the mean ± SD of values obtained from 10 mice in each group. The significance of differences from the normal control group was at ##p < 0.01; that from the model group, at *p < 0.05, **p < 0.01.

DISCUSSION

In accordance with our previous reports,[10,11] markedly elevated levels of ALT and AST, two markers of liver damage, were observed in the plasma of mice after restraint stress. Meanwhile, pathological damage, including spotty and focal hepatic necrosis and inflammatory cell infiltration, was also observed in the liver of stressed mice. Oxidative stress, a condition with imbalanced production and detoxification of reactive oxygen species (ROS), was indicated to play a key role in the mechanism responsible for restraint stress-provoked diseases.[5,8,11] In the present study, mice subjected to restraint

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for 18 h showed a significant decrease in the levels of plasma and liver ORAC, which is an indicator of the total antioxidative capacity. There are two major antioxidant defense systems within the cells, which are low-molecular-weight antioxidants, such as vitamins C and E and GSH, and antioxidant enzymes such as SOD, CAT, GPx, and GST.[24,25] SODs (Cu/ZnSOD and MnSOD) are responsible for catalyzing the dismutation of superoxide anions to oxygen and hydrogen peroxide, which can be further cleared by GPx and CAT through converting it into water.[26−28] In parallel, GSH and its related enzymes, such as GPx and GST, play an important role in detoxification, including scavenging ROS and lipid peroxidation.[29] GPx can reduce lipid hydroperoxides to their corresponding alcohols, while GST can catalyze the conjugation of the reduced form of glutathione to xenobiotic substrates. Unlike SOD, CAT, GPx, and GST, XOD contributes to the generation of free radicals by catalyzing the oxidation of hypoxanthine to xanthine.[30] Therefore, we assayed the changes of the activities and gene expression levels of antioxidative enzymes in this study. The results showed that total SOD activity in plasma and the liver was significantly decreased in restraint-stressed mice. A decrease of GPx, CAT, and GST activities as well as an increase of XOD activity in the liver were also caused by restraint stress. In addition, gene expressions of Cu/ZnSOD, MnSOD, GPx, and CAT in the liver were suppressed. All of the above suggested that restraint stress-provoked liver damage was associated with the occurrence of oxidative stress. This notion was further supported by the elevated contents of MDA, an end product of lipid peroxidation, in the plasma and liver of stressed mice.

Theacrine, a purine alkaloid obtained from *C. assamica* var. *kucha*, was found to attenuate restraint stress-provoked liver damage, as reflected by the recovered ALT and AST levels in the plasma, as well as the decreased inflammatory cell infiltration and focal necrosis in the liver. Further investigation found that the protective effect of theacrine was related with the improvement of oxidative stress via its antioxidant activity, as indicated by the elevated ORAC level and reduced MDA content in the plasma and liver of theacrine groups. The antioxidative capacity of theacrine was further confirmed by *in vitro* ORAC and CAA assays. Results of the ORAC assay showed that the antioxidative capacity of theacrine was much lower than ascorbic acid at the same concentration. However, results of the CAA assay showed that theacrine possessed comparable antioxidant activity to ascorbic acid at the same dose within the cells. These results together suggested that theacrine possessed strong antioxidative capacity, while this capacity was not due to the direct action of free radical clearance. The antioxidative activity might be related to the strengthening of the antioxidant system *in vivo*, as reflected by the elevated activities of SOD, GPx, and CAT, along with the reduced activity of XOD by theacrine treatment. Meanwhile, theacrine could also up-regulate gene expressions of these antioxidant enzymes in stressed mice. Thus, it can be inferred that the anti-inflammation action of theacrine was related with the regulation of antioxidant enzymes from a gene level.

In addition to induction of direct cell injury and apoptosis in the liver, ROS can affect the pathophysiology indirectly by enhancing the formation of pro-inflammatory mediators.[31,32] TNF-α is a cytokine product of monocytes and macrophages that is suggested to be implicated in cellular injury[33] and is thought to be a critical driving force of inflammatory damage in liver diseases.[34] In addition, IL-1β is another pro-inflammatory factor, which was reported to induce the synthesis of several proteins with important physiological functions during inflammatory responses.[35] Moreover, IL-6 is a circulating cytokine known to be secreted from a number of different cells during inflammation, including activated macrophages and lymphocytes.[36] Studies had shown that psychological stress could elevate circulating concentrations of IL-6.[37,38] Apart from the above mediators, INF-γ was also indicated to be a trigger of liver disease.[39,40] Clinical observations indicated that IFN-γ was positively associated with elevated levels of ALT in patients with chronic hepatitis.[41] Accordingly, in this study we examined the changes of these pro-inflammatory mediators at gene levels by PCR. It was found that restraint stress significantly up-regulated the expressions of TNF-α, IL-1, IL-6, and IFN-γ in the liver. However, when stressed mice were pretreated with theacrine, mRNA levels of these pro-inflammation mediators were significantly decreased. These results demonstrated that theacrine possessed anti-inflammation ability, which also contributed to its attenuation on liver damage induced by restraint stress.

In summary, the present work proved that oral administration of theacrine could attenuate restraint stress-provoked liver damage, and the mechanism was associated with its antioxidative and anti-inflammatory activities. Theacrine was found to strengthen the antioxidant system and promote pro-inflammatory mediator expression *in vivo*, rather than clearing free radicals directly. Since theacrine is commonly found in *C. assamica* var. *kucha*, theacrine might be helpful in the prevention of stress-related diseases, and this natural active compound is a potential therapeutic candidate for hepatic diseases.

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Notes

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