Dietary quercetin ameliorates TPT-induced hepatic oxidative damage and apoptosis in zebrafish

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Abstract

The objective of this study was to determine the effects of quercetin on oxidative stress and apoptosis induced by TPT in zebrafish. 240 fish were divided into 4 groups with three repeats. D1: fish fed with the basal diet as the control group. D2: fish fed with basal diet and exposed in 10 ng/L TPT. D3: fish fed diets containing 100 mg/Kg quercetin and exposed in 10ng/L TPT. D4: fish fed diets containing 100 mg/Kg quercetin. The results showed that quercetin could ameliorate oxidative stress, which decreased MDA, NO levels and improved antioxidant enzyme activities. The key apoptotic gene expressions, including caspase3, Bax and caspase9 mRNA expression were significantly induced by TPT exposure as compared with the control group, while notably decreased the Bcl-2 gene. However, dietary quercetin prevented a significant increase in Bax, caspase3 and caspase9 mRNA levels induced by TPT exposure, but increased Bcl-2 mRNA levels. The results of our study also demonstrated that 10 ng/L TPT significantly up-regulated TNF-α, IL-1β, IL-8, and NF-kB p65 gene expression and down-regulated IL-10 and IkB expression compared to the control group. However, TPT-induced inflammation was significantly mitigated in the quercetin treatment group. In conclusion, our findings suggested that quercetin might alleviate hepatic oxidative damage and apoptosis induced by TPT.

1. Introduction

Triphenyltin (TPT), an organotin compound, is widely used in agriculture and industry (Hoch 2001). Although the use of TPT has been banned in many countries, there are still some reports of TPT contamination in coastal areas of China (Gao et al. 2017). It has reported that the seafood (fish, sea vegetables, shellfish) contained TPT levels are higher than the TBT levels (Lee et al. 2016). There has been a lot of concern about toxicological and eco-toxicological properties of organotin compounds (Clasen et al. 2017; Chen et al. 2019). An eco-toxicological evaluation of TPT has indicated that aquatic animals might face serious risks with long-term exposure to TPT (Wen et al. 2018). The aquatic products have posed the most exposure risk to humans. Thus, exploring the mechanism of TPT and protective measures is of great importance. In the present study, TPT was used to induce oxidative stress, and evaluated the antioxidant effect of quercetin. This information will be with great interest to explain a well understanding of the toxicology of TPT and the functional effects of quercetin.

Oxidative stress is due to the excessive production of free radicals and the decrease of the activity of the antioxidant defense system, which interferes the equilibrium state of the pro-oxidation system and the antioxidant system (Kennedy et al. 2005). The antioxidants play a pretty crucial role in the health of fish. Excess reactive oxygen species (ROS) are toxic because they can attack and destroy a variety of biological molecules, such as DNA, carbohydrates, proteins and membrane lipids, leading to cell death and tissue damage. ROS is often used as a biomarker of oxidative stress, and its excessive production can induce oxidative damage and apoptosis (Patten et al. 2010). The influence of ROS on the health of fish has become of increasing interest. Many studies have shown that TPT can induce oxidative stress in different aquatic organisms, such as copepods (*Tigriopus japonicus*) (Yi et al. 2014), green microalgae (*Scenedesmus quadricauda*) (Xu et al. 2011), rotifers (*Brachionus koreanus*) (Yi et al. 2016), male

guppies (*Poecilia reticulata*) (Zhang et al. 2019) and abalones (*Haliotis diversicolor*) (Lu et al. 2016). TPT was found to inhibit the activities of the antioxidant enzymes such as glutathione S-transferases (GST), superoxide dismutase (SOD) and catalase (CAT) (Zhang et al. 2019). The cellular cytophysiological actions are regulated by the balance between the antioxidant capacity and the production of reactive oxygen species (ROS) (Parellada et al. 2012). Antioxidants such as quercetin, Vc, VE, can protect cells by scavenging free radicals. Antioxidant system includes different types of components, both enzymatic and non-enzymatic. The SOD, glutathione peroxidase (GPx) are both antioxidant enzymes, and other enzymatic antioxidants such as CAT can quickly remove the ROS, which can protect the body from their harmful effects (Li et al. 2013). However, how the TPT toxicity inhibits the antioxidants and the quercetin alleviate this response are not clear.

Apoptosis is the death process of cells in response to the physiological and pathological stimulus signal that plays a crucial role in the health of fish. It is an indispensable component of various cellular processes that mediate the phagocytosis to remove dying or infected cells, and remodel the flamed sites by reducing the release of pro-inflammatory cytokines (Luo et al. 2017). Apoptosis is strictly regulated. Many factors including pro-apoptotic factors (Bax, Casp-3 and Casp-9), anti-apoptotic factors such as Bcl-2 could affect the process of apoptosis. So, these genes could serve as indicators to study the effect of TPT in zebrafish liver and the ameliorating effect of quercetin.

Quercetin is an important bioactive compound found abundantly in many vegetables, fruits, fruit juices and herbal dietary supplements (Wach et al. 2007). It is known to have anti-inflammatory, antioxidant, neuro-protection, anti-carcinogenic, antidiabetic, and anti-proliferative effects in vitro and in vivo (Meng et al. 2018; Dokumacioglu et al. 2018; Laura et al. 2018). In one study, quercetin was reported to scavenge ROS and can effectively inhibit neuronal damage by regulating the oxidative stress (Adedara et al. 2017). In addition, quercetin is considered to be a natural flavonoid for the prevention of hepatotoxicity (Oliveira et al. 2014). However, the molecular mechanisms of TPT-induced liver injury and protective effects of quercetin are not yet completely understood. The aim of this study was to fill these gaps. Thus, the antioxidant and anti-inflammatory capability of quercetin were evaluated in this study. In general, this research contributes to a better understanding of the regulatory of quercetin and its role in the management of liver injury.

2. Materials And Methods

2.1. TPT and quercetin

In this experience, TPT chloride (98%) was obtained from Sigma-Aldrich (USA). The concentrations of TPT was 10 µg Sn/mL (33.1 n M/mL), which was prepared in the 95% ethanol. At the time of exposure, the solutions were added directly to the water. Quercetin was also purchased from Sigma-Aldrich with a purity more than or equal to 95%.

2.2. Experimental diets

The proximate composition and ingredients of the basal diets are shown in Table 1. The dosage of quercetin was 100 mg/kg and then was mixed with the basal diet. The feed material were ground into fine powder with a shredder, and then mixed with appropriate water to form a soft dough. The dough was granulated (no steam injected) using a 0.3 mm diameter pellet grind. The experimental feed was dried overnight at 50℃ and stored in airtight bags at 4℃ until use.

Table 1
Ingredients and proximate composition of the basal diet.

Ingredients	(%)	proximate composition	(% air-dry basis)
Fish meal	8	Moisture	11.32
Soybean meal	30	Crude protein	33.42
Cottonseed meal	16	Crude lipid	6.28
Rapeseed meal	16	Energy (MJ/Kg)	14.26
Soybean oil	2		
Fish oil	2		
Wheat bran	5		
Wheat flour	18		
$Ca(H_2PO4)_2$	1.8		
Premix	1		
NaCl	0.2		
Note: Premix supplied the following minerals (g/kg) and vitamins (IU or mg/kg):$CuSO_4 \cdot 5H_2O$, 2.0g, $FeSO_4 \cdot 7H_2O$, 25g, $ZnSO_4 \cdot 7H_2O$, 22g, $MnSO_4 \cdot 4H_2O$, 7g, Na_2SeO_3, 0.04g, KI, 0.026g, $CoCl_2 \cdot 6H_2O$, 0.1g, Vitamin A, 900000IU, Vitamin D, 200000IU, Vitamin E, 4500mg, Vitamin K3, 220mg, Vitamin B1, 320mg, Vitamin B2, 1090mg, Vitamin B5, 2000mg, Vitamin B6, 500mg, Vitamin B12, 1.6mg, Vitamin C, 5000mg, Pantothenate, 1000mg, Folic acid, 165mg, Choline, 60000 mg.			

2.3. Fish and experimental design

Zebrafish were provided from a commercial fish dealer (Guangzhou, China). Before the feeding trial, fish were acclimated for two weeks at the Aquarium Science Laboratory in Henan University of Science and Technology. During the period of acclimation, the fish were feeding a commercial diet with two times per day, which contained 11.32% moisture, 33.42% crude protein and 6.28% crude lipid. After the acclimation, the fish were starved for 48 h. Fish were randomly distributed into four groups, each with 60 fish. Every tank was placed 20 fish (mean weight of 2.05g ± 0.03g) in 60 L glass tanks. The tank contains 40 L dechlorinated tap water. The control group was fed the basal diet (D1) and the following three treatments were implemented. D2: fish fed with basal diet and exposed in 10 ng/L TPT. D3: fish fed with

experimental diets and exposed in 10 ng/L TPT. D4: fish fed with experimental diets. In the process of experiment, fish were fed twice a day at 7:30, 18:30 respectively and water was exchanged by 25% daily at 8:30. The experiment lasted for 8 weeks. Water temperature was controlled at 26℃ to 27℃. pH :7.0 -7.5, The dissolved oxygen was maintained approximately at 6.0 mg/L.

24. Sampling procedure

The fish were anaesthetized by MS-222 (pH 7.6, ethyl 3-aminobenzoatemethane-sulphonic acid, 1 g/L). Fish were dissected on ice. The livers were removed, frozen in liquid nitrogen and stored at -80°C for further enzymological assays and RNA quantification.

2.5. Assessment of oxidative stress

Frozen liver tissues were homogenized in 0.5 mL ice-cold 0.86% physiological saline with 10x volume (pH = 7.4) and centrifuged at 12,000 g for 10 min at 4°C. Then, supernatants were collected for various biochemical analyses. Oxidative stress induced by TPT was examined via measures of the MDA and NO levels. The MDA level was measured using the thiobarbituric acid. The quantification of NO release was detected by DAF FM DA fluorescence assay as described Choi (Choi et al.2012). The glutathione (GSH) level was determined using the method of Sedlak and Lindsay with some modifications (Sedlak and Lindsay 1968). The GSH levels were expressed as nmol/g tissue. The activity of SOD was measured using a SOD kit (Randox labs. Crumlin, UK), as described by Breinholt et.al (Breinholt et al. 2000). The activities of GPx and CAT were determined with the detection kits (Nanjing Jian-Cheng Bioengineering Institute, China) according to the manufacturer's protocol. Using bovine serum albumin as a standard, the total protein concentrations in the supernatants were determined by Bradford method (Bradford 1976).

2.6. Gene expression analysis

Total RNA was isolated from the hepatopancreas using TRIzol reagent (Takara), following the manufacturer's instructions. Using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA) determined the RNA concentration by obtaining the UV absorbance at 260nm. The absorbance ratio at 260nm/280nm was determined to evaluate the RNA Purity. cDNA was synthesized from RNA with the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The reaction tube was placed on the PCR amplification instrument for 60min at 37℃ and 15min at 70℃ for denaturation. The reverse transcripts were stored at 4℃ for later use.

The TNF-α, IL-1β, IL-8, NF- kBp65, IL-10 and IkB, Casp-3, Casp-9, B*ax* and Bcl-2 expression were determined using Real-time PCR. PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems) in 96-well plates (Axygen). The reaction system contained 20 μL mixtures using SYBR Green I dye. PCR amplifications contained 1 μL cDNA template, 1 μL forward and reverse primers (concentration of 1pM), 6.8 μL of SYBR green PCR master mix and 10.2 μL nuclease-free water and. The PCR reaction tube was put into the DNA amplification instrument for 94 ℃ for 4 min, and then the thermal cycle was carried out under the following conditions: 95 ℃ for 45 s, 57 ℃ for 1 min, 72 ℃ for 1 min. The PCR was carried out for 40 cycles. Details of primers used in this study were shown in Table 2. Genes expression

was expressed as multiple change relative to the control. They were calculated by $2^{-\Delta\Delta Ct}$ method with three replicates for treatment (Livak and Schmittgen). The expression profiles of target genes were normalized with β-actin (housekeeping or conserved gene).

Table 2
Primers used in this study.

Primer	Sequence(5'-3')	GenBank accession no.
Bcl-2 F1	TGGCTACAGCGACAGCTCTG	NM_200317.1
Bcl-2R1	GCATTCCAGCGTTCTCTCGA	NP_001116737.1
Bax F1	AGGGCTTCACTCTCTGCAAC	AY735397.1
Bax R1	CACACTGTGCAGCAGGTTTC	AB018218.1
Caspase3F1	CCTTGCGTGGTCACTACACT	
Caspase3R1	CACCTGGAGCCTACAACCTG	
Caspase9F1	TCGCTGCTTCTTCTGTCAGG	
Caspase9R1	ACAGCCGTCCATTTTGGCTT	
TNF-αF1	CTGCTTCACGCTCCATAAGA	AY427649.1
TNF-αR1	GCCTGGTCCTGGTCATCTC	
IL-8F1	GGTGAGAGACGGAGAGATGGAT	JN698962.1
IL-8R1	CACGCTGGAGAAGTTGAACAG	
IL-1βF1	ACAGAATGAAGCACATCAAACC	AY340959.1
IL-1βF1	ACAGAATGAAGCACATCAAACC	
NF-κB P65F1	GGCAGGTGGCGATAGTGTT	AY735398.1
NF-κB P65R1	CATTCCTTCAGTTCTCTTGCG	
IL-10F1	GCAGGCCTGACCCTACATTT	NM_001171592.1
IL-10R1	CCCCAGTGGAACACAGAGTC	
IkBαF1	CAAACTGGTGGTTCAAGCCG	NM_001123265.1
IkBαR1	CACTCACTGGACTGCGAACT	
β-actinF1	TCGTCCACCGCAAATGCTTCTA	AY222742.1
β-actinR1	CCGTCACCTTCACCGTTCCAGT	

2.7. Statistical analysis

SPSS 18.0 software (SPSS, Chicago, IL, USA) was used to analyze the data. The results of treatments significantly differed from the control group were performed with One-way analysis of variance following Tukey's test. The results of analysis are presented as Means ± S.D. P values < 0.05 were accepted as significant.

3. Results

3.1. Effects of TPT and quercetin on hepatic oxidative damage parameters in the liver of zebrafish

The effects of TPT and treatment with quercetin and their combination on lipid peroxidation and liver oxidative parameters are shown in Fig. 1. After exposure to TPT, the levels of MDA and NO were significantly increased, while GSH, GPX, SOD and CAT levels were significantly decreased compared with these indexes in control group ($P < 0.05$). However, the dietary quercetin significantly ($P < 0.05$) reduced the toxic effects of TPT on hepatic GSH, GPx, SOD, CAT, MDA and NO, and these values were higher or lower ($P < 0.05$) than the TPT group values. Interestingly, quercetin group showed significant elevation in the SOD and GSH levels compared with control group ($P < 0.05$).

3.2. Effects TPT and quercetin on apoptosis-related gene expression in the liver of zebrafish

The effects of quercetin on TPT-induced Bcl-2, Bax, caspase3 and caspase9 mRNA expression changes are shown in Fig. 3. Compared with the control treatment, TPT treatment significantly increased the mRNA expression of Bax, caspase3, and caspase9, while significantly decreased the expression level of Bcl-2 ($P < 0.05$). However, dietary quercetin prevented a marked increase in liver Bax, caspase3 and caspase9 mRNA expression induced by TPT exposure, but increased Bcl-2 mRNA levels ($P < 0.05$).

3.3. Effects TPT and quercetin on inflammatory response-related gene expression in the liver of zebrafish.

The effects of TPT and treatment with quercetin on inflammatory response-related gene expression are shown in Fig. 2. Compared with the control group, TPT significantly increased the levels of TNF-α, IL-8, IL-1β and NF- kBp65 mRNA expression levels of in zebrafish liver ($P < 0.05$). However, compared with the TPT group, dietary quercetin significantly decreased the mRNA expression levels of TNF-α, IL-8, IL-1β and NF- kBp65. The IL-10 and IkB mRNA expression were down-regulated by TPT exposure ($P < 0.05$). Quercetin decreased the changes in the expression of IL-10 and IkB decreased by TPT. In addition, administration of quercetin alone showed no significant difference ($P > 0.05$) compared to control groups (Fig. 3).

4. Discussion

Oxidative stress is unbalanced between the oxidation and antioxidation, which is considered an important role in many pathological and physiological phenomena of the body. Concerning oxidative stress indices, our study showed that fish exposure to TPT had significantly higher levels of the MDA and NO in liver tissue in zebrafish. On the other hand, MDA levels in liver tissues decreased after the application of 100 mg/kg quercetin compared to the TPT group. MDA is a scientifically recognized

indicator of oxidative stress, which is the product of polyunsaturated fatty acid peroxidation (Parvez and Raisuddin 2005). MDA levels rise when oxidative stress occurs (Del 2005). In general, the decrease of GSH level or GSH/GSSG ratio can express excess ROS (Jeroen et al. 2015). The liver synthesizes key enzymes that remove ROS and produce the systemic antioxidants, including GSH, SOD, GPx and CAT (Todorova et al 2005). Antioxidant enzymes are considered as the main defense system to inhibit the production of ROS and protect macromolecules from oxidative damage. SOD and GSH are the main antioxidant enzymes in the liver, and are also the main elements to enhance immunity to prevent diseases. While GPx is the main protective enzyme that can effectively scavenge free radicals in organisms, thus protecting cells from oxidative damage (Yildirim and Kilic 2011). In the present study, the antioxidant enzymes were examined after exposure to TPT. Based on the obtained results, it was shown that GSH level, GPx, SOD and CAT activities decreased in the TPT group. However, these enzyme activities in fish fed 100mg/kg quercetin were significantly increased in liver tissue. These results suggest that quercetin can protect liver against TPT-induced oxidative stress by regulating antioxidant enzyme activities. Quercetin effectively mitigates oxidative status by reducing lipid peroxidation and increasing various antioxidant enzymes in the liver of zebrafish. In previous study, quercetin could also scavenge oxidative radicals and increase the level of antioxidant defense (Livingstone 2001). In previous study, it was reported that quercetin increased the level of GSH, decreased the ROS and MDA levels, and regulated antioxidant enzyme activities in ethanol-exposure rats (Molina et al. 2003). Consistent with our experiment, a study showed that quercetin can elevate SOD and GPx activities and ameliorate MDA level (Adedara et al. 2017). Quercetin sweep free radicals off and thereby reduces oxidative and cytotoxic effects may be due to its molecular structure including several hydroxyls which can interact with free radicals to inhibit oxidative stress (Zhang et al. 2019). Whereas in another study, it was reported that quercetin attenuated the increases of antioxdant activities induced by dichlorvos (Salem et al. 2015). These different results might be related to animal species, breed conditions and animal state. This study demonstrated that dietary quercetin can effectively inhibit oxidative stress caused by free radicals.

The hepatotoxicity of TPT is closely related to the formation and apoptosis of ROS in hepatocytes. The production of oxidative stress and ROS are important signals of apoptosis (Todorova et al. 2005). The increased levels of ROS might impair the mitochondria functions, including changes of mitochondrial permeability, respiration and oxidative phosphorylation. Then, The mitochondria release cytochrome C, which activates caspase-3 and caspase-9, leading to cell apoptosis (Chen et al. 2014). It has also been reported that ROS mediate the mitochondria-dependent apoptosis in a variety of cells (Singh et al. 2007). Additionally, oxidative stress can damage DNA and directly or indirectly cause cell apoptosis (Tyor and Pahwa 2017). Bcl-2 is an anti-apoptotic gene and can inhibit cell apoptosis by preventing the release of cytochrome c from mitochondria. But Bax induce release of cytochrome c to trigger a pro-apoptotic pathway (Deng et al. 2009). If the Bax/Bcl2 ratio increases, it indicates the induction of cell apoptosis (Whiteman et al. 2007). In this study, it showed that several apoptotic related genes, such as Bax, caspase3 and caspase9, were up-regulated, while the mRNA expression of anti-apoptotic Bcl2 genes was decreased in the TPT group. We hypothesize that TPT lead to liver cell apoptosis by inducing of oxidative and activating p53 expression, which leads to the transcription of genes encoding pro-apoptotic proteins.

Cas3 (caspase-3) plays an irreplaceable role in cell apoptosis, which could be activated by Cas9 (caspase-9) and is a very important terminal splicing enzyme in the process of apoptosis (Deng et al. 2009). However, dietary quercetin attenuated cell apoptosis with downregulation of *Bax, Casp-3* and *Casp-9* as well as upregulation of *Bcl-2*. Quercetin has anti-apoptosis effect in various tissues (Kumar et al. 2014; Hu et al. 2015; Lei et al. 2015). Similar results were also found that 100 μ M of quercetin decreased apoptosis, while 1000 μ M of quercetin increased apoptosis (Ahn and Jeon 2015). We suggested that in the dietary quercetin group, the decrease of apoptosis might be related to the restrain of pro-inflammatory cytokines in liver. However, the underlying mechanism in fish remains unclear, which require further research.

Numerous researches have shown that organotin caused inflammation and pathological alterations (Zhang et al. 2015; Zhang et al. 2017; Zhang et al. 2018). TNF-α, IL-1β and IL-8 are major pro-inflammatory cytokines and, they are often used as markers of the inflammatory response (Secombes 2016). But the anti-inflammatory cytokines, such as IL-10 and TGF, may reduce the overreaction on inflammatory response in fish (Kemenade et al. 2009). Paralleled with the oxidative stress parameters, the TNF-α, IL-8 and IL-1β mRNA levels were significantly increased compared to the control. But, dietary quercetin remarkably alleviated the inflammatory response induced by TPT, showing lower gene expressions of pro-inflammatory cytokines, indicating that quercetin might alleviate the inflammation in the liver of fish. Although several previous studies indicated that quercetin attenuated animal inflammation mediated by inhibiting the expression of pro-inflammation gene (Julie et al. 2012; Mehta et al 2017). No studies of the possible effects of quercetin on fish have been reported. The quercetin regulated the pro-inflammatory cytokines, which might be related to the related signaling such as NF-kB. NF-kB plays an important role in the regulation of pro-inflammatory cytokines such as IL-8 and TNF-a (Neurath and Pettersson 1997). In this study, compared with the TPT group, quercetin decreased the mRNA expression of NF- kB p65 in the liver of zebrafish. Further contrastive analysis showed that the TNF-a, IL-1β and IL-8 were positively correlated with the mRNA expression of NF-kB p65, which suggested that quercetin inhibited the levels of TNF-a, IL-1β and IL-8 might be related to the decrease of NF- kB gene expression. In addition, NF- kB inhibitor protein (IkB) can directly inhibit NF- kB in fish (Iwasaki et al. 2011). In mice, it has been reported that the up-regulation of NF- kB mRNA was regulated by the down-regulation of IkB gene expression (Beg et al. 1995). In this study, the author firstly showed that TPT down-regulated IkB mRNA levels in the liver of zebrafish, where quercetin up-regulated this gene. Further analysis indicated that the IL-8, TNF-a mRNA levels and NF- kB p65 were negatively correlated with the IkB mRNA levels, suggesting that quercetin might be through up-regulation the IkB mRNA level and down-regulation the NF-kB mRNA level to prevent inflammatory response in fish. Similar results were also observed in mice (Jung et al 2012).

This result indicated that TPT exposure could induce accumulation of MDA and NO in tissue, inhibiting the antioxidant system. Quercetin could protect the zebrafish liver against TPT-induced injury by decreasing ROS production, alleviating cell apoptosis, renewing antioxidant enzymes activities, and attenuating inflammation.

Declarations

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Author Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The procedures of this study involving animals and their care were approved by the Animal Care and Use Committee of Henan University of Scientific and Technology (the number of committees was 9).

The data and materials are ture and they are available in the manuscript.

Authors' contributions

Chuannuan Zhang: Conceptualization, Methodology, Writing, Reviewing, Editing and Revising. Yuheng Wang: Data curation, Writing, Original draft preparation and Revising. Hongtao Ren*:* Investigation, Writing – Reviewing. Junhui Wang: Resources, Writing – Reviewing. Dongxue Jiang and Xiaoyu Yuan: Project administration, Writing- Reviewing.

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Tables

Table 1 Ingredients and proximate composition of the basal diet.

Ingredients	(%)	proximate composition	(% air-dry basis)
Fish meal	8	Moisture	11.32
Soybean meal	30	Crude protein	33.42
Cottonseed meal	16	Crude lipid	6.28
Rapeseed meal	16	Energy (MJ/Kg)	14.26
Soybean oil	2		
Fish oil	2		
Wheat bran	5		
Wheat flour	18		
$Ca(H_2PO4)_2$	1.8		
Premix	1		
NaCl	0.2		

Note: Premix supplied the following minerals (g/kg) and vitamins (IU or mg/kg):$CuSO_4 \cdot 5H_2O$, 2.0g, $FeSO_4 \cdot 7H_2O$, 25g, $ZnSO_4 \cdot 7H_2O$, 22g, $MnSO_4 \cdot 4H_2O$, 7g, Na_2SeO_3, 0.04g, KI, 0.026g, $CoCl_2 \cdot 6H_2O$, 0.1g, Vitamin A, 900000IU, Vitamin D, 200000IU, Vitamin E, 4500mg, Vitamin K3 , 220mg, Vitamin B1, 320mg, Vitamin B2, 1090mg, Vitamin B5, 2000mg, Vitamin B6, 500mg, Vitamin B12, 1.6mg, Vitamin C, 5000mg, Pantothenate, 1000mg, Folic acid, 165mg, Choline, 60000 mg.

Table 2 Primers used in this study.

Primer	Sequence(5’-3’)	GenBank accession no.
Bcl-2 F1	TGGCTACAGCGACAGCTCTG	NM_200317.1
Bcl-2R1	GCATTCCAGCGTTCTCTCGA	NP_001116737.1
Bax F1	AGGGCTTCACTCTCTGCAAC	
Bax R1	CACACTGTGCAGCAGGTTTC	AY735397.1
Caspase3F1	CCTTGCGTGGTCACTACACT	
Caspase3R1	CACCTGGAGCCTACAACCTG	AB018218.1
Caspase9F1	TCGCTGCTTCTTCTGTCAGG	
Caspase9R1	ACAGCCGTCCATTTTGGCTT	
TNF-αF1	CTGCTTCACGCTCCATAAGA	AY427649.1
TNF-αR1	GCCTGGTCCTGGTCATCTC	
IL-8F1	GGTGAGAGACGGAGAGATGGAT	JN698962.1
IL-8R1	CACGCTGGAGAAGTTGAACAG	
IL-1βF1	ACAGAATGAAGCACATCAAACC	AY340959.1
IL-1βF1	ACAGAATGAAGCACATCAAACC	
NF-κB P65F1	GGCAGGTGGCGATAGTGTT	AY735398.1
NF-κB P65R1	CATTCCTTCAGTTCTCTTGCG	
IL-10F1	GCAGGCCTGACCCTACATTT	NM_001171592.1
IL-10R1	CCCCAGTGGAACACAGAGTC	
IkBαF1	CAAACTGGTGGTTCAAGCCG	NM_001123265.1
IkBαR1	CACTCACTGGACTGCGAACT	
β-actinF1	TCGTCCACCGCAAATGCTTCTA	AY222742.1
β-actinR1	CCGTCACCTTCACCGTTCCAGT	

Figures

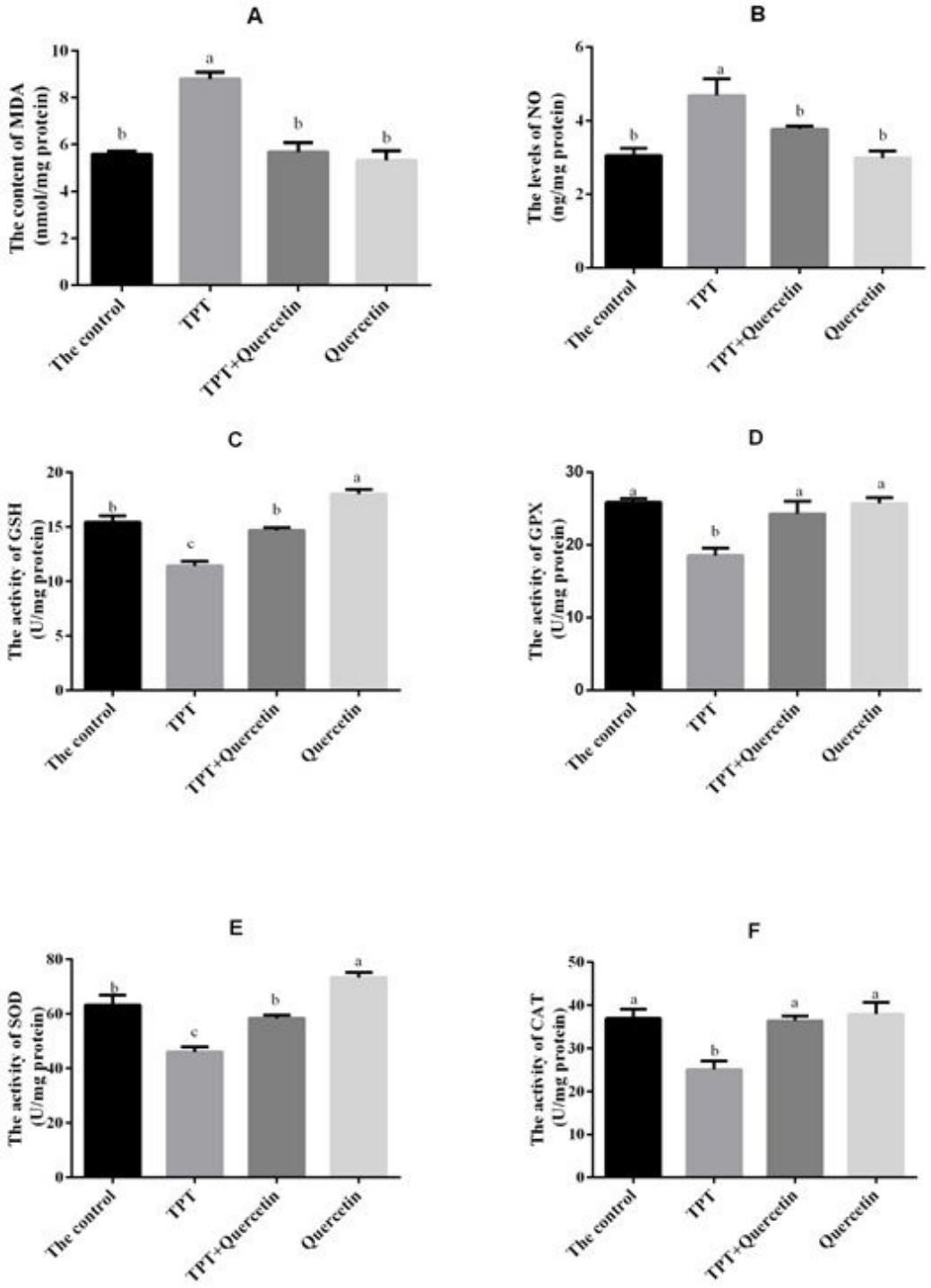

Figure 1

Effects of TPT and quercetin on hepatic oxidative damage parameters ((A) MDA, (B) NO, (C) GSH, (D) GPX, (E) SOD and (F) CAT) in the liver of zebrafish. Data were expressed as mean ± SEM and analyzed by One-way ANOVA, followed by turkey's multiple range test (n=9). Different letters denote significant difference ($P < 0.05$)..

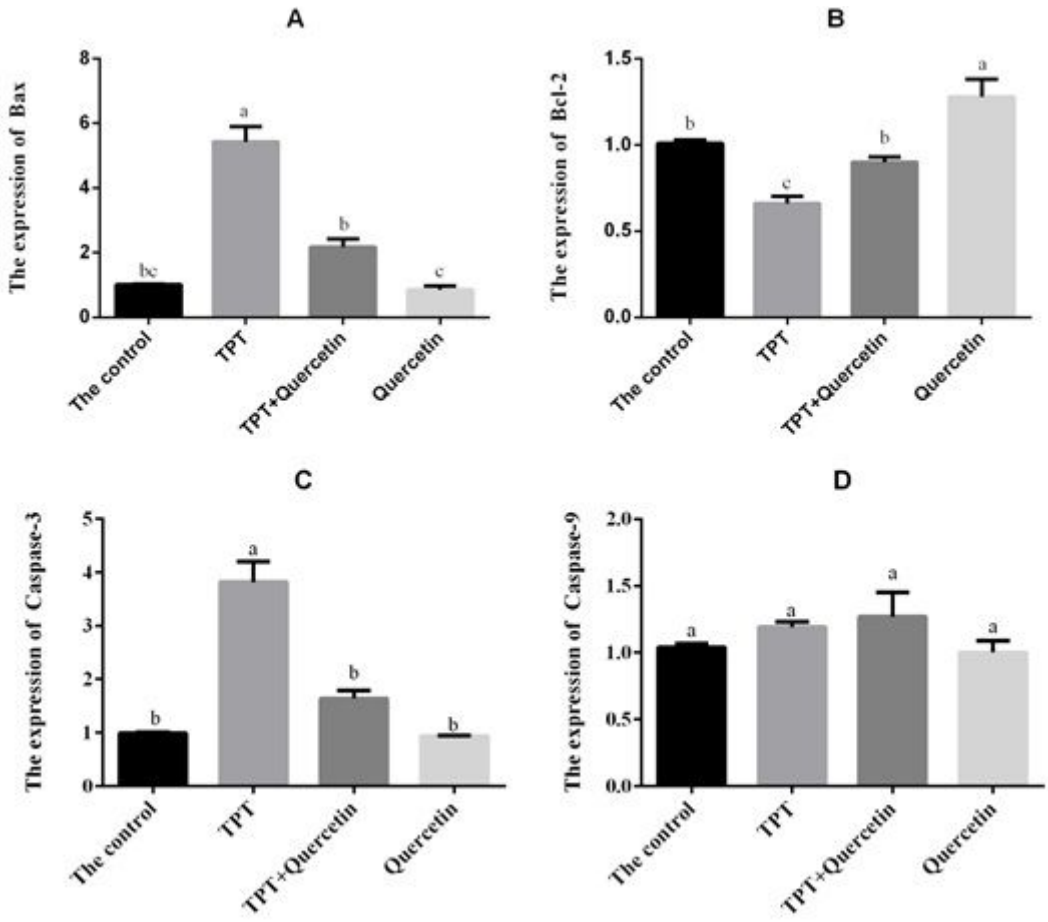

Figure 2

Effects TPT and quercetin on apoptosis-related gene expression ((A) Bcl-2, (B) Bax, (C) caspase3, and (D) caspase9) in the liver of zebrafish. Data were expressed as mean ± SEM and analyzed by One-way ANOVA, followed by turkey's multiple range test (n=9). Different letters denote significant difference ($P < 0.05$).

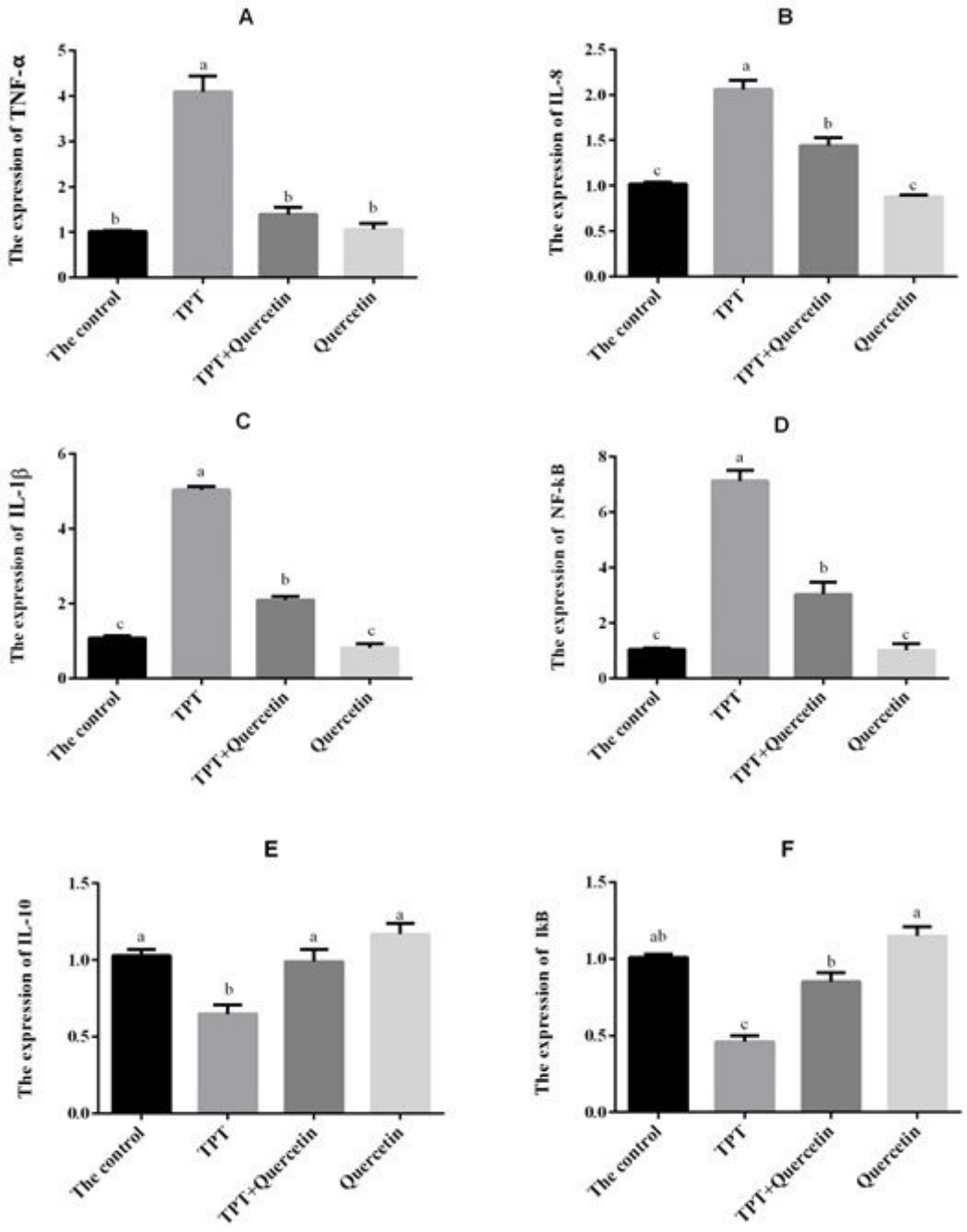

Figure 3

Effects TPT and quercetin on TNF-α, IL-8, IL-1β, NF- kBp65, IL-10 and IkB gene expression in the liver of zebrafish. Data were expressed as mean ± SEM and analyzed by One-way ANOVA, followed by turkey's multiple range test (n=9). Different letters denote significant difference ($P < 0.05$).