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ORIGINAL RESEARCH

Aqueous Extract of Rhubarb Promotes Hepatotoxicity via Facilitating PKM2-Mediated Aerobic Glycolysis in a Rat Model of Diethylnitrosamine-Induced Liver Cancer

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Objective: To identify the polar parts in Rhubarb that cause hepatotoxicity and explore the underlying mechanisms.

Methods: The rat model of liver cancer was established by gavage of diethylnitrosamine (DEN; 0.002 g/rat) for 14 weeks. Starting from the 11th week, Rhubarb granule (4 g/kg), aqueous, ethyl acetate and n-butanol extract of Rhubarb or Rhein equivalent to a dose of 4 g/kg Rhubarb granule were administered intragastrically for 4 consecutive weeks. Liver tissues from rats treated with DEN and Rhubarb granules were used for non-targeted metabolomics analysis. The correlation between pyruvate kinase isozyme type M2 (PKM2) expression level and the progress and prognosis of hepatocellular carcinoma (HCC) was evaluated through bioinformatics analysis based on TCGA database. Liver tissues and blood samples from rats treated with DEN and aqueous, ethyl acetate and n-butanol extract of Rhubarb were used for the screening of hepatotoxic polar parts of Rhubarb. The liver injuries were evaluated by the changes in pathology, liver function, and the expression levels of proliferating cell nuclear antigen (PCNA) and transforming growth factor beta1 (TGF-β1). The mechanism studies focus on PKM2 expression, and the metabolic reprogramming via detecting the activities of lactate dehydrogenase A (LDHA) and isocitrate dehydrogenase (ICDH). Furthermore, molecular docking analysis was performed to validate the target interaction between Rhein and PKM2, and the hepatotoxicity of Rhein was evaluated by testing liver function in the DEN-induced liver cancer model.

Results: The non-targeted metabolomics analysis revealed that Rhubarb promoted aerobic glycolysis in the rat model of DEN-induced liver cancer. And bioinformatics analysis revealed that high PKM2 expression was closely related to the progression and poor prognosis of HCC. In vivo studies indicated that the aqueous extract of Rhubarb, but not ethyl acetate and n-butanol extract, promoted the liver injuries induced by DEN. The mechanism study showed that the aqueous extract of Rhubarb increased the expression of PKM2 and promoted aerobic glycolysis. Moreover, Rhein had a strong binding affinity for PKM2 and aggravated liver injury in the DEN-induced liver cancer model.

Conclusion: Aqueous extract of Rhubarb promoted hepatotoxicity via facilitating PKM2-mediated aerobic glycolysis in the rat model of DEN-induced liver cancer.

Keywords: aqueous extract of rhubarb, hepatotoxicity, PKM2, aerobic glycolysis, Rhein

Introduction

Rheum palmatum L. (Rhubarb, Dahuang), a traditional Chinese herbal medicine, belongs to the *Polygonaceae* family, and is usually used to treat constipation, damp-heat dysentery, gastrointestinal bleeding, and other diseases.[1,2] Rhubarb

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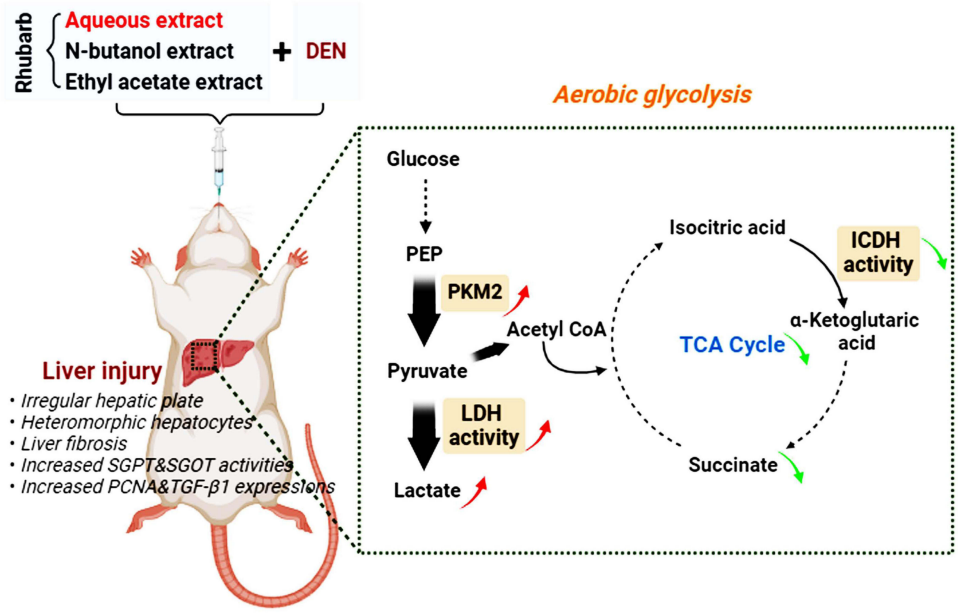
Graphical Abstract



was first recorded in the classic Materia Medica, *Shen Nong Ben Cao Jing*,[3] and has been recorded in the Chinese Pharmacopoeia.[4] Recently, numerous studies showed that Rhubarb, especially the free anthraquinone components, could alleviate various types of liver diseases, such as hepatitis, liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC).[5–8]

Nevertheless, the safety of Rhubarb in the treatment of liver diseases is still controversial. The toxic effects of Rhubarb on liver have been verified in vitro and in vivo.[9,10] The hepatotoxicity of Rhubarb was affected by diverse factors, such as the dosage and duration of administration, dosage form, processing method, and compatibility of formula. Rhubarb causes hepatotoxicity in a dose-dependent manner within a certain range.[11,12] The routine daily dosage of Rhubarb in the 2015 Chinese Pharmacopoeia is 3–15 g, and the clinical usual dosage is 10 g. However, excessive use of Rhubarb frequently occurs in clinical treatment of liver diseases. For instance, the dosages of Rhubarb in Jiawei Sanjia Powder and Dahuang ZheChong Pill are equivalent to 6 and 30 times the usual dosage. Therefore, when Rhubarb is used for clinical diseases treatment, the hepatotoxicity of Rhubarb needs to be taken seriously.

Individual physiological and pathological states remarkably affect the hepatotoxic or hepatoprotective activity of Rhubarb. Recent studies reported that active ingredients of Rhubarb may lead to liver injury through synergistic effects with hepatotoxic reagents.[13,14] Moreover, our previous study revealed that Rhubarb granule at a dosage of 4 g/kg (equivalent to 4 times the clinical usual dosage) did not cause liver injury in normal rats but promoted hepatocarcinogenesis in the DEN-treated rats.[15] This finding indicated that the dose-hepatotoxicity pattern of Rhubarb in the DEN-induced liver cancer model is different from that in the normal physiological conditions. However, which active ingredient in Rhubarb causes liver injury in the rat model of DEN-induced liver cancer was still not clear.

The process of DEN-induced hepatocarcinogenesis in rats largely mimics the pathological process of human HCC, which characterized as liver injury, chronic inflammation, abnormal proliferation of hepatocytes, liver fibrosis and altered immune microenvironment in the liver.[16] During this process, metabolic reprogramming is one of the typical hallmarks of tumor evolution and progression.[17,18] Tumor cells prefer converting glucose into lactate even when oxygen is available, which is termed as aerobic glycolysis or the Warburg effect.[19] The production of ATP through aerobic glycolysis is inefficient, but the produced ATP through accelerated glucose metabolism is sufficient for the rapid growth of tumor cells.[20] Importantly, aerobic glycolysis contributes to the progression and drug resistance of HCC.[21] Three rate-

limiting enzymes in the glycolytic pathway, including hexokinase 2 (HK2), phosphofructokinase 1 (PFK1) and PKM2, play important roles in the regulation of aerobic glycolysis. Our previous study revealed that Rhubarb induced the expression of PKM2 in the DEN-induced liver cancer model, and inhibiting PKM2 expression by using resveratrol could reduce the production of lactate and attenuate liver injury in the Rhubarb-DEN-treated rats.[22] Our results suggested that PKM2-mediated aerobic glycolysis was involved in the hepatotoxicity of Rhubarb. However, the underlying mechanisms need to be further explored.

In the present study, we analyzed alterations of metabolites in rats treated with Rhubarb and DEN by non-targeted metabolomics, and evaluated the role of PKM2 in the progression of HCC by bioinformatics analysis. Moreover, we assessed the hepatotoxicity of different polar parts of Rhubarb in the DEN-induced liver cancer model. Our results indicated that aqueous extract of Rhubarb played a major role in promoting hepatotoxicity induced by Rhubarb-DEN via facilitating PKM2-mediated aerobic glycolysis. Furthermore, we found that the hepatotoxicity had the potential to be relevant to Rhein in Rhubarb. Our work will help establish and improve evaluation methods and indicators for hepatotoxicity of Rhubarb in liver cancer treatment and provide a preliminary basis for the study of high-dose Rhubarb formulations for the treatment of liver cancer.

Material and Methods

Herbal Medicine

Rhubarb granules (batch number: 9045611; Guangdong Yifang Pharmaceutical Co., Ltd.) and raw Rhubarb (batch number: 211100199; producing area: Qingyang, Gansu Province, China; Kangmei Bozhou Century Chinese Medicine Co., Ltd.) were purchased from the First Affiliated Hospital of Anhui University of Chinese Medicine, and authenticated by Professor Peng Huang (College of Pharmacy, Anhui University of Chinese Medicine).

Experimental Animals

Male Sprague-Dawley rats (170 ± 20 g) were provided by the Laboratory Animal Center in Anhui University of Chinese Medicine, and were housed under a specific-pathogen-free (SPF) environment with a 12 h light/dark cycle. All animal experiments were performed following the Anhui University of Chinese Medicine and national guidelines and regulations. And this study was approved by the Academy.

Animal Experimental Ethical Committee at the Anhui University of Chinese Medicine.

(Code of Ethics: AHUCM-rats-2023035).

Hepatic Non-Targeted Metabolomics Analysis

The metabolic changes in the liver tissues of rats treated with DEN, Rhubarb or DEN-Rhubarb were analyzed by non-targeted metabolomics analysis which was performed as previously described.[15]

Bioinformatic Analysis

The levels of PKM2 transcript in HCC and normal liver tissues, as well as survival data of HCC patients, were all obtained from The Cancer Genome Atlas (TCGA) database. The online tool UALCAN (http://ualcan.path.uab.edu./analysis.html) was used to analyze the relationship between PKM2 expression and the progression of HCC.

Extraction of Different Polar Parts of Rhubarb

The raw Rhubarb in powdered form was successively dissolved in an appropriate amount of petroleum ether, ethyl acetate, n-butyl alcohol and distilled water. After adding each reagent, the Rhubarb suspension was heated under reflux for 2 h. The correspondingly different polar parts of Rhubarb were obtained through slightly cooling, filtration, rotary evaporation and drying. The extraction ratios of aqueous extract, ethyl acetate extract and n-butanol extract of Rhubarb were 1.5%, 0.58% and 0.98%, respectively.

Drug Administration

In our previous study, two dosages of Rhubarb granule (4 g/kg and 8 g/kg body weight) were used to treat rats in combination with DEN, and we found that Rhubarb combined with DEN-induced liver injury in a dose-dependent manner. Rhubarb granule at a dosage of 4 g/kg combined with DEN is sufficient to cause liver injury and hepatocarcinogenesis.[15] In the present study, we treated rats with 4 g/kg body weight of Rhubarb granule or equivalent dosage of different polar parts of Rhubarb or Rhein in combination with DEN.

For screening of hepatotoxic polar parts of Rhubarb, the rats were randomly divided into five groups. Group 1 (Control; n = 8) and Group 2 (DEN; n = 8) were, respectively, given normal saline solution (1 mL; Xinhe Yuansheng Pharmaceutical Co., Ltd., China) and DEN (1 mL; 0.002 g/rat; Sigma-Aldrich, St Louis, MO, USA) by gavage four times a week in the afternoon until 14 weeks. Starting at week 11, all of the rats in Group 1 and Group 2 were given normal saline solution (3 mL) by gavage once a day in the morning for 4 weeks. Group 3 (Aqueous extract-DEN; n = 8), Group 4 (Ethyl acetate extract-DEN; n = 8) and Group 5 (N-butanol extract-DEN; n = 8) were all given DEN (1 mL; 0.002 g/rat) by gavage four times a week in the afternoon until 14 weeks. Starting at week 11, the rats in Group 3, Group 4 and Group 5 were, respectively, given aqueous extract (3 mL), ethyl acetate extract (3 mL) and n-butanol extract (3 mL) of Rhubarb by gavage once a day in the morning for 4 weeks. The dosage of each polar part of Rhubarb used was equivalent to 4 g/kg body weight of Rhubarb granule according to extraction ratio.

To investigate the role of Rhein (MCE, Shanghai, China) in promoting hepatotoxicity in the model of DEN-induced liver cancer, the rats were randomly divided into four groups. Group 1' (Control; n = 5) and Group 2' (DEN; n = 5) were same as above Group 1 and Group 2. Group 3' (Rhein; n = 5) and Group 4' (Rhein-DEN; n = 5) were, respectively, given normal saline solution (1 mL) and DEN (1 mL; 0.002 g/rat) by gavage four times a week in the afternoon until 14 weeks.

Starting at week 11, all of the rats in Group 3' and Group 4' were given Rhein (3 mL) by gavage once a day in the morning for 4 weeks. The extraction ratio of Rhein is 0.3%, and the dosage of Rhein used was equivalent to 4 g/kg body weight of Rhubarb granule.

Histologic Examination

Liver tissues were fixed with 10% formalin followed by paraffin embedding. Sections of liver tissues (3 μm) were deparaffinized and rehydrated. The prepared tissue slides were stained with hematoxylin and eosin (H&E; Beyotime Institute of Biotechnology, Jiangsu, China). Collagen fibers were visualized with Masson's trichrome staining (Solarbio, Beijing, China). The liver tissues were examined microscopically (100× and 200× magnification) on an Axioskop 2 microscope (Carl Zeiss, Jena, Germany).

Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) Assays

The activities of ALT and AST was measured using ALT assay kit (Solarbio) and AST assay kit (Solarbio), respectively, as previously described.[15]

Western Blot

Liver tissues from rats were frozen in liquid nitrogen, ground into powder, and then lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology). Proteins were separated with SDS-PAGE and transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany). Five percent skim milk (Beyotime Institute of Biotechnology) was used to block the PVDF membranes. The various primary antibodies (Cell Signaling Technology, Beverly, MA, USA) used in the experiments were as follows: anti-PKM2 (1:1000 dilution), anti-proliferating cell nuclear antigen (PCNA; 1:1600 dilution), anti-transforming growth factor beta1 (TGF-β1; 1:1000 dilution), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000 dilution). After incubation with primary antibodies and washing with TBST, membranes were incubated with secondary antibodies (Cell Signaling Technology) as follows: goat anti-rabbit IgG (1:5000 dilution) and goat anti-mouse IgG (1:5000 dilution). The enhanced chemiluminescence method was used to assay the immunoreactive bands. Western blot was performed at least three times and each repeat was performed as a separate, independent experiment. The results were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Lactate Dehydrogenase (LDH) Activity Assay in Liver Tissues

LDH activity in liver tissues was measured by LDH Activity Assay Kit (Solarbio) according to the manufacturer's instructions. The result was detected spectrophotometrically (450 nm) with the Thermo Varioskan Flash (Thermo Electron Corporation, Vantaa, Finland).

Isocitrate Dehydrogenase (ICDH) Activity Assay in Liver Tissues

ICDH activity in liver tissues was measured by cytoplasmic ICDH Assay kit (Solarbio) according to the manufacturer's instructions. The result was detected spectrophotometrically (340 nm) with the Thermo Varioskan Flash (Thermo Electron Corporation).

Molecular Docking

Molecular docking was performed to predict the interaction between PKM2 and Rhein. The molecular structure of Rhein and proteins were, respectively, obtained from the TCMSP database (https://old.tcmsp-e.com/tcmsp.php) and the RCSB protein database (http://www.rcsb.org). The crystal structure of the protein was imported into Pymol 2.5.7 Software (https://pymol.org/2/) to remove water molecules and ligands and add hydrogen atoms and charges. The molecules were next imported into AutoDock to define the interaction between Rhein and its targets. Targets with lower binding energy and better conformation were selected for presentation.

Statistical Analysis

Data were presented as mean ± SD. Differences were evaluated by Analysis of Variance (ANOVA) followed by Duncan's multiple range tests with SPSS v21.0 software (SPSS, USA), and $p<0.05$ was considered a statistically significant difference.

Results

Rhubarb Promotes Aerobic Glycolysis in the Rat Model of DEN-Induced Liver Cancer

Our previous study suggested that PKM2-mediated aerobic glycolysis might be involved in hepatic precancerous injury induced by Rhubarb combined with DEN in rats.[22] To further confirm the role of aerobic glycolysis in Rhubarb-DEN-induced liver injury and comprehensively understand the effects of Rhubarb-DEN on metabolism in liver tissues, we detected total metabolites by non-targeted metabolomics. The control, Rhubarb-treated, DEN-treated and Rhubarb-DEN-treated groups were analyzed by ultra-high performance liquid chromatography (UHPLC) system in electrospray ionization (ESI)-positive and ESI-negative ion modes. Through non-targeted metabolomics, we revealed a range of different metabolites among these groups.[15] Among these different metabolites, the relative amount of lactate was increased following Rhubarb and DEN treatment (Figure 1A). Moreover, Rhubarb-DEN-treated group revealed a significantly higher level of lactate than DEN-treated group. The lactate in Rhubarb-DEN-treated group was higher but without significance (p = 0.071) when compared to that in Rhubarb-treated group (Figure 1A). Interestingly, the relative amount of succinate changed in various groups in contrast to the change in lactate. We observed that a remarkable decrease in succinate in the Rhubarb-treated and DEN-treated cohorts in comparison to the control group, and Rhubarb-DEN-treated group showed a significantly lower level of succinate than Rhubarb-treated and DEN-treated groups (Figure 1B). Given lactate is the product of glycolysis and succinate is an intermediate product of the tricarboxylic acid (TCA) cycle, the liver in Rhubarb-DEN treated group exhibited a metabolic state of accelerated glycolysis and weakened oxidative phosphorylation, which is a feature of aerobic glycolysis in the process of cellular malignant transformation. Taken together, our results indicated that Rhubarb caused liver injury in rats treated with DEN by enhancing aerobic glycolysis of hepatic cells.

PKM2 Expression is a Promising Biomarker for the Progression of Hepatocellular Carcinoma

To elucidate the role of PKM2 in Rhubarb-DEN-induced hepatocarcinogenesis, we analyzed the expression level of PKM2 in human HCC tissues and its correlation with the tumor progression and prognosis of HCC patients. As shown in

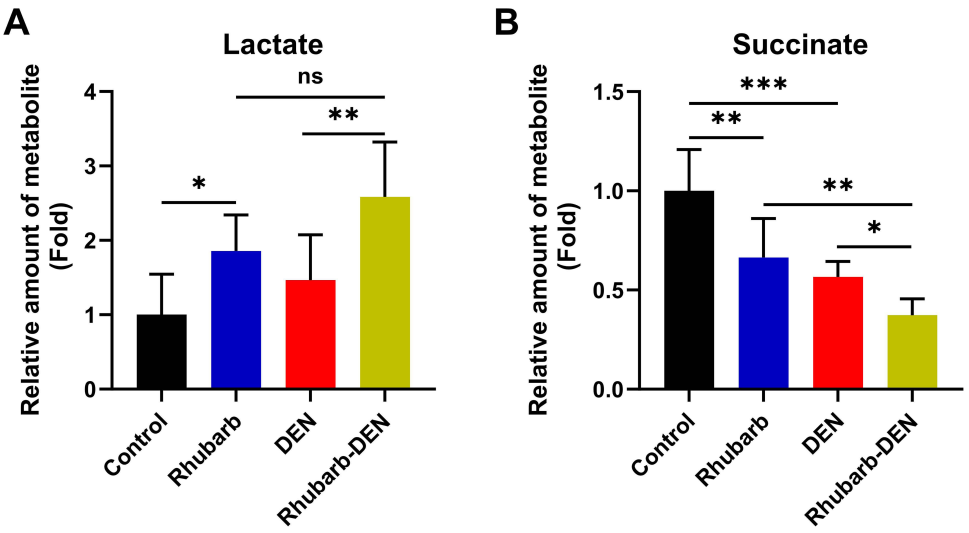


Figure 1 The glucose metabolism analyzed by non-targeted metabolomics. (**A**) The relative amount of lactate in indicated groups. (**B**) The relative amount of succinate in indicated groups. The rat model of liver cancer was established by gavage of DEN (0.002 g/rat) for 14 weeks. Starting from the 11th week, Rhubarb granule (4 g/kg) was administered intragastrically for 4 consecutive weeks. Values are presented as mean ± SD (n = 8 for each group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: non-significant.

Figure 2A, the patterns of PKM2 mRNA expression were analyzed in 371 HCC and 50 normal tissue specimens. In HCC primary tumor specimens, PKM2 mRNA expression was significantly higher than in normal tissue specimens (Figure 2A). Moreover, the relationship between PKM2 expression and clinical parameters of HCC was determined by Wilcoxon signed-rank sum test. We found higher pathological cancer stages and histological tumor grade were associated with the higher PKM2 expression (Figure 2B and C). Furthermore, the effect of PKM2 on patient survival was measured by log rank test. Kaplan–Meier survival curves showed that HCC patients with high-PKM2 expression had a significantly shorter survival time than HCC patients with low/medium-PKM2 expression (Figure 2D). Taken together, the expression level of PKM2 is closely related to the occurrence, development and poor prognosis of HCC, and can be served as a promising biomarker for the diagnosis of HCC.

Aqueous Extract of Rhubarb Promotes Hepatotoxicity in the Rat Model of DEN-Induced Liver Cancer

After determining the hepatotoxic effect of Rhubarb, it is necessary to clarify the hepatotoxic components of Rhubarb in order to further explore the underlying mechanism. Therefore, we next treated the rats with aqueous, ethyl acetate and n-butanol extract of Rhubarb, respectively, to explore which polar parts of Rhubarb promotes hepatotoxicity in the DEN-induced liver cancer model. Pathological changes of liver injury such as irregular hepatic plate and heteromorphic hepatocytes with deep-dyed large nuclei and obvious nucleoli were observed in H&E staining images of liver tissues in DEN-treated and different polar extracts of Rhubarb-DEN-treated groups. Among these groups, the liver injury was most severe in the aqueous extract of Rhubarb-DEN-treated group (Figure 3A). Given liver fibrosis is another outstanding feature of typical environment in which cancer arises in the model of DEN-induced hepatocarcinogenesis,[16] we next evaluated the liver fibrosis in each group using Masson's trichrome staining. Consistent with the pathological changes mentioned above, we observed typical morphological features of liver fibrosis such as increased collagen deposition around the portal vein in aqueous extract of Rhubarb-DEN-treated rats (Figure 3B). Furthermore, we also estimated the function of liver by testing the activities of ALT and AST, the enzymes located in the hepatic cells and leak out from the damaged liver.[23] As shown in Figure 3C, the ALT activity in DEN-treated group and different polar extracts of Rhubarb-DEN-treated groups were increased compared with control group. In particular, the aqueous extract of Rhubarb-DEN-treated group showed the highest activity of ALT, which was significantly higher than that of the DEN-treated group. Moreover, the trend of AST activity change in each group was similar to that of ALT (Figure 3D).

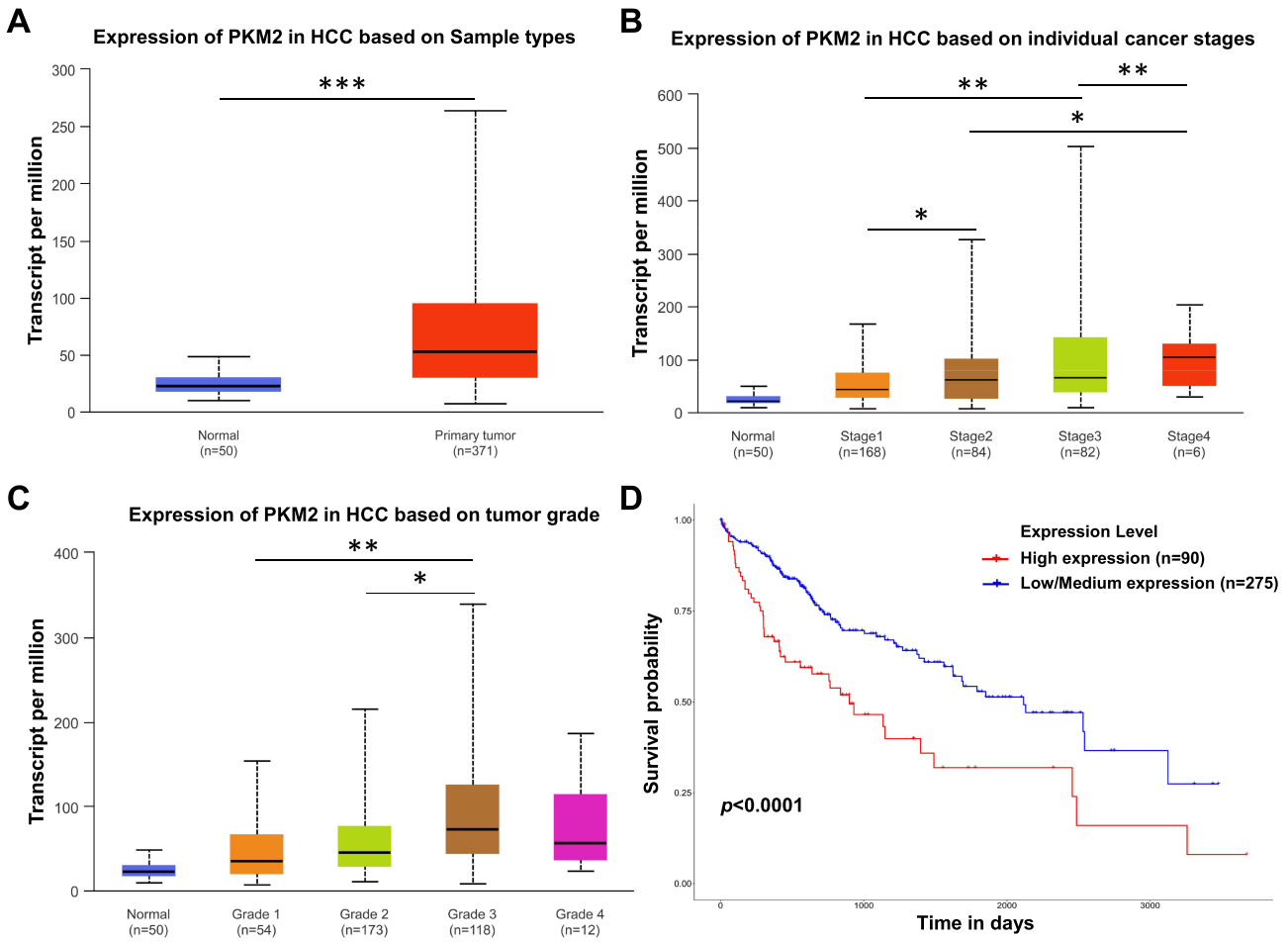


Figure 2 The association between PKM2 expression level and the progression and prognosis of HCC. (**A**) The PKM2 gene expression level in normal (n = 50) and primary tumor (n = 371) samples. (**B**) The PKM2 gene expression level in HCC with different cancer stages. (**C**) The PKM2 gene expression level in HCC with different tumor grades. (**D**) The survival of HCC patients with high and low/medium PKM2 expression. All data are from TCGA database. *p < 0.05, **p < 0.01, ***p < 0.001.

Considering the malignant transformation of hepatocytes is an important process of hepatocarcinogenesis induced by DEN,[16] we also detected the expressions of marker proteins of malignant transformation to explore the roles of different polar parts of Rhubarb in DEN-induced hepatocarcinogenesis. PCNA is a key factor in DNA replication and cell cycle regulation, and the increasing level of PCNA was related to the uncontrolled proliferation of malignant hepatocytes.[24] Consistent with previous researches, DEN markedly increased the expression of PCNA in liver tissues (Figure 3E and F). Interestingly, DEN-induced PCNA expression was significantly enhanced by aqueous extract of Rhubarb but not by ethyl acetate and n-butanol extracts of Rhubarb (Figure 3E and F). We further evaluated the degree of malignant transformation by detecting the expression of TGF-β1. TGF-β1, a cytokine that regulates the differentiation and growth of transforming cells, plays a vital role in accelerating the progression of liver cancer.[25] The expression of TGF-β1 is significantly increased at the early stage of hepatocarcinogenesis of rat.[26] As shown in Figure 3G and H, aqueous extract of Rhubarb-DEN caused the most significant increase in TGF-β1 expression, while DEN alone as well as ethyl acetate and n-butanol extracts of Rhubarb-DEN slightly increased the TGF-β1 expression in liver tissues. Taken together, these results indicated that the water-soluble part of Rhubarb plays a major role in promoting hepatotoxicity under DEN treatment.

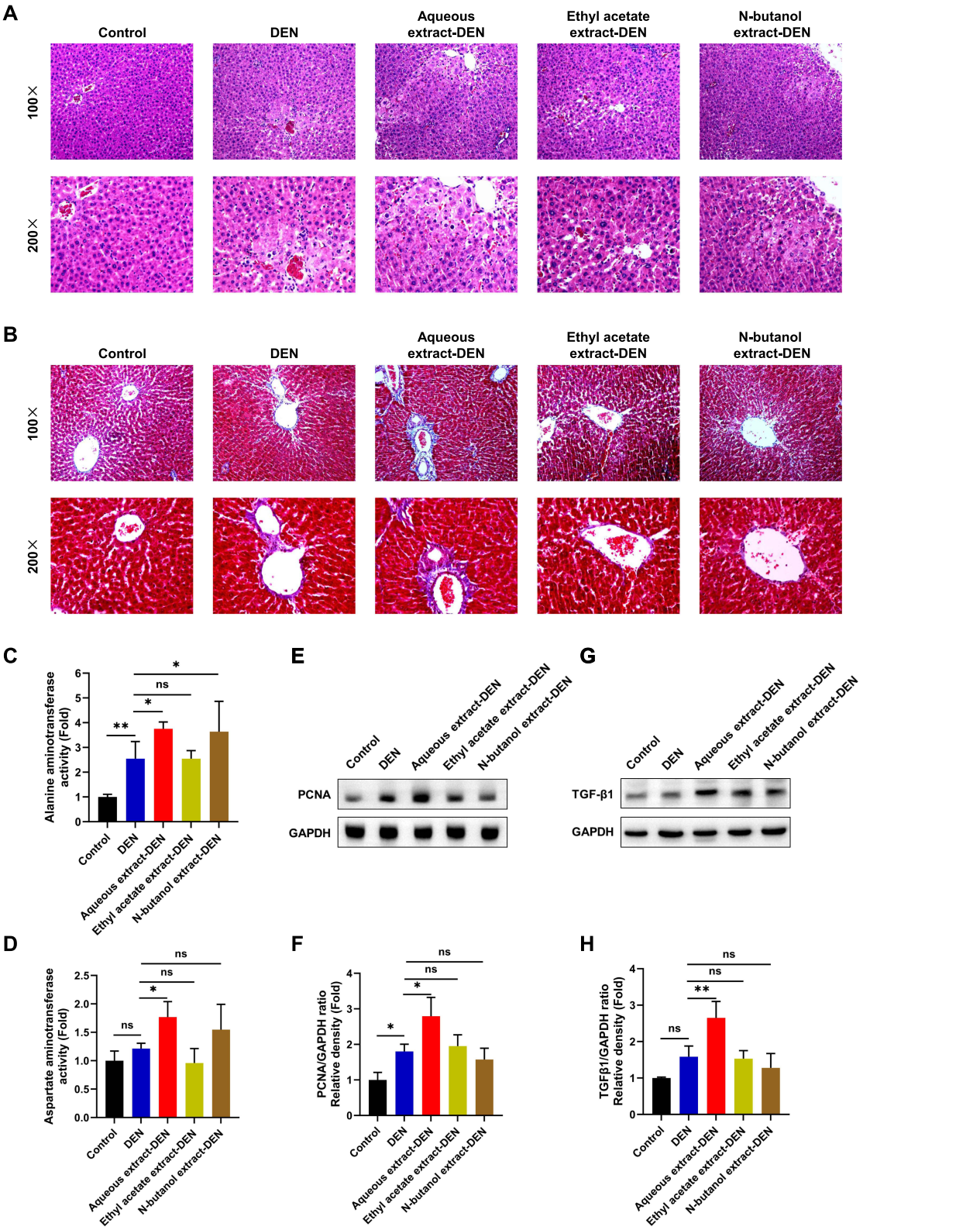


Figure 3 Aqueous extract of Rhubarb promotes DEN-induced hepatotoxicity. Representative images of H&E staining (**A**) and Masson's trichrome staining (**B**) of the liver sections from indicated groups. The activity of ALT (**C**) and AST (**D**) in indicated groups (Values are presented as mean ± SD; n ≥ 5 for each group). (**E**) Western blot of PCNA in the rat liver samples from indicated groups. Quantification of PCNA expression is depicted in (**F**). (**G**) Western blot of TGF-β1 in the rat liver samples. Quantification of TGF-β1 expression is depicted in (**H**). All samples were collected from rats at week 14 after initiation of DEN treatment. Western blot was performed at least three times and each repeat was performed as a separate, independent experiment. *$p < 0.05$, **$p < 0.01$, ns: non-significant.

Aqueous Extract of Rhubarb Promotes PKM2-Mediated Aerobic Glycolysis in the Rat Model of DEN-Induced Liver Cancer

As mentioned above, PKM2-mediated aerobic glycolysis is the key cause of liver injury induced by Rhubarb-DEN. Therefore, in order to further verify that water-soluble components are the dominant components in Rhubarb causing liver injury, we examined the effects of aqueous extract of Rhubarb on PKM2 expression and aerobic glycolysis in the DEN-induced liver cancer model. The result of Western blot revealed that DEN significantly increased expression of PKM2 in liver tissues, and the DEN-induced PKM2 expression was significantly enhanced by aqueous extract, but not by ethyl acetate and n-butanol extracts of Rhubarb (Figure 4A and B).

Furthermore, we examined the effects of different polar parts of Rhubarb on aerobic glycolysis by analyzing the activities of LDHA (an enzyme facilitates glycolytic process by converting pyruvate to lactate) and ICDH (an essential metabolic enzyme for the TCA cycle). As shown in Figure 4C and D, DEN slightly increased LDHA activity and

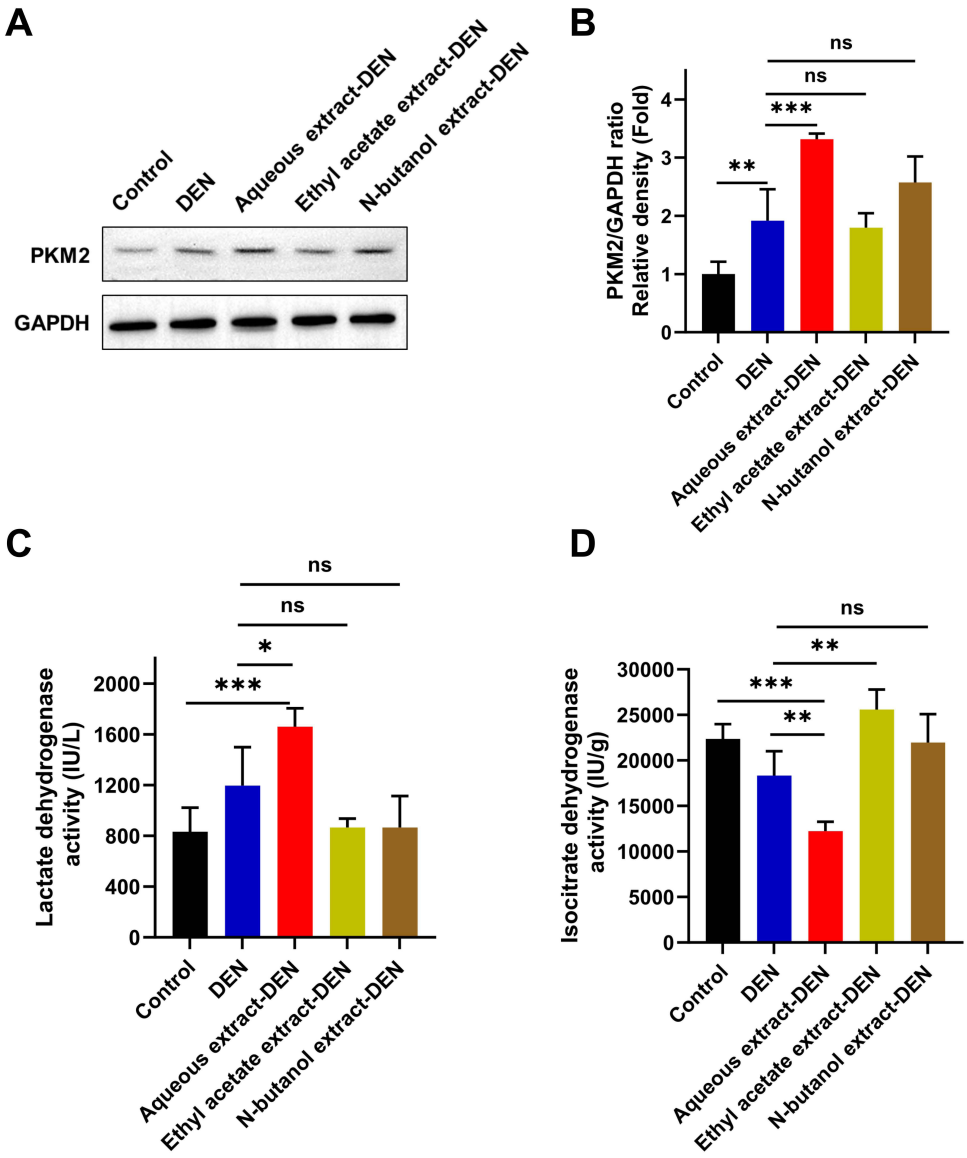


Figure 4 Aqueous extract of Rhubarb promotes DEN-induced aerobic glycolysis in liver tissues. (**A**) Western blot of PKM2 in the rat liver samples from indicated groups. Quantification of PKM2 expression is depicted in (**B**). Western blot was performed at least three times and each repeat was performed as a separate, independent experiment. The activities of LDHA (**C**) and ICDH (**D**) in indicated groups (Values are presented as mean ± SD; n = 4 for each group). Liver tissue samples were collected from rats at week 14 after initiation of DEN treatment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: non-significant.

decreased ICDH activity without significant difference. Interestingly, both the upregulation of LDHA activity and the downregulation of ICDH activity triggered by DEN were greatly enhanced by aqueous extract of Rhubarb. However, there was no significant difference in LDHA activity and ICDH activity among the control, ethyl acetate extract-DEN-treated and n-butanol extract-DEN-treated groups (Figure 4C and D). According to these findings, the treatment of aqueous extract of Rhubarb combined with DEN enhanced glycolysis but inhibited TCA cycle in liver tissues, which indicated that aqueous part of Rhubarb promotes aerobic glycolysis induced by DEN in rats.

Taken together, aqueous extract is the main polar part of Rhubarb causing hepatotoxicity via promoting aerobic glycolysis, which is mediated by the upregulated expression of PKM2 in the rat model of DEN-induced liver cancer.

Rhein Promotes Hepatotoxicity in the Rat Model of DEN-Induced Liver Cancer

Rhein, one of the main active components of Rhubarb aqueous extract, is a 1, 8-dihydroxy anthraquinone derivative. Recently, multiple studies have shown that Rhein has the risk of hepatotoxicity.[27,28] Herein, we explored whether Rhein is the main component in aqueous extract of Rhubarb causing hepatotoxicity in the rat model of DEN-induced liver cancer. Considering that PKM2 plays a vital role in mediating hepatotoxicity of Rhubarb, molecular docking analysis was performed to validate the target interaction between Rhein and PKM2. Binding energy values represented the affinity of Rhein for PKM2. Lower absolute and relative energy values indicate that a ligand is closer to its docking site. Rhein showed the highest binding affinity for PKM2 with a binding energy of −7.04 Kcal/mol. 3D diagrams of molecular docking displayed that Rhein interacted with amino acids of PKM2 protein through hydrogen bonds and hydrophobic interactions (Figure 5A). Overall, molecular docking analysis revealed that Rhein had a strong binding affinity for PKM2. Moreover, we also evaluated the effect of Rhein on liver function by testing the activities of ALT and AST. As

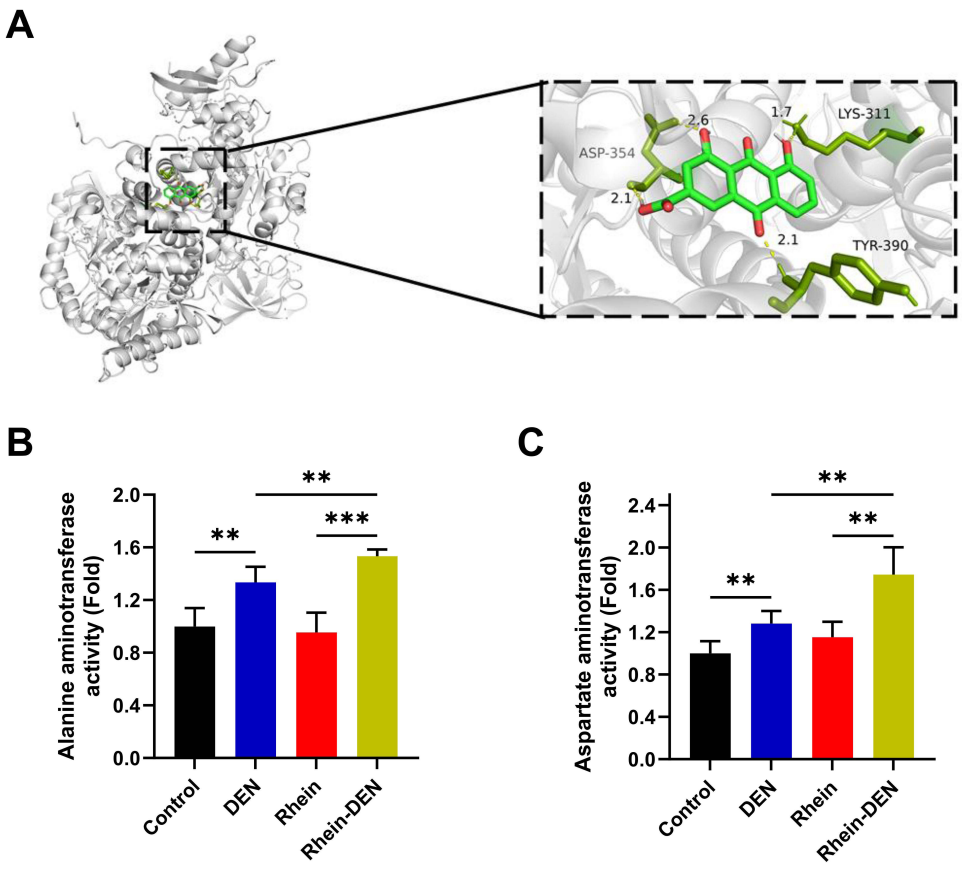


Figure 5 Rhein promotes DEN-induced hepatotoxicity. (**A**) Rhein interacts with PKM2 molecular docking verification. The activities of ALT (**B**) and AST (**C**) in indicated groups (Values are presented as mean ± SD; n = 5 for each group). Liver tissue samples were collected from rats at week 14 after initiation of DEN treatment. $^{**}p < 0.01$, $^{***}p < 0.001$.

shown in Figure 5B, the ALT activity was significantly increased in DEN-treated group, but was not affected by Rhein in the absence of DEN. In comparison to the DEN-treated group, the ALT activity was significantly higher in the Rhein-DEN-treated group. Moreover, the change of AST activity under DEN and/or Rhein treatmet was shown a similar tread as the ALT activity (Figure 5C), indicating that Rhein promotes hepatotoxicity in the presence of DEN. Taken together, our results revealed that Rhein is one of the active ingredients in aqueous extract of Rhubarb causing hepatotoxicity by targeting PKM2 in the rat model of DEN-induced liver cancer.

Discussion

Rhubarb, one of the most widely used Chinese herbs, has been shown to possess properties of hepatoprotection. However, following the development of clinical medicinal trial, the hepatotoxicity of Rhubarb has gradually exposed and attracted widespread attention. Our previous study revealed that Rhubarb promoted liver injury and hepatocarcinogenesis in DEN-induced liver cancer model in rat.[15,22] These findings strongly indicate that the two-way effect of Rhubarb on liver should be considered comprehensively when using Rhubarb to treat liver diseases. Herein, we observed that aqueous extract of Rhubarb caused the most severe hepatocellular damage and liver fibrosis, and significantly increased the activities of ALT and AST, which suggested that the water-soluble components in Rhubarb might account for the hepatotoxicity of Rhubarb in the rat model of DEN-induced liver cancer.

Traditional Chinese medicine emphasizes the influence of physiological status on the efficacy of Chinese medicine. Studies have shown that Rhubarb is different in hepatotoxicity in normal and disease animal models.[12,29] It is reported that a high dose range of total extracts from prepared Rhubarb has a significant protective effect to animals with liver fibrosis induced by carbon tetrachloride (CCl_4), but has a certain hepatotoxic effect to normal animals.[12] Moreover, under a modest inflammatory stress induced by lipopolysaccharide (LPS), doses of emodin previously thought to have a protective effect on the liver exerted a promoting effect on liver injury.[29] Unlike these studies, we evaluated the effect of different polar parts of Rhubarb on liver in a classical rat model of DEN-induced liver cancer. In this model, chronic exposure to DEN leads to hepatocyte damage, chronic inflammation and fibrosis, which is followed by the formation of HCC after 14–20 weeks.[30,31] We observed both significant liver injury and upregulation of PCNA and TGF-β1 expression at 14 weeks after DEN treatment, indicating that the rat model was in the early stage of HCC. In such a specific pathological state, we found that both of Rhubarb granule and aqueous extract of Rhubarb aggravated liver damage and promoted liver tumorigenesis. Our results emphasized that the safety issue of using Rhubarb or water-soluble components of Rhubarb in liver cancer treatment should not be ignored.

Metabolic reprogramming is an important molecular biological feature that drives hepatocarcinogenesis.[32] Our previous work demonstrated that Rhubarb at a low dose combined with DEN promoted hepatic precancerous injury by PKM2-mediated aerobic glycolysis in rats.[22] The upregulated level of lactate and downregulated level of succinate in Rhubarb-DEN-treated rats determined by non-targeted metabolomics together further validated the role of aerobic glycolysis in regulating the hepatotoxicity of Rhubarb. Moreover, we found that aqueous extract of Rhubarb enhanced the suppression of ICDH and activation of LDHA induced by DEN, which indicated that the DEN-induced aerobic glycolysis metabolic phenotype was promoted by aqueous extract in Rhubarb. Mitochondrial dysfunction has been shown to mediate the hepatotoxicity of active ingredients in Rhubarb, and the mechanisms are related to depolarization of mitochondrial membrane potential and the suppression of mitochondrial respiratory chain complex.[9,33] According to these studies, Rhubarb impairs mitochondrial metabolic capacity by causing mitochondrial dysfunction or directly targeting the key enzymes in TCA cycle. Furthermore, accelerated glycolysis may act as a compensatory metabolic pathway to provide adequate energy to hepatocytes undergoing malignant transformation following the treatment of DEN and aqueous extract of Rhubarb.

PKM2, one of the rate-limiting enzymes in aerobic glycolysis, is overexpressed in various tumors including HCC.[21,34] Recently, the expression level of PKM2 was considered as a biomarker for the early diagnosis of liver cancer.[35] Consistent with this view, the data form TCGA database revealed that the PKM2 expression level is closely related to the progress and poor prognosis of human HCC. Moreover, we found that DEN significantly increased the expression of PKM2, and this effect was enhanced by aqueous extract of Rhubarb. The change of PKM2 expression was in line with the hepatotoxicity induced by aqueous extract of Rhubarb-DEN, indicating that PKM2 played a vital role in the

hepatotoxicity induced by aqueous extract of Rhubarb-DEN. Therefore, abnormal expression of PKM2 in liver tissue may serve as a predictor of hepatotoxicity induced by Rhubarb in the treatment of primary liver cancer. However, in future studies, we need to further elucidate the mechanism of aqueous extract of Rhubarb promoting the expression of PKM2 in the rat model of DEN-induced liver cancer.

To further identify the hepatotoxic components in aqueous extract of Rhubarb, we focused on the effects of Rhein on the liver. Rhein is one of the most abundant water-soluble components in the anthraquinones of Rhubarb, and possesses a variety of hepatoprotective pharmacological activities.[36,37] However, numerous studies have shown that Rhein has potential liver toxicity as well.[27,38] A recent cytotoxicity assay performed in rat primary hepatocytes revealed that Rhein is the most toxic ingredient of anthraquinones, followed by emodin, aloe-emodin, physcion and chrysophanol.[39] The bidirectional regulation of Rhein on liver is closely related to the dosage and duration of administration. When Rhein exerts the hepatoprotective effect, the administration dosage varies from 20 to 150 mg/kg, and the duration of administration is usually less than 14 days.[37] In the present study, we observed that Rhein, at a dosage of 0.012 g/kg, which is equivalent to 4 g/kg body weight of Rhubarb granule, caused remarkable hepatotoxicity in the DEN-induced liver cancer model. However, the same dose of Rhein did not cause hepatotoxicity in normal rats. Our results indicated that the hepatotoxicity of Rhein was not only affected by the dosage and duration of administration but also affected by the certain physiological status. Obviously, the toxicity of Rhein to liver exhibits the same characteristics as that of Rhubarb granule. Hence, we concluded that Rhein plays a vital role in causing hepatotoxicity in the rat model of DEN-induced liver cancer. Certainly, whether other components of anthraquinone in Rhubarb contribute to the hepatotoxicity of Rhubarb needs to be further explored.

Conclusion

In summary, the present study revealed that enhanced aerobic glycolysis mediated by PKM2 was a potential biomarker for predicting hepatotoxicity of Rhubarb. In addition, we demonstrated that aqueous extract of Rhubarb played a major role in promoting hepatotoxicity via facilitating PKM2-mediated aerobic glycolysis in the rat model of DEN-induced liver cancer. Moreover, we found that the hepatotoxicity had potential to be relevant to Rhein in Rhubarb. Logically, reducing the hepatotoxic components of Rhubarb identified in this study is expected to reduce the hepatotoxicity and enhance the hepatoprotective effect of Rhubarb. Furthermore, our results are helpful in guiding the rational use of Rhubarb in the clinical treatment of liver cancers.

Abbreviations

DEN, diethylnitrosamine; PKM2, pyruvate kinases type M2; HCC, hepatocellular carcinoma; TCGA, the cancer genome atlas; PCNA, proliferating cell nuclear antigen; TGF-β1, transforming growth factor beta1; LDHA, lactate dehydrogenase A; ICDH, isocitrate dehydrogenase; HK2, hexokinase 2; PFK1, phosphofructokinase 1; SPF, specific-pathogen-free; H&E, hematoxylin and eosin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; ICDH, isocitrate dehydrogenase; TCMSP, traditional Chinese medicine systems pharmacology; UHPLC, ultra-high performance liquid chromatography system; ESI, electrospray ionization; TCA, tricarboxylic acid; CCl_4, carbon tetrachloride; LPS, lipopolysaccharide.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding authors on request.

Ethics Approval

All animal studies were conducted according to protocols approved by the Academy Animal Experimental Ethical Committee at the Anhui University of Chinese Medicine (Code of Ethics: AHUCM-rats-2023035).

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Disclosure

The authors declare that there are no conflicts of interest in this work. This paper has been uploaded to SSRN as a preprint: https://ssrn.com/abstract=4736236 or http://dx.doi.org/10.2139/ssrn.4736236

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