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The ameliorative role of phlorotannin on aflatoxin B_1-induced liver oxidative stress and mitochondrial injury is related to the activation of Nrf2 and Nrf1 signaling pathways in broilers

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Abstract

Background Aflatoxin B_1 (AFB_1) risks animal and human health, and the liver is considered the most crucial detoxification organ. Phlorotannin (PT) is a polyhydroxy phenol that has a wide range of biological activities, including antioxidation and hepatoprotection, which can promote the ability of liver detoxification. This study aimed to elucidate the protective effect of PT on AFB_1-induced liver damage in broilers.

Results In vivo experiment showed that the PT reduced AFB_1 content and AFB_1-exo-8,9-epoxide DNA (AFBO-DNA) concentration in serum and liver ($P < 0.05$), improved the histomorphology of liver and hepatic mitochondria, and activated nuclear factor erythroid 2-related factor 2 (Nrf2)-related antioxidant and detoxification pathway by upregulating the activities of antioxidant enzymes (catalase [CAT], glutathione S-transferase [GST]) and total antioxidant capacity (T-AOC) level ($P < 0.05$), and inhibited the mRNA expression of *CYP1A1* (cytochrome P450 family 1 subfamily A member 1) and phase II detoxification enzyme related genes (*GPX1*, *GSTT1*, and *NQO1*) of broilers exposed to AFB_1 ($P < 0.05$). Meanwhile, PT upregulated the Nrf1 pathway-related mitochondrial biosynthetic genes (*Nrf1*, mitochondrial transcription factor A [*TFAM*], mitofusin 1 [*MFN1*]) in broilers fed AFB_1 contaminated diet ($P < 0.05$). In vitro verification study suggested that the use of Nrf2/Nrf1 inhibitors suppressed the ameliorative role of PT on AFB_1-induced liver injury of broilers, which was manifested in the mRNA expression of *Nrf2*, *NQO1*, *GSTT3*, *Nrf1*, *TFAM*, and other genes decreasing ($P < 0.05$), and down-regulation of the protein expression of Nrf2, total and nucleus p-Nrf2, and total and nucleus p-Nrf1 ($P < 0.05$).

Conclusion The PT ameliorates oxidative stress and hepatotoxicity by activating the Nrf2-mediated phase II detoxification enzymes pathway and maintains mitochondrial homeostasis by activating the Nrf1 signaling pathway in broilers exposed to AFB_1.

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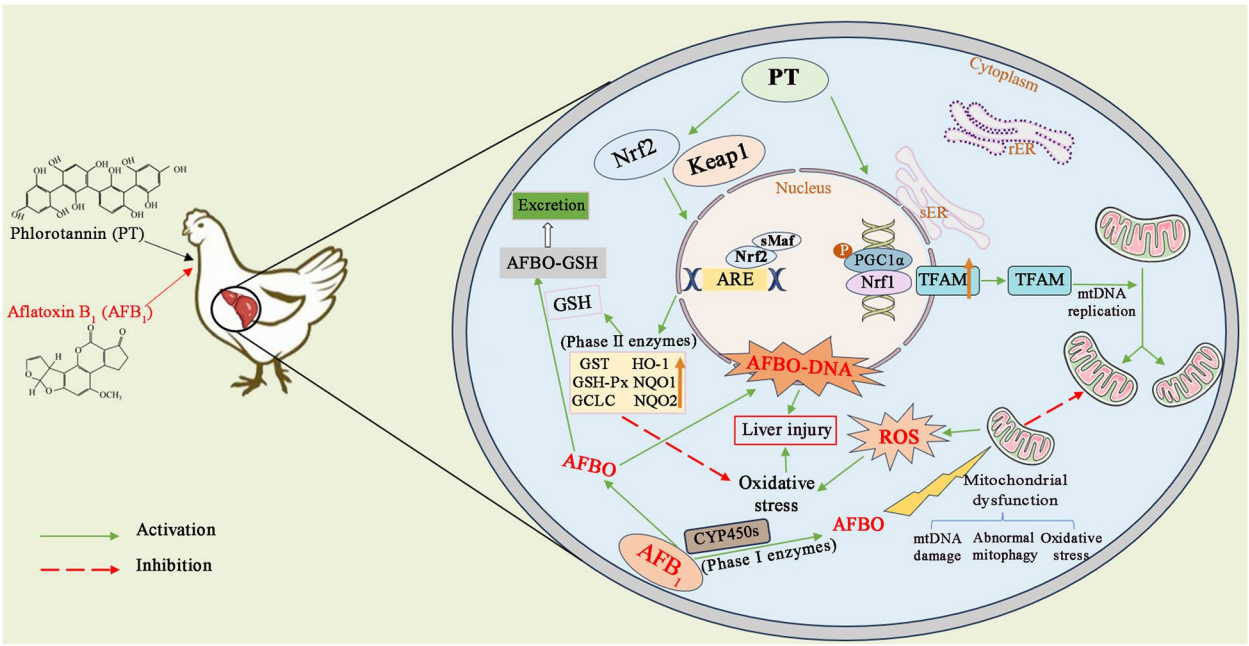
Keywords Aflatoxin B_1, Biological detoxification, Broiler chickens, Liver injury, Phlorotannin

Graphical Abstract



Introduction

Mycotoxin contamination in foodstuffs and feed ingredients causes substantial economic losses in the food and agricultural industry, and the accumulation of mycotoxin has significant toxic effects on humans and animals [1]. Among these, aflatoxin B_1 (AFB_1) is one of the most prevalent; it is a secondary metabolite produced by *Aspergillus flavus* and *Aspergillus parasiticus* [2, 3]. According to the global data reported in the past ten years, the incidence and the highest level of aflatoxin in cereals are 55% and 1,642 μg/kg, respectively [4]. Feeding AFB_1-contained diets negatively impacts livestock production and causes a significant threat to food security [5, 6]. Chicken is currently the main animal-derived food worldwide. It has been reported that broiler chickens are sensitive to AFB_1, and AFB_1 induces oxidative stress and hepatotoxicity in broilers, which is detrimental to broiler production and supply [7, 8].

The AFB_1 is classified as a carcinogen because it produces harmful product AFB_1-exo-8,9-epoxide (AFBO) through the biotransformation pathway of metabolism and detoxification. AFBO can bind to DNA and proteins in cells to form adducts, resulting in DNA mutations and liver damage [9, 10]. It has been found that when the body is exposed to AFB_1, it will not only lead to the oxidative phosphorylation decoupling of the mitochondrial respiratory chain and disturb the oxidative balance of hepatocytes but also reduce the mitochondrial membrane potential, cause mitochondrial swelling and induce mitochondrial dysfunction [11, 12]. It has been proved that the activation of AFB_1 into AFBO is regulated by cytochrome P (CYP) enzymes (phase I metabolic enzymes), which mainly include CYP1A1, CYP1A2, CYPA6, CYP3A4 [13]. Subsequently, AFBO is detoxified by conjugation to reduced glutathione (GSH), which is catalyzed by glutathione S-transferase (GST; phase II detoxification enzyme) [14]. As is known, the liver is the main organ for the metabolism and detoxification of AFB_1 [15]. When the content of AFB_1 exceeds the detoxification threshold of the liver, excessive AFB_1 produces a large amount of reactive oxygen species (ROS) during the metabolic process in the liver, thus causing oxidative stress and impairing the hepatic function [16, 17]. The nuclear transcription factor is closely related to the

process of cell oxidation, in which nuclear factor erythroid 2-related factor 2 (Nrf2) plays an irreplaceable role in regulating the process of oxidative stress [18]. On the other hand, mitochondria are important organelles in the hepatocytes and are involved in producing energy and ROS [19, 20]. It has also been reported that the metabolism and detoxification of AFB_1 are closely related to mitochondria [21, 22]. Several transcription factors can regulate the biogenesis and function of mitochondria, such as peroxisome proliferators-activated receptor γ coactivator 1 alpha (PGC-1α), nuclear respiratory factor 1 (Nrf1), and mitochondrial transcription factor A (TFAM) [23]. Exposure to AFB_1 impairs the structure of mitochondria, leading to the disorder of its biosynthesis function and further accelerating liver damage [24].

Many studies have shown that several bioactive substances can alleviate AFB_1-induced liver injury by reducing oxidative stress and mitochondrial-mediated apoptosis [25–27]. Polyphenols are natural antioxidants because they contain one or more hydroxyl groups in the structures and have antioxidant, anti-tumor, and anti-inflammatory functions [28, 29]. Polyphenols are abundant in plants and have been reported to relieve AFB_1-induced hepatotoxicity, nephrotoxicity, reproductive toxicity, inflammatory reaction, and apoptosis in vitro and in vivo by reducing oxidative stress [15, 30]. With the continuous development of marine resources, the development and utilization of seaweed resources have made remarkable progress in recent years. As a secondary metabolite of brown algae, phlorotannin (PT) is a polyhydroxy phenol that has a wide range of biological activities [31], including anti-oxidation [32], anti-inflammation, and hepatoprotection [33]. A previous study confirmed that PT plays a preventive role in liver injury induced by toxins [34]. However, it remains unclear whether PT exerts a beneficial role in AFB_1-induced liver injury in broilers, especially the mitochondrial protective effect of PT in the liver is still unknown. Therefore, this study was conducted to evaluate the effects of PT on the liver injury of broilers exposed to AFB_1 and to illustrate the molecular mechanisms to provide new strategies for alleviating the hepatotoxicity of AFB_1 in broilers.

Materials and methods

Experimental birds and diets (in vivo)

All experimental procedures were conducted with the approval of the Animal Care and Use Committee of College of Coastal Agricultural Sciences of Guangdong Ocean University (Approval No. 20221008, Zhanjiang, Guangdong, China). The PT was obtained from Shaanxi Baichuan Biotechnology Co., Ltd. (Xi'an, China). The AFB_1 was purchased from Sigma-Aldrich (St. Louis, MO, USA). A total of 360 one-day-old Abor Acres (AA) male broilers (Charoen Pokphand Group, Zhanjiang, China) were randomly and equally divided into 6 groups: Control (basal diet), AFB_1 (0.1 mg/kg AFB_1), AFB_1 + PT_{200} (0.1 mg/kg AFB_1 + 200 mg/kg PT), AFB_1 + PT_{400} (0.1 mg/kg AFB_1 + 400 mg/kg PT), AFB_1 + PT_{600} (0.1 mg/kg AFB_1 + 600 mg/kg PT), AFB_1 + PT_{800} (0.1 mg/kg AFB_1 + 800 mg/kg PT). There were 6 replicates and 10 birds per replicate. Birds of each replication were housed in wire cages (80 cm length × 70 cm width × 40 cm height) equipped with nipple waterer, for a feeding period of 21 d. The doses and the experimental cycle were chosen on the basis of previous studies, which reported that dietary consumption of 0.1 mg/kg AFB_1 for 21 d induced liver damage in broiler chickens [35, 36]. The temperature remained around 33–35 °C for the first three days, then reduced gradually (2–3 °C every week) from 35 to 26 °C, and the relative humidity was maintained at 60%–70%. The basal diets were formulated to meet the nutrient requirements of broilers (NRC, 1994) [37] and feeding standards for the AA [38]. AFB_1 is dissolved in methanol to make a solution, and then evenly sprayed into the basal diet and mixed to obtain the 0.1 mg/kg AFB_1-contaminated diet [39]. The equivalent methanol was sprayed evenly on the normal feed to obtain the basal diet. The treatment concentration of PT was calculated and added uniformly into the diet and mixed evenly. All the diets were evaporated at 37 °C and mixed with a vertical mixer. The ingredient composition and nutritional levels of the basal diet are shown in Table 1.

Table 1 Compositions and nutrient levels of experimental basal diet (as-fed basis)

Ingredients	Contents, %	Nutrient levels	Contents, %
Corn	53.00	ME, MJ/kg	12.42
Soybean meal	33.00	Crude protein	20.87
Wheat bran	4.60	Ca	1.00
Fishmeal	2.00	Total P	0.68
Soybean oil	3.00	Available P	0.46
Limestone	1.50	Lys	1.22
$CaHPO_4$	1.60	Met	0.53
L-Lysine	0.10	Total Met + Cys	0.87
DL-Methionine	0.20		
NaCl	0.30		
Choline chloride	0.20		
Mineral premix[1]	0.30		
Vitamin premix[2]	0.20		

[1] Premix provided per kilogram of diet: 8 mg of Cu ($CuSO_4$), 80 mg of Fe ($FeSO_4$), 85 mg of Mn ($MnSO_4$), 80 mg of Zn ($ZnSO_4$), 0.2 mg of Se (Na_2O_3Se), 0.15 mg of I [$Ca(IO_3)_2$]

[2] Premix provided per kilogram of diet: 9,000 IU of vitamin A, 3,240 IU of vitamin D_3, 6 IU of vitamin E, 0.75 mg of vitamin K_3, 1.5 mg of vitamin B_1, 4.5 mg of vitamin B_2, 10.5 mg of vitamin B_3, 1.5 mg of vitamin B_6, 0.45 mg of folic acid, and 9 mg of pantothenic acid

Sample collection

At the end of 21 d, one bird was randomly selected from each replicate ($n = 6$/treatment). The blood samples were collected into anticoagulation tubes from broiler's wing vein. Then, the birds were sacrificed and quickly dissected and liver samples were collected. The individual serum sample was separated by centrifuging at 3,000 r/min for 15 min and then stored at −80 °C until further analysis. The liver samples were placed in an enzyme-free tube with 4% formaldehyde solution and stored at room temperature to prepare tissue sections. In order to be used for the subsequent determination of liver function, metabolite content, antioxidant, and gene expression, the remaining samples were placed in a sterile frozen tube and quickly frozen with liquid nitrogen for storage at −80 °C until further analysis.

Growth performance analysis

During the feeding trial, broilers were weighed, and feed consumption was recorded weekly to calculate the average daily gain (ADG), average daily feed intake (ADFI), and the feed conversion ratio (FCR). In addition, the organ weight of the liver is taken after slaughter to calculate the liver index. The formula is as follows:

$$\text{Liver index (\%)} = \left[\text{liver weight (g)}/\text{body weight (g)}\right] \times 100\%$$

H&E staining

Liver tissue samples were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (H&E). The slices were placed under a 200 × microscope, and 20 visual fields were evaluated with a score of 0 for no lesions, 1 point for less than 3 lesions, 2 points for more than 3 and less than 5 lesions, and 3 points for more than 5 lesions. Images were observed with a fluorescence microscope (GD-30REL) under 200 × and 400 × magnification and captured by CapStudio software (Version No. 3.8.6).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

The single apoptotic nucleus or apoptotic body was stained in situ by fluorescence TUNEL assay. First, paraffin sections were dewaxed to water, and then protease K working solution (original solution:PBS = 1:9) was dripped to cover the tissues. After a series of steps, such as breaking the membrane, balancing at room temperature, adding TUNEL reaction solution, DAPI counterstaining the nucleus, and sealing the slices, the sections were viewed under a fluorescence microscope (GD-30REL), and the apoptosis rate was calculated. Principally, the nucleus stained by DAPI is blue under the excitation of ultraviolet light, the TUNEL kit (Wuhan Sevier Biotechnology Co., Ltd., Wuhan, China) is labeled with TMR fluorescein, and the positive apoptotic nucleus is red.

Transmission electron microscope (TEM)

The fresh liver tissues were cut into 2 mm^3 portions and fixed overnight at 4 °C with 2.5% glutaraldehyde. Then, washed three times with PBS for 15 min, and 1% osmium tetroxide at 4 °C for 2 h to fix. The slices were made by dehydration, embedding, sectioning, and staining. Finally, the mitochondrial structures were observed and recording using a TEM.

Hepatic function, AFB_1 metabolite content, and antioxidant indexes

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and the concentrations of total protein (TP) and albumin (ALB) were determined using the corresponding kits from the Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China). The contents of AFB_1 residues and AFBO-DNA adducts in the serum and liver of broilers were detected by ELISA kits produced by Jiangsu Meimian Industrial Co., Ltd. (Nanjing, China). The content of malondialdehyde (MDA), the activities of catalase (CAT), glutathione peroxidase (GSH-Px), glutathione S-transferase (GSH-ST), total superoxide dismutase (T-SOD), and the level of total antioxidant capacity (T-AOC) in the liver were measured using the commercial kits provided by Nanjing Jiancheng Bioengineering Research Institute Co., Ltd. (Nanjing, China). The heme oxygenase 1 (HO-1) and cytochrome P450 (CYP450) were determined by ELISA kits from the Jiangsu Meimian Industrial Co., Ltd. (Nanjing, China) according to the manufacturer's instructions.

Quantitative real-time PCR analysis

The total RNA was extracted from about 0.1 g liver sample using 1 mL RNA lysate (TRIzol reagent) following the manufacturer's instructions and transcribed into cDNA using a reverse transcription kit from the Novizan Co., Ltd. (Nanjing, China). RT-qPCR was conducted using qPCR SYBR Green Master Mix kit from Novizan Co., Ltd. (Nanjing, China) and Bio RAD CFX Connect PCR instrument (Hercules, CA, USA). The qPCR reaction system was as follows: Mix (10 μL), H_2O (8.2 μL), cDNA (1 μL), F/R (0.4/0.4 μL). The PCR procedure was as follows: 95 °C for 30 s, and then 40 cycles using a step program (95 °C for 5 s and 60 °C for 34 s), followed by 1 cycle of

95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The expression of the target genes was determined using the $2^{-\triangle\triangle CT}$ method, and the mRNA level of the housekeeping gene β-actin was used as an endogenous reference control. The primer sequence information is listed in Table S1.

Leghorn Male Hepatoma cell line culture

The Leghorn Male Hepatoma (LMH) cell line was obtained from iCell Bioscience, Inc. (Shanghai, China). The LMH cells were grown on 0.1% gelatin-coated culture dishes in DMEM/F12 (Gibco, New York, USA), supplemented with 10% fetal bovine serum (FBS) (SERANA, Shanghai, China), and 1% antibiotics (penicillin and streptomycin; Gibco, New York, USA) at 37 °C with 5% CO_2.

Study on the toxic effect of PT on LMH cells

The LMH cells were cultured in 96-well plates until 70%–80% confluence. Cells were treated with 0.025% DMSO as control and with PT at various concentrations (1.25, 2.5, 5, 10, 20, and 40 μg/mL). After treatment, 10 μL of the cell counting Kit-8 assay solution (CCK-8) (ZETA life, Shanghai, China) was added to each well and incubated for another 2 h. Then, the optical densities were read on a microplate reader (Bio Tek, Winooski, VT, USA) at 450 nm. At last, cell viability was calculated relative to the control group.

Study on the effect of PT on LMH cells under the action of AFB_1

To verify the effect of different concentrations of PT on the survival rate of LMH cells induced by AFB_1, the experiment was divided into the following 7 groups, including the control group containing 0.025% DMSO, the AFB_1 group containing 0.1 μg/mL AFB_1 and the AFB_1 + PT groups were containing 0.1 μg/mL AFB_1 + PT (0, 1, 2, 3, 4, 5 μg/mL). According to previous studies, treating with 0.1 μg/mL AFB_1 to the medium and incubating for 12 h had some damage to LMH cells [35]. The detection method was the same as those described in "Study on the toxic effect of PT on LMH cells".

In vitro verification of PT's action pathway on AFB_1-induced liver damage

To verify whether PT alleviates AFB_1-induced injury of LMH cells through the Nrf2 or Nrf1 pathway, the cells were divided into 4 groups: control group (containing 0.025% DMSO), AFB_1 group (0.1 μg/mL AFB_1), 0.1μg/mL AFB_1 + 1 μg/mL PT group and 0.1 μg/mL AFB_1 + 1 μg/mL PT + 1.9 μmol/L ML385 group or 0.1 μg/mL AFB_1 + 1 μg/mL PT + 10 μmol/L WRR139 group. ML385 is an Nrf2 inhibitor and WRR139 is an Nrf1 inhibitor; both were purchased from MedChemExpress (Shanghai, China). The well-grown LMH cells were seeded in 6-well plates at 70%–80% confluence, and the cells were treated for 12 h. Then, the experimental steps of qPCR were the same as described above.

Western blot analysis of protein expression in vitro

The cells were lysed with RIPA buffer (containing protease and phosphatase inhibitors; Servicebio, Wuhan, China). SDS-PAGE gels were used to electrophorese the protein samples, which were then transferred to PVDF membranes (Millipore, Burlington, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered-saline with Tween (TBST) for 2 h at room temperature, then incubated with primary antibodies overnight at 4 °C. After three washes with TBST, the membranes were incubated with a secondary antibody for 2 h at room temperature. Finally, the membranes were washed with TBST three times, and a Tanon 4600 System (Shanghai, China) was used to detect the expression of proteins with an enhanced chemiluminescence (ECL) kit (Tanon, Shanghai, China). The protein bands were scanned and quantified based on optical densities using ImageJ software (Version No. 1.5.4) and normalized to β-actin (Total protein) or Lamin B (Nuclear protein). Primary antibodies used were rabbit anti-Nrf2, rabbit anti-phospho-Nrf2 (ser 40), rabbit anti-Nrf1, rabbit anti-β-actin, and rabbit anti-Lamin B. Secondary antibody was used Goat Anti-Rabbit IgG (H + L). The antibody information is shown in Table S2.

Statistical analysis

All data were analyzed by one-way ANOVA using SAS 9.4 software (Cary, NC, USA), after checking the normal distribution and homogeneity of variances, and the difference between the mean values were analyzed by Duncan's multiple comparison. $P < 0.05$ indicates a significant difference, and $0.05 \leq P < 0.10$ indicates that it tends to be significant.

Results

PT improves growth performance and hepatic function in broilers exposed to AFB_1

Compared with the control group, the ADFI and FCR of broilers in the AFB_1 group increased, while the liver index decreased ($P < 0.05$). Compared with AFB_1, ADFI was decreased by adding 400, 600, and 800 mg/kg PT, and FCR was decreased by adding 400 mg/kg PT ($P < 0.05$). In addition, dietary supplementation of 600 mg/kg PT increased liver index (Fig. 1B). Hepatic function showed

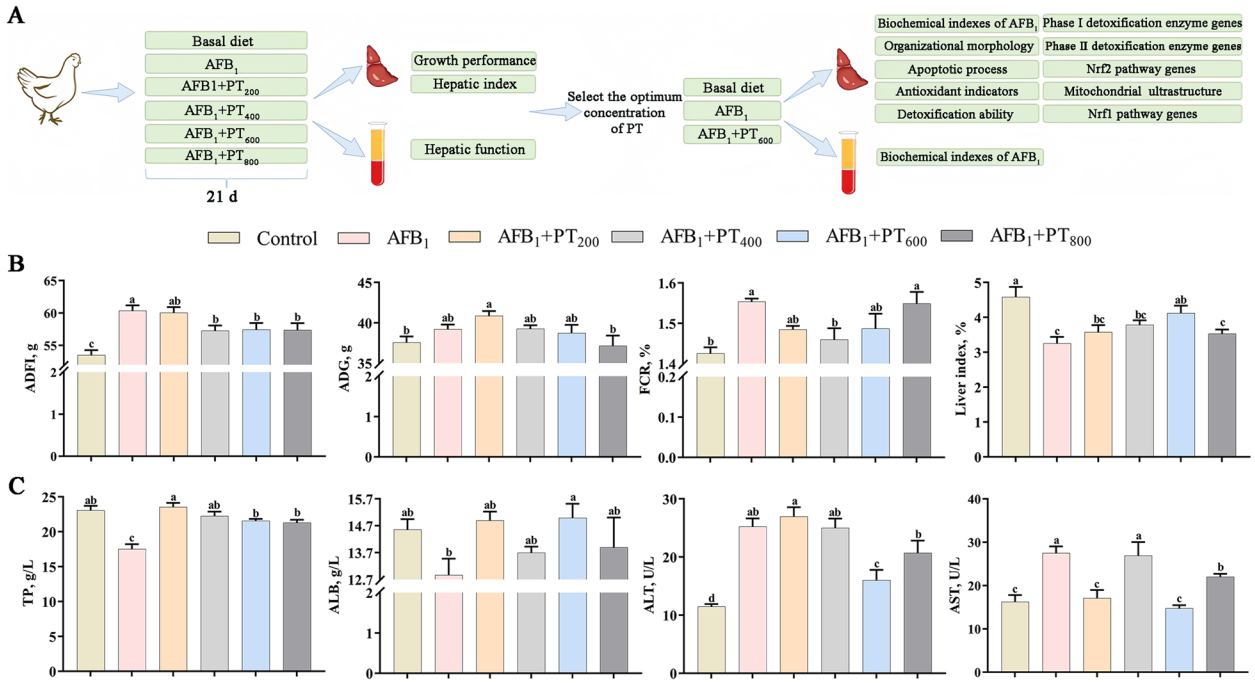


Fig. 1 Effect of phlorotannin on AFB_1-induced the growth performance and hepatic function in broilers. **A** Outline of experimental procedures to examine the role of PT in AFB_1-induced broilers. **B** Growth performance and liver index. ADFI, Average daily feed intake; ADG, Average daily gain; F/G, Average daily feed intake/Average daily gain. **C** Serum TP and ALB contents and serum ALT and AST activity in the indicated broilers. TP, Total protein; ALB, Albumin; ALT, Alanine aminotransferase; AST, Aspartate transaminase. AFB_1, Aflatoxin B_1; PT, Phlorotannin; Control, basal diet; AFB_1, basal diet + 0.1 mg/kg AFB_1; $AFB_1 + PT_{200}$, basal diet + 0.1 mg/kg AFB_1 + 200 mg/kg PT; $AFB_1 + PT_{400}$, basal diet + 0.1 mg/kg AFB_1 + 400 mg/kg PT; $AFB_1 + PT_{600}$, basal diet + 0.1 mg/kg AFB_1 + 600 mg/kg PT; $AFB_1 + PT_{800}$, basal diet + 0.1 mg/kg AFB_1 + 800 mg/kg PT. Data are shown as mean ± SEM ($n = 6$). $^{a–d}$Different superscript letters indicate significant differences ($P < 0.05$)

that the serum activities of ALT and AST of broilers in the AFB_1 group were higher than those in the control group, while the content of TP was reduced, and the content of TP was increased by supplementation of 200, 400, 600 and 800 mg/kg PT ($P < 0.05$). Supplementation of 600 mg/kg PT increased the ALB content and decreased the activity of ALT and supplementation of 200, 600, and 800 mg/kg PT decreased the activity of AST ($P < 0.05$; Fig. 1C). Based on the above results, it can be found that 600 mg/kg PT supplementation has more obvious effects on the liver index and liver function of broilers induced by AFB_1. Therefore, PT (600 mg/kg) was selected as the best active dose.

PT alleviates liver histomorphology and apoptosis in broilers exposed to AFB_1

The H&E staining revealed that the liver cells were arranged in a compact and normal shape and structure in the control group. Compared with the control group, the AFB_1 group had poorly demarcated of hepatocytes, many cells showed inflammatory infiltration, some cytoplasm was loose, and there were a lot of round vacuoles with different sizes and well-demarcated, while after PT supplementation, the inflammatory infiltration of hepatocytes was reduced, the vacuoles in the cytoplasm disappeared, and the morphology and structure of hepatocytes were relieved (Fig. 2A). The scores of inflammations were higher in the AFB_1 group than in the control group. The degree of inflammation decreased after supplementation of 600 mg/kg PT ($P < 0.05$; Fig. 2B). Using the TUNEL staining assay, it clearly observed that the apoptosis of liver cells in AFB_1 group was more than that in the control group (Fig. 2C). The apoptosis of liver cells was decreased when PT was supplemented ($P < 0.05$; Fig. 2D).

PT boosts hepatic antioxidant capacity and Nrf2-mediated phase II detoxification enzyme ability in broilers exposed to AFB_1

The results revealed elevated AFB_1 and AFBO-DNA contents in serum and liver of the AFB_1 group compared with the control group, which decreased in 600 mg/kg PT treatment ($P < 0.05$; Fig. 3A). Through detecting antioxidant indexes and detoxification ability, AFB_1 reduced T-AOC level, CAT, T-SOD, GST, GPX and HO-1 activities and increased MDA content in broiler liver compared with the control group

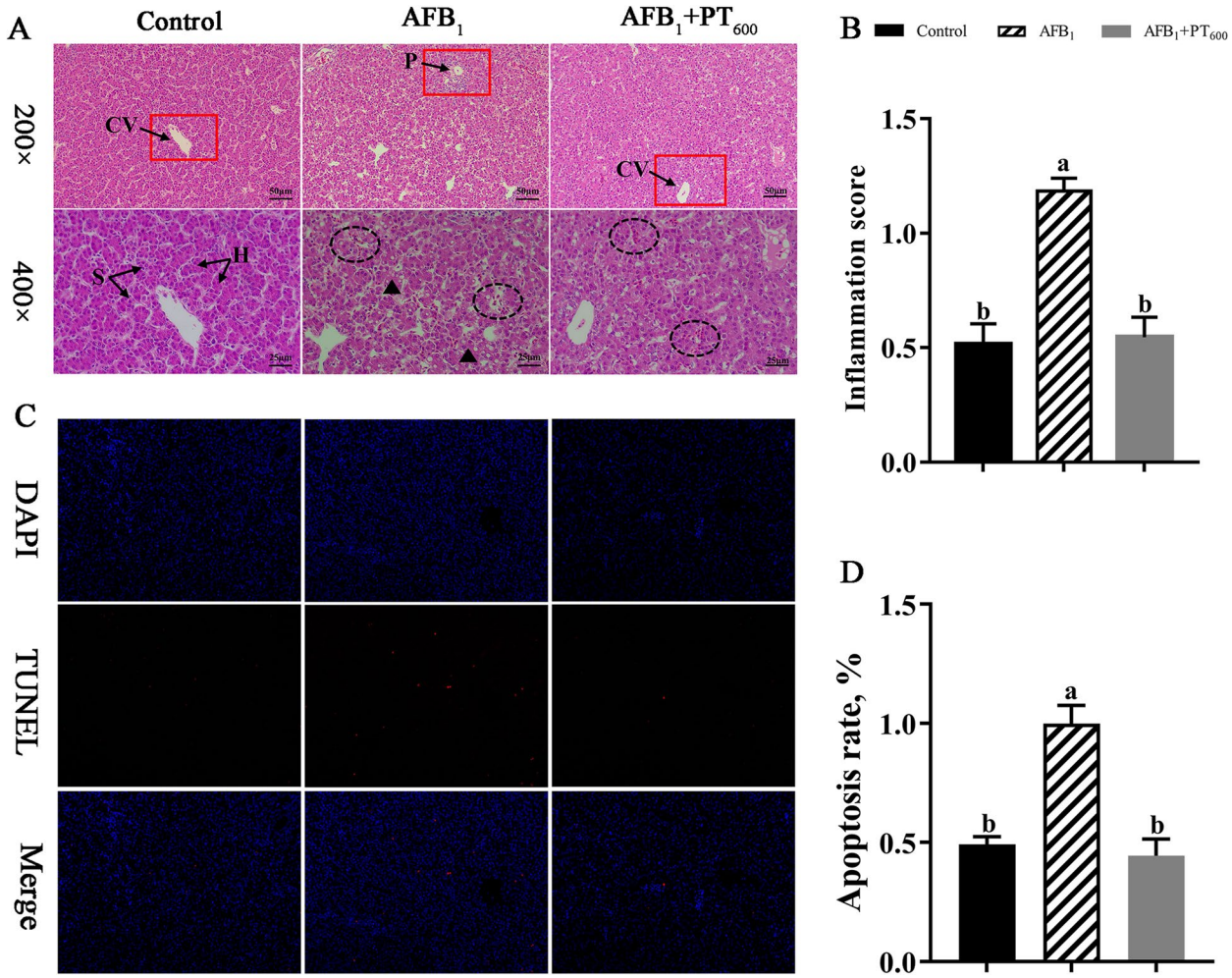


Fig. 2 Effect of phlorotannin on AFB_1-induced liver structure and apoptosis of broilers. **A** H&E staining images of liver sections in indicated broilers. CV: Central vein; H: Hepatocytes; S: Hepatic sinusoid; P: Portal area. Black triangle represents steatosis. Black ellipse represents inflammatory infiltration. The scar bar is 50 μm of 200 × and 25 μm of 400 ×. **B** Inflammation score by H&E staining images. **C** Representative image of immunofluorescence staining for TUNEL in broiler's liver. The scar bar is 200 ×. **D** Apoptosis rate by TUNEL staining images. AFB_1, Aflatoxin B_1; PT, Phlorotannin; Control, basal diet; AFB_1, basal diet + 0.1 mg/kg AFB_1; AFB_1 + PT_{600}, basal diet + 0.1 mg/kg AFB_1 + 600 mg/kg PT. Data are shown as mean ± SEM ($n = 6$). ^{a,b}Different superscript letters indicate significant differences ($P < 0.05$)

($P < 0.05$). Compared with the AFB_1 group, the level of T-AOC, the activities of CAT, GST, and GPX in the liver of broilers were increased, and the content of MDA was decreased after supplementation of 600 mg/kg PT ($P < 0.05$; Fig. 3B). The q-PCR results of phase I and II detoxification enzyme related genes showed that AFB_1 upregulated *CYP1A1* and *CYP2A6*, downregulated *GPX3* and *GSTA3* relative expression compared with the control group ($P < 0.05$). Compared with the AFB_1 group, PT treatment was downregulated *CYP1A1*, *CYP1A2*, *CYP2A6*, and *CYP3A4*, and relative expression amounts of *GPX1*, *GPX3*, *GSTT1*, *GSTA3*, *GSTO1*, *NQO1*, and Glutamate-cysteine Ligase (*GCLM*) were upregulated ($P < 0.05$; Fig. 3C and D). Nrf2 pathway-related gene expression showed that AFB_1 decreased the relative expression of *MafF*, *MafK*, and *Nrf2* and upregulated the relative expression of *Keap1* ($P < 0.05$). In contrast, the relative expression of *MafG*, *MafK*, *SOD2*, and *Nrf2* increased, and the relative expression of *Keap1* decreased after supplementation of 600 mg/kg PT ($P < 0.05$; Fig. 3E).

PT attenuates mitochondrial damage of the liver and upregulated the Nrf1 pathway in broilers exposed to AFB_1

TEM images showed that the structure of mitochondria was normal, the double-layer membrane was intact and clear, and the arrangement of mitochondrial cristae was

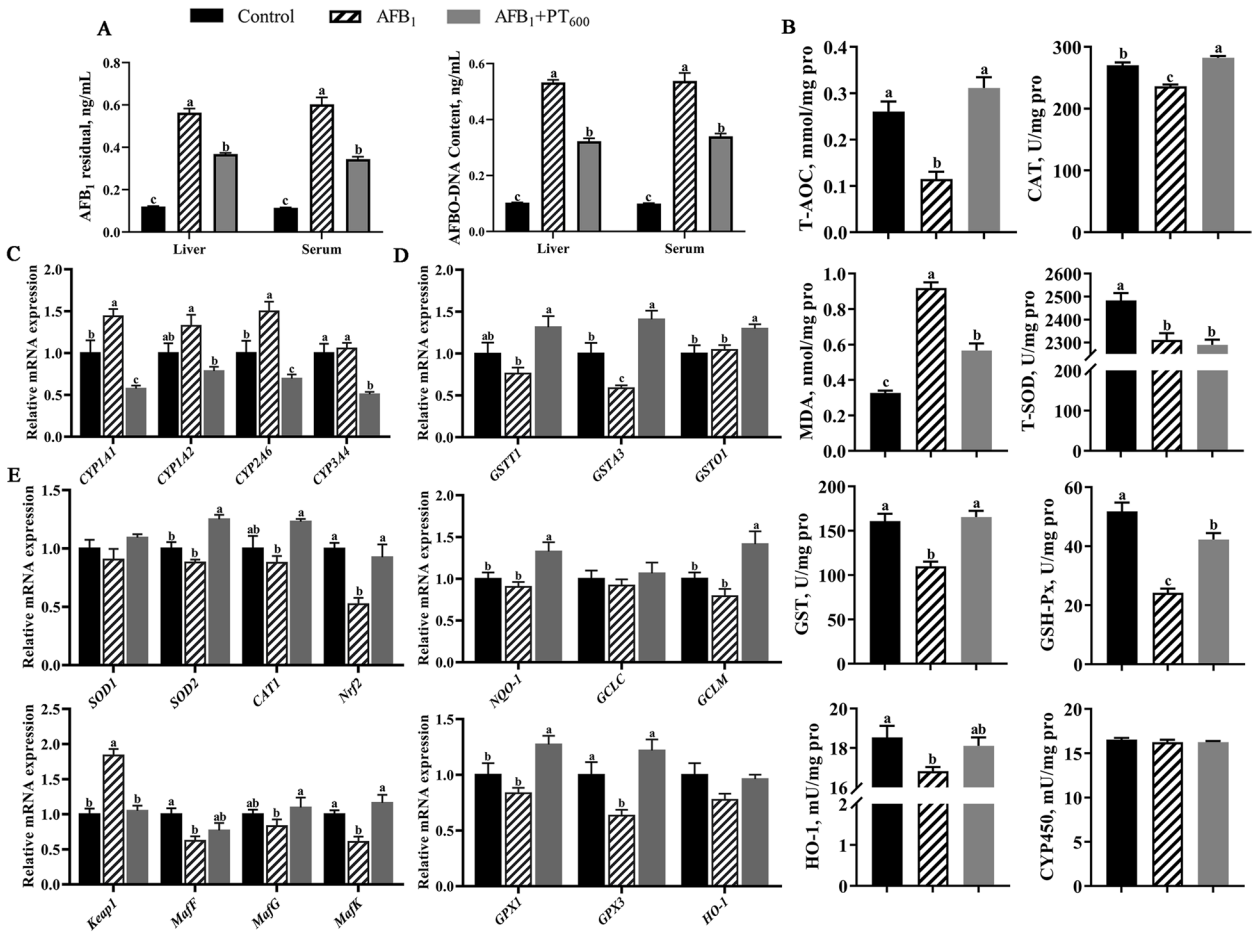


Fig. 3 Effect of phlorotannin on AFB_1-induced liver antioxidant capacity and Nrf2-mediated phase II etoxification enzyme ability of broilers. **A** Liver and serum AFB_1 and AFBO contents in broilers. **B** Liver antioxidant levels and detoxification ability of broilers. **C** Relative mRNA levels of Phase I detoxification enzyme in broilers liver. **D** Relative mRNA levels of Phase II detoxification enzyme in broilers liver. **E** Relative mRNA levels of Nrf2 pathway-related gene in broilers liver. AFB_1, Aflatoxin B_1; PT, Phlorotannin; Control, basal diet; AFB_1, basal diet + 0.1 mg/kg AFB_1; $AFB_1 + PT_{600}$, basal diet + 0.1 mg/kg AFB_1 + 600 mg/kg PT. Data are shown as mean ± SEM ($n = 6$). $^{a–c}$Different superscript letters indicate significant differences ($P < 0.05$)

normal in the control group. In contrast, in the AFB_1 group, the swelling of mitochondria was severe, the structure of the double-layer membrane was blurred or even disappeared, and the arrangement of Cristae was disordered. Compared with the AFB_1 group, PT (600 mg/kg) could reduce the swelling of mitochondria and restore the structure of most mitochondrial bilayer membranes (Fig. 4A and B). Nrf1 pathway-related gene expression showed that AFB_1 reduced the relative expression of *TFAM*, *Nrf1,* Mitofusin 1 *(MFN1)*, and *MFN2* and upregulated the relative expression of *Mff* ($P < 0.05$), compared with the control group. After supplementing PT, relative expression of *TFAM, Nrf1*, optic atrophy 1 (*OPA1*), and *MFN1* were upregulated, and *Mff* and *DRP1* were down-regulated ($P < 0.05$; Fig. 4C).

Optimal concentration of mitigative effect of PT on LMH cell injury induced by AFB_1

About 1.25 and 2.5 μg/mL PT increased cell survival rate compared with the control group, while concentrations of PT (10, 20, and 40 μg/mL) resulted in a survival rate declined ($P < 0.05$; Fig. 5B). Compared with the control group, AFB_1 decreased cell survival rate, while concentrations of PT (1 and 2 μg/mL) result in increased cell survival rate ($P < 0.05$), and the cell survival rate was the highest when the concentration of PT was 1 μg/mL (Fig. 5D). Consequently, 1 μg/mL of PT was further examined for its impact on AFB_1-induced LMH cells.

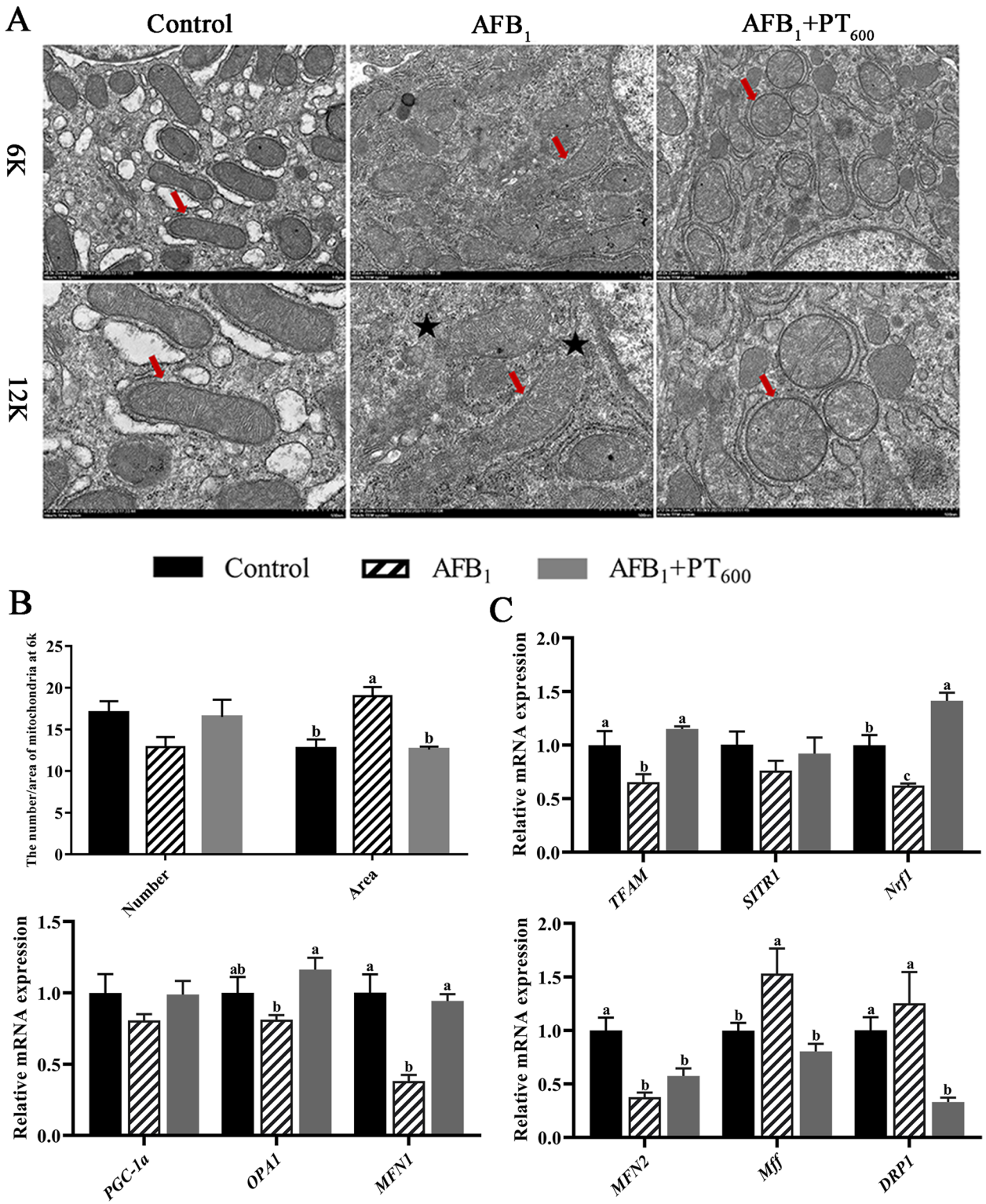


Fig. 4 Effect of phlorotannin on AFB_1-induced liver mitochondrial damage of broilers. **A** Transmission electron microscope image of broiler's liver mitochondria. The scar bar is 6 K and 12 K. Red arrow represents the mitochondrial bilayer membrane structure. Black pentagram represents the blurring or disappearance of the mitochondrial bilayer membrane structure. **B** The number and area of mitochondria at 6 K. The unit of area is μm^2. **C** Relative mRNA levels of Nrf1 pathway-related gene in broilers liver. AFB_1, Aflatoxin B_1; PT, Phlorotannin; Control, basal diet; AFB_1, basal diet + 0.1 mg/kg AFB_1; $AFB_1 + PT_{600}$, basal diet + 0.1 mg/kg AFB_1 + 600 mg/kg PT. Data are shown as mean ± SEM ($n = 6$). $^{a–c}$Different superscript letters indicate significant differences ($P < 0.05$)

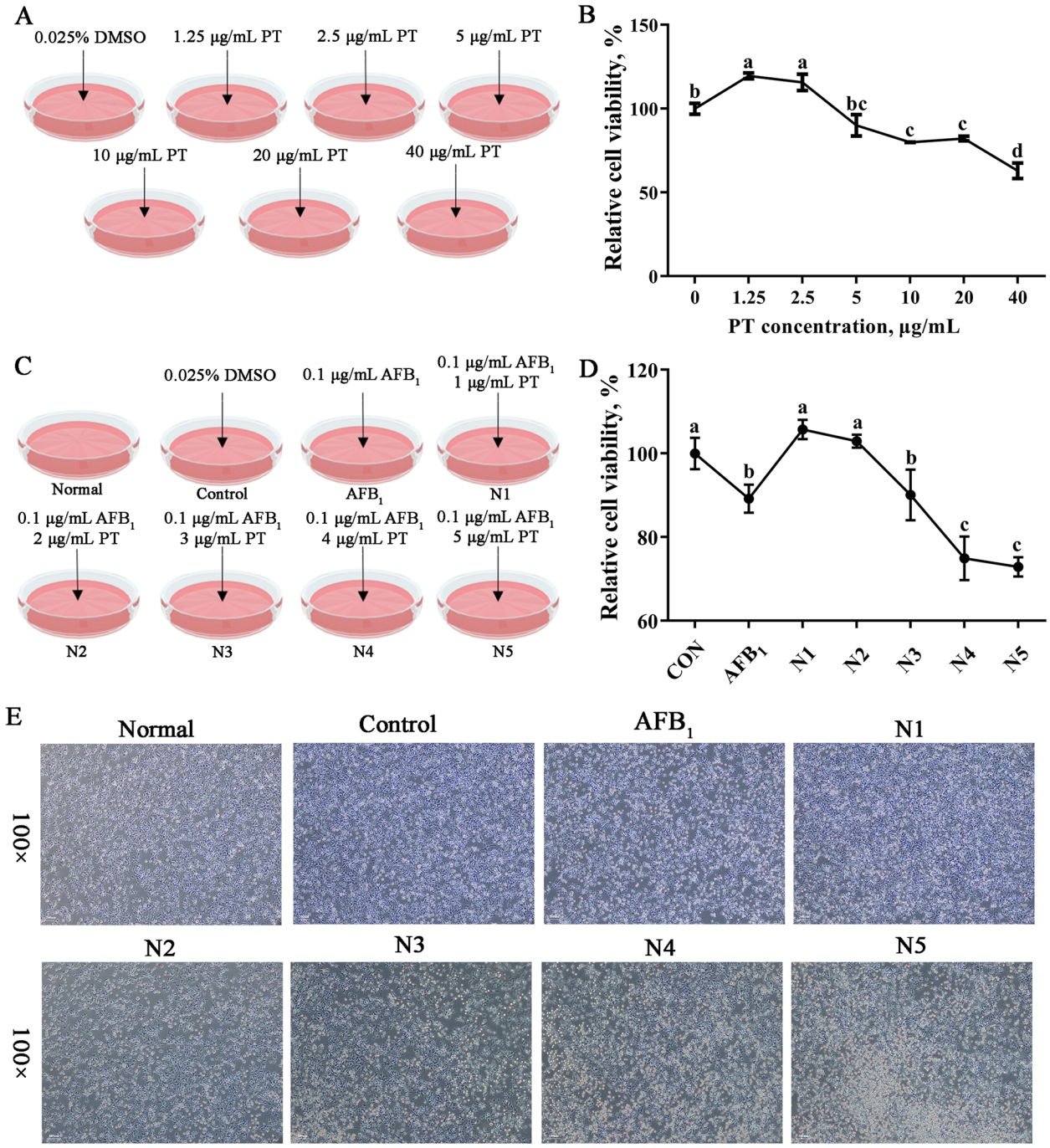


Fig. 5 Effects of phlorotannin with different concentrations on AFB_1-induced LMH cells. **A** Outline of the experimental grouping of different concentrations of PT on the survival rate of LMH cells. **B** Survival rate of LMH cells treated with different concentrations of PT for 12 h. **C** Outline of the experimental grouping of different concentrations of PT on the survival rate of AFB_1-induced LMH cells. **D** Survival rate of AFB_1-induced LMH cells treated with different concentrations of PT for 12 h. **E** LMH cell state diagram. The scar bar is 100 ×. AFB_1, Aflatoxin B_1; PT, Phlorotannin; Normal, 10% complete medium; Control, 10% complete medium + 0.025% DMSO; AFB_1, 10% complete medium + 0.1 μg/mL AFB_1; N1, 10% complete medium + 0.1 μg/mL AFB_1 + 1 μg/mL PT; N2, 10% complete medium + 0.1 μg/mL AFB_1 + 2 μg/mL PT; N3, 10% complete medium + 0.1 μg/mL AFB_1 + 3 μg/mL PT; N4, 10% complete medium + 0.1 μg/mL AFB_1 + 4 μg/mL PT; N5, 10% complete medium + 0.1 μg/mL AFB_1 + 5 μg/mL PT. Data are shown as mean ± SEM ($n = 6$). $^{a–d}$Different superscript letters indicate significant differences ($P < 0.05$)

PT reduces AFB_1-induced oxidative stress and hepatotoxicity of LMH cells through Nrf2 pathway

Compared with the control group, AFB_1 treatment significantly downregulated the relative expression of *GSTT1*, *GPX4*, *GSTO1*, *NRF2*, *GSTA3*, and *GPX3* and upregulated the expression of *Keap1* in LMH cells ($P < 0.05$). About 1 μg/mL PT treatment promoted the expression of *GSTT1*, *GPX4*, *Nrf2*, *GSTT1*, and *GPX4* and decreased the relative expression of *Keap1* compared with the AFB_1 group. Nrf2 inhibitor (ML385) treatment reduced the relative expression of *GSTT1, Nrf2, GPX3, HO-1*, and *NQO1* and increased the relative expression of *Keap1* ($P < 0.05$; Fig. 6A). By western blotting, compared with the control group, AFB_1 treatment reduced the expression of p-Nrf2 protein and decreased the nuclear incorporation of p-Nrf2 ($P < 0.05$). PT treatment increased the expression of total Nrf2, p-Nrf2, and nuclear p-Nrf2 compared to the AFB_1 group, but Nrf2 inhibitor (ML385) treatment reversed the influence compared to the PT group ($P < 0.05$; Fig. 6B and C).

PT alleviates AFB_1-induced mitochondrial damage of LMH cells via Nrf1 pathway

The mRNA levels of *TFAM* and *MFN1* were decreased in the presence of AFB_1 compared with the control ($P < 0.05$). When pretreated with 1 μg/mL PT, the mRNA levels of *Nrf1, TFAM,* and *MFN1* were dramatically increased compared with the AFB_1 group, but Nrf1 inhibitor (WRR139) treatment inhibited the effect of PT ($P < 0.05$; Fig. 7A). By western blotting, compared with the control group, the total and nuclear Nrf1 protein expression levels of LMH cells in AFB_1 group were down-regulated ($P < 0.05$). The total and nuclear Nrf1 protein expression levels were upregulated in PT treatment compared with the AFB_1 group ($P < 0.05$); such an elevation was reduced when exposed to an Nrf1 inhibitor (WRR139) ($P < 0.05$;Fig. 7B and C).

Discussion

Contamination of AFB_1 is a challenge to the livestock feed industry. Poultry as one of the most sensitive animals to AFB_1, particularly the chicks, their liver detoxification ability is relatively weak, making them susceptible to AFB_1 [40]. The investigation report of animal feed pollution in China showed that the detection rate of AFB_1 in poultry diets was 99.6%, and the maximal concentration was 54 μg/kg [4]. The Chinese feed hygiene standards set the maximum limit of AFB_1 in broiler feed at 10–20 μg/kg [41]. It has been reported that when exposed to 50 μg/kg AFB_1, the ADG of broilers decrease by 5%–10%, and when exposed to 100 μg/kg AFB_1, the ADG decrease by 10%–20% in broilers [42]. Gao et al. [43] found that broiler chicks exposed to 0.1 mg/kg of AFB_1 for 21 d had reduced growth performance and liver damage. Ma et al. [44] found that the FCR of AA broiler with 0.1 mg/kg AFB_1 in the basal diet was higher than that of the control group, and reached 1.87%. In our study, after feeding 0.1 mg/kg of AFB_1 for 21 d, the ADFI and FCR of broilers increased, and the liver index decreased. It can be seen that feeding broiler chickens 0.1 mg/kg AFB_1 has a negative effect on their growth performance and liver. However, currently, there is limited research on the use of PT in broilers, but other polyphenols have been proven to improve the growth performance of poultry [45]. Jiang et al. [46] showed that curcumin can reduce the adverse effects of AFB_1 and promote the growth and development of ducklings. Similarly, Yang et al. [47] found that resveratrol could improve the growth performance by increasing the ADG and final weight of ducklings. We found that supplementation of 400, 600, and 800 mg/kg PT decreased the ADFI, and 400 mg/kg PT decreased the FCR of broilers. In addition, supplementation of 600 mg/kg PT can significantly increase the liver index of broilers. To sum up, the results showed that PT can alleviate the growth performance decline of broilers caused by AFB_1 to some extent. It can be attributed to PT improving the liver function damage of broilers induced by AFB_1 and promoting the metabolism of nutrients and excretion of non-nutrients.

As the main target organ of AFB_1, AFB_1 can cause severe damage to the liver and hepatocytes [1]. The damage to the liver can lead to abnormal metabolism of nutrients, which can be reflected by blood biochemical indexes. However, the activities of AST, ALT, and ALP in serum are commonly used to detect whether the liver function is normal [48]. AST and ALT are synthesized by liver cells [49]. Exposure of broilers to AFB_1 causes serious hepatic injury, which leads to the release of aminotransferase from liver cells into the blood when the permeability of liver cells increases, thus improving the serum activities of ALT and AST, which is consistent with the results of this study [50]. A previous study has shown that resveratrol can improve the oxidative stress of the liver induced by AFB_1, such as reducing the activities of ALT, AST, and ALP in the liver of broilers [51]. Supplementation of 200, 400, 600, and 800 mg/kg PT in this study increased the TP content. Supplementation of 200, 600, and 800 mg/kg PT decreased the activity of AST. It is worth noting that supplementation of 600 mg/kg PT increased the ALB content and decreased the ALT activity. Therefore, based on some indexes, we considered 600 mg/kg PT as the best dose to alleviate the liver damage induced by AFB_1 in broilers. A previous study reported that AFB_1 can cause histopathological changes in the liver, such as necrosis, degeneration, and inflammation [52]. In addition, AFB_1 can induce apoptosis and increase

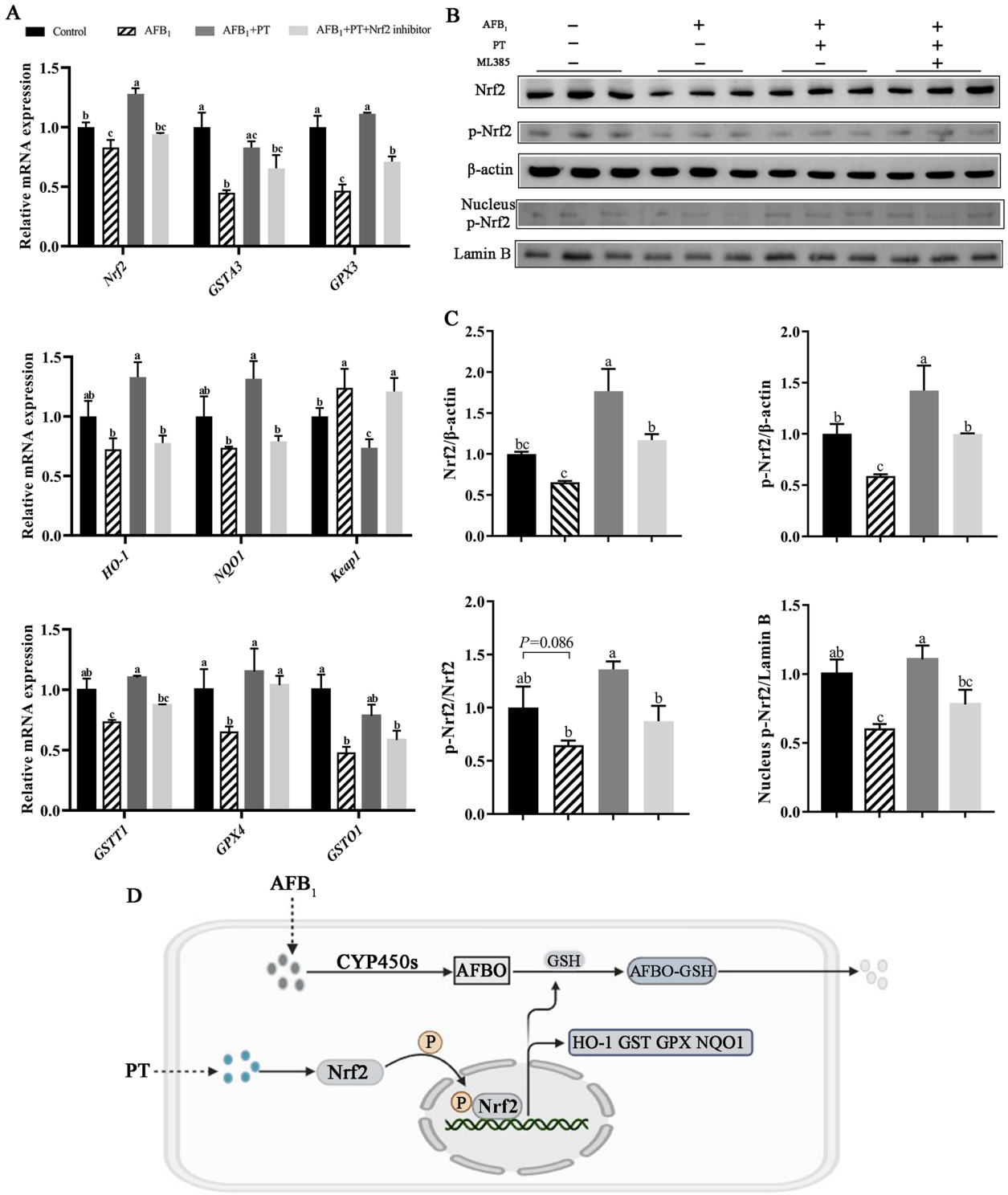


Fig. 6 Effect of phlorotannin on AFB_1-induced oxidative stress of LMH cells through Nrf2 pathway. **A** Relative mRNA levels of Nrf2 pathway-related gene in AFB_1-induced LMH cells. **B** Western blot analysis of Nrf2, total and nuclear p-Nrf2 in AFB_1-induced LMH cells. Lamin B and β-actin served as loading control. **C** Quantification of protein expression Nrf2, total and nuclear p-Nrf2. **D** Schematic diagram of AFB_1 metabolism and PT detoxification pathway. AFB_1, Aflatoxin B_1; PT, Phlorotannin; Control, 10% complete medium + 0.025% DMSO; AFB_1, 10% complete medium + 0.1 μg/mL AFB_1; AFB_1 + PT, 10% complete medium + 0.1 μg/mL AFB_1 + 1 μg/mL PT; AFB_1 + PT + Nrf2 inhibitor, 10% complete medium + 0.1 μg/mL AFB_1 + 1 μg/mL PT + 1.9 μmol/L ML385 (Nrf2 inhibitor).– for not adding, + for adding. Data are shown as mean ± SEM ($n = 6$). $^{a–c}$Different superscript letters indicate significant differences ($P < 0.05$)

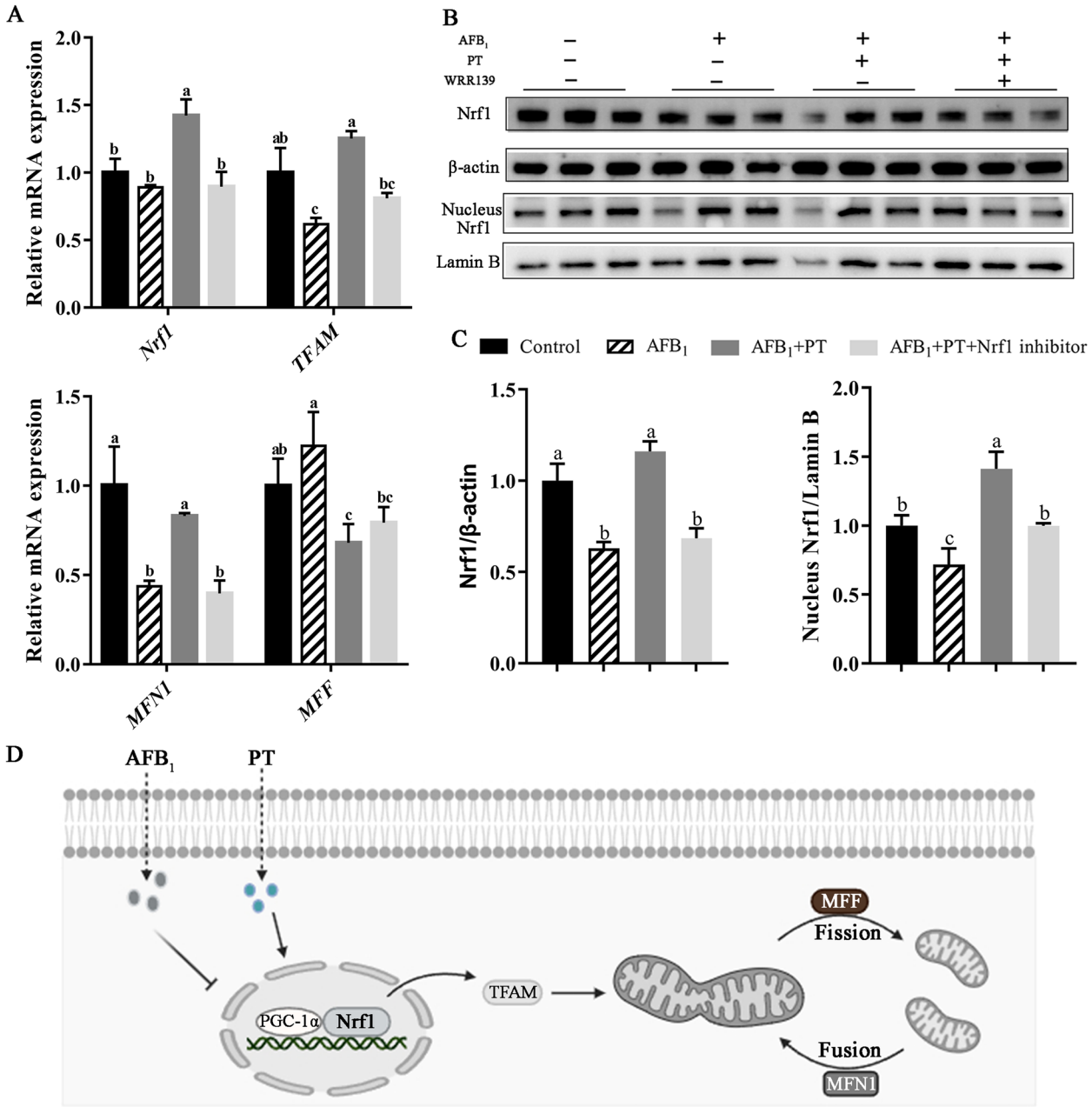


Fig. 7 Effect of phlorotannin on AFB_1-induced mitochondrial damage of LMH cells via Nrf1 pathway. **A** Relative mRNA levels of Nrf1 pathway-related gene in AFB_1-induced LMH cells. **B** Western blot analysis of total and nuclear Nrf1 in AFB_1-induced LMH cells. Lamin B and β-actin served as loading control. **C** Quantification of protein expression total and nuclear Nrf1. **D** Schematic diagram of mitochondrial function mediated by AFB_1 and PT on Nrf1. AFB_1, Aflatoxin B_1; PT, Phlorotannin; Control, 10% complete medium + 0.025% DMSO; AFB_1, 10% complete medium + 0.1 μg/mL AFB_1; AFB_1 + PT, 10% complete medium + 0.1 μg/mL AFB_1 + 1 μg/mL PT; AFB_1 + PT + Nrf1 inhibitor, 10% complete medium + 0.1 μg/mL AFB_1 + 1 μg/mL PT + 10 μmol/L WRR139 (Nrf1 inhibitor). – for not adding, + for adding. Data are shown as mean ± SEM ($n = 6$). $^{a–c}$Different superscript letters indicate significant differences ($P < 0.05$)

the percentage of apoptosis, thus causing liver damage. Li et al. [45] found that broilers fed AFB_1-contaminated diets showed inflammatory cell infiltration, hepatic lobule structure, and hepatic cord disorder in the liver, and these pathological changes were improved after curcumin supplementation. Similar to the above research results, with supplementation of 600 mg/kg PT, the overall pathological changes of the liver were alleviated, the inflammatory infiltration of hepatocytes was significantly reduced, the vacuoles in the cytoplasm disappeared, and

TUNEL staining revealed a reduction in AFB_1-induced hepatocyte apoptosis. Also, in vitro assays found that the survival rate of hepatocytes significantly increased. It suggests that PT could protect the liver from the damage caused by AFB_1 and maintain the normal structure and function of hepatocytes.

It is well known that the liver is the main organ for the accumulation and metabolism of AFB_1, mainly mediated by phase I and phase II detoxification enzymes, and phase I and II detoxification are two different types of metabolic processes [53]. Phase I reaction is mainly oxidation, reduction, and hydrolysis, which is usually a coupling reaction as a chemical construction of phase II, and Nrf2 is not only a key transcription factor to regulate oxidative stress response but also a key transcription factor to regulate phase II detoxification enzymes [53]. For the hepatotoxicity induced by AFB_1, Nrf2 can enhance antioxidant capacity by promoting the expression of phase II etoxification enzymes. GSTs, as the key of phase II detoxification enzyme, which isozymes are divided into classes of Alpha (GSTA), Pi (GSTP), Theta (GSTT), Omega (GSTO) and so on [54]. Studies have shown that the expression of GSTA3, GSTO1, and GSTT1 play an important role in regulating redox balance and metabolizing exogenous toxins [55, 56]. It is believed that the ability of AFBO to undergo conjugate binding reaction in vivo depends on the content, type, and activity of GST, which catalyzes the coupling of AFB_1 with glutathione (GSH) to produce a non-toxic product (AFBO-GSH), which are excreted in urine [52]. In this study, AFB_1 resulted in the decreased T-AOC level, activities of GST, GSH-Px, and HO-1, and relative expression of *MafF*, *MafK*, and *Nrf2* in broilers liver, and PT supplementation upregulated the activities of antioxidant enzymes and phase II detoxification enzymes and the expression of Nrf2 signaling pathway-related genes. Zhang et al. [57] reported that curcumin alleviated AFB_1-induced liver damage in broilers by promoting the expression of Nrf2 pathway-related genes, which is consistent with our findings.

We conducted in vitro trials to verify our results and to determine whether PT can alleviate AFB_1-induced liver injury through the Nrf2 signaling pathway. The results showed that by adding 1 μg/mL PT to LMH cells exposed to AFB_1, the relative expression of Nrf2-related genes and proteins was significantly upregulated after supplementing PT. The Nrf2 signaling pathway was affected after supplementation of Nrf2 inhibitor (ML385), and the nucleation of p-Nrf2 protein was reduced. Luo et al. [33] showed that nano-ions based on phlorotannin effectively attenuated carbon tetrachloride (CCl_4)-induced liver damage, oxidative stress, and inflammation in mice liver by modulating the Nrf2/HO-1 signaling pathway. Park et al. [59] found that phlorotannin increased the phosphorylation of Nrf2 in myoblasts and upregulated the expression of *HO-1* and its enzymatic activity, which was similar in this study. Our results confirm that PT inhibited AFB_1-induced liver damage in broilers by activating the phase II detoxification enzyme pathway mediated by Nrf2 signaling pathway. The antioxidant system of the body is regulated by antioxidant enzymes and the glutathione redox system [60]. Antioxidant enzymes are an important factor in maintaining redox balance, and the elimination of free radicals in tissues depends on a variety of antioxidant enzymes, including SOD, CAT, and GSH-Px [61]. Studies have confirmed that the damage of AFB_1 to the liver is mainly due to the destruction of the antioxidant system of the liver, which leads to liver damage and dysfunction [12]. In this study, AFB_1 decreased the level of T-AOC, decreased the activities of CAT, T-SOD, GST, GSH-Px, and HO-1, and increased the content of MDA in the broiler's liver, which was consistent with a previous study [62]. Wang et al. [63] showed that the addition of catechin effectively reduced the level of MDA and increased the level of T-AOC and the activity of CAT enzyme. Jin et al. [64] showed that adding curcumin to the diet could restore the activity of antioxidant enzymes in duck liver induced by AFB_1 to the normal level. Consistent with the above research, supplementation of 600 mg/kg PT in this study effectively alleviated the oxidative stress induced by AFB_1. Therefore, supplementation of PT can regulate the expression of Nrf2 to alleviate the oxidative stress induced by AFB_1 and promote the expression of phase II detoxification enzyme to play a detoxification role.

Mitochondria are crucial for energy metabolism in cells and are often called the "energy pool" of the cell [65]. As a central metabolic organ, the liver contains a large number of mitochondria [66], and as such, mitochondria are essential for metabolic homeostasis in the liver, and their dysfunction is also a major cause of liver disease [67]. However, the normal structure of mitochondria is the basis of its various functions, and the damage to mitochondrial structure relates to its functions [24]. AFB_1, as an important inducer of mitochondrial antioxidant dysfunction, can induce a large number of ROS, trigger oxidative stress, and further induce DNA and mitochondrial damage and inflammatory reaction [22]. When ROS is produced excessively, it disturbs the steady state of cells, leads to excessive mitochondria fusion and division, causes mitochondrial dysfunction, and leads to cell damage and even death [68]. In addition, AFB_1 can also decrease the level of mitochondrial membrane potential and induce the increase of mitochondrial permeability by uncoupling mitochondrial oxidative phosphorylation. The ultrastructure of hepatic mitochondria

was observed by transmission electron microscope. The structure of mitochondria was damaged by AFB_1, and most of the mitochondrial bilayer membrane was fuzzy and cristae were disordered, and the double-layer membrane structure was restored. These outcomes are similar to those of a previous study, which reported that when curcumin was added to the feed of broilers exposed to AFB_1, mitochondrion was clearly visible and evenly distributed, and its structure returned to normal levels [69]. Studies have shown that AFB_1 produces excess ROS, induces mitochondrial DNA damage and autophagy, reduces its membrane potential, and induces protein and lipid oxidation [1, 69, 70]. The liver is a highly oxygen-consuming organ, and its rich mitochondria account for about 18% of the cell volume. Therefore, the damaged mitochondria can be used as a marker of liver cell damage, and severe mitochondrial damage affects vital functions such as metabolism and detoxification in the liver [71]. There are evidence that AFB_1 reduces mitochondrial quality and interferes with mitochondrial biogenesis [24, 72]. Li et al. [2] found that the structure of mitochondria in the liver of broilers exposed to AFB_1 was obviously damaged, and the mitochondria showed severe swelling or vacuolization and disappearance of the mitochondrial crest, which is consistent with our findings. Mitochondrial biosynthesis is very important for repairing mitochondrial structure and maintaining its function, and this process is regulated by the key transcription activator PGC-1α [73]. Activation of PGC-1α can promote mitochondrial biosynthesis by activating the Nrf1/TFAM pathway [74]. However, as a key regulator of mitochondrial biosynthesis and function, Nrf1 is released from the endoplasmic reticulum to the cytoplasm by a series of complex reversal, modification, and processing processes, and finally into the nucleus to regulate the expression of downstream genes, such as mitochondrial transcription factor a (TFAM), Cytochrome c [75]. A series of studies have shown that PT and other polyphenols play key roles in ameliorating hepatotoxicity, interacting with Nrf1 signaling pathways to inhibit the damage of hepatic mitochondria [76, 77]. Huang et al. [78] found that the mitochondrial structure of mice exposed to AFB_1 was damaged, which showed that the mRNA expression levels of *PGC-1α*, *Nrf1*, *TFAM*, *DRP1*, *FIS1*, *MFN1* and *OPA1* decreased. Our study proved that the characteristics of mitochondrial dysfunction by PT in AFB_1-induced hepatotoxicity were related to the upregulated Nrf1/TFAM pathway. That is, AFB_1 downregulated the Nrf1/TFAM pathway, while PT upregulated it; after Nrf1 was silenced, the role of PT was partially inhibited. Therefore, the present study suggests that PT can alleviate AFB_1-induced mitochondrial damage in hepatocytes by activating Nrf1 signaling pathway, thereby further alleviating liver damage in broilers.

Conclusion

Supplementation of 600 mg/kg phlorotannin alleviated AFB_1-induced liver damage in broilers, and 1 μg/mL phlorotannin has a hepatoprotective role for hepatocytes exposed to AFB_1 in vitro. This effect is mainly through activating the Nrf2 signal pathway, inhibiting the activity of phase I detoxification enzymes and increasing the activity of phase II detoxification enzymes, thus promoting the excretion of AFB_1 in the liver. Also, phlorotannin could upregulate the Nrf1 signaling pathway to alleviate AFB_1-induced mitochondrial damage, thereby further alleviating liver injury. These findings provide novel insights into studying phlorotannin as a new type of biological detoxification substance to protect against liver damage caused by AFB_1.

Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
AFB_1	Aflatoxin B_1
AFBO	AFB1-exo-8,9-epoxide
ALB	Albumin
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CAT	Catalase
CCK- 8	Cell counting Kit-8
CYP1A1	Cytochrome P450 family 1 subfamily A member 1
CYP1A2	Cytochrome P450 family 1 subfamily A member 2
CYP2A6	Cytochrome P450 family 2 subfamily A member 6
CYP3A4	Cytochrome P450 family 3 subfamily A member 4
FBS	Fetal bovine serum
FCR	Feed conversion rate
GCLM	Glutamate-cysteine ligase
GSH	Glutathione
GSH-PX	Glutathione peroxidase
GSH-ST	Glutathione S-transferase
H&E	Hematoxylin and eosin
HO-1	Heme oxygenase 1
LMH	Leghorn Male Hepatoma
MDA	Malondialdehyde
MFN1	Mitofusin 1
MFN2	Mitofusin 2
Nrf1	Nuclear respiratory factor 1
Nrf2	Nuclear factor erythroid 2-related factor 2
NQO1	NAD(P)H:quinone dehydrogenase 1
OPA1	Optic atrophy 1
PGC-1α	Peroxisome proliferators-activated receptor γ coactivator l alpha
PT	Phlorotannin
ROS	Reactive oxygen species
T-AOC	Total antioxidant capacity
TFAM	Mitochondrial transcription factor A
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
TP	Total protein
T-SOD	Total superoxide dismutase

Supplementary Information

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Additional file 1: Table S1 Primer sequences for hepatic gene expression. Table S2 Antibody information.

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Authors' contributions
YXQ: Writing–original draft, Methodology, Formal analysis, Data curation, Conceptualization. YYY: Methodology, Formal analysis. YQH: Methodology. HMY: Methodology. BB: Writing—review and editing. RJ: Writing—review and editing, Supervision. LWC: Writing—review and editing, Supervision, Project administration, Conceptualization, Funding acquisition. All authors read and approved the final manuscript.

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Data availability
The basic data of this paper will be shared by the corresponding authors under reasonable requirements.

Declarations

Ethics approval and consent to participate
All animal experimental procedures were conducted with the approval of the Animal Care and Use Committee of College of Coastal Agricultural Sciences of Guangdong Ocean University (Approval No. 20221008, Zhanjiang, Guangdong, China).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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