Liver metabolomics study reveals protective function of *Phyllanthus urinaria* against *CCl₄*-induced liver injury

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[ABSTRACT] *Phyllanthus Urinaria* L. (PUL) is a traditional Chinese medicine used to treat hepatic and renal disorders. However, the mechanism of its hepatoprotective action is not fully understood. In the present study, blood biochemical indexes and liver histopathological changes were used to estimate the extent of hepatic injury. GC/MS and LC/MS-based untargeted metabolomics were used in combination to characterize the potential biomarkers associated with the protective activity of PUL against *CCl₄*-induced liver injury in rats. PUL treatment could reverse the increase in ALT, AST and ALP induced by *CCl₄* and attenuate the pathological changes in rat liver. Significant changes in liver metabolic profiling were observed in PUL-treated group compared with liver injury model group. Seventeen biomarkers related to the hepatoprotective effects of PUL against *CCl₄*-induced liver injury were screened out using non-parametric test and Pearson’s correlation analysis (OPLS-DA). The results suggested that the potential hepatoprotective effects of PUL in attenuating *CCl₄*-induced hepatotoxicity could be partially attributed to regulating L-carnitine, taurocholic acid, and amino acids metabolism, which may become promising targets for treatment of liver toxicity. In conclusion, this study provides new insights into the mechanism of the hepatoprotection of *Phyllanthus Urinaria*.

[KEY WORDS] Metabolomics; Hepatic protection; *Phyllanthus Urinaria*; Carbon tetrachloride


Introduction

Acute and chronic liver injuries are attributed to various pathological factors, such as viruses, alcohol and other chemical reagents, causing extremely high mortality and morbidity around the world [ref]. Liver is a multirunctional organ involved in a variety of metabolic activities. Therefore any injury to liver by hepatotoxic reagents would lead to serious disturbance in the whole body metabolism [1]. The chemical hepatic injury induced by Carbon tetrachloride (*CCl₄*) in rat and mouse models has been found to be similar to that in humans [2]. Therefore *CCl₄* has been widely used to investigate the mechanism of chemical-induced hepatic injury in animal model since 1928 [3]. Although the mechanism of liver injury is not completely revealed, it is clear that reactive oxygen species (ROS) plays an important role in pathological changes in liver [ref]. In liver cells, *CCl₄* activates cytochrome P-450 enzyme (CYP) system, generates ROS, initiates lipid peroxidation and causes oxidative stress, which further leads to liver cell injury [4].

*Phyllanthus Urinaria* L. (PUL) (Euphorbiaceae), a
well-known traditional Chinese medicine (TCM), has been used to treat a number of diseases, including dysentery, jaundice, urinary tract infection, and malnutritional stagnation. Recent pharmacological studies have illustrated that PUL has many pharmacological effects, such as anti-HBV, antimetastatic, hepatoprotective, cardioprotective, anti-hypertensive, anti-cancer, and anti-HSV activities. Although PUL has been proven to have protective effects against CCl4-induced liver injury in rats, there is little knowledge regarding the specific mechanism of its action. Understanding the molecular process and the systematical metabolic response to PUL by identifying altered metabolic pathways and networks in a whole living biosystem represents a greater potential because multilayered metabolic regulation may be involved.

Metabolomics, a powerful omics science rapidly developed in systems biology, has been widely applied in comprehensively analyzing the changes of small molecule metabolites (< 1 kDa) and the correlation between physiological pathology and metabolites in complex biochemical networks. Metabolomics involves analytical techniques with good specificity, high sensitivity and selectivity, super reliability, statistical pre-processing classification models and identifying potential biomarker. In recent years, metabolomics, which is based on high-throughput and highly sensitive technology platform such as nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS), has developed rapidly. Metabolomics is a promising approach that has been widely applied in disease diagnosis, molecular pathology, biomarker discovery, toxicity evaluation, and pharmacological researches.

In the present study, for the first time to our knowledge, a combined GC/MS and LC/MS based metabolomics approach was employed to reveal the mechanism of PUL’s hepatoprotective effects in rats. Metabolites related to the protective effects of PUL against CCl4-induced liver injury were screened out. Comprehensive studies have demonstrated that hepatic injury, to a certain extent, could be resolved or regressed by PUL through partially regulating the perturbed metabolic pathways. Results from our study provided a new metabolomics evidence to support the therapeutic value of PUL for liver injury.

**Material and Methods**

**Chemicals and reagents**

Acetonitrile, ethylacetate, and methanol of HPLC grade were purchased from Honeywell (Burdick & Jackson, USA). Deionized water was prepared by a Milli-Q system (Millipore, MA, USA). Methoxymine hydrochloride (MIX), N-methyl-N-trifluoroacetamide (MSTFA), and pyridine were purchased from Sigma-Aldrich (St louis, MO, USA). Other chemicals were of analytical grade. Sodium carboxymethylcellulose (CMC-Na) was obtained from the Sinopharm Chemical Reagent (Shanghai, China). Carbon tetrachloride was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Olive oil was purchased from Chinese Medicine Group Chemical Reagent Co., Ltd. (Shanghai, China). Diagnostic kits for alanine aminotransaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), cholinesterase (CHE), glutathione S-transferase (GSH-ST), total protein (TP), urea (BUN), and γ-glutamyl transferase (γ-GT) were purchased from Nanjing Jiancheng Technology Co., Ltd. (Nanjing, China).

**Phyllanthus urinaria L.** was collected in October 2014 from Guangxi Province, China and identified by Professor LIU Bu-ming at Guangxi Key Laboratory of Traditional Chinese Medicine Quality Standards (Nanning, China).

**Preparation of PUL**

The dried powder of PUL (150 g) was extracted twice with 3 000 mL of ethanol/water (80 : 20, V/V) by ultrasonic extraction at 40 °C for 40 min. The two extracts were combined and filtered to collect the filtrate. Then solvent was evaporated using a rotary evaporator. The residue was reconstructed in 0.5% CMC-Na aqueous solution to obtain crude extract at a concentration of 2 g·mL⁻¹. The overall quality of the extract of Phyllanthus urinaria L. was controlled by chromatographic fingerprints, and the concentration of four lignans, including hypophyllanthin, phyllanthin, nirtetralin, and niranthin, were determined.

**Animal handling and sample collection**

All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals and was authorized by the Animal Ethics Committee of China Pharmaceutical University. Twenty four male Sprague-Dawley (SD) rats (weighing 160 ± 20 g) were purchased from the Experimental Animal Center of Qinglong Mountain (Nanjing, China) and housed in a temperature-controlled environment (22 ± 2 °C) under 12 h/12 h-dark/light cycle, and relative humidity of 50% ± 5%. The rats were acclimatized in plastic cages for 7 days, with a standard rodent diet and water available ad libitum. After acclimatization, they were randomly divided into three groups with six rats in each group: the normal control (N), CCl4-induced liver injury model (M), and PUL treatment (P) groups. The rats in the N and M groups were administrated with 0.05% CMC-Na (W/V) in water for seven consecutive days twice a day from the second day to fifth day’s night and sacrificed on the sixth day. The rats in M and P groups were intraperitoneally (i.p.) injected with 10 % CCl4 (2 mL·kg⁻¹ body weight) diluted in olive oil solution on the zero day (8:00 a.m.) and the third day (8:00 p.m.) to cause liver injury. The rats in N group received a corresponding administration of saline. PUL (2 mL/kg body weight) suspended in 0.05 % CMC-Na was given intragastrically to the rats in P group twice per day for 4 days. Animals in N group received equivalent volume of Milli-Q-filtered water.

The blood samples collected daily (at 7:00 a.m.) from the rats were allowed to clot at 4 °C for 60 min and then centri-
fuged at 8 000 r·min⁻¹ (Eppendorf 5430R, Germany) for 10 min at 4 °C to remove any precipitates. The liver was collected and used for histological examination and metabolomic analyses.

**Biochemical Analysis and Histopathology examination**

Serum ALT, AST, ALP, TBIL, CHE, GSH-ST, TP, BUN and γ-GT levels were measured on a Tecan Infinite 200 PRO automatic analyzer (Switzerland), according to the instructions of assay kits.

Partial liver sections were fixed in 4% (V/V) buffered formalin solution, embedded in paraffin wax, stained with hematoxylin-eosin (H&E), and then examined by light microscopy for histopathology assessment.

**Sample preparation for metabolic profiling**

Liver homogenates were thawed before preparation at room temperature, 100 μL of methanol (containing 5 μg·mL⁻¹ heptadecanoic acid, IS) was added to 10 μL of liver homogenate and the solution was vortex-mixed for 1 min at room temperature to precipitation protein and extract metabolites. Then the solution was centrifuged twice (14 000 r·min⁻¹, 10 min, 4 °C). 25 μL of pyridine (containing 10 mg·mL⁻¹ methoxyamine hydrochloride) was added to 80 μL of the supernatant and the mixture was then vortexed for 1 min and oxidized at 1 200 r·min⁻¹ for 90 min at 37 °C. Then the mixture was evaporated to dryness by the CentriVap Centrifugal Concentrator (Labconco, Kansas, MO, USA) at 45 °C for 2 h. Finally, 120 μL of 50% MSTFA in ethyl acetate was added to each sample and the mixture was vortexed for 1 min. After incubation for 2 h at 37 °C, the mixture was analyzed by GC/MS [27].

The liver homogenate (20 μL) was mixed with 100 μL of acetonitrile and 40 μL of glibenclamide (5 μg·mL⁻¹, IS) and vortexed thoroughly for 5 min. The mixture was centrifuged twice (14 000 r·min⁻¹, 10 min, 4 °C) and the supernatant was transferred into an autosampler vial and prepared for LC/MS analysis [27].

**GC/MS analysis**

Gas chromatography analysis was performed on a GC-MS QP2010 Ultra (Shimadzu Co., Kyoto, Japan) equipped with a fused silica capillary column (Rtx-5MS; 30 m × 0.25 mm i.d., film thickness 0.25 m, Thames Restek, UK). Separation was achieved with a temperature program where the temperature was held at 70 °C for 2 min, then gradually increased at rate of 10 °C/min to 320 °C and kept at 320 °C for 2 min. Helium was employed as the carrier gas and the flow rate was set at 1 mL·min⁻¹. Sample injection volume of 1 μL was used with the split ratio of 50: 1. The mass spectrometer was operated in EI mode (70 eV) with an acquisition range of m/z 45–600 in scan mode. The injector, MS interface and ion source were set at 250 °C, 250 °C and 200 °C, respectively [27].

**LC/MS analysis**

Liver samples were also analyzed by using a Shimadzu Ultrafast LC-ion trap time-of flight MS system. A 5 μL sample was loaded onto a Phenomenex Kinelex C18 column (100 mm × 2.1 mm, 2.6 μm) (Phenomenex, USA). The column and sample glass vials were maintained at 40 °C and 4 °C, respectively. Separation was achieved with a gradient involved 5%–95% acetonitrile (0.1% V/V) formic acid) –aqueous formic acid (0.1% V/V) formic acid) at a flow rate of 0.4 mL·min⁻¹. Positive ion mode and negative ion mode mass spectra were acquired simultaneously by switching the interface voltage between 4.5 and –3.5 kV in a full-scan operation with a scan range of 50–900 m/z. The flow rate of nebulizing gas (N₂) and pressure of drying gas were 1.5 L·min⁻¹ and 100 kPa. The temperature of heat block and curved desorption line (CDL) were both set at 200 °C and the detector voltage of the TOF analyzer was set at 1.70 kV. Mass spectra and chromatograms were acquired and processed with LC/MS solution version 3.0 (Shimadzu, Japan) [27].

**Data pretreatment and multivariate statistical analysis**

The original chromatogram data were processed by Profiling Solution version 1.1 (Shimadzu) for peak deconvolution and alignment. The primary parameters were set as follows: ion m/z tolerance (500 mDa) for GC/MS and (25 mDa) for LC/MS analysis, ion retention time tolerance (0.05 min) for GC/MS and (0.3 min) for LC/MS analysis, and ion intensity threshold both (10 000 counts). Other parameters were set as default. A resulting matrix consisting of matched ion features with retention time, m/z value, and corresponding intensities was generated and exported to an excel table. The data were processed according to “80% rule in test samples” and “30% rule in quality control (QC) samples”: only the variables with values above zero in at least 80% of each group were kept for the following analysis [28]. The individual ion fragment intensity was normalized to the intensity of the sum intensity of all peaks in the relative chromatogram. Then the variables with relative standard deviation (RSD) lower than 30% in QC samples were kept for multivariate statistical analysis. Unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analyses (OPLS-DA) were performed using SIMCA-P version 13.0 (Umetrics, Sweden) after pareto scaling. Further, according to corresponding variable importance in the projection (VIP) values calculated in the OPLS-DA model and P-values in nonparametric tests (Wilcoxon, Mann–Whitney test), variables with VIP > 1.0 and P < 0.05 were selected as potential biomarker for further identification [29].

**Potential biomarker identification and metabolic pathway reconstruction**

Preliminary compound identification in GC/MS by using the NIST and Willey El mass spectral library search was performed with the Shimadzu GC/MS solution software (version 2.7). Peaks with more than 70% similarity index were assigned compound names. For identification of the potential biomarkers, chromatographic and mass spectrometric information such as retention times (tR), m/z, and MS/MS fragmentation patterns were used to compare with that commercially available reference standards or data published in literatures or databases (HMDB, METIN). Pearson’s correlation
analysis between metabolites and biochemical parameters were performed by SPSS 19.0 to identify potential relations between altered metabolites and the AUC of hepatic enzyme activities in seven consecutive days. The heat map of Pearson correlations was used to visualize using MultiExperiment Viewer v.4.8. Finally, the potential metabolites were connected by Metaboanalyst for pathway analysis. The most biologically vital and correlative pathways involved in the metabolic modulations were under investigation.

Results and Discussion

Biochemical parameters

Nine serum hepatic enzymes activities were measured over seven continuous days as indexes of liver damage. Compared with the normal control (N) group, highly significant increases in serum ALT, AST and ALP levels were observed in the liver injury (M) group. The administration of PUL significantly reduced the serum levels of ALT, AST, and ALP activities (Fig. S1A-C).

In order to take cumulative effect into consideration, the AUC (area under the curve) of serum ALT, AST and ALP levels over seven continuous days were calculated. As shown in Figs. 1A-C, in the rats treated with PUL, the elevated AUC of serum ALT, AST and ALP induced by CCl4 were reduced significantly, which suggested that PUL could effectively attenuate CCl4-induced hepatotoxicity.

Liver histopathology

Liver histopathological examination was performed for detection of abnormalities on the structure of the hepatocytes, and the results revealed that CCl4-induced liver injury was attenuated by PUL. As shown in Fig. 1D, the liver samples obtained from the normal control (N) group rats showed basically complete organelle structure and well structural integrity of nuclei. The specimens in the liver injury group (M), showed several pathological damages (Fig. 1E), including inflammatory cell infiltration, steatosis or microvesicular fatty degeneration. The specimens from the rats treated with PUL showed less steatosis or microvesicular fatty degeneration or mild inflammatory cell infiltration (Fig. 1F). The above results suggested PUL could remarkably ameliorated CCl4-induced liver injury, which were consistent with that of biochemical parameters.

Multivariate statistical analysis

At the first step of statistical analysis, PCA was employed to visualize grouping trends in samples. PCA and PCA scores of the liver samples are shown in supplementary Fig. S2A-C. Clear group separation of sample points suggested that exposure to CCl4 made a vast difference in liver metabolic profiles of the N and M groups, and at the same time indicated that the liver injury model was established successfully in the present study. The QC samples clustered tightly, indicating that the GC/MS and LC/MS systems were stable throughout the whole run. To highlight the metabolite difference between groups, feature selections were performed by using OPLS-DA. In OPLS-DA analysis, the value of R2Y describes the fraction of Y variance by a specific model component, while the value of Q2 describes the predictive accuracy of the model. Typically, models with Q2 values greater than or equal to 0.5 are generally considered to have good predictive capability [30-31]. In the present study, a series of OPLS-DA were applied to discover variables that account for group separation between the N and M groups, as well as M and P groups, respectively. Figs. 2A-C display the results of the OPLS-DA, showing an appreciable separation of the data pertaining to the three groups. These results suggested PUL altered CCl4-induced hepatotoxicity in liver metabolic network, which was consistent with the results obtained from the assay of serum hepatic enzymes activities and the histopathological examination of liver.

Correlation analysis between marker metabolites and biochemistry parameters

Seventeen biomarkers related to the hepatoprotective effects of PUL against CCl4-induced liver injury were screened...
out using OPLS-DA (VIP > 1) and nonparametric test ($P < 0.05$). In order to identify the potential links between these marker metabolites and biochemistry parameters, Pearson correlation analysis was then conducted. As shown in Fig. 3, the heatmap revealed a wide range of correlation coefficients among the metabolites and biochemical parameters, ranging from 1.0 (maximum positive correlation) to $-1.0$ (maximum negative correlation), with 0 indicating no correlation. ALT activity level was positively related to AST (pearson pairwise correlation coefficients 0.986) and ALP (pearson pairwise correlation coefficients 0.848) as well as amino acids (AAs), lysophosphatidylethanolamine (LysoPE), fatty acids, and taurocholic acid. On the contrary, liver injury parameters (ALT, AST, and ALP) had a negative relationship with L-carnitine and sugars. Additionally, L-carnitine was strongly and positively correlated with sugars, whereas both were negatively correlated with amino acids, LysoPE, and taurocholic acid. The correlation analysis results further confirmed that these seventeen biomarkers were in close relationship with liver injury and the regulation of PUL.

**Effects of PUL on metabolic changes in CCl₄-induced liver injury**

Acute chemical liver injury has become an important risk factor for morbidity and mortality in addition to viral hepatitis in the world [ref]. It is of high value to find out effective therapy in liver injury. Although great efforts have been made to search for efficient hepatoprotective therapies, no effective hepatoprotective chemical drugs are available until now. Chinese herbal medicine plays a major role in the treatment of hepatic disorders. A number of medicinal plants and their formulations are used to cure hepatic disorders [32-33]. Despite there are many pharmacological researches on the hepatoprotective effects of *Phyllanthus Urinaria* L. [34-35], the evidence for specific mechanism of its hepatoprotective action is still lacking. Metabolomics reflects the most downstream metabolite information, which is the direct response to pathophysiological changes. In the present study, we investigated the metabolic changes of metabolites using CCl₄-induced liver injury model and demonstrated that PUL conferred a hepatoprotective effect. The trends of the screened out marker metabolites induced by CCl₄ in this study was consistent with published researches [36-37]. As a result, fourteen of seventeen marker metabolites which were perturbed by CCl₄ were found to be restored to normal level by PUL (Fig. 4). The concentrations of L-carnitine and sugars were significant decreased after CCl₄ exposure, whereas the other potential biomarkers exhibited the opposite tendency. As shown in supplementary Fig. S3, metabolic pathway analysis revealed that pathways associated with liver injury included phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine biosynthesis, as well as taurine and hypotaurine metabolism. PUL could ameliorate CCl₄-induced liver injury through restoring complex responses from multiple interconnected metabolic pathways (Fig. 5).

Liver is the center organ of the amino acid metabolism,
any injury to liver function could result in disturbance of amino acids' concentrations [38]. In the present study, we found that almost all amino acids levels were increased after CCl₄ exposure, which may be associated with decreased protein synthesis, amino acid metabolism, and increased protein catabolism. Leucine (Leu) and Isoleucine (Ile), which belong to branch chain amino acids (BCAAs), cannot be synthesized endogenously and classified as essential amino-acid [39]. It has been reported that BCAA catabolism has an important function in regulating lipogenesis and protein biosynthesis, as well as inducing a significant reduction in inflammation and preventing liver injury owing to the induction of antioxidation and free-radical scavenger activities [40-41]. The elevation of liver Leu and Ile in CCl₄ induced liver injury groups implied that the catabolism of those amino acids had been suppressed. PUL’s hepatoprotective activity against CCl₄ might through relieving perturbed BCAA catabolism and inducing free-radical scavenger activities. Proline, considered as “functional amino acids”, plays important roles in protein synthesis, metabolism (particularly the synthesis of polyamines, arginine, and glutamate via pyrroline-5-carboxylate), as well as antioxidative reactions [42]. The metabolic process of proline can effectively induce anti-oxidative system against the oxidative stress caused by liver injury [43-44]. In the present study, the increased proline level induced by CCl₄ was significantly down-regulated by PUL, which suggested that PUL may reverse CCl₄-induced hepatotoxicity by relieving perturbed metabolic changes related to proline and effectively inducing antioxidative reactions. Ornithine, a non-protein amino acid, plays an essential role in the urea cycle. The synthesis of urea is one of the unique functions of liver [45]. The ammonia in the blood is converted to urea mainly through urea cycle and then excreted from the body. The suppression of urea cycle can lead to ammonia intoxication in liver damage [46]. Compared...
with the normal group, a significant increase in the level of ornithine in CCl4 group indicated that CCl4 induced hepatotoxicity which disturbs the urea cycle. Furthermore, we also found that PUL administration could restore the increased ornithine concentration to normal level. Phenylalanine is an essential aromatic amino acid, playing an important role in the synthesis hormones and neurotransmitter mainly in the liver [ref]. It has been reported that liver damage could cause phenylalanine metabolic disorders leading to the significant elevation of phenylalanine levels [47], which was also found in our study. The data in our study unveiled that it could be regulated to normal by PUL through partially regulating the perturbed metabolic pathways of phenylalanine. These changes of amino acids in CCl4 group indicated that acute CCl4 treatment caused oxidative stresses, as well as altered metabolic and osmotic functions of liver. Our data suggested that PUL could protect the liver against CCl4-induced damage, and this hepatoprotective effect was at least in part due to its ability through relieving effect of depression factors and regulating these amino acids to normal level.

Bile acids (BAs), produced only in the liver as the major end products of cholesterol catabolism, not only acts the classic function of promoting hepatobiliary secretion of endogenous metabolites, but also plays equally important roles in regulating the metabolism of glucose and lipids in the enterohepatic system, and energy expenditure in peripheral tissues [48-49]. BAs, especially some conjugated forms such as taurocholic acid and chenodeoxycholic acid, can be considered as sensitive biomarkers of chemical induced liver injury [50-51]. It has been reported that CCl4 exposure could lead to the significant elevation of taurocholic acid [52], which was also found in our study. Furthermore, we also found that PUL administration could restore the increased taurocholic acid concentration to normal level. Thus, we speculated that PUL’s hepatoprotective activity against CCl4 might through relieving perturbed primary bile acid biosynthesis and cholesterol metabolism.

L-carnitine, which is synthesized by lysine and methionine in the liver, has a critical role in shuttling free fatty acids from the cytosol into mitochondria matrix and producing ATP for the metabolic process of fatty acids β-oxidation in mitochondria [53]. More likely, it may function as an antioxidant [54]. The effect of L-carnitine on lipid peroxidation and antioxidant capacity has been uncovered [55-57]. L-carnitine is found at a lower level in hepatotoxicity and the supplementary administration of L-carnitine could be of hepatoprotective effect against liver injury [58-59]. In the present study, the decreased L-carnitine level induced by CCl4 was significantly up-regulated by PUL, which suggested that PUL may reverse CCl4-induced hepatotoxicity though relieving perturbed metabolic changes related to L-carnitine and inhibiting free radical generation.

As revealed by the metabolomic profiling analyses in our study, the levels of L-carnitine, taurocholic acid, and amino acids in the liver of CCl4 induced hepatotoxic rats treated with PUL were similar to that of the normal control rats.

**Conclusion**

The present study utilized a liver metabolomics approach, combined with serum biochemical and histopathological tests, to investigate the hepatoprotective effect of PUL on the CCl4-induced liver injury. Significant changes were found in both GC/MS and LC/MS liver metabolic profiles. Seventeen biomarkers related to the hepatoprotective effects of PUL against CCl4-induced liver injury were discovered. Comprehensive studies demonstrated that hepatic injury, to a certain extent, could be resolved or regressed by PUL through partially regulating the perturbed metabolic pathways. Our study provided new insights into the mechanism of the hepatoprotection of Phyllanthus Urinaria L.. This work also proved that comprehensive metabolomics analysis could greatly facilitate and provide useful evidence to further comprehensively understand the mechanism of action for TCM.

**References**


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