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The hepatoprotective effects of *Salvia plebeia* R. Br. extract in zebrafish (*Danio rerio*)

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ABSTRACT

Salvia plebeia R. Br. is a traditional Chinese medicinal herb that has been widely used for the treatment of many inflammatory diseases such as hepatitis. However, the underlying molecular mechanism about the hepatoprotective effects of *S. plebeia* remains largely unknown. Here, we investigated the antioxidant activities and anti-inflammatory effects of ethanol extracts of *S. plebeia* (SPEE) in the zebrafish model. Firstly, we determined the chemical compositions of SPEE and identified three major constituents by using GC-MS analysis. After that, SPEE exhibited significantly antioxidant properties in the LPS-induced zebrafish embryos, and the enzyme activities of ROS, CAT and SOD were obviously inhibited in a dose-dependent manner. Secondly, SPEE greatly reduced fat vacuoles (HE staining), lipid accumulation (Oil O staining) and hepatocyte fibrosis (Gemori staining) in the thioacetamide (TAA)-induced hepatocyte injury of adult zebrafish. Meanwhile, the NO contents and lipid metabolism-related genes were substantially down-regulated after SPEE exposure. Thirdly, we used RNA-Seq analysis to identify the differentially expressed genes (DEGs) after SPEE exposure in adult zebrafish liver. The results showed that 1289 DEGs including 558 up-regulated and 731 down-regulated were identified between the TAA + SPEE and TAA groups. KEGG pathway and GO functional analysis revealed that steroid biosynthesis, oxidation-reduction and innate immunity were significantly enriched. Mechanistically, SPEE can considerably reduce the cell apoptosis of hepatocytes and promote the translocation of Nrf2 protein from the nucleus to the cytoplasm in TAA-induced zebrafish. Moreover, SPEE can modulate various inflammatory cytokines and immune genes both in the control and H_2O_2-stimulated conditions. The pro-inflammatory cytokines such as IL-1β and TNF-α was markedly up-regulated but the anti-inflammatory cytokines such as TGF-β was greatly down-regulated after SPEE treatment. In addition, some key genes in the TLR signaling were also activated in the H_2O_2-stimulated conditions. In summary, our results suggested that SPEE had an important role in the antioxidant and anti-inflammatory effects in zebrafish in the near future. Some of the components identified in this study may be served as potential sources of new hepatoprotective compounds for the treatment of inflammatory diseases.

1. Introduction

Salvia plebeia R.Br. (Labiatae), an annual or biennial herb that widely distributed in many countries such as China, India, Korea and Australia [1,2]. In the past decades, *S. plebeia* is used as a folk medicine for the treatment of a variety of inflammatory diseases including hepatitis, cough, diarrhea, tumors and hemorrhoids [3,4]. Previous phytochemical studies have revealed that *S. plebeia* mainly contains flavonoids like hispidulin, homoplantaginin, nepetin, nepetin-7-glucoside, luteolin-7-glucoside and luteolin [5,6]. Ethanol extracts of *Salvia plebeia* (SPEE) displays strong antioxidant activities and anti-inflammatory effects on the *in vitro* experimental models [7]. However, the role and regulatory mechanism of SPEE in aquatic vertebrates especially fish remains still unclear.

The zebrafish (*Danio rerio*) possesses many unique features such as small size, external fertilization, rapid development, and extreme

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sensitivity to chemicals, making it an ideal vertebrate model for studying inflammatory diseases such as hepatitis and even hepatocellular carcinoma [8,9]. Although the pathogenesis and molecular mechanism of liver damage or hepatocyte fibrosis in zebrafish remains largely unknown, excessive oxidative stress and inflammation are considered to be involved in this process [10,11]. Over-production of reactive oxygen species (ROS) and inflammatory cytokines triggered by environmental or pathological factors will ultimately lead to cellular dysfunction or death [12,13]. Therefore, maintaining oxidative stress and inflammation at an appropriate level has long been a traditional therapeutic strategy to reduce or prevent these diseases [14]. On the other hand, some natural components extracted from many medicinal plants have favorable antioxidant and anti-inflammatory effects, which may be served as valuable candidates for the treatment of various inflammatory diseases [15,16]. In recent years, zebrafish has gradually become a prominent model for screening and evaluating these natural drugs [17,18].

The zebrafish innate immune system is homologous to the humans, which the conservation of innate immune genes between zebrafish and humans is as high as 87% [19]. During the initiation and progression of inflammatory diseases, the TLR signaling is the major pathway for regulating the expression of downstream genes [20]. Zebrafish Toll-like receptors (TLRs) can recognize pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS), which will activate TLR signaling and transduce intracellular signals through MyD88/NF-κB cascades [21]. Meanwhile, it will also induce the translocation of NF-κB from cytoplasm into the nucleus and promote the expression of many pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin 6 (IL6), interleukin 1β (IL-1β), and interferon γ (IFN-γ) [22,23]. Furthermore, ROS is a by-product of normal oxygen metabolism and plays an important role in organic development and cellular function [24,25]. ROS is involved in the maintenance of cellular 'redox homeostasis', while excessive ROS results in membrane damage through the formation of lipid peroxide and ultimately leads to cell apoptosis [26,27]. To avoid the potentially negative effects of oxidative stress, individual organisms possess several antioxidant enzyme such as superoxide dismutase (SOD) and catalase (CAT), which can convert H_2O_2 into H_2O and remove the redundant ROS [28,29].

Previous studies have suggested that the pure compounds extracted from *S. plebeia* possess potent antioxidant and anti-inflammatory activities. For example, the homoplantaginin isolated from the *S. plebeia* has a protective effect on oxidative stress-induced hepatocyte injury *in vivo* and *in vitro* [30,31]. The flavonoid hispidulin has been observed to prevent bromobenzene-induced liver injury in mice [32]. In addition, the SPEE significantly decreased induced nitric oxide synthase (iNOS) and ROS in the LPS-stimulated RAW264.7 cells [33,34]. Recent studies revealed that *S. plebeia* also possessed potent anti-inflammatory activity in a TPA-induced ear edema model, and the methanol extracts from which can inhibit NO and PGE2 production, and reduce the protein expression of iNOS and COX-II through the activation of heme oxygenase-1 (HO-1) and nuclear factor erythroid 2-related factor2 (Nrf2) [35,36].

In this work, we further evaluated the antioxidant and anti-inflammatory properties of ethanol extracts of *S. plebeia* (SPEE) in LPS-stimulated zebrafish embryos and TAA-induced liver damage in adult zebrafish. Next, we performed the RNA-Seq analysis to identify the differentially expressed genes and signaling pathways potentially involved in the hepatoprotective effects of *S. plebeia* in zebrafish. Besides, we also examined the expression of immunity-related and apoptosis-related genes that were regulated by SPEE exposure, and identified the expression patterns of inflammatory cytokines in the H_2O_2-stimulated conditions. Taken together, our study will provide insights into the molecular mechanism of the hepatoprotective effects of *S. plebeia* in zebrafish.

2. Materials and methods

2.1. Chemical reagents

Lipopolysaccharides (LPS) was purchased from Sigma-Aldrich (CAS No. L2880, St. Louis, USA), and the purity was greater than 98%. The stock solution (1 mg/mL) of LPS was dissolved in deionized water, and the corresponding diluted solution was prepared to use immediately prior to each experiment. Thioacetamide (TAA) was purchased from Sangon Biotech Co., Ltd. (CAS No. A600940, Shanghai, China), and the H_2O_2 was purchased from Aladdin Biotech Co., Ltd. (CAS No. H112517, Tianjin, China). All other chemicals utilized in this study were of analytical grade.

2.2. Preparation the ethanol extracts of Salvia plebeia R.Br. (SPEE)

The plant materials of *Salvia plebeia* R.Br. were collected from Xi'an, Shanxi Province, China, in July 2018. Prof. Jun'e Zhang, a corresponding author in the Jiangxi Normal University, was responsible for performing botanical identification, and a voucher specimen was deposited at the Center of Developmental Biology, Jinggangshan University. The aerial parts of *S. plebeia* were air-dried and ground into a fine powder, and then extracted with 95% ethanol at 70 °C for 5 h. The crude extracts were concentrated by a rotary vacuum evaporator until all the solvents had been completely removed. The final lyophilized extracts (SPEE, 1:10 w/w) were yielded and stored at 4 °C for experimental use.

2.3. Identification the components of SPEE by GC-MS

The gas chromatography/mass spectrometry (GC/MS) method was used to screen for the major bioactive compounds in the ethanol extracts of *S. plebeia* (SPEE). Briefly, 20 mg of lyophilized SPEE was redissolved in 50 mL of methanol and then filtered through a 0.45 μm membrane (Millipore, USA). Next, 10 μl of the solution was injected into the Agilent GC-MS chemstation system. Helium was used as a carrier gas. The following temperature programming was used: initial temperature, 60 °C; ramping rate, 3 °C/min; final temperature, 243 °C; run time, 61 min. Interpretation of the mass spectrum was conducted using the database of the National Institute Standard and Technology (NIST). The spectra of unknown components were compared with the spectra of the known components stored in the NIST library. The name, molecular weight and structure of the components in the SPEE were ascertained.

2.4. Maintenance of zebrafish

Zebrafish husbandry was performed according to the standard protocols previously established in our lab [37–39]. Briefly, the wild type (AB strain) zebrafish were raised in a circulating aquarium system at an environmentally controlled room (28 ± 1 °C with 80% humidity). The photoperiod was set to a 14 h light/10 h dark cycle. The larval and adult fish were fed with live brine shrimp twice a day. For experiments, fertilized eggs were collected and chosen under a stereomicroscope (Leica, M165FC, Germany) at 6 hpf (hour post-fertilization). Moreover, all zebrafish embryos and adult fish were handled according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Jinggangshan University.

2.5. Anti-inflammatory effects of SPEE in the LPS-induced zebrafish embryos

Normally developed zebrafish embryos at 6 hpf were randomly divided into five groups in 6-well culture plates and incubated with E3 embryo culture medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, pH 7.4). Then, the embryos were added with control

(1x PBS) or LPS (4 μg/mL) for 24 h to induce inflammatory responses. Each treatment was performed in at least triplicates and 40 embryos per well in a plate was set as a biological replicate. After treatment, the LPS-induced zebrafish embryos were exposed to different concentrations of SPEE with 0, 100, 250 and 500 μg/mL for 48 h, respectively. At 72 hpf, all tested embryos were collected and lysed, and the antioxidant enzyme activities together with the expressions of inflammatory genes were measured according to the manufacturer's instructions. In addition, the fresh solutions were completely replaced every 24 h, and dead embryos/larvae were removed daily.

2.6. Detection of antioxidative activities and NO contents

Approximately 20 embryos in each treatment were collected and washed with ice-cold PBS for three times. Then, they were suspended and homogenized in 200 μl of cold lysis buffer. The homogenates were centrifuged with 4000 × *g* at 4 °C for 15 min to obtain the supernatants, and the protein concentrations were measured by using the Bradford assay. The antioxidative enzyme activities of catalase (CAT, CAS No. A007-1) and superoxide dismutase (SOD, CAS No. A001-3) were determined according to the manufacturer's protocols (Jiancheng Institute of Biotechnology, Nanjing, China). Besides, the ROS (CAS No. CA1410, Solarbio, Beijing, China) were quantified by using dichlorofluorescein-diacetate (DCFH-DA) probes, and the fluorescence intensities were evaluated under a stereomicroscope (Leica M205 FA, Germany).

On the other hand, The content of nitric oxide (NO) was detected according to the manufacturer's instructions (CAS No. BC1475, Solarbio, Beijing, China). Briefly, equal volumes of homogenate supernatants with Griess reagents were allowed to react for 15 min, and the nitrite contents were measured by determining the absorbance of the mixture at 540 nm (SpectraMax iD3, MD, USA). The calibration curve was also constructed by using known concentrations of sodium nitrite.

2.7. RNA extraction and quantitative real-time PCR

For each sample, total RNA was extracted from zebrafish embryos or liver tissues using TRIzol reagent (Invitrogen, CAS No.15596018, USA) according to the manufacturer's protocols. Approximately 1 μg of total RNA was reverse transcribed by using M-MLV reverse transcriptase to synthesize the first-strand complementary DNA (cDNA). After that, quantitative real-time PCR amplifications were performed on the ABI StepOne Plus system (Applied Biosystems, USA) by using SYBR Green Master Mix reagent kit (Novoprotein Biotech, CAS no. E094-01A, China) as our previous described [40]. The thermal cycle was as follows: denaturation for 2 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 30 s. Relative gene expression was normalized to an endogenous control gene (β-actin) and was calculated using the comparative Ct method formula $2-\Delta\Delta Ct$ (four biological triplicates in each sample). The sequences of the primers used in this study were listed in Supplemental Table S3.

2.8. Histopathological examination of the liver tissues in adult zebrafish

We induced steatohepatitis in adult zebrafish by using thioacetamide (TAA), and examined the hepatoprotective effects of SPEE in zebrafish. Randomly selected adult zebrafish (three months old) were exposed to Control, SPEE (0.5 mg/mL), TAA (5 mg/mL), and TAA (5 mg/mL) + SPEE (0.5 mg/mL), respectively. Next, the fish were euthanized in 0.1% tricaine, and the liver tissues were subsequently dissected. The samples were fixed in 4% paraformaldehyde (PFA) at 4 °C overnight. The next day, the tissues were embedded in OCT reagents and cut into approximately 8 μm sections using the frozen section system (Leica, CM3050S, Germany). Selected liver slides were placed on a constant temperature incubator at 37 °C until completely dried. Then, the slides were stained with the hematoxylin and eosin (H&E) staining (histological structure), Oil O staining (fatty droplet accumulation), and Gomori staining (reticular fibers) according to the manufacturer's instructions. Finally, the stained slides were mounted and covered with a coverslip and the sections were observed under a positive optical microscope (Zeiss Scope A1, Germany), and photomicrographs were taken using a Zeiss Axiocam 512 colour digital camera.

2.9. Library construction and high-throughput RNA-Seq sequencing

The liver tissues from each treatment were homogenized in liquid nitrogen, and total RNA was extracted by using the RNeasy Mini Kit (Qiagen, CAS No. 74134, Germany) following the manufacturer's protocols. The quantity and quality of RNAs were measured on the NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer. Only RNA samples with high quality ($OD_{260/280} \geq 1.8$ and RIN ≥ 7) were selected for the subsequent experiments. RNA-Seq sequencing libraries were prepared by using the TruSeq™ RNA sample preparation kit (lllumina, San Diego, CA, USA) following the manufacturer's proposals. Four paired-end libraries were sequenced on the Illumina HiSeq X-ten platform and the length is 2 × 150 bp (Novogene Biotech, Tianjing, China). Finally, approximately 5G of raw data were generated from each library.

2.10. Bioinformatics analysis

Firstly, the low-quality reads and adaptors were filtered out from the fastq format raw data by Trimmomatic packages [41], and the remaining clean and high-quality reads were obtained and used for downstream analysis. Next, all clean reads were mapped onto zebrafish genome (GRCz11) using the HISAT2 package [42]. The accurate gene expression abundances were calculated by the normalized FPKM value (fragments per kilobase of transcript per million fragments mapped) that estimated by RSEM software [43]. Subsequently, differentially expressed genes (DEGs) between in the control and treated libraries were effectively identified based on the significance level (p value < 0.001) using the DEGseq package with the default parameters [44]. The expression difference is more than or less than 2 fold that we considered as differentially expressed genes (DEGs).

After that, hierarchical clustering analysis of DEGs was performed by using the heatmap.2 function within the gplots package in the R language, and *Pearson* correlation method was adopted to calculate the distance between both rows and columns as our previously described [45]. For principal component analysis (PCA), PCs, PC variances and PC scores were calculated using eigen function in the R language [46]. For gene functional annotation, gene ontology (GO) enrichment analysis of DEGs was implemented using the DAVID online tool (https://david.ncifcrf.gov/). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classifications were performed by the KOBAS software [47]. Besides, the genes of lipid metabolism, redox process and inflammatory responses were identified based on the KEGG classification and further validated by the BLAST against the NCBI non-redundant nucleotide database.

2.11. TUNEL staining of hepatic apoptosis

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of apoptotic cells in zebrafish liver tissues was performed using “Click-iT™ Plus TUNEL Assay” (Invitrogen, Cas no. C10619, USA) according to manufacturer's instructions. Briefly, randomly selected liver sections in each group were washed with 1xPBS for three times and fixed with 4% paraformaldehyde (PFA) at 4 °C for overnight, followed by incubation with 50 μl of TdT reaction mixture at 37 °C for 1 h in a hybridization chamber. After 1xPBS wash, Click-iT Plus reaction was performed at 37 °C for 30 min, and then the fluorescence was observed with Alexa Fluor 647 dyes by the laser-scanning

confocal microscope (Leica TCS SP8, Germany).

2.12. Immunofluorescence and western blot analysis

The experiments of immunofluorescence were performed as previously described [48]. Briefly, the fresh zebrafish liver tissues were cutted approximately 6 μm thickness, and the citric acid buffer (CW0128S; CWBIO) together with the microwave treatment were used to repair the corresponding antigens. Next, the slides were probed with anti-Nrf2 antibodies (Cell Signaling, Cas no.12721) overnight at 4 °C and then were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Code#A11034) for 1 h. After that, the photographs were recorded under the laser-scanning confocal microscope and ten fields from each treatment were analyzed.

For immunoblot analysis, zebrafish liver samples were lysed in RIPA buffer (Cwbiotech, Cas no. CW2333S) supplemented with protease and phosphatase inhibitors. Protein concentrations were determined using the BCA protein assay kit (Sangon Biotech, Cas no. C503021). 10 μg of proteins in each group were fractionated on 12% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked in Tris-buffered saline containing 5% defat milk for 2 h at room temperature. Next day, the membranes were hybridized with the following primary antibodies at 4 °C overnight: anti-Bcl-2 (Abcam, Cas no. ab94720), NF-kB p65 (Cell Signaling, Cas no. 8242) and anti-Acetyl-Histone H3 (Abcam, Cas no. ab47915) and anti-GAPDH (Cell Signaling, Cas no. 2118). After that, the proteins were visualized by the addition of the peroxidase-conjugated secondary antibody, and specific bands were detected by the ChemiDoc Touch Imaging System (Bio-Rad).

2.13. The expression profiling of inflammatory genes in the H_2O_2-induced conditions

We mimicked the procedures of hepatocyte injury by exposure of H_2O_2 in zebrafish, and measured the expressions patterns of immune genes after SPEE exposure. The adult zebrafish were pre-treated with control, SPEE, TAA, TAA + SPEE for 48 h, respectively. Next, the fish were then exposed to 750 μM H_2O_2 for another 24 h. And Non-H_2O_2 treated zebrafish were incubated under the same conditions as those used in the experimental protocols with H_2O_2. After that, the liver tissues were dissected and the total RNAs were extracted from each sample. Finally, the inflammatory cytokines or immune genes under various conditions were quantified by qRT-PCR experiments.

2.14. Statistical analysis

Statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA) and *P* values were analyzed using Student's *t*-test. The results were considered statistically significant at $*p < 0.05$, and $**p < 0.01$. All values were presented as means ± standard deviation (SD).

3. Results

3.1. Effects of S. plebeia on LPS-induced inflammatory responses in zebrafish embryos

S. plebeia has been considered as a natural medicinal plant that is used for the prevention or treatment of various inflammatory diseases. The morphological characteristics of this plant and the ethanol extracts of *S. plebeia* (SPEE) are shown in Fig. 1A. To explore and screen for the major constituents in the SPEE, GC-MS method was employed to generate chromatographic profiles. As shown in Fig. 1B and 55 different substances were identified in the SPEE including tetradecanoic acid (Rt = 13.310 min), octadecanoic acid (Rt = 14.247 min) and exanedioic acid (Rt = 15.263 min), which were the main three components in this extracts. It is worth mentioning that the two main bioactive ingredients (hispidulin and luteolin) that found in the previous studies were also identified in our results.

To further investigate the antioxidant activities of SPEE in zebrafish, LPS (4 μg/mL) was used to induce oxidative stress in zebrafish embryos at 6 hpf, and then to evaluate the free radicals scavenging capacities of SPEE exposure. As shown in Fig. 1C, our results suggested that LPS can significantly increase the level of oxidative stress, while SPEE can greatly reduce the related antioxidative activities. It is showed that the levels of ROS were markedly decreased when exposed to various concentrations of SPEE. In addition, SPEE can inhibit the enzymatic activities of CAT and SOD within the LPS-induced zebrafish embryos in a dose-dependent manner. On the other hand, we also evaluated the anti-inflammatory effects of SPEE in zebrafish embryos. Our results demonstrated that the relative mRNA levels of two pro-inflammatory cytokines (TNF-α and IL-1β) were significantly down-regulated in a concentration-dependent manner (Fig. 1D). On the contrary, the stress-induced heme oxygenase-1 (HO-1), the expression level of which was greatly up-regulated after SPEE treatment. Taken together, the above data indicated that SPEE has a powerful antioxidant and anti-inflammatory properties in zebrafish embryos.

3.2. Hepatoprotective effects of SPEE in the TAA-induced adult zebrafish

To further evaluate the protective effects of SPEE in hepatocytes, acute liver damage was induced by thioacetamide (TAA) in adult zebrafish. As shown in Fig. 2A, TAA (5 mg/mL) induced a significant increase on the hepatic damage for 72 h by using hematoxylin and eosin (H&E) staining. Meanwhile, the number of large fat vacuoles in the hepatocytes was obviously more than that of the control group. Furthermore, it is demonstrated that the inflammatory response was greatly alleviated in TAA-stimulated zebrafish that was further exposed to 0.5 mg/mL SPEE for another 24 h, which the inflammatory infiltration and necrotic size was significantly reduced. On the other hand, histopathological analysis revealed that a large number of lipid droplets have been deposited in the liver tissues in the TAA-induced group by Oil red O staining. Similar to the results described above, the number of lipid droplets in the TAA + SPEE group was significantly decreased when compared with TAA-stimulated group (Fig. 2B). Besides, we also examined the degrees of liver fibrosis by using Gemori staining. Our results suggested that the level of liver fibrosis induced by TAA was significantly increased, while SPEE greatly ameliorated the hepatic steatosis of adult zebrafish in the TAA + SPEE group (Fig. 2C).

In addition, we also detected the relative amounts of nitric oxide (NO) in the control, SPEE, TAA, and TAA + SPEE, respectively. The results showed that NO production of TAA-treated group was greatly increased when compared with control or SPEE-exposed group. Moreover, the level of NO was significantly inhibited in the TAA + SPEE group when compared with the TAA-stimulated conditions (Fig. 2D). Additionally, we also examined whether SPEE affected the expression of lipid metabolism associated genes. The results indicated that relative mRNA levels of genes such as CytoP450, ApoE and PPAR-γ, were also significantly decreased after SPEE treatment in the TAA-induced zebrafish (Fig. 2E). In summary, these results further suggested that SPEE had potent anti-inflammatory and hepatoprotective effects in adult zebrafish.

3.3. The gene expression profiling after SPEE treatment was determined by RNA-Seq

In order to identify the gene expression levels in adult zebrafish after SPEE exposure, four libraries from Control, SPEE, TAA, TAA + SPEE were constructed and sequenced by high-throughput RNA sequencing on the Illumina HiSeq X-ten platform. After removal of adaptor sequences and low-quality or low-complexity reads (Q < 20), these libraries yielded 32.6, 34.7, 30.1 and 31.7 million high-quality clean reads with 5.54, 5.70, 5.12, and 5.3 Gb data, respectively

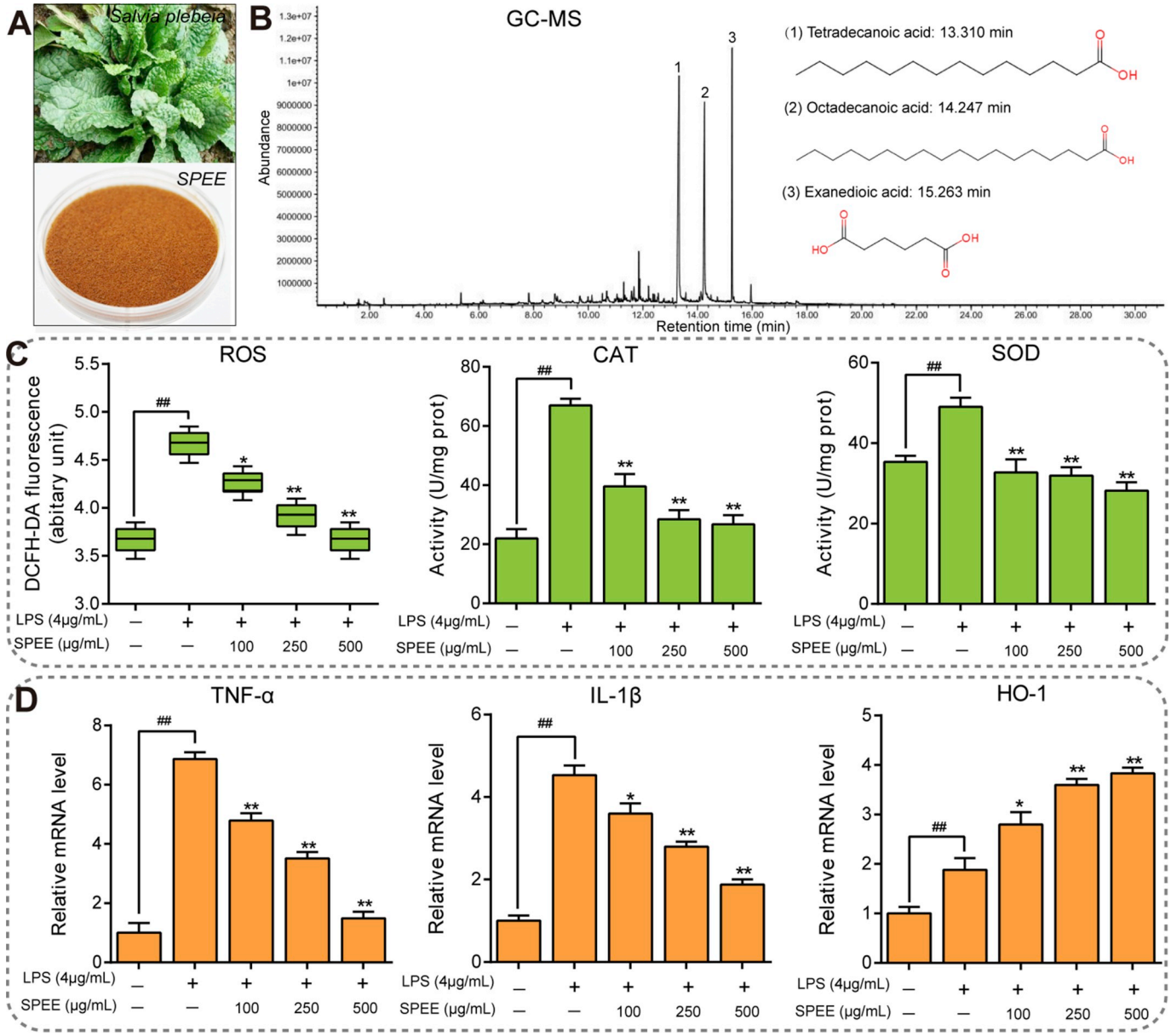

Fig. 1. The antioxidant and anti-inflammatory effects of SPEE in LPS-induced zebrafish embryos. (A) The photograph of *Salvia plebeia* was shown on the top pannel and SPEE was shown on the lower pannel. (B) Representative GC-MS chromatogram of the ethanol extracts of *S. plebeia* (SPEE). The main compounds were identified as tetradecanoic acid (Rt = 13.310 min), octadecanoic acid (Rt = 14.247 min) and exanedioic acid (Rt = 15.263 min). The corresponding molecular formulas were shown in the top right corner. (C) The antioxidant enzyme activities of ROS, CAT and SOD were determined after SPEE treatment. The zebrafish were first incubated with LPS (4 μg/mL) for 24 h and exposed to 0, 100, 250, 500 μg/mL of SPEE, respectively. And the corresponding enzyme activities were measured at 72 hpf. (D) The relative mRNA levels of pro-inflammatory genes after SPEE exposure in LPS-induced zebrafish. Each column represents the mean ± SD of three independent experiments. (Student's *t*-test, *, $p < 0.05$, **, $p < 0.01$, when compared with the LPS-treated group; ##, $p < 0.01$, when compared with the control group).

(Supplemental Table S1). Next, all the clean reads were separately mapped to the reference zebrafish genome (GRCz11) by using the HISAT2 package, and the average mapping rate was higher than 80%. Furthermore, the flowchart of transcriptome sequencing and bioinformatics analysis was shown in Supplemental Fig. S1.

After that, to gain insights into the transcriptional regulation after SPEE exposure in zebrafish liver, we used RSEM package to calculate the gene expression abundance estimated by FPKM value. Then, we performed pairwise comparisons between these libraries to identify the differentially expressed genes (DEGs) by the DEGseq package. The threshold for screening DEGs is 2 fold or more changes between two libraries was discriminated upon the criteria of the corrected p value < 0.05. The detailed gene annotations and expression levels of DEGs within the pairwise comparisons including SPEE vs. Control, TAA vs. Control, TAA + SPEE vs. Control, and TAA + SPEE vs. TAA were listed in Supplemental Table S2. Our results showed that there were 2466 differentially expressed genes in the comparison between the TAA and control groups, of which 909 (36.8%) genes were up-regulated and 1557 (63.2%) genes were down-regulated (Fig. 3A). Moreover, when TAA + SPEE compared with TAA group, there were 1289 DEGs (558 up-regulated and 731 down-regulated) were identified in zebrafish.

In order to compare the gene expression levels in zebrafish liver after SPEE exposure, we performed hierarchical clustering analysis of DEGs and identified discrete clusters in each group that were relevant to the SPEE treatment. The results showed that SPEE induced significant down-regulation in a number of DEGs when compared with

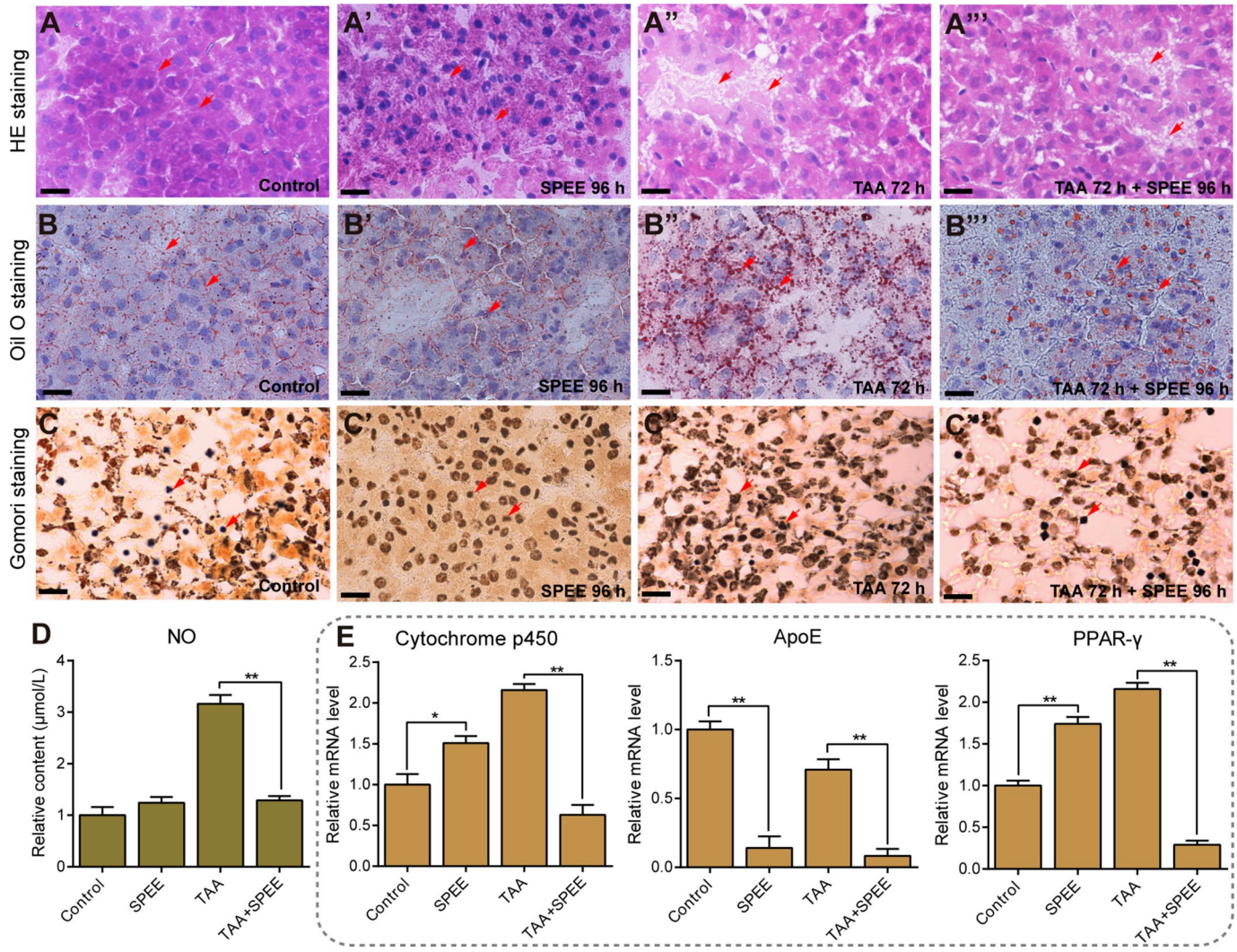

Fig. 2. The hepatoprotective effects of SPEE in the TAA-induced adult zebrafish. (A) Histopathological examination of liver sections by using HE staining. The adult zebrafish were exposed to 5 mg/mL TAA for 72 h, and then incubated with 0.5 mg/mL SPEE for another 24 h. The photographs of the control, SPEE, TAA and TAA + SPEE treated groups were recorded. Magnification: 400x. Scale bar = 50 μm. (B) Lipid accumulation was evaluated in the liver tissues using Oil O staining. The blue arrow marks the lipid droplets. (C) The extent of liver fibrosis was assessed using Gomori staining. The blue arrow marks the reticular fibres in black and the collagen fibres in yellow-brown. (D) The inhibitory effects of SPEE on NO production in TAA-treated liver tissues. (E) The relative mRNA levels of lipid metabolism-related genes in each group. The column represents the mean ± SEM of three independent experiments. (Student's *t*-test, *, $p < 0.05$, **, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

control or TAA-treated conditions (Fig. 3B). Besides, venn diagram indicated that 532 genes (11.4%) were exclusively differentially regulated in the TAA + SPEE group, while 367 genes (7.9%) were specifically differentially regulated in the TAA-induced group (Fig. 3C). In addition, the comprehensive data showed that there were consistent up-regulation of 82 genes in all libraries, and most of these genes were related to immunity or lipid metabolism. Moreover, we performed the principal component analysis (PCA) analysis to compare the global gene changes in all the libraries, and the results suggested that SPEE exposure induced dramatic alterations in the gene expression profiling on the zebrafish liver tissues (Fig. 3D). There was an obvious variation between the treated and control groups, and it demonstrated that the difference between TAA + SPEE and TAA group was much more significant than that between the SPEE and control groups.

3.4. KEGG and GO analysis of differentially expressed genes

To better characterize the regulation of gene expression induced by SPEE in adult zebrafish liver, DEGs were functionally sorted into the canonical signaling pathways based on KEGG pathway analysis. As a result, there was a remarkable difference among the major functional genes between the TAA + SPEE vs. TAA and SPEE vs. CK group (Fig. 4A). It suggested that the terms of “steroid biosynthesis”, “biosynthesis of antibiotics”, “metabolic pathways”, “carbon metabolism” and “PPAR signaling pathway” were the top 5 significantly regulated pathways according to p values ($p < 0.05$) when the TAA + SPEE compared with TAA group. Besides, the terms of “primary bile acid biosynthesis” and “starch and sucrose metabolism” were significantly enriched pathways when SPEE compared with CK group.

To further analyze the potential functions of these DEGs, Gene Ontology (GO) enrichment analysis was performed by using the DAVID online tool. All the DEGs were successfully assigned into three main categories including biological process (BP), cellular component (CC) and molecular function (MF). The majority of the GO terms for biological processes that were significantly enriched in proteolysis, steroid metabolic process, and oxidation-reduction process (Fig. 4B). With regard to the categories of cellular components, “extracellular space” and “high-density lipoprotein particles” were the significantly enriched GO terms in these DEGs (Fig. 4C). In addition, the molecular functions of most DEGs were associated with oxidoreductase activity, lipid

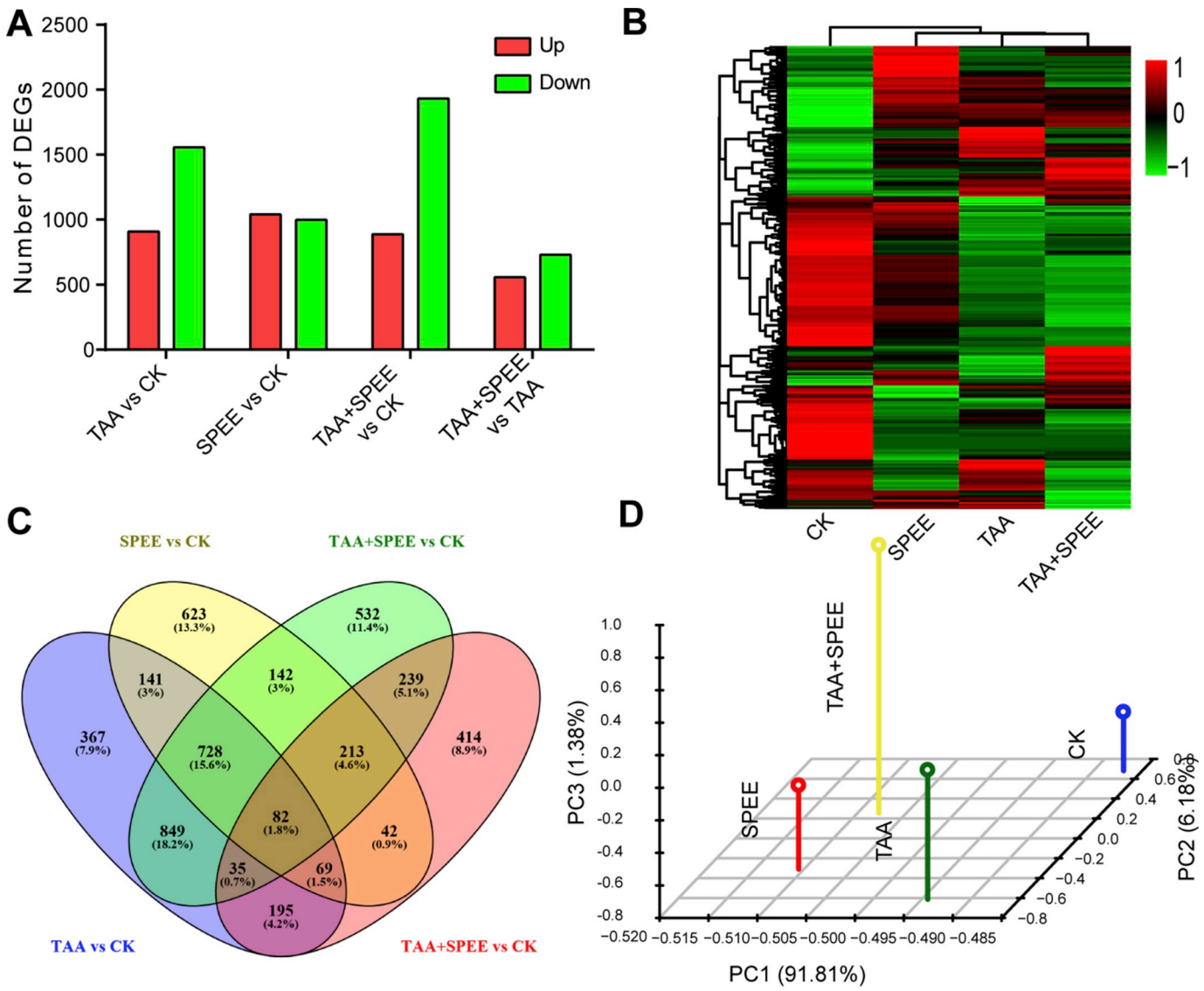

Fig. 3. Distinct transcriptome profiles elicited by SPEE treatment in zebrafish liver. (A) The number of differentially expressed genes (DEGs) identified by pairwise comparison in RNA-Seq. Red represents up-regulated genes, while green represents down-regulated genes. (B) Hierarchical clustering analysis of DEGs in CK, SPEE, TAA and TAA + SPEE groups, respectively. (C) Venn diagram showed that the shared and unique DEGs in each library. The number and percentage of genes in each region were identified in the graph. Blue, green, yellow and purple represent TAA vs. CK, TAA + SPEE vs. CK, SPEE vs. CK, and TAA + SPEE vs. CK, respectively. (D) Principal component analysis (PCA) analysis of global gene expression in each group based on data from four sequencing libraries. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transporter activity and peptidase activity (Fig. 4D).

Furthermore, hierarchical cluster analysis was performed on those genes involved in lipid metabolism, redox process and inflammatory response (Fig. 4E). Our data demonstrated that the expression of lipid metabolism genes was substantially up-regulated after TAA treatment. For example, methylsterol monooxygenase 1 (msmo1) and insulin induced gene 1(insig1) that associated with cholesterol biosynthesis, and the relative mRNA levels of which were increased approximately 1.5 fold and 3.6 fold in TAA group when compared with control group, respectively. Meanwhile, oxidation-reduction genes were significantly up-regulated in the TAA + SPEE group, such as apoptosis inducing factor mitochondrion associated 1 (aifm1), phosphogluconate dehydrogenase (pgd), arachidonate 5-lipoxygenase b (alox5b), and so on. It is worth mentioning that the inflammatory genes including mpx, IL-1β and CXCL11.7 were greatly up-regulated only in the SPEE group. Taken together, all these results further demonstrated that SPEE can regulate the genes of lipid metabolism, oxidative stress and innate immunity in adult zebrafish at the transcriptome level.

3.5. Cell apoptosis and immune genes expressions after SPEE treatment

To test whether SPEE affects cell apoptosis in zebrafish liver, the adult fish was exposed to Control, SPEE, TAA, and TAA + SPEE groups and we detected the apoptosis cells by TUNEL staining. Our results suggested that SPEE can inhibit cell apoptosis, while TAA can significantly increase the apoptotic level of hepatocytes. Most importantly, SPEE can reduce the apoptotic level induced by TAA in the TAA + SPEE group (Fig. 5A). Besides, Nrf2 is a nuclear transcription factor that regulates the expression of antioxidant proteins, which protect the host against oxidative damage and inflammation. We also detected the distribution of Nrf2 in the zebrafish liver when exposed to SPEE treatment. As shown in Fig. 5B, Nrf2 is mainly located in the cytoplasm in normal cells, and TAA can partly translocate of Nrf2 from cytoplasm entry into the nucleus. Meanwhile, SPEE treatment can greatly reduce the nucleation rate of Nrf2 protein in the TAA + SPEE group. We further tested the protein levels of apoptosis-related Bcl-2 and pro-inflammatory nuclear factor kappa B (NF-κB). The results

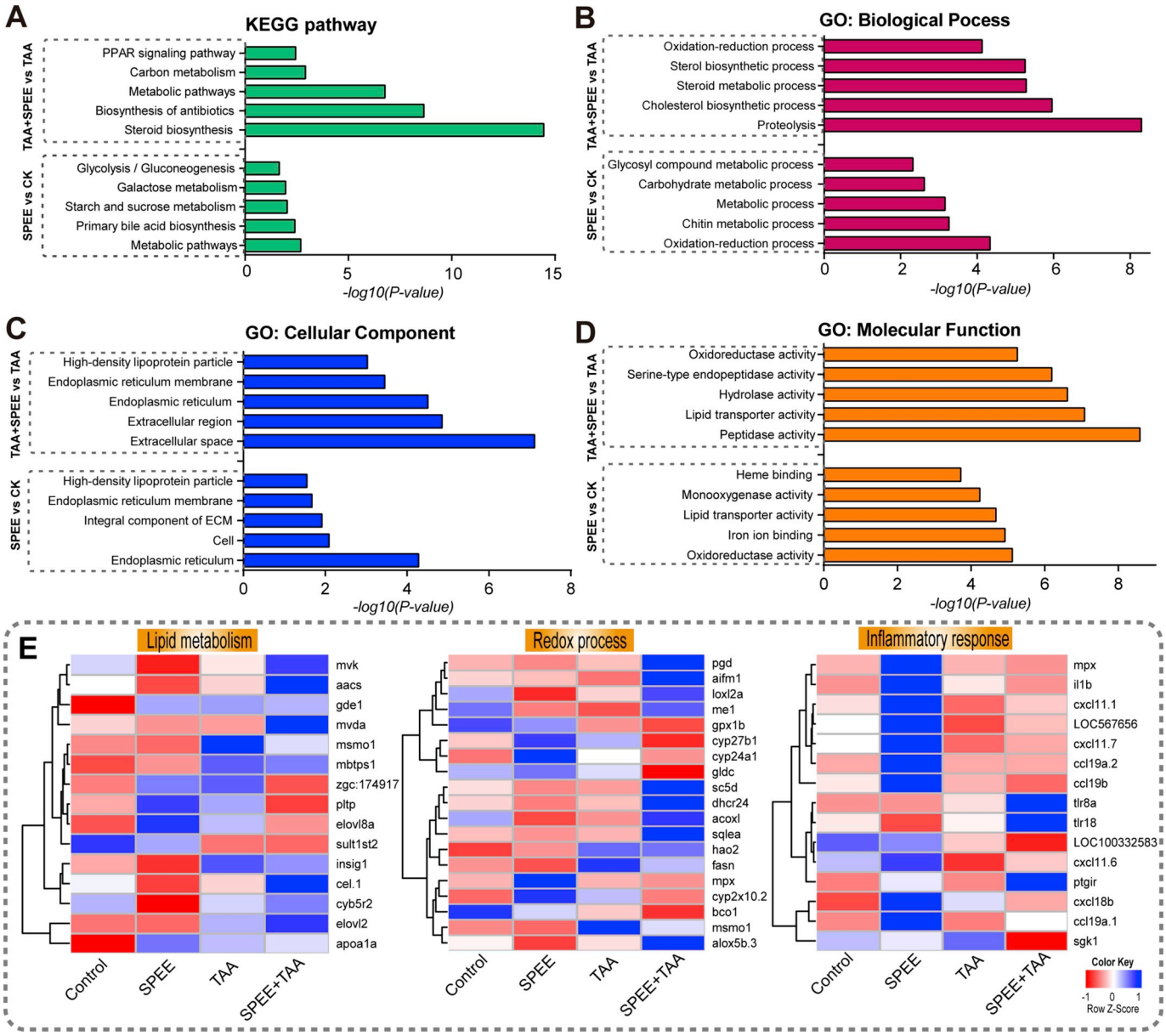

Fig. 4. Comparative transcriptome analysis revealed that lipid metabolism, redox and immune genes were significantly enriched after SPEE treatment. (A) Distribution of KEGG functional groups between TAA + SPEE vs. TAA and SPEE vs. CK. (B) The top five GO terms of biological process were presented between TAA + SPEE vs. TAA and SPEE vs. CK. (C) The top five GO terms of cellular component were presented between TAA + SPEE vs. TAA and SPEE vs. CK. (D) The top five GO terms of molecular function were presented between TAA + SPEE vs. TAA and SPEE vs. CK. (E) Cluster analysis showed the expression profiles of DEGs in zebrafish liver, which were organized into three groups: lipid metabolism, redox process and Inflammatory response. Blue represents the up-regulated and red represents the down-regulated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

revealed that the expression of Bcl-2 and NF-κB p65 were significantly down-regulated after SPEE exposure both in the mock and TAA-induced conditions (Fig. 5C, D, and E). On the other hand, previous studies reported that several immune genes can be acetylated in liver inflammation. We also detected the levels of acetylated histone H3, on the contrary, which was substantially increased in the SPEE and TAA + SPEE groups (Fig. 5C and F).

3.6. Dynamic expression profiles of immune genes induced by H_2O_2 exposure

Hydrogen peroxide (H_2O_2) is one of the main reactive oxygen species, which is well known to cause lipid peroxidation in various cell types including hepatocyte cells. To further elucidate the expression patterns of immune genes regulated by SPEE exposure, we detected the expression levels of several immune genes and inflammatory cytokines both in the mock and H_2O_2-induced conditions. Our results revealed that pro-inflammatory cytokine IL-1β was significantly up-regulated when exposed to SPEE or TAA treatment, which was sharply decreased under the H_2O_2 treatment. Interestingly, IL-6 was only increased in the control and SPEE groups induced by H_2O_2. Besides, TNF-α was up-regulated in the TAA and down-regulated in the TAA + SPEE under H_2O_2 conditions. It should be pointed out that the anti-inflammatory cytokine TGF-β were significantly down-regulated in the control, SPEE and TAA groups under H_2O_2-induced conditions (Fig. 6A).

In addition, the antiviral-related genes, including IRF8 and IFN-γ, were also differentially regulated after SPEE exposure both in the mock and H_2O_2-stimulated conditions (Fig. 6B). The genes of kelch-like ECH-associated protein 1(keap1) and NAD(P)H dehydrogenase, quinone 1(NQO1) that associated with Nrf2 signaling pathway, which was

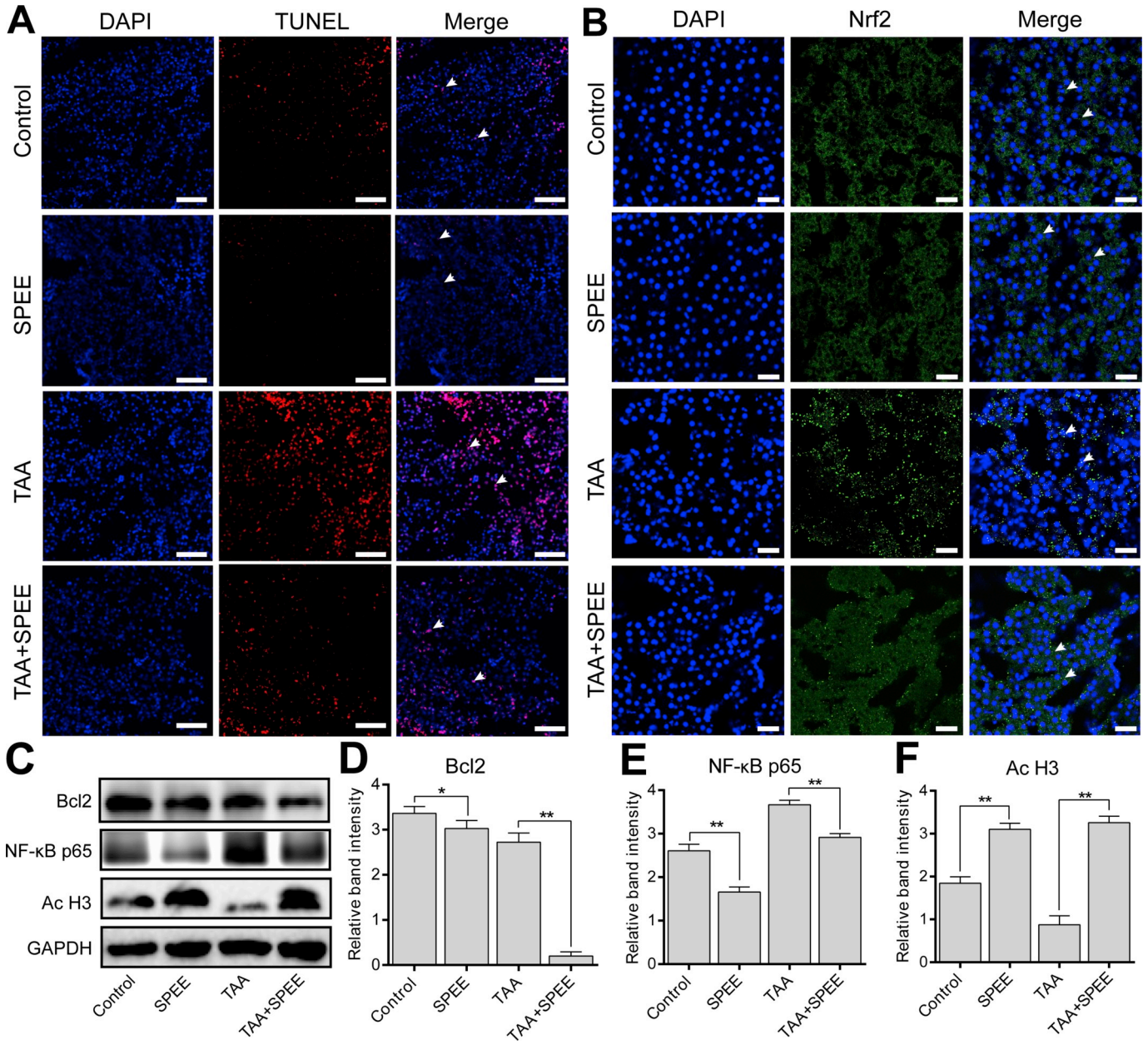

Fig. 5. SPEE regulates cell apoptosis and immune genes in zebrafish liver. (A) Cell apoptosis of zebrafish liver in each group was detected by TUNEL staining. The white arrow indicates the apoptotic cells, in which the red fluorescence (Alexa Fluro 647 dyes) was overlaid with blue fluorescence (DAPI). For all experiments, at least 3 samples were used for each treatment. Scale bar = 50 μm. (B) The localization of Nrf2 protein in hepatocytes after SPEE treatment was observed by immunofluorescence staining. The green marks the nrf2 protein and blue marks the nucleus. (C) The expression of Bcl-2,NF-κB p65 and acetylated histone H3 (Ac H3) were detected by western blotting. The GAPDH was served as a loading control. (D, E, F) Quantitation of the expression levels of Bcl-2, NF-κB p65 and AcH3 by ImageJ software. Data were expressed as fold-changes of SPEE-treated groups compared under the control or TAA conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

significantly decreased in the TAA + SPEE group induced by H_2O_2. Finally, TLR4/MyD88/NF-κB cascades is a major signaling pathway involved in the inflammatory response. We also investigated whether SPEE affected the activation of TLR pathway. Our results suggested that TLR4 and MyD88 were considerably activated both in the control and SPEE-treated groups, but were significantly decreased in the TAA or TAA + SPEE groups under H_2O_2 treatment (Fig. 6C). Additionally, the levels of NF-κB and IκBα were greatly increased in the H_2O_2-stimulated zebrafish. In sum, these results demonstrated that SPEE can modulate inflammatory genes and innate immune genes in adult zebrafish.

4. Discussion

The traditional Chinese medicinal herb, *S. plebeia*, possessed a variety of anti-inflammatory and antioxidant bioactive substances by accumulated studies [49]. However, few studies has explored the anti-inflammatory effects from this herb in aquatic vertebrates. In the present study, we firstly investigated the major components in the ethanol extracts of *S. plebeia* (SPEE) by using GC-MS methods. Our results demonstrated that SPEE contained three major compounds: tetradecanoic acid, octadecanoic acid and exanedioic acid. These substances were also existed in *S.plebeia* and other plant materials from previous studies [50,51]. Then, we further assessed whether these bioactive ingredients have antioxidant effects in zebrafish. SOD and CAT act coordinately to

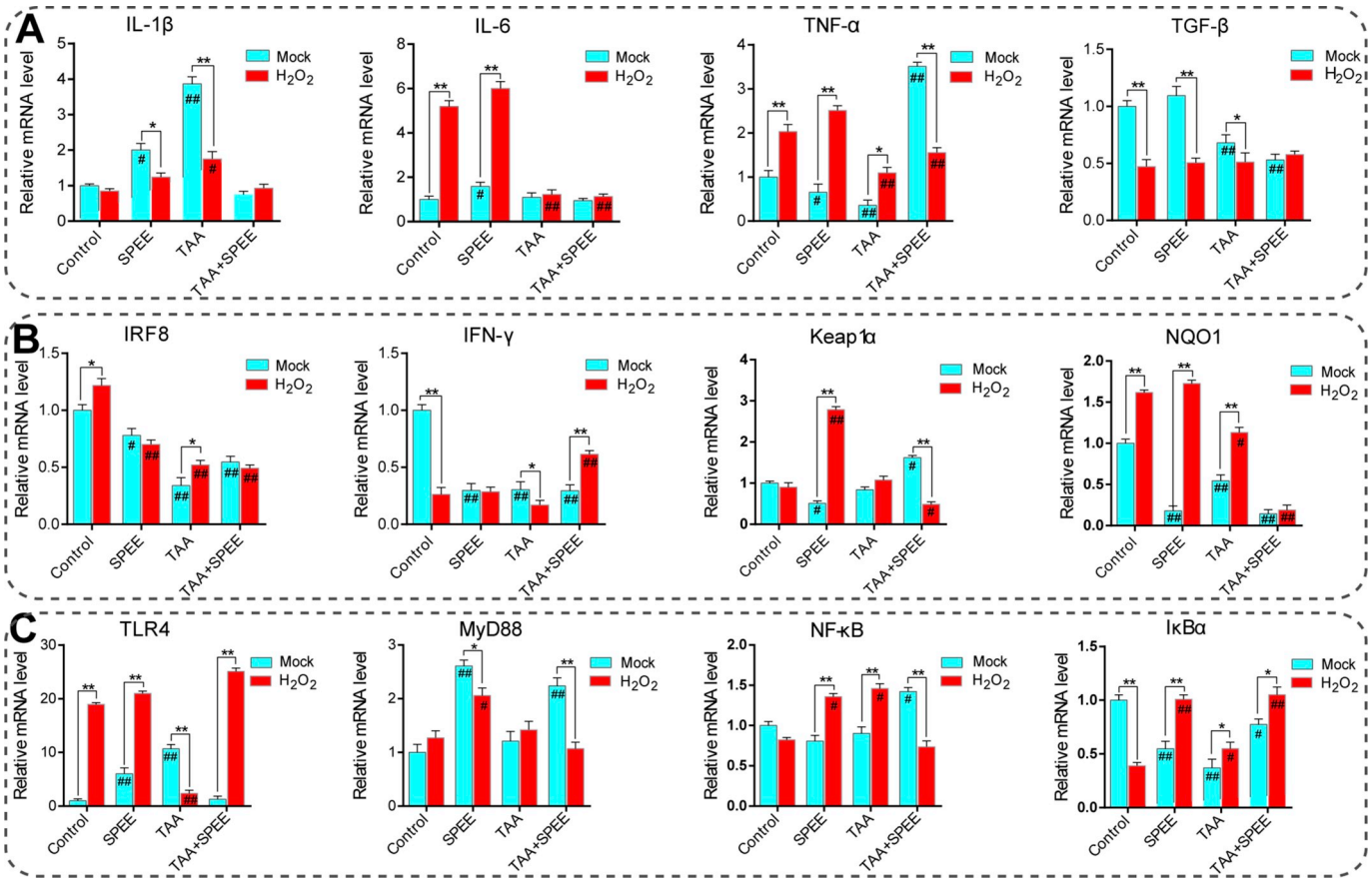

Fig. 6. The expression patterns of the immunity-related genes in the mock and H_2O_2-induced conditions. (A) The pro-inflammatory and anti-inflammatory genes were differentially expressed in each group under mock and H_2O_2-induced conditions. (B) The interferon and Nrf2 signaling-related genes were differentially expressed in each group under mock and H_2O_2-induced conditions. (C) The key genes in TLR signaling pathway were differentially expressed under mock and H_2O_2-induced conditions. For all experiments, the relative mRNA levels are represented as the mean ± S.D. (n = 4). *, $p < 0.05$; **, $p < 0.01$ (inter-group difference); $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$ (intra-group difference).

control ROS levels, and SOD catalyzes the reaction of two superoxide anions, resulting in the formation of hydrogen peroxide, which is less reactive and can be degraded by enzymes such as CAT [52]. Our results indicated that SPEE markedly inhibited the antioxidant enzyme activities of CAT and SOD in LPS-induced zebrafish embryos. Meanwhile, ROS was also significantly down-regulated after SPEE treatment. Similar results were also found from other Korean medicinal plants [53]. Besides, we further detected the expression levels of some pro-inflammatory cytokines such as TNF-α and IL-1β in LPS-induced zebrafish embryos. These genes were significantly decreased after SPEE exposure in a dose-dependent manner. These results were in accordance with previous studies of *S.plebeia* in the LPS-stimulated RAW 264.7 cells [54]. Interestingly, HO-1 is a stress-induced gene that was obviously increased after SPEE exposure, which implied that HO-1 has a unique role in the SPEE-mediated anti-inflammatory effect.

Next, We also evaluated the hepatoprotective effects of SPEE in adult zebrafish. Hepatic steatosis can be induced by thioacetamide (TAA), which is considered as a good liver injury model in zebrafish [55]. Our results showed that SPEE can significantly alleviate vacuolar degeneration, lipid accumulation and liver fibrosis in TAA-induced zebrafish liver. In addition, lipid metabolism-related genes such as *ApoE* and *PPAR*-γ were all sharply decreased after SPEE exposure both in the mock and TAA-stimulated conditions. Previous studies suggested that dysregulation of these genes will affect coronary heart disease risk or atherosclerosis [56]. Besides, nitric oxide (NO) is a signaling molecule that plays a crucial role in the pathogenesis of inflammation [57]. The inhibitory effects of SPEE on NO production were also confirmed in TAA-treated zebrafish model. These results further indicated that SPEE seems to ameliorate hepatic steatosis by reducing the expression of lipid metabolism genes and NO contents in TAA-induced liver injury.

In order to elucidate the global gene expression patterns in SPEE-exposed zebrafish, we used high throughput RNA-Seq to investigate the transcriptional landscapes of zebrafish liver after SPEE treatment both in the mock and TAA-induced conditions. Bioinformatics analysis revealed that 2, 040 genes were differentially expressed including 1041 up-regulated and 999 down-regulated in the SPEE compared with control group. The number of DEGs is much more than the data of the previous study, in which 382 DEGs in the brain and 926 DEGs in the spleen of zebrafish infected with spring viremia of carp virus [58]. KEGG pathway analysis revealed that steroid biosynthesis, biosynthesis of antibiotics and metabolic pathways were significantly enriched after SPEE exposure. This results further confirms that SPEE mainly affects lipid metabolism and immune response in zebrafish. Moreover, GO enrichment analysis showed that peptidase activity and oxidoreductase activity were also significantly regulated after SPEE exposure. These changes appear to reflect disruption of enzyme activities in liver injury, similar to a study in human liver disease showing that xanthine oxidoreductase activity was significantly increased [59]. Previous studies reported that oxidative stress and Inflammation were involved in liver injury [60,61]. Besides, cluster analysis showed that the genes in lipid metabolism, oxidation-reduction and immune response were differentially regulated after SPEE treatment. For example, sterol-C5-desaturase (sc5d) was increased approximately 19.8-fold in the SPEE + TAA-treated group. These results agreed with a previous study that oxidative stress can contribute to the progression of nonalcoholic steatohepatitis (NASH) by stimulating both humoral and cellular immune responses

[62].

Next, we examined whether the level of apoptosis is affected after SPEE treatment. TUNEL assay revealed TAA significantly increased hepatocyte apoptosis and SPEE could rescue this phenotype. Meanwhile, apoptosis-related Bcl-2 gene was greatly reduced after SPEE treatment both in the mock and TAA-induced conditions. On the other hand, several studies have demonstrated that Nrf2 contributes to the anti-inflammatory process by orchestrating the recruitment of inflammatory cells and regulating gene expression through the antioxidant response element (ARE) [63,64]. Our results suggested that Nrf2 is mainly distributed in the cytoplasm by immunofluorescence localization. Inflammation such as TAA treatment can induce Nrf2 into the nucleus while SPEE can reduce the ratio. At the same time, the classical NF-κB pathway is activated by a variety of inflammatory signals, resulting in coordinate expression of multiple inflammatory and innate immune genes [65,66]. Western blot further revealed that NF-κB were greatly reduced after SPEE treatment. It is worth mentioning that histone acetylation can also regulate NF-κB transcriptional activity [67]. On the contrary, acetylated histone H3 was significantly increased both in the SPEE and SPEE + TAA treated groups. These results further demonstrated that SPEE can regulate liver injury through apoptosis and classical immune signaling pathways.

Furthermore, the inflammatory cytokines could be induced in response to oxidative stress [68]. We detected the expression levels of several inflammatory or immune genes in the H_2O_2-induced conditions. TNF-α is secreted by activated macrophages and IL-1β activates neutrophils and macrophages [69]. IL-6 promotes the induction of acute phase proteins and IFN-γ belongs to the antiviral cytokines and mediates immune and inflammatory responses [70]. Our results showed that IL-6 and TNF-α were greatly up-regulated after SPEE treatment in the H_2O_2-induced conditions. However, IFN-γ was substantially decreased after TAA treatment while it was partially increased in the TAA + SPEE groups. Besides, we also tested the Nrf2 and Keap1 in ARE-mediated NQO1 expression. The results indicated that Keap1 and NQO1 were mostly activated under different conditions. Finally, the key genes in TLR signaling pathway such as TLR4, MyD88 and NF-κB were obviously activated in response to SPEE exposure both in the mock and H_2O_2-induced conditions. The expression patterns of these immune genes were similar with our previous studies regarding clethodim immunotoxicity in the zebrafish embryos [71].

From all above results, we further elucidated the molecular mechanism of antioxidant and anti-inflammatory effects of SPEE in zebrafish. Bioassays verified that SPEE exhibited much potent inhibition for antioxidant enzyme activities and inflammatory genes in zebrafish embryos. In TAA-induced liver injuries of adult zebrafish, SPEE significantly activated lipid metabolism and redox process, while most of inflammatory responses were inhibited. Studies of anti-inflammatory mechanisms demonstrated that cell apoptosis and the pro-inflammatory mediators including TNF-α, IL6 and IFN-γ were affected through regulation the NF-kB and Nrf2 signaling pathways. In conclusion, these information brings new insights into the hepatoprotective effects of SPEE in zebrafish. Present results suggested that the extracts in *S. plebeia* could be a potential candidate for anti-inflammation, and may validate the folk use of *S. plebeia* in inflammatory diseases.

5. Conclusion

In summary, our findings indicated that the ethanol extract of *S. plebeia* (SPEE) had potentially antioxidant and anti-inflammatory effects in zebrafish embryos. Meanwhile, SPEE reduced lipid deposition and liver fibrosis in the TAA-induced adult zebrafish. RNA-Seq analysis revealed that SPEE significantly regulated the expressions of genes involved in lipid metabolism, redox process and inflammatory response. Further studies found that SPEE inhibited the expressions of immune and apoptotic genes. In addition, several inflammatory cytokines and some key genes in TLR pathway were also differentially expressed in the H_2O_2-induced conditions. Given the observed hepatoprotective effects of SPEE in zebrafish, further studies should focus on the specific molecular mechanisms of lipid metabolism and immune inflammation from different perspectives. These informations provided a better understanding of *S.plebeia* therapeutic application in inflammatory diseases.

Declaration of competing interest

The authors declared that they have no conflicts of interest to this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.040.

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