**Glabridin attenuates paracetamol-induced liver injury in mice via CYP2E1-mediated inhibition of oxidative stress**

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**ABSTRACT**

CYP2E1 plays a crucial role in the bio-activation of toxic substances leading to liver damage. In this context, CYP2E1 converts paracetamol (PCM) to N-acetyl-p-benzoquinone imine (NAPQI), which is prone to cause hepatotoxicity. Hence, we aimed to explore the protective effect of glabridin on widely used PCM-induced liver injury model in the present study and, after that, correlated with the role of CYP2E1 toward its efficacy. Glabridin was isolated from *Glycyrrhiza glabra* and characterized before the investigation in an *in-vivo* mice model of PCM-induced liver injury. Glabridin after oral treatment at 5–20 mg/kg showed a considerable improvement in serum biochemical parameters (ALT and AST) and oxidative stress markers (MDA, GSH, SOD, and catalase) in comparison to only PCM-treatment. Histopathological examination of the liver depicted that glabridin exhibited substantial protection from PCM-induced liver injury compared to the disease control group. Significant down-regulation of CYP2E1 protein and its mRNA expression levels were observed in the glabridin-treated groups compared to PCM-induced respective elevation of CYP2E1. Moreover, activation of NF-κB was significantly inhibited by glabridin. Therefore, glabridin has the potential to protect PCM-induced liver injury through CYP2E1 inhibition-mediated normalization of oxidative stress. Further research is warranted to establish glabridin as a phytotherapeutics for liver protection for which no effective and safe oral drug is available to date.

**GRAPHICAL ABSTRACT**

**Introduction**

The liver is vulnerable to drug-induced liver injury (DILI) as it is the primary site of drug metabolism. Several drugs are either withdrawn from the market like ibufenac, iproniazid, nimesulide, sulfathiazole, troglitazone, etc. or issued a black box warning by the United States Food and Drug Administration (USFDA) like amiodarone, flutamide, isoniazid, methotrexate, propylthiouracil, etc. due to their potential to cause liver toxicity (DILI rank Dataset, USFDA). However, numerous drugs prone to cause hepatotoxicity are even available in the market because of the non-availability of suitable alternative therapy. DILI is a growing concern worldwide that leads to severe clinical conditions like acute/chronic hepatitis, acute cholestasis, nonalcoholic steatohepatitis, etc.
(Kaplowitz 2004). Xenobiotics & chemicals can cause hepatoxicity and even cause cancer by activating pro-carcinogen by liver enzymes (Hewawasam et al. 2004; Jayaweera et al. 2017). In this context, cytochrome P450 2E1 (CYP2E1)-mediated oxidation plays a vital role in the direct or indirect elimination of toxic substances from the liver. Despite the beneficial effects of CYP2E1 in the metabolism of alcohol, endogenous fatty acids, etc., diverse nutritional and physiological factors elevate the CYP2E1 enzyme (Wang et al. 2003; Khemawoot et al. 2007). Furthermore, toxic substances are inadvertently bio-activated in the process of CYP2E1-mediated oxidation and lead to oxidative stress (Trafalis et al. 2010; Koneru et al. 2018). Therefore, CYP2E1 inhibition can be an important target to combat DILI. Research works are ongoing to develop an effective and safe drug, but there is hardly any drug currently available in the market for use. Under these circumstances, plant secondary metabolites, especially flavonoids, have immense potential toward liver protection based on traditional system of medicine (Yang et al. 2013; Tsai et al. 2018). The present study deals with glabridin, a phytoconstituent from Glycyrrhiza glabra, a widely used medicinal plant. Based on the previous in-vitro studies, glabridin was found to inhibit CYP2E1 in human liver microsomes (Bhatt et al. 2021). We aimed here to investigate any protective role of glabridin in DILI through CYP2E1 regulation using paracetamol (PCM)-induced liver injury model. PCM is metabolized by glucuronidation and sulfonation (McGill and Jaeschke 2013) and to some extent by CYP2E1 leading to the production of N-acetyl-p-benzoquinone imine (NAPQI). Lower levels of NAPQI are detoxified by reduced glutathione (GSH), but its excessive production at the high dose of PCM exhausts the hepatic GSH level and eventually leads to oxidative stress & liver injury (Dai et al. 2006; Bunchorntavakul and Reddy 2013).

Under these circumstances, the objectives of the present study were assessment of the effect of glabridin on serum biochemical parameters [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)], oxidative stress markers [malondialdehyde (MDA), GSH, superoxide dismutase (SOD), catalase (CAT)], histopathology of liver, CYP2E1 protein expression & CYP2E1 mRNA expression, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation.

**Materials and methods**

**Chemicals and reagents**

PCM (≥98%) and CAT assay kit were procured from Cayman (Michigan, USA). SOD assay kit was purchased from Sigma-Aldrich (Darmstadt, Germany). Anti-Cytochrome P450 2E1 antibody and Phospho-NF-kB p65 (Ser536) monoclonal antibody were purchased from Abcam (Massachusetts, USA) and Invitrogen (Illinois, USA), respectively. Total RNA isolation kit and First Strand cDNA synthesis kit was purchased from Qiagen (Hilden, Germany), and Thermo Scientific (California, USA), respectively. All other chemicals/reagents were of analytical grade or above. Ultrapure water was obtained from the water purification system (Merck, Molesheim, France).

**Isolation, purification, and characterization of glabridin**

Glabridin was isolated from roots of Glycyrrhiza glabra, purified by column chromatography, and characterized by 1H NMR, 13C NMR, HRMS, and HPLC (Supporting Information). The purity of glabridin was >99%.

**Animal model and ethical prerequisite**

Experimentation was carried out using healthy adult male Swiss mice with a bodyweight of 25–30 g (6–8 weeks age). The study was performed using male mice, which is preferred over female mice for PCM-induced liver injury model (Mohar et al. 2014; Jadeja et al. 2015; Yan et al. 2016). Animals were maintained in polypropylene cages under standard laboratory conditions. Animals were fed with the standard pellet diet and water ad libitum. The animal study was performed following ‘Committee for the Purpose of Control and Supervision of Experiments on Animals’ (CPCSEA) guidelines with prior approval from our Institutional Animal Ethics Committee (IAEC approval no: 1704/75/8/2019).

**Dose formulation**

Dose formulations for both PCM and glabridin were prepared separately in a common vehicle which was an aqueous suspension containing 0.25% (w/v) sodium carboxymethylcellulose. Each dose was prepared freshly before dose administration.

**Study design**

Total thirty animals were randomly divided into five specific groups (n = 6), as mentioned below. PCM was used at 200 mg/kg through an intraperitoneal route to induce liver injury (Mohar et al. 2014; Jadeja et al. 2015; Yan et al. 2016; Lu et al. 2017). Glabridin was used at 5–20 mg/kg orally. We selected these dose levels of glabridin considering the following aspects: (a) content of glabridin in Glycyrrhiza glabra extract is 1.86–10.03% (Lim 2016); (b) the usual daily dose of Glycyrrhiza glabra containing capsule for human use is 0.5–1.5 g, and can be increased up to 4 g on thrice daily basis (www.medicinenet.com); (c) we observed earlier that glabridin at 20 mg/kg in Balb/C mice could alter oral exposure of CYP2E1 substrate (chlorozoxazone) by 1.9 folds (Bhatt et al. 2021). Treatment protocols are as follows: Group 1 (control group): only vehicle orally for seven days; Group 2 (diseased control group): only vehicle orally for six days followed by PCM on the 7th day; Groups 3 (test group): glabridin at 5 mg/kg for seven days followed by PCM on the 7th day; Groups 4 (test group): glabridin at 10 mg/kg for seven days followed by PCM on the 7th day; Groups 5 (test group): glabridin at 20 mg/kg for seven days followed by PCM on the 7th day. 12 h after receiving PCM, withdrawn blood samples from the retro-orbital plexus of each animal. The serum was separated and used for biochemical analysis. Then, sacrificed the animals by carbon dioxide euthanasia, removed the liver, washed with ice-cold normal saline, and blotted dry. A part...
of liver tissue was kept for histopathological examination, whereas the remaining portion of the liver tissue was snap freeze under liquid nitrogen and stored at –80 °C for further studies.

**Determination of serum biochemical parameters**

Serum samples were analyzed to determine activities of AST (Sensitivity: 2.5 U/L; Detection range: 2.5–1000 U/L) and ALT (Sensitivity: 3.0 U/L; Detection range: 3.0–1000 U/L) [Manufacturer: ERBA Diagnostics Mannheim, Germany & Transasia Bio-Medicals, HP, India] using an automated biochemical analyzer (Make: ERBA Mannheim Germany & Transasia Bio-Medicals, HP, India; Model: EM360).

**Estimation of oxidative stress markers: MDA, GSH, SOD, CAT**

MDA content was measured in liver tissue homogenates, prepared at 100 mg/mL using 1.15% KCl in water (w/v). The study was performed following earlier reported protocol, and MDA content was expressed as nmol/g of tissue (Uchiyama and Mihara 1978). A detailed methodology is provided in Supporting Information.

GSH content was measured in liver tissue homogenates, prepared using 50 mg of tissue each in 100 mM of sodium phosphate buffer containing 5 mM of ethylenediamine tetra-acetic acid (EDTA) (750 μL) and 25% H₃PO₄ (200 μL). The study was executed following previously reported protocol, and GSH content was expressed as μg/g of tissue (Hissin and Hilf 1976). A detailed methodology is provided in Supporting Information.

The SOD activity was measured in the homogenate of each liver tissue containing 5 mM of ethylenediamine tetra-acetic acid (EDTA) (750 μL) and 25% H₃PO₄ (200 μL). The activity of SOD was determined according to the manufacturer’s protocol, where data was expressed as % inhibition rate (Muniz et al. 2015; Lee et al. 2018).

The CAT activity was measured in the homogenate of each liver tissue, which was prepared at 200 mg/mL using 50 mM of potassium phosphate buffer (pH 7.4) containing 1 mM of EDTA. The activity of CAT was measured according to the manufacturer’s protocol, where activity was expressed as nmol/min/mL (Wang et al. 2017).

**Examination of liver histopathology**

Liver tissue samples were fixed in 10% neutral buffered formalin solution, embedded in paraffin, sectioned using a microtome, dehydrated using ethanol: water composition, and stained using hematoxylin-eosin (H & E) dye. After that, slides were evaluated for liver injury under the light microscope (Make: Magnus Opto Systems, Noida, India; Model: INVI). Further, histopathological scoring was performed, and assessment was expressed as the sum of individual score grades from normal (zero), mild (one), moderate (two), to severe (three and above) for each of the following parameters: sinusoidal dilation, inflammatory infiltration, hepatocyte ballooning, hepatocyte necrosis, red blood cells (RBC) congestion and nuclear necrosis (Tag 2015; Kalantar et al. 2019).

**Assessment of CYP2E1 protein expression**

The expression of CYP2E1 protein was assessed by western blot analysis of liver tissue lysates, prepared using RIPA buffer followed by an estimation of protein by the Bradford method. The proteins in the lysates were separated by SDS-PAGE (75 V, 3 h) and transferred to PVDF membrane (100 V, 2 h, 4 °C). The membrane was blocked with 5% skimmed milk for 1 h at room temperature to avoid nonspecific antibody binding. Primary antibodies for CYP2E1 were added with necessary dilution and kept overnight at 4 °C followed by HRP-conjugated secondary antibody for 1 h at room temperature. Chemiluminescent HRP substrate (Millipore, Massachusetts, USA) was used to visualize the specific proteins under ChemiDoc Imaging System (Make: Syngene, Maryland, USA; Model: G:BOX, XT-4). Densitometry analysis of protein was done using ImageJ software.

**Analysis of CYP2E1 mRNA expression**

The mRNA expression level of CYP2E1 was analyzed in liver tissues using the following steps: extracted total RNA from liver tissue homogenates as per the manufacturer’s protocol; given RNase free-DNase treatment to total RNA; synthesized cDNA from DNA-free RNA using oligo dT primer as per the manufacturer’s protocol; analyzed gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Model: Applied Biosystems, California, USA; Make: StepOnePlus). After qRT-PCR reaction, the test gene (CYP2E1) and reference gene (GAPDH) expression levels were estimated in the form of CT values which were further used to calculate the relative fold change in expressions compared to the control using 2(-Delta Delta CT) (Livak and Schmittgen 2001; Dogra et al. 2020). A detailed methodology is provided in Supporting Information.

**Evaluation for NF-κB protein expression**

Expression of NF-κB protein was estimated in liver tissue lysates following the same study protocol for CYP2E1 protein expression except the use of NF-κB primary antibody instead of CYP2E1 antibody.

**Statistical evaluation**

Statistical analysis was performed by One-way analysis of variance (ANOVA) followed by Turkey’s test using GraphPad Prism software and p values of less than 0.05, 0.01, and 0.001 was considered statistically significant.
Results

Glabridin lowered hepatic marker enzymes: AST & ALT

Animals treated with PCM showed a markedly elevated level of AST (Figure 1(A)) and ALT (Figure 1(B)) compared to control animals. Treatment with glabridin significantly lowered AST and ALT, respectively, at 5–20 mg/kg (Figure 1(A,B)). A high dose of PCM is known to cause elevation of AST and ALT during liver injury, as found in the present study. Lowering AST and ALT levels upon treatment with glabridin indicates that it may protect the membrane of the liver against leakage of marker enzymes into the systemic circulation. Therefore, glabridin can restrict the rise in two hepatic marker enzymes associated with PCM-induced liver injury.

Glabridin improved oxidative stress markers: MDA, GSH, SOD & CAT

MDA can serve as a marker of oxidative stress due to its formation linked to lipid peroxidation (Smilkstein et al. 1991). The marked enhancement of MDA content was observed due to PCM treatment compared to the control group. There was a significant decrease in the MDA level by glabridin at 5–20 mg/kg compared to the PCM alone group (Figure 1(C)). Therefore, results suggest that glabridin can prevent the generation of oxidative stress due to PCM-induced liver injury.

Treatment of PCM considerably depleted the GSH content in the disease control group’s liver tissues compared to the control group. Noticeable improvement in the GSH content was observed for the glabridin treated groups at all the dose levels compared to PCM alone (Figure 1(D)). As GSH protects cells by neutralizing ROS due to its antioxidant properties, it can be stated that glabridin can protect PCM-induced liver injury by reducing ROS formation.

SOD and CAT are two key enzymes for cellular protection from oxidative damage. SOD scavenges the superoxide radical to H₂O₂ which CAT further converts into H₂O and O₂ that are nontoxic. Here, SOD was substantially dropped by PCM treatment compared to control group. Glabridin treatment at 5–20 mg/kg displayed that SOD activity was maintained at levels close to the control group (Figure 1(E)). Similarly, the CAT level was significantly decreased in the diseased control group, and glabridin treatment significantly increased the CAT level (Figure 1(F)). The remarkable recovery of these enzymes by glabridin treatment suggests that it can decrease the free radicals generation linked to a high dose of PCM. Furthermore, it can subsequently contribute to restrict ROS generation leading to oxidative stress.

Glabridin restricted alteration in liver histopathology

Histological examinations of liver tissue were performed to obtain more information and better understanding. Liver tissues from control group showed normal lobular architecture (Figure 2(A–C)). Treatment of PCM caused injury to the liver with increasing incidence of the following liver histopathological alteration: infiltration of inflammatory cells, RBC congestion, nuclear necrosis, hepatocyte ballooning, sinusoidal dilation, hepatocyte necrosis (Figure 2(D–F)). In contrast, liver
architecture was markedly improved in the animals treated with glabridin at 5, 10, and 20 mg/kg as depicted in Figure 2(G–I), Figure 2(J–L), and Figure 2(M–O), respectively. Figure 2(A–O) are presented with the scale bar in Figure S6–S20 (Supporting Information). In the PCM treated group, the liver structure was destroyed and showed significant aggravation in dilatation of sinusoids (Figure 3(A)), inflammatory infiltration (Figure 3(B)), hepatocytes ballooning (Figure 3(C)), hepatocyte necrosis (Figure 3(D)), RBC congestion (Figure 3(E)), and nuclear necrosis (Figure 3(F)) as compared to control group. Parameters like sinusoidal dilation and hepatocyte necrosis were reduced significantly at all doses of glabridin in comparison to the diseased control group. Glabridin showed a marked effect in reducing inflammatory infiltration, RBC congestion and nuclear necrosis at 10 and 20 mg/kg. Hepatocyte ballooning was significantly reduced at 20 mg/kg of glabridin treatment. Therefore, glabridin has the significant potential to protect against PCM-induced liver injury.

**Glabridin decreased CYP2E1 protein & its mRNA expression**

CYP2E1 protein expression level is enhanced by a variety of physiological conditions and interferes with the normal hemostasis. Here, CYP2E1 protein levels in the liver tissues of PCM treated animals were significantly higher than the control group, suggests an up-regulation of CYP2E1 protein expression. Elevated expression of CYP2E1 in the PCM treated animals was significantly decreased in glabridin treated animals at 5–20 mg/kg (Figure 4(A)). CYP2E1-mediated higher NAPQI generation exhausts the GSH level and increases
mitochondrial dysfunction, oxidative stress, and DNA damage (Xie et al. 2014). Eventually, it causes liver damage involving hepatocyte toxicity, apoptosis, and inflammation in the liver. Therefore, treatment with glabridin in the present study results in inhibition of CYP2E1 protein expression in liver tissues that subsequently reduced the PCM treatment linked higher oxidative stress leading to liver injury. The qRT-PCR analysis was performed additionally to assess the mRNA expression levels of CYP2E1 protein in the liver tissues (Figure 4(B)). PCM treatment caused a significant elevation in the mRNA expression of hepatic CYP2E1 protein compared to the control group. In contrast, glabridin treatment significantly decreased the mRNA expression level of CYP2E1 protein compared to only PCM treatment, which helps to prevent the PCM treatment associated higher generation of oxidative stress leading to liver injury.

**Glabridin downregulated activation of NF-κB protein**

NF-κB plays an important role in cell survival. In the present study, p-NF-κB protein levels in the liver tissues of PCM-treated animals were significantly higher than in the tissues from control animals, which indicates activation of NF-κB protein expression due to PCM treatment. Elevated expression of p-NF-κB in the hepatic tissues of PCM treated animals was significantly decreased in glabridin treated animals at the dose level of 10–20 mg/kg (Figure 5). It has been reported in the literature that CYP2E1 plays a fundamental role in the regulation of NF-κB or NF-κB is the main mediator in the CYP2E1 expression (Martinez-Gil et al. 2020). Therefore, reduction in NF-κB protein level by glabridin may be beneficial to manage oxidative stress and the inflammatory response during liver injury.

**Discussion**

In the quest to explore the hepatoprotective effect of glabridin in PCM-induced liver injury model and thereby correlating its role toward CYP2E1, results of present investigations reveal that glabridin can improve the PCM-induced alteration in biochemical parameters (AST & ALT), oxidative stress markers (MDA, GSH, SOD, & CAT), CYP2E1 protein & mRNA expression.
expression and NF-κB protein in mice model. Similar effects of normalization on PCM-induced biochemical parameters and oxidative stress markers through intraperitoneal route are also reported for glycyrrhizin, another phytoconstituent of *Glycyrrhiza glabra* but did not significantly decrease AST and ALT levels orally at 50–100 mg/kg (Yan et al. 2016). However, glabridin in the present study is found to control the same enzyme levels orally at lower dose levels. Additionally, a parallel group of only glabridin treatment at its highest dose without any PCM treatment can be helpful for better insights into its action. Considering the above-mentioned parameters along with the histopathological changes, glabridin at the highest experimental dose level (20 mg/kg) is found to be most appropriate for its ameliorating effect against PCM-induced liver injury.

CYP2E1 shows low enzymatic activity under normal physiological conditions and facilitates direct or indirect excretion of toxic chemicals from the body (Liu and Baliga 2003). A high dose of PCM upon CYP2E1-mediated metabolism generates NAPQI, which causes an increased ROS level. The elevated ROS formation induces lipid peroxidation (Dai et al. 2006). We also observed here a similar effect of PCM on ROS-linked markers. Further, glabridin showed protection through the reduction in PCM-induced ROS generation. These *in-vivo* observations on ROS generation are in line with our *in-vitro* effects where glabridin was itself found to be safe up to 30 μM and prevented significantly PCM-induced ROS generation in HepG2 cell lines (Figure S21, Supporting Information). PCM-induced increased ROS generation leads to

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**Figure 4.** (A) Representative western blot analysis of liver tissue lysates and densitometry data of CYP2E1 protein expression normalized by β-actin; (B) mRNA expression levels of CYP2E1 in the liver tissues by qRT-PCR. Significance level: *p < 0.05 versus control group and #p < 0.05, ##p < 0.01 and ###p < 0.001 versus only PCM treated group.

**Figure 5.** Representative western blot analysis of liver tissue lysates and densitometry data of pNF-κB normalized to β-actin. Significance level: **p < 0.01 versus control group; #p < 0.05 and ##p < 0.01 versus only PCM treated group.
the induction of CYP2E1 protein and its mRNA expression. However, treatment with glabridin restricts the CYP2E1 protein and its mRNA expression levels to normalize the PCM-induced higher ROS formation leading to higher oxidative stress. Glycyrrhizin can protect against hepatotoxicity, but it does not affect CYP2E1 (Yan et al. 2016). Here, glabridin is found to significantly inhibit CYP2E1 that can be beneficial toward liver protection. This is the first report of glabridin for its hepatoprotective effect by minimizing ROS formation involving CYP2E1 regulation toward lessening oxidative stress leading to hepatotoxicity. Higher oxidative stress due to PCM treatment activates the NF-κB pathway that leads to up-regulation of inflammatory genes. We observed that treatment of glabridin caused down-regulation of NF-κB protein. Further investigations for the attenuating effect of glabridin are required to illustrate the subsequent involvement of key pathway(s) toward the management of oxidative stress, inflammation, and apoptosis. In this context, glabridin, obtained from the plant, Glycyrrhiza glabra, which is considered as Generally Recognized As Safe (GRAS) substances by the USFDA (Select Committee on GRAS substances, USFDA) and by Food Safety and Standards Authority of India (FSSAI) as nutraceutical (Food Safety and Standards Regulations 2016), therefore, there may be a huge potential to be developed as phytotherapeutics against drug-induced liver injury.

Conclusion

Based on the present investigational results, glabridin is found to significantly protect PCM-induced liver injury where inhibition of CYP2E1 plays a major role in lowering of oxidative stress linked to a high dose of PCM. This is the first report of glabridin toward liver protection through regulating CYP2E1 and should be further explored to develop as phytotherapeutics for liver protection.

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Disclosure statement

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