Effects of quercetin on bisphenol A-induced mitochondrial toxicity in rat liver

Masoud Mahdavinia [1]*, Said Alizadeh [1], Atefeh Raesi Vanani [1], Mohammad Amin Dehghani [1], Maryam Shirani [1], Meysam Alipour [2], Hedayat Allah Shahmohammadi [2], Sirous Rafiei Asl [3]

[1] Department of Toxicology, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2] Nutrition and Metabolic Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3] Department of Clinical Pathology, School of Veterinary Medicine, University of Shahid Chamran, Ahvaz, Iran

ARTICLE INFO	ABSTRACT
	Objective(s): Recognized as a distinguished environmental and global toxicant, Bisphenol A (BPA) affects the liver, which is a vital body organ, by the induction of oxidative stress. The present study was designed to investigate the protective effect of quercetin against BPA in hepatotoxicity in Wistar rats and also, the activity of mitochondrial enzymes were evaluated. ***Materials and Methods:*** To this end, 32 male Wistar rats were divided into four groups (six rats per group), including control, BPA (250 mg/kg), BPA + quercetin (75 mg/kg), and quercetin (75 mg/kg). ***Results:*** The BPA-induced alterations were restored in concentrations of alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST) due to the quercetin treatment (75 mg/kg) (all $P<0.001$). While the levels of mitochondrial membrane potential (MMP), reactive oxygen species (ROS), and malondialdehyde (MDA) decreased by the quercetin treatment in the liver mitochondria ($P<0.001$), catalase (CAT) and glutathione (GSH) increased ($P<0.001$). ***Conclusion:*** According to the results, the potential hepatotoxicity of BPA can be prevented by quercetin, which protects the body against oxidative stress and BPA-induced biochemical toxicity. Moreover, the reproductive toxicity of BPA after environmental or occupational exposures can be potentially prohibited by quercetin.

Introduction

Bisphenol A (BPA), which was identified by a Russian chemist (Alexander Dianin) in 1891 (1), is a material used in the production of polycarbonate bottles in the plastics industry, fungicides, heat-resistant materials, and the epoxy layer of canned foods (2). Application of this chemical compound in industries is on the rise, such that each year, more than 2.2 million tons of these products are manufactured worldwide (3). However, this has increased the exposure of human beings, especially due to greater use of polycarbonate plastic and food packaging containers (4). Pervasiveness or BPA in water, air, and soil and consumer products has occurred due to the increased production of this compound (5). Therefore, a circulating level of BPA in the range of 10-100 nM has been caused due to the absorption, inhalation, and ingestion of the chemical by humans upon exposure (6). Previous assessments have confirmed the presence of BPA in human tissues (7). Given the fact that BPA in the liver is converted to a less toxic compound called bisphenol A-glucuronide (BPAG), there is a possibility of presence of a higher concentration and toxicity of this compound in the mentioned organ (8).

As a vital metabolic organ, the liver has the responsibility of keeping the internal environment constant (homeostasis). Moreover, this organ has many important functions, including xenobiotics detoxification, glycogen storage, metabolism, and production of proteins, cholesterol, and bile (9). The harmful impact of exposure to BPA has been reported in various studies. For example, treatment of rats by BPA in a study led to increased levels of ALT, AST, and LDH. Furthermore, this compound caused defects in the morphology of the liver (10). In another study on animals, BPA interacted and connected with DNA in the liver tissue (11). It is known that BPA affects the liver, which is a vital body organ, through the induction of oxidative stress (12, 13). Cytotoxic agents of hydrogen peroxide, hydroxyl and peroxy radicals, and superoxide are ROS with the ability to stimulate oxidative stress through causing defects in antioxidant/pro-oxidant balance (14, 15). Mitochondrial damage is mainly caused by impairment of the electron transport chain and induction of oxidative stress (16). Moreover, impairment of the electron transport chain seriously damages the tissues (17). According to the literature, exposure to BPA causes defects in oxidative phosphorylation through the inhibition of the first complex of the electron transfer chain (18).

Liver cells contain a large number of mitochondria and are involved in liver diseases, chronic hepatitis C, steatosis (fat accumulation), and oxidative damage to mitochondria (19). There is a number of ROS in biological

systems, including hydroxyl radicals, superoxide anion, and hydrogen peroxide (20). Studies have shown that mitochondrial ROS can be applied in the induction of production of a proinflammatory cytokine in cells as signaling molecules (21). Most researchers attribute the harmful effects of BPA to disturbing the body's oxidant/antioxidant balance. However, the molecular mechanism of increased production of active oxygen species in exposure to BPA and disturbance of oxidant/ antioxidant balance are still not clear (22). Generally, phenol antioxidants protect the human body from free radicals and ROS proteins (23).

Ubiquitously occurring in plant-based foods, flavonoids are a big group of phenolic compounds and have many benefits for our health (24). A member of this family is quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone, Que), which is widely found in cereals, vegetables, and fruits that are commonly eaten by human beings. Characterized by the presence of a phenyl benzo(c) pyrone-derived structure, quercetin is an essential flavonoid and can be extensively found in various vegetables and fruits (25). This compound has many pharmacological features, including anti-ischemic, antiallergenic, anti-cancerogenic, anti-inflammatory, and antiviral (26). Moreover, quercetin protects the liver and kidneys against melphalan-induced oxidative stress (27). Several epidemiologic studies have confirmed the association of quercetin consumption with diseases that are significantly affected by inflammation and oxidative stress, such as cardiovascular disease, lung cancer, asthma, and other respiratory disorders (28). Compared to β-carotene and vitamins E and C, quercetin is a more effective antioxidant and can form a chelate with metal ions (e.g., iron). Therefore, it is able to prevent the reaction of iron-catalyzed Fenton (29).

Liver damage is prevented by quercetin treatment, which is also able to overturn overexpression of the inducible form of nitric oxide synthase (iNOS) induced by different inflammatory stimuli (30). BPA affects various body systems by disturbing the antioxidant/oxidant balance of the body, which is associated with the oxidation of lipids, proteins, and nucleic acids. Therefore, it seems that application of compounds with strong antioxidant features, such as quercetin, can reduce the production of free radicals created due to the toxicity of BPA in the body and play a significant role in the correction of antioxidant/oxidant balance. With this background in mind, this research aimed to evaluate the effect of PBA-induced mitochondrial toxicity in the rat liver.

Materials and Methods
Chemicals
All of the compounds used in the study, including thiobarbituric acid (TBA), reduced GSH, 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES), quercetin, tetraethoxypropane (TEP), sucrose, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dithiobis-2-nitrobenzoic acid (DTNB), Coomassie blue G, D-mannitol, 2′,7′-dichlorofluorescin diacetate (DCF-DA), bovine serum albumin (BSA), ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) and rhodamine 123 (Rh 123), were provided by the Sigma Chemical Co. (St. Louis, Missouri, USA). Other

compounds applied in the current research had the highest available grade of purity.

Animals
For the tests, the male Wistar rats (180–210 g) were provided by Ahvaz Jundishapur University of Medical Sciences. With free access to drinking water and standard rodent chow, the animals were maintained in polypropylene cages at a controlled temperature (25+2 °C) and on a 12:12-hr light-dark regimen. The ethical protocols and standards confirmed by the Committee of Animal Experimentation of Ahvaz Jundishapur University of Medical Sciences (ethical code: IR.AJUMS. REC.1395.632) were adhered to by the researchers throughout all of the tests.

Experimental design
The rats were randomly classified into four groups (n=6) and were administered with one dose of BPA per day for 14 days. In this research, group I included the control animals administered placebo (olive oil). On the other hand, group II contained rats treated with olive oil + 250 mg/kg of BPA. The rats in group III received olive oil + 250 mg/kg BPA + quercetin 75 mg/kg, and the animals in group IV were administered with olive oil + quercetin 75 mg/kg. All treatments were orally administered for 14 days. The experiment was performed according to the Guidelines of the Animal Ethics Committee for the Use of Experimental Animals (31, 32).

Biochemical assays
In this part of the research, we estimated some liver function test essential enzymes in the serum so that the BPA-induced toxicity of the liver could be evaluated. In addition, Reitman and Frankel assay method was applied to assess the activities of AST and ALT (33). Moreover, the technique presented by Wenk and Fernandis was exploited to measure the activity of ALP (34). It should be noted that ALP, AST, and ALT's activities were expressed as international units per milliliter.

Mitochondrial preparation
Differential centrifugation of rat-liver was carried out to prepare mitochondria (35). After the removal and mincing of the liver by a small scissor, the liver pieces were placed in a freshly prepared cold mannitol solution encompassing 10 mM HEPES-potassium hydroxide, 70 mM sucrose, 0.1% (w/v) BSA, 200 mM d-mannitol, and 1 mM EGTA at pH of 7.4. A glass homogenizer was applied to mildly homogenize the minced liver, followed by sedimentation of the broken cell debris and nuclei by centrifugation at 600xg and 4 °C for 10 min. Moreover, centrifugation of the supernatants was carried out in 10,000xg for 15 min. Afterward, we carefully removed the upper layer and washed the pellet through mild suspension in the isolation buffer, followed by the centrifugation of the compound at 10,000xg for 10 min. Using the mannitol solution, the resulting mitochondrial pellets were suspended. Moreover, the Coomassie blue protein-binding method was exploited to determine the protein concentrations according to the descriptions by Bradford (36).

Mitochondrial ROS level assay
Applying the fluorescent probe DCF-DA, we measured the mitochondrial ROS. In addition, incubation of the isolated mitochondria (0.5 mg protein/ml) was carried

Table 1. Effects of quercetin on the serum, relative liver weight (mg/kg), liver concentration, initial and final body weights, and liver function tests in BPA-induced toxicity

Group	Initial body weight (g)	Final body weight (g)	Relative liver weight (mg/kg)	Liver density
Control	205.8 ± 4.5	219.5 ± 3.87	23.50 ± 1.04	61.27 ± .84
BPA (mg/kg)	194.8 ± 2.85	231.2 ± 4.68	37.17 ± 1.05 ***	51.6 ± .78 ***
BPA + quercetin (mg/kg)	204.2 ± 4.52	221.8 ± 3.32	32.58 ± 1.35 ###	55.57 ± .41 ###
Quercetin (mg/kg)	197.3 ± 6.21	211.7 ± 4.84	25.02 ± .93	59.68 ± .71

Data expressed as mean±SEM (n=6). *significantly different from the control group (*** $P<0.001$) and # significantly different from the BPA group (### $P<0.001$) applying the one-way ANOVA test. BPA: Bisphenol A

out at 37 °C for 10 min with 1.6 μ M DCF-DA. At the emission and excitation wavelengths of 500 and 520 nm, respectively, we exploited the Perkin Elmer LS-50B luminescence fluorescence spectrophotometer (California, USA) to measure the fluorescence (37).

MMP assay
In this research, MMP was determined using the mitochondrial uptake of the cationic fluorescent dye Rh 123. For incubation of the mitochondrial suspensions (0.5 mg protein/ml), the tubes were mildly shaken at 37 °C with 1.5 μM Rh 123 for 10 min. Following that, the Elmer LS-50B luminescence fluorescence spectrophotometer was applied at emission and excitation wavelengths of 490 and 535 nm, respectively to estimate the fluorescence (38).

Measurement of GSH content
In isolated mitochondria, DTNB was applied by the spectrophotometric technique to determine the GSH contents (39). Afterward, 0.04% DTNB was mixed with the mitochondrial suspensions (0.5 mg protein/ml) in 0.1 mol/l of phosphate buffers (pH 7.4). Using a spectrophotometer (UV-1650 PC), we read the development of the color yellow at 412 nm. It is notable that the content of GSH was expressed as μg of protein per mg of tissue.

Lipid peroxidation assay
The technique introduced by Zhang *et al.* was used to determine lipid peroxidation (MDA) (40). Moreover, we applied 0.25 ml sulfuric acid (0.05 M) and 0.3 ml 0.2% TBA for incubation of mitochondrial suspensions (0.5 mg protein/ml). For 30 min after that, the tubes were maintained in a bath filled with boiling water. Finally, the tubes were transferred to an ice bath and butanol (0.4 ml) was poured into each tube. In the next stage, centrifugation of the tubes was carried out at 3500×g for 10 min. Following that, assessment of the total quantity of MDA shaped in each sample was carried out using a spectrophotometer (UV-1650 PC) to measure the supernatant's absorbance at 532 nm. Furthermore, MDA was expressed as nmol/mg protein and TEP was applied as the standard.

Measurement of CAT activity
Catalase (Cat) activity assay was performed according to the method reported by Khodayar *et al* (41). In brief, mitochondrial suspensions were added to Tris-HCl (0.05 mM) and H_2O_2 (0.01 M), mixed, and incubated for 10 min. Subsequently, ammonium molybdate (4%) was added, and absorbance was measured at 410 nm.

Statistical analysis
All results were statistically analyzed using GraphPad Prism (ver. 5.04) as mean ± SEM with one-way ANOVA test followed by Tukey's *post hoc* analysis and *P-value* less than 0.05 was considered significant.

Results
Changes in body and organ weight
There were no significant differences in the body weights of the groups compared with the control group. The final average body weights of the groups statistically increased compared with the initial average body weights. There was a significant difference in liver weight among the four experimental groups ($P<0.001$) (Table 1).

Effects of the treatment on ALT, AST, ALP, and LDH levels
As shown in Table 1, ALT, AST, ALP, and LDH levels significantly increased in the BPA group compared with the control group. In the groups that were administered the antioxidant, the AST and the ALT levels decreased when compared with the BPA group ($P<0.001$, Table 2).

Quercetin pretreatment could reduce the elevating effects of BPA on the mitochondrial ROS production
According to the results, there was a significant reduction in the ROS level of the BPA-receiving group, compared to the control group ($P<0.001$). Administration of 75 mg/kg of quercetin significantly decreased the level of ROS, compared to the BPA-receiving group ($P<0.001$) (Figure 1).
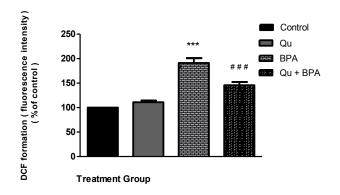

Figure 1. Effect of quercetin on ROS in rat liver mitochondria homogenate exposed to BPA. Values are means±SEM (n=6). *significantly different from the control group (*** $P<0.001$) and #significantly different from the BPA group (### $P<0.001$) applying the one-way ANOVA test
ROS: Reactive oxygen species; DCF: Dichlorofluorescin

Table 2. Effects of quercetin on the serum ALT, AST, ALP, and LDH activity, liver function tests in BPA induced toxicity

Group	ALT (U/l)	AST (U/l)	ALP (U/l)	LDH (U/l)
Control	20.83 ± 1.75	82.67 ± 3.98	78.17 ± 6.04	434.3 ± 37.30
BPA (mg/kg)	68.83 ± 3.63 ***	160.2 ± 5.30 ***	237.7 ± 8.11 ***	949.7 ± 57.91 ***
BPA + quercetin (mg/kg)	39.5 ± 2.14 ###	120.7 ± 3.88 ###	142.8 ± 6.22 ###	671.2 ± 41.08 ###
quercetin (mg/kg)	23.17 ± 1.47	85.50 ± 4.35	83.33 ± 4.59	444.7 ± 34.24

Levels of ALP, AST, ALT, and LDH activity are expressed as nmol NADH oxidized/min/mg protein and IU/mL of serum, respectively. Data expressed as mean±SEM (n=6). *significantly different from the control group (***P<0.001) and # significantly different from the BPA group (###*P*<0.001) applying the one-way ANOVA test. ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; BPA: Bisphenol A
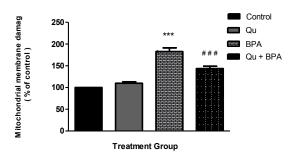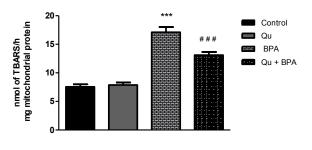

Figure 2. Effect of quercetin on MMP (ΔΨm) in liver mitochondria of rats via homogenate exposure to BPA. Values are means±SEM (n=6). *significantly different from the control group (***P<0.001) and # significantly different from the BPA group (###*P*<0.001) applying the one-way ANOVA test
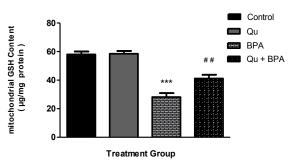BPA: Bisphenol A; Qu: Quercetin; MMP: Mitochondrial Membrane Potential

Figure 3. Effect of quercetin on GSH in liver mitochondria of rats through homogenate exposure to BPA. Values are means±SEM (n=6). *significantly different from the control group (***P<0.001) and # significantly different from the BPA group (###*P*<0.001) applying the one-way ANOVA test
BPA: Bisphenol A; Qu: Quercetin; MMP: Mitochondrial Membrane Potential

BPA induced MMP damage can be reduced with pretreatment by quercetin

According to the results, a significant increase was observed in MMP, known as ΔΨm, of the samples in the BPA receiving group (*P*<0.001). It seems that 75 mg/kg of quercetin significantly decreased MMP, compared to the BPA-receiving group (*P*<0.001) (Figure 2).

The increasing impact of BPA on the mitochondrial GSH can be reduced by pretreatment with quercetin

In the BPA-receiving group, the GSH level significantly decreased, compared to the control group (*P*<0.001). Administration of 75 mg/kg of quercetin significantly increased the GSH level, compared to the BPA group (*P*<0.001, Figure 3).

The increasing impact of BPA on the MDA can be reduced by pretreatment with quercetin

In the BPA group, the MDA level significantly increased, compared to the control group (*P*<0.001). Administration of 75 mg/kg of quercetin significantly reduced the MDA level, compared to the BPA receiving group (*P*<0.001, Figure 4).

The increasing impact of BPA on the mitochondrial CAT activity can be reduced by pretreatment with quercetin

Evaluation of the level of CAT activity showed that BPA significantly decreased the activity of this enzyme, compared to the control group (*P*<0.001). Moreover, administration of quercetin significantly increased the CAT activity level, compared to the BPA receiving group (*P*<0.001) (Figure 5).

Figure 4. Effect of quercetin on MDA in liver mitochondria of rats with homogenate exposure to BPA. Values are means±SEM (n=6). *significantly different from the control group (***P<0.001) and # significantly different from the BPA group (###*P*<0.001) applying the one-way ANOVA test
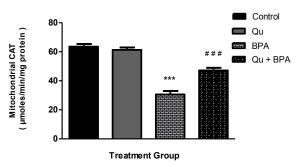BPA: Bisphenol A; Qu: Quercetin; MDA: Malondialdehyde

Figure 5. Effect of quercetin on activity in liver mitochondria (0.5 mg/mL) of rats with homogenate exposure to BPA. Values are means ± SEM (n=6). *significantly different from the control group (***P<0.001) and #significantly different from the BPA group (###*P*<0.001) applying the one-way ANOVA test
BPA: Bisphenol A; QU: Quercetin

Discussion

BPA is a broadly applied industrial endocrine-disrupting chemical produced in large volumes, exposure to which by humans has currently attracted significant attention. Given the fact that there is exposure to this chemical compound during prenatal life due to its presence in amniotic fluid, cord blood, and maternal serum and transfer to fetus via placental tissues and at birth, it is safe to say that exposure to BPA is a universal event (42). There is a higher level of vulnerability in the liver toward the low BPA doses, compared to other organs, owing to the initial metabolism of BPA by the liver via glucuronic acid conjugation (43). Despite having an endogenous antioxidant defense system, it seems that the liver is the main part exposed to oxidative stress and toxicants. Therefore, specific tests have been performed on the BPA impacts on the liver. In a study on rats, it was concluded that the impact of BPA on the liver antioxidant/oxidant balance was the main cause of damage to liver function after BPA exposure (10). Generally, fat is partially infiltrated, oxidative stress is induced, and cytochrome P450 isoforms in the liver of rats is inhibited by BPA (44). Evaluations in this regard have made it clear that the lipophilic nature of BPA is responsible for the mechanism introduced for the elevation of oxidative stress level (45). In general, there is a tendency in BPA to start inducing lipid peroxidation and formation of hydroxyl radicals by interacting with the hydrophobic cell plasma membrane (46). Mitochondria-mediated apoptosis is induced in hepatic cells by BPA, reported by Xia *et al* (47). According to other studies which included *in vivo* experiments, liver tumors, metabolic syndromes, and hepatic steatosis are caused by BPA exposure (48). Moreover, this compound elevates the formation of ROS (43). ROS, which act as regulators and mediators of calcium signaling, are scavenged and produced in mitochondria, the main area for these acts, and are applied to control the function of mitochondria (49). According to the results of the current research, the liver tissue mitochondria are damaged by BPA exposure through elevation in the number of ROS. Moreover, BPA exposure may activate apoptosis, pathway of oxidative stress, and other cytotoxicity pathways. After measuring the end product of lipid peroxidation, we found that creation of ROS increased by BPA. In addition, there was an elevation in the concentration of MDA at three various pubertal times. In this regard, our findings are in line with the results obtained by other studies, which indicated increased tissue MDA due to BPA exposure (50). According to these reports, the functions of the liver are impaired by the overproduction of ROS, which is associated with elevated levels of serum AST, ALT, and ALP (51).

In the current research, administration of BPA led to a significant increase in the activities of ALT and AST, which might be due to oxidative damage to the liver induced by BPA, which results in hyperactivity of the liver or the release of hepatic enzymes in the blood (12). We applied the tests of liver performance (e.g., LDH, AST, ALT, and ALP) to assess liver disease or damage. As one of the most prominent dietary antioxidants, quercetin might be regarded as an effective therapeutic agent in patients with damage to the liver (52). According to several studies, environmental contaminant-induced oxidative stress in the liver is caused by the antioxidant activities of quercetin (53). In addition, direct free quenching of lipid peroxides and free radicals and increase in the formation of non-enzymatic antioxidants and antioxidant enzyme activities under *in vivo* conditions are carried out by quercetin (54). According to the results of the present research, hepatotoxicity induced by BPA was ameliorated by regulation of levels of ALT, MDA, and AST following a 14-day pretreatment of the samples with quercetin. Furthermore, there was a reduction in the histological changes induced by BPA in the liver tissue after treatment with quercetin.

For the first time, our findings were indicative of the significant hepatoprotective effect in hepatotoxicity in rats induced by BPA after 14 consecutive days of quercetin pretreatment. It seems that the regulation of several functions greatly depends on mitochondrial glutathione. In fact, free radicals are quenched by this process (55). According to our observations, the GSH level significantly decreased in the liver mitochondria of rats treated with BPA. With regard to the increase in the production of superoxide in rats treated with BPA, the reduction of the level of GSH demonstrated its application in the detoxification of free radicals. Mitochondria may be significantly stressed due to the decrease in the GSH levels along with reduced activities in antioxidant enzymes (56). In the current research, there was a significant reduction in the levels of GSH in the BPA group, compared to the control group. Moreover, there was a significant increase in the levels of GSH in the BPA + quercetin group, compared to the BPA group, which is most probably due to the antioxidant feature of quercetin. By donating electrons, which are able to end the electron chain reaction, quercetin and its metabolites can neutralize the ROS from BPA (57). In addition, BPA significantly increases the serum levels of ALT, AST, ALP, and LDH (22). Oxidative damage to MMP is caused by shaping of hydroxyl radicals via building of a pro-oxidative status by GSH oxidation (58). Over the last decade, while researchers have determined the mitochondrial motility machinery, the signaling mechanism that underlies the specificity and the spatiotemporal control of the mitochondrial movements is still not clear. Given the inhibition of mitochondrial transport by depolarization of mitochondria with agents, $\Delta\psi_m$ is crucial for mitochondrial movement (5). Early studies found that mitochondria with high $\Delta\psi_m$ were shown to preferentially move anterograde (59).

In the current research, MMP significantly increased in the BPA group, compared to the control group. On the other hand, there was a significant reduction in the MMP of the BPA+ quercetin group, compared to the BPA group, which might be due to the antioxidant feature of quercetin. In addition, it is the most effective agent in protection against mitochondrial dysfunction among different structurally related naturally occurring flavonoids (60). Our results were also indicative of a significant decrease in the level of activities of antioxidant enzyme CAT in liver mitochondria of the BPA group. Generally, CAT is an essential antioxidant enzyme that changes hydrogen peroxide into water and oxygen, which leads to the mitigation of toxic impacts (61). In this regard, our findings are in congruence with the results obtained on the down-regulation of gene

expression of antioxidant enzyme CAT and their reduced activities in the BPA-exposed liver of male rats (62).

Conclusion

According to the results of the current research, the activities of mitochondrial electron transport chain (ETC) enzyme complexes in the liver were significantly affected by BPA. This compound also impacted the antioxidant milieu of mitochondria and caused oxidative stress. Mitochondrial damage of the liver, which is induced by BPA, is protected by quercetin by decreasing the oxidative stress and the formation of its factors. However, it is suggested that other studies be conducted so that we are able to more efficiently comprehend quercetin's protective action mechanism.

Acknowledgment

Conflicts of Interest

All authors declare that they have no conflicts of interest.

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