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Quercetin and Catechin Protects Leptin-Deficient Lep$^{ob/Ob}$ Mice Against Alloxan-Induced Diabetes and Hepatotoxicity via Suppression of Oxidative Stress and Inflammation

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Abstract

Background: The study focuses on evaluating the combined effects of quercetin (QCT) and catechin (CAT), both plant-based antioxidants, on alloxan-induced liver toxicity and diabetes in leptin-deficient (Lep$^{ob/ob}$) mice. Diabetes is a metabolic disorder characterized by high blood glucose levels due to inadequate insulin secretion or insulin resistance.

Methods: Thirty mice were divided into five groups of 6, including: normal control, diabetic control, diabetic mice treated with 150 mg/kg CAT, diabetic mice treated with 150 mg/kg QCT, and diabetic mice treated with 150 mg/kg CAT, and 150 mg/kg QCT for seven days. Mice were anesthetized after overnight fasting on the 8th day, and the blood sample was collected and the levels of antioxidants and pro-inflammatory factors in serum, and the expression of ADP-ribose polymerase (PARP) protein were measured, and histological studies were performed.

Results: The results showed that diabetic mice receiving QCT and CAT showed lower liver enzymes, fasting blood sugar (FBS), blood urea nitrogen (BUN), creatinine (Cr), cholesterol, triglyceride, low-density lipoprotein (LDL), TNF-α, and thiobarbituric acid reactive substances (TBARS) levels and increased high-density lipoprotein (HDL), total thiol, catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) levels in the liver compared to the ALLO group alone ($P<0.001$). The level of PARP protein significantly declined in the ALLO group compared to the control group.

Conclusion: The findings of this study demonstrated that QCT, and CAT are reasonably effective in preventing hepatotoxicity and diabetes in mice.

Keywords: Alloxan, Catechin, Quercetin, Diabetes, Hepatotoxicity, Mice.

Introduction

Diabetes is considered a chronic disease that about 25% of the world's population suffer from it (1,2). Diabetes is a metabolic disorder that is caused by insufficient secretion of insulin by the cells of the islets of Langerhans of the pancreas or insulin resistance, which ultimately leads to increase of blood glucose (3). As a result of the activity of two intestinal enzymes, alpha-amylase and alpha-glucosidase, carbohydrates are broken down into disaccharides and monosaccharides, which eventually increases blood glucose (4).

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Diabetes is associated with increased blood lipids due to the disruption of metabolic pathways leads to hyperglycemia and hyperlipidemia, which ultimately causes many structural and metabolic disorders in different organs of the body, including the liver (5). Oxidative stress is the leading cause of diabetes and related diseases. Reactive oxygen species (ROS) lead to changes in the structure and function of proteins, lipids and nucleic acids. As a result of lipid oxidation, malondialdehyde is produced, which is an important finding in measuring oxidative stress (6). Uncontrolled diabetes leads to complications such as coronary artery diseases, peripheral vascular diseases, cerebrovascular diseases, retinopathy, neuropathy, and nephropathy (7).

Researchers constantly try to find a safe and effective therapeutic approach to treat diabetes. Many chemical agents are available to control diabetes, but no complete cure for the disease has been reported. Alloxan (ALLO) is a urea derivative that causes selective necrosis of pancreatic islet cells. By using ALLO, conditions similar to diabetes are created in mice. Alloxan is known as a hepatotoxic and diabetic agent by activating oxidant and inflammatory factors. Consequently, the primary approach to ameliorate ALLO-induced hepatotoxicity and diabetes is using antioxidants (8). Antioxidants play a significant role in reducing the complications of diabetes.

Herbal treatments are usually cheaper, more accessible and available compared to chemical treatments and have less side effects. So far, more than 1200 herbal medicines have been identified that can be effective in treating diabetes. Flavonoids are a group of polyphenolic compounds that are found in significant amounts in plant materials, especially tea, onions, and berries (9). Green tea is one of the most famous traditional drinks around the world, which contains different amounts of catechin (CAT) that have the potential to treat diabetes (10). Some human and animal intervention studies show that green tea extract can benefit blood sugar control and lead to the removal of ROS and reduction of oxidative stress (11-13). A clinical trial study showed that green tea extract supplementation reduced glycosylated hemoglobin in diabetic patients (14). Quercetin (QCT) is a plant substance belonging to the flavonoid family that has antioxidant effects and the ability to reduce free radicals in the body (15). Quercetin is found in fruits and vegetables, including onions, blueberries, and broccoli. The results of numerous tests show that QCT has beneficial physiological effects such as antioxidant, anti-inflammatory properties and increasing glucose absorption (16). Quercetin inhibits xanthine oxidase, lipid peroxidation, cyclooxygenase, and lipoxygenase. Loggia et al. shown that QCT can protect pancreatic β cells against inflammatory damage (17). Another essential function attributed to QCT is the ability to reduce aldose reductase (which is involved in converting glucose to sorbitol through the polyol pathway). Accumulation of sorbitol in different organs of the body leads to various complications, such as diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy. (18). Based on biochemical and pharmacological studies, QCT is a potent scavenger of ROS that reduces the risk of cardiovascular and renal diseases (19-21). According to past studies, excessive energy consumption in response to excessive production of ROS and inflammation, leads to excessive activation of ADP-ribose polymerase (PARP) enzymes (22) and the inhibition of these enzymes leads to the protection of mice against diabetes. It becomes (23). Considering the antioxidant effects of QCT, and CAT, which have been proposed in various studies, it seems that these compounds may effectively reduce oxidative stress and diabetes in the liver. In this study, we investigated the combined effect of QCT, and CAT on ALLO-induced hepatotoxicity and diabetes in $Lep^{ob/ob}$ mice.

Materials and Methods

Chemicals

Quercetin (CAS: 117-39-5, Purity ≥95%), catechin (CAS: 154-23-4, Purity: ≥98%), alloxan (CAS: 2244-11-3, Purity: ≥98%) were purchased from Sigma Aldrich Company, USA. Fasting blood sugar (FBS), blood urea

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nitrogen (BUN), creatinine (Cr), cholesterol (Cho), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) assay kits were purchased from Pars Azmoon Company, Iran. Thiobarbituric acid reactive substances (TBARS), total thiol, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) assay kits were purchased from the ZellBio Company, Germany. TNF-α (tumor necrosis factor alpha) kit was purchased from the Bioassay technology laboratory in Shanghai, China. Expression of PARP protein was evaluated by Western blot.

Animals

Thirty male mice (24 Lep ob/ob and 6 normal mice), weighing 27±2 g were subjected to a light/dark cycle of 12 h/12 h, temperature of 23±2 °C, relative humidity and free access to sufficient water and food. All ethical principles for the use of animals were based on the protocols of Ahvaz Jundishapur University of Medical Sciences (ethics number: IR.AJUMS.ABHC.REC.1401.052).

Induction of diabetes

Conditions similar to type 1 diabetes were induced in mice by a single intraperitoneal injection of ALLO monohydrate 150 mg/kg in physiological saline in a volume of 0.1 ml. In 72 hours after the ALLO injection, blood glucose level was measured using a glucometer (Germany) in the tail vein area. The animals with fasting blood sugar levels more than 270 mg/dL were considered as experimental diabetic (24).

Experimental design

This study was conducted for 14 days. A total of thirty male mice (24 diabetic mice, six normal mice) into five groups of 6 were used for the experiment. The groups were as follows: group 1, normal control (150 ml/kg normal saline); group 2, diabetic control (150 mg/kg ALLO intraperitoneal); group 3, 4, and 5, diabetic mice treated with 150mg/kg QCT, 150 mg/kg CAT, and 150 mg/kg QCT, and 150 mg/kg CAT for seven days by gavage. Quercetin (25), CAT (12), and ALLO (26) doses were selected based on previous research. After 7 days and one night of fasting, FBS was measured with a glucometer. After mice were anesthetized with ketamine (90 mg/kg)/xylazine (10 mg/kg), blood samples were collected directly from the heart and stored in gel tubes. After separating the serum, the sample was stored at –20 °C until the tests were done. The liver was extracted and washed with normal saline. A part of the liver was kept in 10% formalin solution for histopathological examination. The other part of the liver was kept at –70 °C to assay oxidative, and inflammatory factors.

Biochemical analysis of the serum

The serum levels of FBS, BUN, Cr, Cho, TG, LDL, HDL, ALP, ALT, and AST were determined using commercial detection kits (Pars Azmoon Company, Iran) with a Hitachi 912 auto-analyzer (Japan).

Preparation of liver tissue homogenate and biochemical analysis

For this purpose, one part of the liver tissue was homogenized in phosphate buffer, using a homogenizer and centrifuged. The supernatant was separated and stored at −70 °C to measure tissue factors (TBARS, total thiol, CAT, SOD, GPx, TNF-α, and expression of PARP protein). The Bradford's protein assay method was used to determined protein concentration in the liver tissue homogenates supernatants. The absorbance was measured at 595 nm (27).

Total thiol content was determined using Ellman's reagent (DTNB), and the formation of the yellow TNB (28). The absorbance was read at 412 nm with a spectrophotometer and expressed as nmol/mg protein.

The level of TBARS was measured using the Kei method (29). The absorption of the resulting pink color was measured at 532 nm, and reported as nmol/mg protein.

The activity of superoxide dismutase (SOD), and glutathione peroxidase (GPx) and catalase (CAT) enzymes, were measured

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using ZellBio colorimetric assay kits, and were recorded as nmol/mg protein.

The liver tissue level of TNF-α was evaluated using an enzyme-linked immunosorbent assay kit, and the absorbances was measured at 450 nm and was recorded as pg/mg protein.

The homogenized liver tissue samples were transferred to polyvinylidene difluoride membranes. The membranes were probed with the primary antibody, PARP (Cat No. 13371-1-AP), overnight at 4 °C. The next day, it was incubated with a secondary antibody. Finally, the obtained protein band was analyzed using Image J software. GAPDH enzyme (code no: 1.2.1.12) was used as a loading control in Western blotting.

Histopathological analysis

One part of the liver tissue was fixed in 10% formalin and cut into tissue blocks by the cutting method. Sections of 5 micron-thick were obtained from these blocks and stained with hematoxylin/eosin and observed under a light microscope (30). For each mouse, six microscopic slides were examined for histological assessment. The histological features of the liver to evaluate infiltration of inflammatory cells and fatty change were classified in the four groups: normal (0), mild (1), moderate (2), and severe (3).

Statistical analysis

Statistical analysis was performed by GraphPad Prism version 8 statistical software and the normality of the data was confirmed using the Kolmogorov-Smirnov test. The mean ± SEM of the data in each group was calculated and finally the results were analyzed using one-way ANOVA and Tukey's post hoc test for multiple comparisons. Values of $p<0.05$ were considered significant.

Results

The effect of QCT, and CAT on the levels of BUN, and Cr

Measurement of the BUN, and Cr at the end of the experiment showed that the serum levels of BUN, and Cr were significantly increased in the ALLO treated group compared to the control group ($p<0.001$). Treatment with QCT, and CAT reduced these biomarkers in a dose dependent manner (Fig. 1).

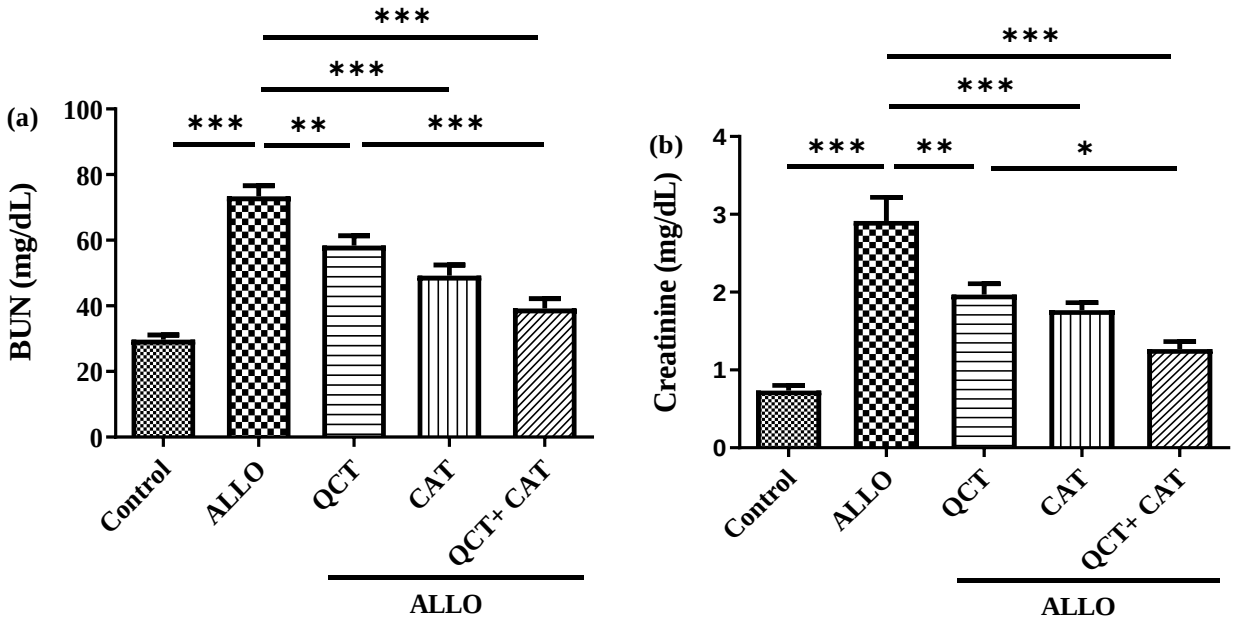

Fig. 1. The effect of quercetin (QCT), and catechin (CAT) on the levels of blood urea nitrogen (BUN), and creatinine (Cr) in diabetic mice. Data are expressed as mean ± SEM (n=6). (*P<0.05, **P<0.01 and *** P<0.001).

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The effect of QCT, and CAT on the liver function markers

Elevated serum levels of liver enzymes, including ALT, AST, and ALP could be a symptom of liver damage (Fig. 2). In the ALLO group increased the activities of ALT, AST, and ALP compared to the control group (P<0.001). However, ALLO supplemented with QCT, and CAT significantly decreased the activities of these enzymes compared to ALLO alone (P<0.001).

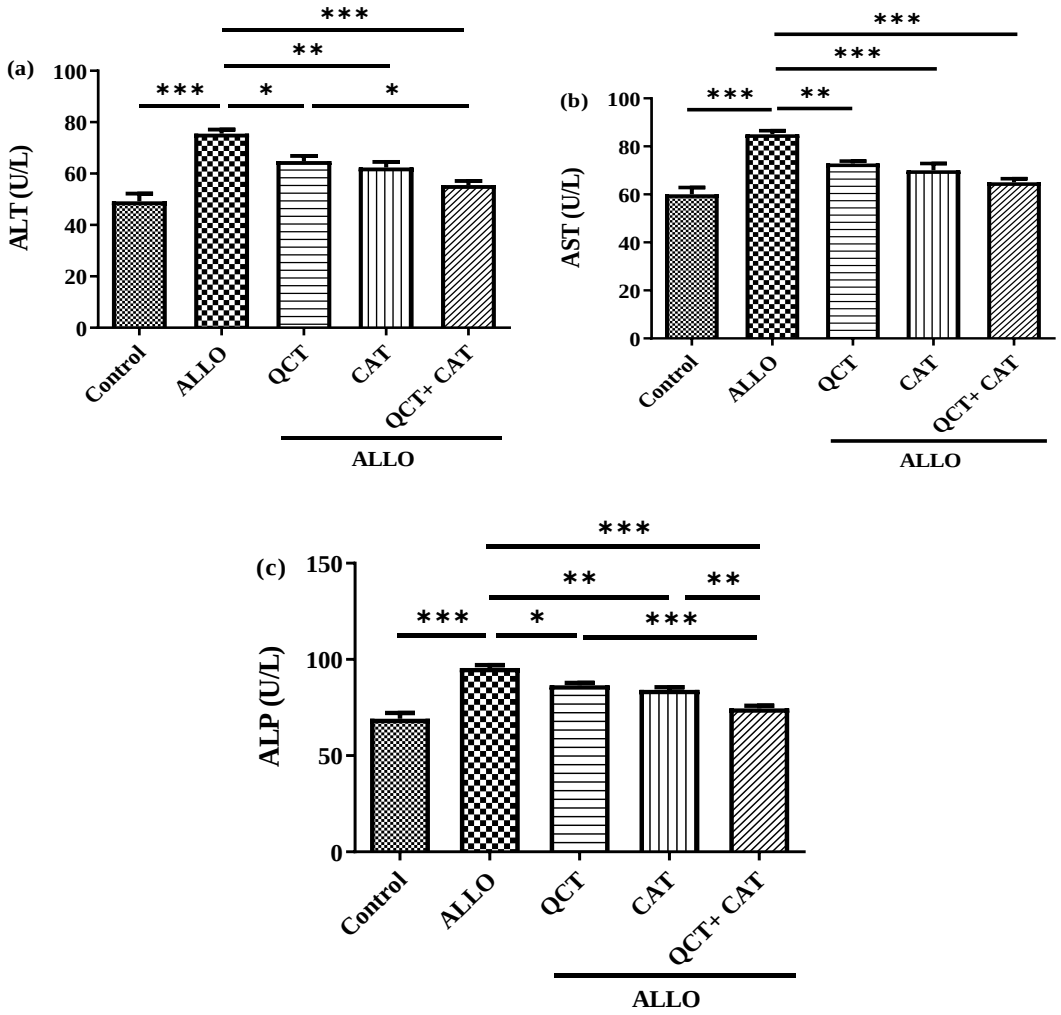

Fig. 2. The effect of quercetin (QCT), and catechin (CAT) on the liver function markers of ALT (alanine aminotransferase), AST (aspartate aminotransferase), and ALP (alkaline phosphatase) in diabetic mice. Data are expressed as mean ± SEM (n=6). (*P<0.05, **P<0.01 and *** P<0.001).

The effect of QCT, and CAT on the biochemical markers of the serum

The level of FBS increased in the diabetic control group. The treatments with QCT, and CAT decreased the level of FBS compared to diabetic control. In the ALLO with QCT and ALLO with CAT groups, we observed a significant decrease in the serum levels of cholesterol, triglyceride, and LDL (P<0.001) an increase in the serum levels of HDL (P<0.001). In contrast, receiving QCT, and CAT in diabetic mice led to a significant decrease (P<0.001) in cholesterol, triglyceride, and LDL compared to the diabetic control group. Eventually, in the ALLO with QCT, and CAT group compared to ALLO with QCT or CAT groups, a further reduction in cholesterol, triglyceride, and LDL, and a further increase in HDL was observed, that showed the better effect of QCT, and CAT compared to QCT or CAT alone in diabetic mice (Fig. 3).

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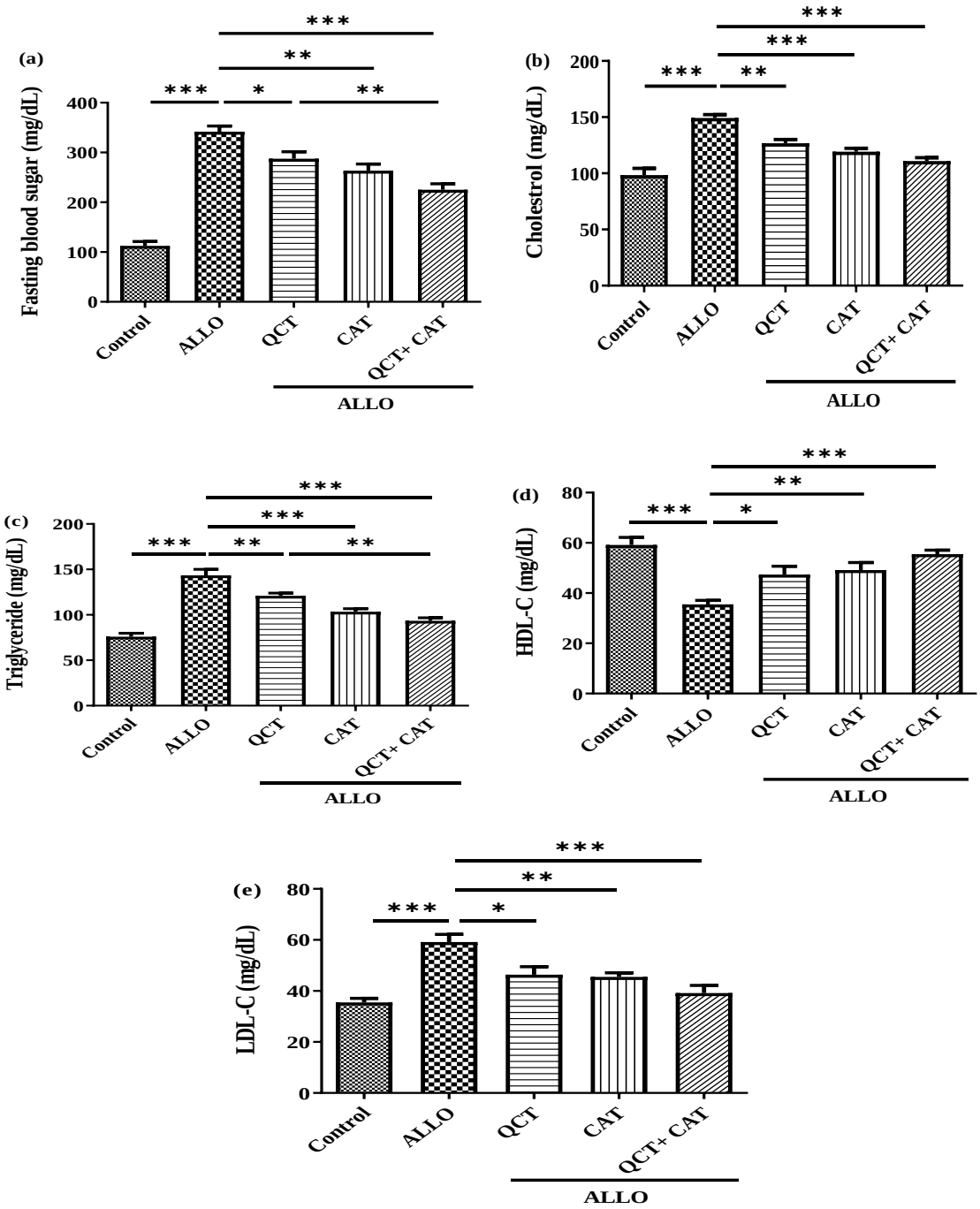

Fig. 3. The effect of quercetin (QCT), and catechin (CAT) on the levels of FBS (fasting blood sugar) and lipid profiles of cholesterol, triglyceride, HDL (high-density lipoprotein), and LDL (low density lipoprotein) in diabetic mice. Data are expressed as mean ± SEM (n=6). (*P<0.05, **P<0.01 and *** P<0.001).

The effect of QCT, and CAT on oxidative stress parameters

The levels of SOD, CAT, total thiol, and GPx in ALLO group were significantly lower than the control group (P<0.001). However, in the group receiving QCT or CAT with ALLO, the levels of SOD, CAT, total thiol, and GPx were significantly higher than ALLO group (P<0.001). Finally, in the ALLO with QCT, and CAT group compared to ALLO with QCT or CAT groups, a more significant increase in SOD, CAT, total thiol, and GPx were observed, that indicates a better effect of QCT, and CAT compared to QCT or CAT alone in

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diabetic mice. Alloxan led to an increase in the level of TBARS compared to the control group (P<0.001). However, in the group receiving QCT or CAT with ALLO, compared to the ALLO group, the amount of TBARS decreased significantly (P<0.001). Finally, in the ALLO with QCT, and CAT group compared to ALLO with QCT or CAT groups, a greater decrease in the level of TBARS was observed, that indicates a better effect of QCT, and CAT compared to QCT or CAT alone in diabetic mice (Fig. 4).

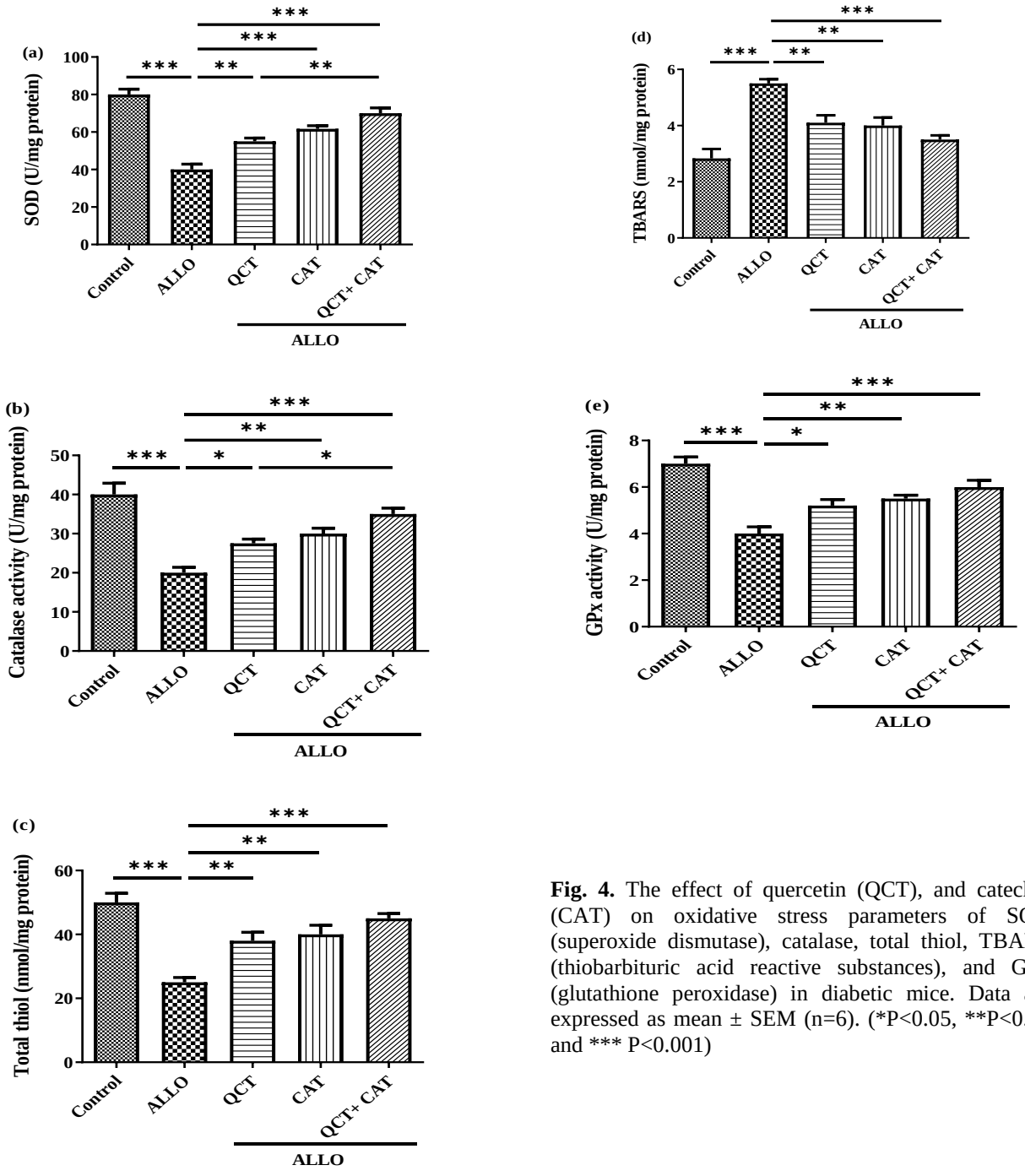

Fig. 4. The effect of quercetin (QCT), and catechin (CAT) on oxidative stress parameters of SOD (superoxide dismutase), catalase, total thiol, TBARS (thiobarbituric acid reactive substances), and GPx (glutathione peroxidase) in diabetic mice. Data are expressed as mean ± SEM (n=6). (*P<0.05, **P<0.01 and *** P<0.001)

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The effect of QCT, and CAT on the level of TNF-α

The results showed that QCT, and CAT improved inflammatory factor and caused a significant decrease TNF-α in the treated groups compared to the control group. Finally, in the ALLO with QCT, and CAT group compared to ALLO with QCT or CAT groups, a more significant decrease TNF-α was observed ($p<0.001$), which indicates a better effect of QCT, and CAT compared to QCT or CAT alone in diabetic mice (Fig. 5).

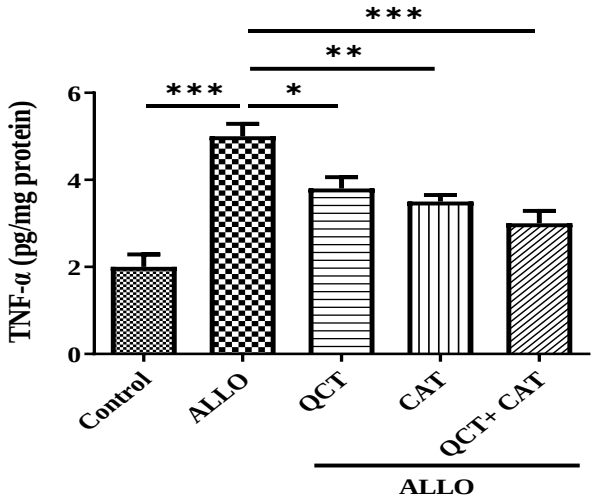

Fig. 5. The effect of quercetin (QCT), and catechin (CAT) on the level of inflammatory biomarker of TNF-α (tumor necrosis factor-alpha) in diabetic mice. Data are expressed as mean ± SEM (n=6). (*P<0.05, **P<0.01 and *** P<0.001).

The effect of QCT, and CAT on the expression of PARP protein in the liver

The level of PARP protein significantly increased in the ALLO group compared to the control group. Co-treatment with QCT, and CAT, remarkably decreased the expression of PARP protein compared to the ALLO group. These findings reveal that QCT, and CAT can reverse ALLO-induced reduction of PARP protein expression (Fig. 6).

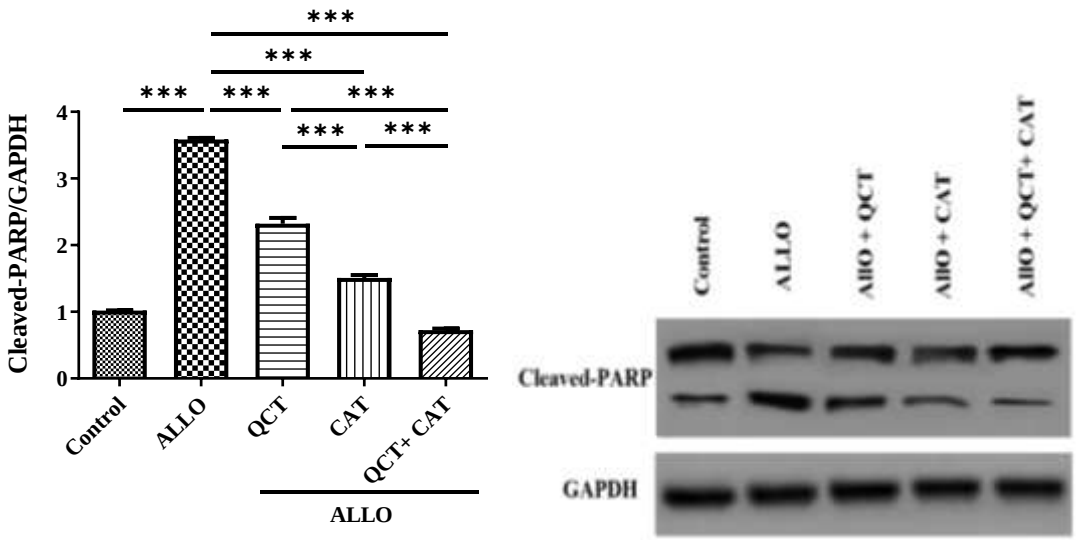

Fig. 6. The effect of quercetin (QCT), and catechin (CAT) on the expression of PARP (poly ADP-ribose polymerase) protein in the liver of diabetic mice. Data are expressed as mean ± SEM (n=6). (*P<0.05, **P<0.01 and *** P<0.001).

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Histopathological examination

In the control group, the lobular structure and shape of the liver tissue looked normal, and the lobular structure of the liver was clear and regular. In the ALLO group, the liver tissue was severely damaged, so that the liver lobules were messed up to a great extent, the port spaces could not be identified, and the sinusoids were dilated in some places. Fatty change was observed as micro vesicular (in the hepatocytes cytoplasm) and macro vesicular (between the hepatocytes). Focal and local infiltration of inflammatory cells in the space around the central vein of liver cells was also observed. In the treated groups, compared to ALLO group, the changes mentioned above, such as the widening of the sinusoids, and the confusion of the liver lobules, were reduced to a large extent, and the fatty change was significantly less than the ALLO group. However, the infiltration of inflammatory cells in the treated diabetic groups did not change significantly compared to the untreated diabetic group (Fig. 7).

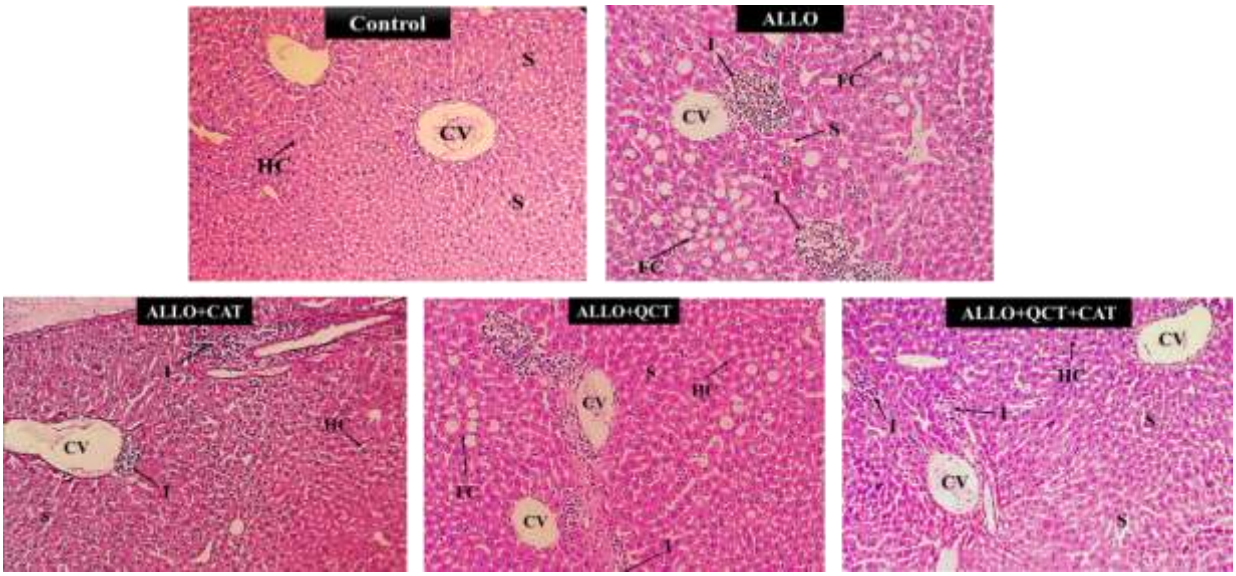

Fig. 7. Light microscopy examination of liver tissue sections that stained by H&E in the groups of control (C), alloxan (ALLO), alloxan+quercetin (ALLO+QCT), alloxan+catechin (ALLO+CAT), alloxan+ quercetin+catechin (ALLO+QCT+CAT). Inflammation (I), Width of sinusoids (S), confusion of hepatocytes (HC) & lobules and fatty change (FC) were observed in the ALLO group (A). In the treated diabetic groups (ALLO+QCT/ALLO+CAT/ALLO+QCT+CAT), compared to the untreated diabetic group (ALLO), the widening of the sinusoids and the confusion of the liver lobules were reduced to a large extent, and the fatty change was significantly less than the ALLO group. However, the infiltration of inflammatory cells in the treated diabetic groups did not change significantly compared to the untreated diabetic group. Magnification: ×400.

Table 1. A semi-quantitative analysis of liver tissue damage in the treated groups (**p<0.01), and (***P<0.001) comparison with the control group. ($^{\#}$P<0.05), and ($^{\#\#\#}$P<0.001) comparison of the ALLO groups receiving QCT or CAT or QCT+CAT with the ALLO group.

Groups	Inflammation	Fatty change (%)
Control	0.11 ±0.03	**0.00 ± 0.00**
ALLO	2.19 ± 0.28***	**7.43 ± 1.54*** **
ALLO+QCT	2.05 ± 0.26**	**7.81± 1.82*** $^{\#}$**
ALLO+CAT	2.14 ± 0.19**	**2.7± 0.34*** $^{\#}$**
ALLO+QCT+CAT	1.15± 0.25* $^{\#}$	**0.12 ± 0.08** $^{\#\#\#}$**

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Discussion

Alloxan is known as a hepatotoxic and diabetic agent by activating oxidant and inflammatory factors. Leptin-deficient $Lep^{ob/ob}$ mice was used in this study. Based on past studies, inflammation in fat tissue leads to increased insulin resistance and disruption of glucose metabolism (31). Consequently, the primary approach to ameliorate ALLO-induced hepatotoxicity and diabetes is using antioxidants. Antioxidants play a significant role in reducing the complications of diabetes (32,33). The flavonoids QCT and CAT, which are essential in the treatment and prevention of complications of diabetes and hepatotoxicity due to their antioxidant properties. Therefore, this research determined the combined effect of QCT and CAT on the reduction of ALLO-induced hepatotoxicity and diabetes in mice. Alloxan significantly increased the levels of ALT, AST, ALP, FBS, Urea, Creatinine, cholesterol, triglyceride, LDL, TBARS, and expression of PARP protein, and decreased the levels of HDL, total thiol, CAT, SOD, and GPx in the liver compared to the control group. The histopathological examinations of the liver tissue confirmed the biochemical results. Alloxan led to hyperglycemia, ultimately increasing glucose oxidation to produce additional oxidative products. Therefore, the decrease in the activity of antioxidant enzymes can be related to the rapid consumption and disruption of their storage in the body during the fight against free radicals produced (29). To investigate the therapeutic effects on the modulation of liver function, this study measured the activity of liver enzymes. It was found that the activity of AST, ALT, and ALP decreased significantly in each of the three treatment groups compared to the ALLO group, which indicates the protective effects of QCT, and CAT on liver function. The study by Hao Yang et al. showed that QCT significantly reduced levels of serum transaminase, and TNF-α, and the levels of cholesterol, triglyceride, superoxide dismutase, catalase, and total thiol increased in the liver, which is consistent with our results (34). In Dhanya's study, QCT affected multiple diabetes targets, regulated vital signaling pathways and showed similar effects to metformin (35). Boots et al.'s results showed that QCT supplementation improved antioxidant defense. In addition, QCT supplementation reduced markers of oxidative stress and inflammation, which was consistent with our study (16). Crespy et al. showed that CAT can have beneficial effects on FBS and lead to the removal of ROS and reduction of oxidative stress (13), which was consistent with the results of our study. The results of the study by Isabele BS Gomes et al. indicated that treatment with QCT leads to reduction in polyuria (~45%) and hyperglycemia (~35%), and hypertriglyceridemia, and a reduction in proteinuria and high levels of plasma urea and creatinine (36). It was consistent with the results of our study. In this study, co-treatment with QCT, and CAT, remarkably decreased the expression of PARP protein compared to the ALLO group. These findings reveal that QCT, and CAT can reverse ALLO-induced reduction of PARP protein expression. According to previous studies, excessive activation of PARP enzymes leads to excessive production of reactive oxygen species and inflammation, and ultimately high energy consumption (22). Another study indicates that the inhibition of PARP enzymes leads to the protection of mice against diabetes (23) which confirms the results of the present study. The histopathological examinations of the liver tissue showed that in the treated groups compared to ALLO group, the widening of the sinusoids and the confusion of the liver lobules were reduced to a large extent, and the fatty change was significantly less than the ALLO group. However, the infiltration of inflammatory cells in the treated diabetic groups did not change significantly compared to the untreated diabetic group. The findings of this study demonstrated that QCT, and CAT could be considered compelling candidates in the treatment of hepatotoxicity and diabetes in mice.

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This study showed that QCT, and CAT have a protective effect against hepatotoxicity and diabetes caused by ALLO because they have antioxidant, anti-inflammatory, and anti-fibrotic activity. The dose of 150 mg/kg of QCT, and 150 mg/kg CAT showed better results. More animal models and clinical trials are needed to confirm the use of QCT, and CAT.

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Conflict of interest
The authors confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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