ORIGINAL ARTICLE

Veterinary Research Forum. 2025; 16 (2): 97 - 105
doi: 10.30466/vrf.2024.2033878.4338
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Hepatoprotective effect of royal jelly on dibutyl phthalate-induced liver injury in rats

Mahdieh Nezami Majd[1], Goudarz Sadeghi-Hashjin[1]*, Hassan Malekinejad[2], Ali Rassouli[1]

[1] Department of Comparative Biosciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [2] Department of Pharmacology and Toxicology, School of Pharmacy, Urmia University of Medical Sciences, Urmia, Iran.

Article Info

Article history:

Received: 28 June 2024
Accepted: 14 August 2024
Available online: 15 February 2025

Keywords:

Dibutyl phthalate
Hepatotoxicity
Inflammation
Insulin resistance
Oxidative stress

Abstract

Phthalate esters, such as dibutyl phthalate (DBP), are extensively utilized and human and animal exposure leads to serious toxic effects, including hepatotoxicity. In the present study the protective effects of royal jelly (RJ) on DBP-induced liver damage was investigated. A total number of 40 Wistar albino rats were randomly divided into eight groups (n = 5): control (corn oil), DBP (500 mg kg^{-1}), RJ (200 mg kg^{1}), Quercetin (QCN; 50.00 mg kg^{-1}), RJ (100 mg kg^{-1}) + DBP, RJ (200 mg kg^{-1}) + DBP, RJ (300 mg kg^{-1}) + DBP, QCN (50.00 mg kg^{-1}) + DBP. After 28 days of daily oral gavage treatment, animals were euthanized. The insulin resistance index, lipid profile and hepatic enzymes were measured on the collected serum samples. Moreover, oxidative and nitrosative stress biomarkers were determined in the liver. Histopathological alterations and ultimately cytochrome P450 2E1 (CYP2E1) activity was also assessed. Data obtained revealed that RJ significantly reduced the insulin resistance index and liver enzymes level in RJ-DBP groups. At the same time, RJ recovered the DBP-induced oxidative stress and restored the DBP-depleted glutathione. Moreover, RJ improved lipid profile and reduced significantly the DBP-induced hepatic CYP 2E1 activity in RJ-DBP groups. Dibutyl phthalate induced-hepatic damage such as necrosis of hepatocytes and scattered bleeding was alleviated in RJ-DBP group. Our data suggested that the administration of RJ could protect the DBP-induced hepatic functional and structural alterations. The RJ protective effects might be attributed to its antioxidant and anti-inflammatory properties and reduced CYP 2E1 activity.

Introduction

Phthalates are environmental toxicants that humans are exposed to on a daily basis all around the world. They are a group of chemical substances composed of alkyl diesters of phthalic acid. Phthalate esters are classified into high molecular weight with 7 - 13 carbon atoms and low molecular weight with 3 - 6 carbon atoms in their backbone.[1,2] Generally, phthalates in their pure form are odorless, colorless, oily liquids with high lipophilic properties and low solubility in water.[1] Phthalates are usually used as plasticizer to increase the pliability, flexibility and elasticity of plastics, and also extensively used in cosmetics, personal care products, food packaging and medical products.[3] Phthalates are non-covalently bound to parent material, hence, they commonly leach from these substances into environmental sources especially under high temperatures, and it leads to human being frequently exposed to these substances through ingestion, inhalation and dermal contact.[4] Phthalates have been detected in water, air, sediments, soil, food, human blood plasma, breast milk and urine.[3] Exposure to phthalates is a public health concern because they have been identified as reproductive and developmental toxicants as well as endocrine-disrupting chemicals .[2]

Epidemiological studies have found close associations between phthalate exposure and insulin resistance.[5,6] For instance, urinary levels of several phthalate metabolites were positively correlated with insulin resistance in the adult males.[7] Animal studies also indicated that phthalate can induce insulin resistance, It was shown that mice exposed to diisononyl phthalate demonstrated increased insulin resistance index [homeostasis model assessment - insulin resistance (HOMA-IR)] and oxidative stress.[8] Previous studies indicated that oxidative stress activated various serine/-threonine kinase, hence, increased serine

***Correspondence:**
Goudarz Sadeghi-Hashjin. DVM, PhD
Department of Comparative Biosciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
E-mail: gsadeghi@ut.ac.ir

phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 which results in the reduction of IRS-mediated insulin signaling and induce insulin resistance.[9]

Moreover, other findings indicated that Di-(2-ethyl-hexyl)-phthalate disrupts the insulin signaling pathway in the liver of Sprague Dawley rats and L02 cells through inducing the activity of peroxisome proliferator-activated receptor (PPAR) γ which have an important role in decreasing protein expression of insulin receptor (IR) and glucose transporter (GLUT) 4. Previous studies also showed that PPARγ forms hetrodimer with retinoid X receptor and is able to hinder the transcriptional activity of the GLUT4 promoter by directly and specifically binding to the GLUT4 promoter region.[10] Reportedly, dibutyl phthalates (DBPs) could decrease insulin synthesis and secretion in rat insulinoma cells through mitochondrial apoptotic signaling pathway and oxidative stress. In the same study, it was shown that DBP could reduce the expression of GLUT2 proteins which plays an important role in insulin secretion in pancreatic β-cells.[11]

Non-alcoholic fatty liver disease (NAFLD) is closely related to insulin resistance in the liver and peripheral organs such as adipose tissue and skeletal muscle. It is worth noting that two-thirds of type 2 diabetes mellitus patients have NAFLD.[9] In peripheral IR, glucose uptake from blood into skeletal muscle is reduced and lipolysis in the adipose tissue is increased. Conversely, hepatic IR is correlated with diminishing of glycogenesis and also an increase of gluconeogenesis and glycogenolysis.[9] Alteration caused by insulin resistance, including increased lipolysis in adipose tissue, which leads excess fatty acids entering to the liver as well as increased *de novo* lipogenesis and impaired fatty acids beta oxidation in mitochondria lead to fat accumulation in the liver and incidence of NAFLD.[12] Consequently, modulation of IR represents a potential strategy for NAFLD treatment.

Royal jelly (RJ) is a yellowish-white and acidic secretion of hypopharyngeal and mandibular glands of nurse bees used to nourish young worker larvae for the first three days and the whole life of queen bees. The main constituents of RJ include water, proteins, sugars, and lipids, minor constituents including minerals, amino acids, vitamins, enzymes, hormones, polyphenols, nucleotides, and minor heterocyclic compounds.[13] Royal jelly has several pharmacological effects such as antioxidant, anti-inflammatory, anti-diabetic, anti-hyperlipidemia, and anti-hypertension.[13] Previous studies showed that RJ could decrease the fructose-induced insulin resistance in rats.[14] In another study, it was shown that RJ increases the mRNA expression of adiponectin and adiponectin receptors and also increases the protein expression of phosphorylated AMP-activated protein kinase leading to a decrease in the expression of glucose-6- phosphatase enzyme, which plays an important role in gluconeogenesis, thus, improves hyperglycemia in obese/diabetic KK-Ay mice.[15] In a recent study, insulin resistance, insulin, cholesterol, triglycerides (TGs), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were increased in rats fed on a high fat diet and the administration of RJ (100 mg kg^{-1}, 8 weeks) led to a decrease in insulin resistance, insulin and an improvement in the lipid profile.[16] Hani M reported that RJ improved oxidative stress conditions in the streptozocin-induced diabetic rats by increasing glutathione, catalase, superoxide dismutase and decreasing malondialdehyde (MDA).[17]

According on RJ pharmaceutical and biological novel effects in this study we aimed to investigate the potential and beneficial effects of RJ on DBP induced hepatotoxicity with focusing on any alterations in insulin resistance and its consequences.

Materials and Methods

Chemicals and assay kits. Chemicals used in this study including: Quercetin (QCN), nicotinamide adenine dinucleotide phosphate (NADPH), ascorbic acid, 5,5′-dithiobis-(2-nitrobenzoic acid), sodium acetate trihydrate were purchased from Sigma-Aldrich (Sigma-Aldrich., St. Louis, USA). Corn oil was obtained from local grocery (Ladan Inc., Tehran, Iran). Royal Jelly was prepared from a local bee keeper (Hamedan, Iran). Dibutyl phthalate, potassium chloride, phosphoric acid (85.00%), thio-barbituric acid, tris-base, ethylene diamine tetra acetic acid, sulphanilamide, n-(1-naphthyl) ethylene diamine-2HCl, folin's reagent, 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ), $FeCl_3.6H_2O$ (Ferric chloride hexahydrate), hydro-chloric acid (HCl), sodium hydroxide, perchloric acid, potassium sodium tartrate, sodium carbonate, Copper (II) sulfate, glacial acetic acid, 4-nitrophenol were purchased from Merck (Darmstadt, Germany). Assay kits including alanine aminotransferase (ALT), aspartate amino-transferase (AST), fast blood sugar, TG, Cholesterol, HDL, LDL were purchased from Delta Darman (Delta Darman Part, Tehran, Iran). Hematoxylin and Eosin staining kits were supplied by Asia Pajohesh (Tehran, Iran).

Animal grouping and study design. Adult Wistar strain albino rats (200 - 250 g) obtained from the Animal department of Urmia University of Medical Sciences, Urmia, Iran. Animals were maintained under standard laboratory conditions (27.00 ± 2.00 ˚C and 12-hr dark-light cycle) and had free access to standard pellet diet and tap water. After 1 week of acclimation they were divided into eight groups of five rats each as follows: control (corn oil), DBP (500 mg kg^{-1}), RJ (200 mg kg^{-1}), QCN (50.00 mg kg^{-1}), RJ (100 mg kg^{-1})+ DBP, RJ (200 mg kg^{-1}) + DBP, RJ (300 mg kg^{-1}) + DBP, QCN (50.00 mg kg^{-1}) + DBP. All treatments were administered once daily. Both QCN and DBP were dissolved in corn oil, and RJ was dissolved in normal saline. All compounds were administered by oral gavage and administration lasted for 28 days. All experimental

protocols were carefully considered and ultimately approved by the Ethics Committee of Tehran University, Tehran, Iran (IR.UT.VETMED.REC.1402.013).

Blood sampling and tissue collection. One day after the last treatment, tissue collection and blood sampling from animals both were performed. Animals were anesthetized with a combination of 90.00 mg kg^{-1} ketamine (Rotexmedica, Aachen, Germany) and 9.00 mg kg^{-1} xylazine (Alfasan, Woerden, The Netherlands) intra-peritoneally. After induction of deep general anesthesia, blood samples were collected *via* direct cardiac puncture and were centrifuged at 1,500 *g* for 5 min to separate blood serum. The collected serum samples were stored at – 20.00 ˚C until further analyses. The liver tissues were rapidly dissected out and cleaned with cold normal saline solution. Subsequently, one part of the liver tissue was fixed in 10.00% formalin for routine histological examination and another part was stored at − 80.00 ˚C for molecular studies. After dissection, liver index (liver weight per bodyweight) was determined.

Biochemical analyses. The serum levels of liver enzymes ALT, AST, lipid profile including HDL, LDL, TG and cholesterol and the level of blood glucose were determined by commercially available kits. Moreover, the serum concentration of lactate dehydrogenase (LDH) was measured by available assay kit (Ziestchem, Tehran, Iran).

Hormonal assay (insulin). The serum level of insulin hormone was measured using assay kits (Siemens, Munich, Germany) according to the manufacturer's procedure. Homeostasis model assessment was used to assess insulin resistance as:[18]

HOMA - IR= (Fasting insulin × Fasting glucose)/405

Total antioxidant capacity of serum (TAC). Assessment of the TAC of collected serum samples was performed by utilizing the ferric ion reducing/antioxidant power assay as a colorimetric method based on the formerly described protocol.[19] According to this assay, conversion of colorless and oxidized Fe^{3+} ions of Fe^{3+}-2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) substrate into blue-colored and reduced Fe^{2+}-tri-pyridyl triazine (TPTZ) in acidic pH (pH 3.60; produced by acetate buffer) is measured by a spectrophotometer (Boitek, Winooski, USA) at 593 nm.

Malondialdehyde level in the liver tissue. To assess the level of lipid peroxidation, the content of MDA in liver samples was detected according to thiobarbituric acid reaction as described by previous methods.[20] Each sample MDA content was expressed as nmol *per* mg of protein.

Nitric oxide (NO) measurement. To assess the nitrosative stress, the content of the NO was assessed in hepatic homogenates by the Griess reaction.[21] In this reaction, NO was converted to nitrite, which is a more stable compound than NO, and then in an acidic environment (created by orthophosphoric acid) converted to HNO_2, which reacts with sulfanilamide to form the diazonium salt. Finally, the Azo dye resulted from the reaction of the diazonium salt with N-(1-naphthyl) ethylenediamine 2HCl was detected at 540 wavelengths. The NO content was expressed as nmol per mg of protein in samples.

Total thiol molecules (TTM) assessment. The concentration of TTM was evaluated according to the previous methods.[22] Briefly, supernatant from homogenized liver tissue was reacted with 5,5′ - dithiobis 2-nitrobenzoic acid (10.00 mM in pure methanol) and incubated at room temperature (25.00 ˚C) for 15 min. Finally, the absorbance of the supernatants was measured at 412 nm. The TTM contents of samples were expressed as nmol per mg of protein.

Hepatic cytochrome P450 2E1 (CYP 2E1) activity measurement. The activity of CYP 2E1 isoenzyme was evaluated based on the hydroxylation of 4-nitrophenol to 4-nitrocatechol according to previous methods.[23] Concisely, the reaction mixtures contained 0.10 mM 4-NP, 1.00 mM NADPH, 0.10 M potassium phosphate buffer (GoldBio, St. Louis, USA), 1.00 mM ascorbic acid, pH 7.00, and hepatic homogenate (1.00 mg mL^{-1} final concentration), in a final volume of 1.00 mL. The reaction was started by adding NADPH at 37.00 ˚C and terminated by adding 0.20 mL of 1.50 M perchloric acid after 45 min of incubation at 37.00 ˚C. To determine the produced 4-nitrocatechol level, 0.10 mL sodium hydroxide 10.00 N was added to the supernatants and its absorbance was read at 510 nm wavelength. The activity was expressed as nmol per min per mg of protein.

Protein content evaluation. Total protein content of the samples was measured according to the Lowry *et al.* method.[24]

Histopathological examination. Formalin-fixed liver tissue sections were dehydrated by a standard graded series of ethanol and then embedded in paraffin and divided into 5.00 µm sections. These sections were then stained using the Hematoxylin and Eosin method after that evaluated using a light microscope (Nikon, Tokyo, Japan).

Statistical analyses. Statistical differences between the test groups were determined by One-way ANOVA followed by Tukey's post hoc test through GraphPad Prism software (version 9.0; GraphPad Software Inc., San Diego, USA). A $p < 0.05$ was considered as statistically significant. All data are presented as mean ± standard deviation.

Results

Body weight (BW) and liver index of rats. As represented in Table 1, any changes of BW and liver index (liver weight *per* BW) were evaluated at the end of the study. There were no differences in the average body and liver weight of rats among the study groups.

Royal jelly and QCN reversed the DBP-induced alterations in hepatic functional enzymes and lipid profile. As shown in Table 2, compared to control group, DBP exposure caused a significant increase in serum level of ALT and AST ($p<0.05$). The RJ-DBP and QCN-DBP treated animals, significantly reduced the serum levels of ALT and AST when compared with DBP group ($p < 0.05$). As represented in Table 3, compared to control group, animals treated with DBP demonstrated a significant increase in serum levels of cholesterol, TG and LDL, along with a significant decrease in serum levels of HDL ($p < 0.05$). The serum levels of cholesterol and TG were significantly reduced in RJ-DBP and QCN-DBP groups when compared to DBP group. In addition, serum levels of LDL remarkably diminished at medium dose (200 mg kg^{-1}) of RJ-DBP group ($p < 0.05$). There was no significant difference in serum HDL levels between DBP group and the groups receiving the combination of DBP with RJ or QCN ($p > 0.05$).

Royal jelly and QCN reversed DBP-induced changes on LDH level. As shown in Figure 1, compared to control group, DBP exposure elevated serum level of LDH ($p<0.05$). Compared to DBP group, medium (200 mg kg^{-1}) and high (300 mg kg^{-1}) doses of RJ-DBP groups and QCN-DBP group demonstrated a significant decrease in serum LDH level ($p < 0.05$).

Royal jelly and QCN reduced the DBP-elevated insulin resistance index. As represented in Table 4, compared to control group, DBP exposure resulted in higher concentrations of blood glucose and insulin and consequently a higher insulin resistance index ($p < 0.05$). Compared to DBP group, RJ-DBP and QCN-DBP treated animals remarkably lowered the DBP-elevated insulin resistance index values ($p < 0.05$). The RJ-DBP group at medium and high dose levels and QCN-DBP group demonstrated a significant decrease in glucose and insulin content ($p < 0.05$).

Royal jelly reversed DBP-induced alterations in TAC. As shown in Figure 2, compared control group, DBP exposure reduced TAC remarkably in DBP group ($p < 0.05$). The serum level of TAC in medium and high doses of RJ-DBP groups significantly increased, when compared with DBP group ($p < 0.05$).

Table 1. Effect of royal jelly on bodyweight, liver weight, and liver index alterations in the control and treatment groups.

Parameters	Control	DBP	RJ	QCN	RJ 100+DBP	RJ 200+DBP	RJ 300+DBP	QCN+DBP
Body weight (g)	217.38 ± 21.12	215.87 ± 9.55	247.94 ± 24.93	217.25 ± 18.24	211.18 ± 21.55	217.63 ± 14.50	211.67 ± 13.12	198.74 ± 42.82
Liver weight (g)	7.82 ± 0.95	8.39 ± 0.49	9.49 ± 1.16	8.36 ± 0.46	7.86 ± 1.01	8.13 ± 0.35	8.14± 0.94	7.27 ± 1.27
Liver index	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00

DBP: Dibutyl phthalate; RJ: Royal jelly; QCN: Quercetin

No significant differences were found among the groups ($p > 0.05$).

Table 2. Effect of royal jelly and quercetin on dibutyl phthalate-induced alterations in liver enzyme.

Parameters	Control	DBP	RJ	QCN	RJ 100+DBP	RJ 200+DBP	RJ 300+DBP	QCN+DBP
ALT (U L^{-1})	78.50 ± 14.85	105.50 ± 7.78*	52.50 ± 0.71	65.00 ± 8.49	95.00 ± 7.07	69.00 ± 5.66#	55.00 ± 7.07#	59.00 ± 2.83#
AST (U L^{-1})	142.00 ± 4.24	192.00 ± 9.90*	135.00 ± 4.24	102.00 ± 16.97	157.00 ± 1.41#	127.50 ± 6.36#	131.00 ± 15.56#	133.50 ± 9.19#

DBP: Dibutyl phthalate; RJ: Royal jelly; QCN: Quercetin

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

* indicates significant differences between the control and DBP-treated animals ($p < 0.05$); # represents significant difference between the DBP-treated animals and groups received combination of DBP with RJ or QCN ($p < 0.05$).

Table 3. Effect of royal jelly and quercetin on dibutyl phthalate induced alterations in lipid profile.

Parameters	Control	DBP	RJ	QCN	RJ 100+DBP	RJ 200+DBP	RJ300+DBP	QCN+DBP
Cholesterol (mg dL^{-1})	52.00 ± 4.00	82.50 ± 1.50*	78.50 ± 1.50	53.00 ± 10.00	71.50 ± 6.50#	71.50 ± 1.50#	73.50 ± 5.50#	52.00 ± 8.00#
TG (mg dL^{-1})	33.50 ± 2.12	143.00 ± 19.80*	57.50 ± 9.19	42.00 ± 11.31	50.50 ± 6.36#	36.50 ± 0.71#	64.00 ± 12.73#	39.00 ± 12.73#
HDL (mgdL^{-1})	51.50 ± 0.71	41.50 ± 6.36*	50.00 ± 4.24	46.50 ± 4.95	31.00 ± 7.07	45.00 ± 14.14	47.00 ± 7.07	30.00 ± 11.31
LDL (mg dL^{-1})	6.00 ± 0.00	9.00 ± 1.41*	7.00 ± 1.41	5.00 ± 1.41	8.50 ± 2.12	5.00 ± 1.41#	8.50 ± 2.12	7.00 ± 0.00

DBP: Dibutyl phthalate; RJ: Royal jelly; QCN: Quercetin

TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

* indicates significant differences between the control and DBP-treated animals ($p < 0.05$); # represents significant difference between DBP-treated animals and groups received combination of DBP with RJ or QCN ($p < 0.05$).

Table 4. Effects of royal jelly and quercetin on serum levels of glucose, Insulin.

Parameters	Control	DBP	RJ	QCN	RJ 100+DBP	RJ 200+DBP	RJ 300+DBP	QCN+DBP
Glucose (mg dL^{-1})	124.50 ± 20.50	229.50 ± 26.50*	161.00 ± 11.00*	148.00 ± 3.00	191.50 ± 15.50	153.00 ± 6.00#	180.00 ± 28.00#	176.50 ± 5.50#
Insulin (μlU mL^{-1})	5.45 ± 0.35	8.50 ± 1.50*	5.50 ± 0.50	4.45 ± 0.45	6.55 ± 0.45	4.40 ± 0.30#	4.50 ± 0.50#	4.25 ± 0.75#
HOMA-IR index	1.66 ± 0.24	4.72 ± 0.42*	2.20 ± 0.49	1.63 ± 0.28	3.11 ± 0.66#	1.67 ± 0.25#	1.97 ± 0.13#	1.86 ± 0.54#

DBP: Dibutyl phthalate; RJ: Royal jelly; QCN: Quercetin

HOMA-IR: Homeostasis model assessment - insulin resistance

* indicates significant differences between the control and DBP-treated animals ($p < 0.05$); # represents significant difference between DBP-treated animals and groups received combination of DBP with RJ or QCN ($p < 0.05$).

Royal jelly and QCN reversed DBP-elevated oxidative/nitrosative biomarker in liver tissue. The content of MDA as an important and common biomarker of lipid peroxidation and the concentration of NO as an indicator of nitrosative stress were measured in liver samples. As shown in Figure 3, comparing with control, DBP exposure elevated the level of hepatic MDA remarkably in DBP group ($p < 0.05$). Comparing with DBP group, the levels of hepatic MDA in RJ-DBP and QCN-DBP groups were significantly decreased ($p < 0.05$). The NO content of liver in the DBP-exposed animals was significantly elevated when compared with control group ($p < 0.05$). The RJ-DBP groups at medium and high doses and QCN-DBP group showed a significant reduction of NO content in the liver (Fig. 4).

At the same time TTM concentration as an index of glutathione resources were evaluated in liver tissues. The concentration of TTM was significantly ($p < 0.05$) reduced in the DBP-received animals, while DBP-RJ groups at medium dose significantly prevented thiol depletion (Fig. 5).

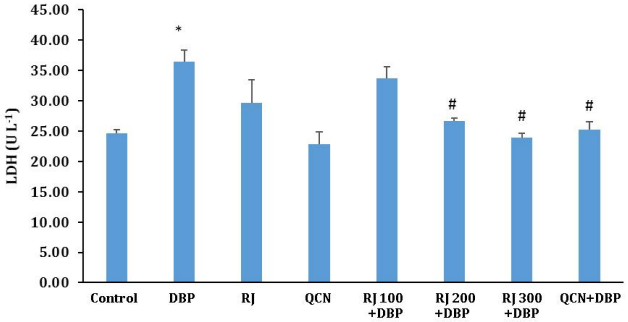

Fig. 1. Effect of royal jelly (RJ) and quercetin (QCN) on dibutyl phthalate (DBP)-induced alterations on lactate dehydrogenase (LDH) serum level.
* indicates significant differences ($p < 0.05$) between the control and DBP-treated animals; # represents remarkable difference between DBP-treated animals and groups received combination of DBP with RJ or QCN ($p < 0.05$).

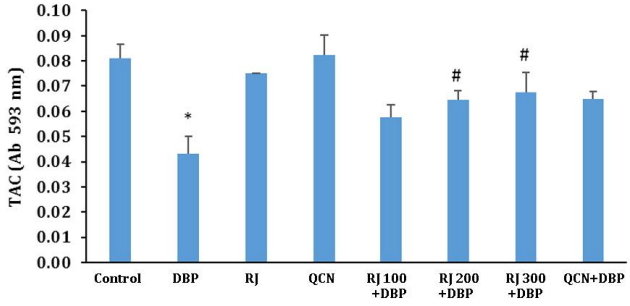

Fig. 2. Effects of royal jelly (RJ) and quercetin (QCN) on dibutyl phthalate (DBP)-reduced alterations on serum total antioxidant capacity (TAC).
* indicates significant differences ($p < 0.05$) between the control and DBP-treated animals; # represents remarkable difference between DBP-treated animals and groups received combination of DBP with RJ or QCN ($p < 0.05$).

Royal jelly and QCN restored the DBP-induced hepatic CYP 2E1 activity. As shown in Figure 6, comparing with control group, DBP exposure significantly elevated the CYP 2E1 activity in DBP group ($p < 0.05$). Comparing with DBP group, CYP 2E1 activity in RJ-DBP groups and QCN-DBP groups significantly decreased ($p < 0.05$).

Histopathological findings. Liver tissue sections from the control group had normal structures with normal hepatocytes. In contrast, the sections from the DBP-exposed group showed abnormal structure, including necrosis of hepatocytes and scatter bleeding. In the group of animals that were treated just with RJ or quercetin, there were no particular pathological alterations. Among the groups receiving the combination of RJ and DBP, the medium dose of RJ showed better results compared to both the low and high doses. Additionally, in the histopathological examinations of the low and high doses of RJ, certain pathological changes, such as necrosis and bleeding, were shown. In groups that received the combination of QCN and DBP, there were also no particular pathological alterations (Fig. 7).

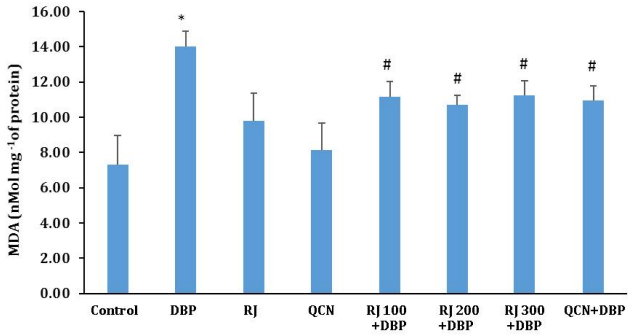

Fig. 3. Effect of royal jelly (RJ) and quercetin (QCN) on dibutyl phthalate (DBP)-induced malondialdehyde (MDA) level in liver.
* indicates significant differences ($p < 0.05$) between the control and DBP-treated animals; # represents remarkable difference between DBP-treated animals and groups received combination of DBP with RJ or QCN ($p < 0.05$).

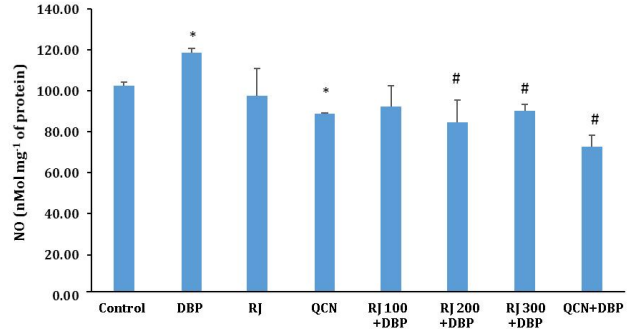

Fig. 4. Effect of royal jelly (RJ) and quercetin (QCN) on dibutyl phthalate (DBP)-induced nitric oxide (NO) level in liver.
* indicates significant differences ($p < 0.05$) between the control and DBP-treated animals; # represents remarkable difference between DBP-treated animals and groups received combination of DBP with RJ or QCN ($p < 0.05$).

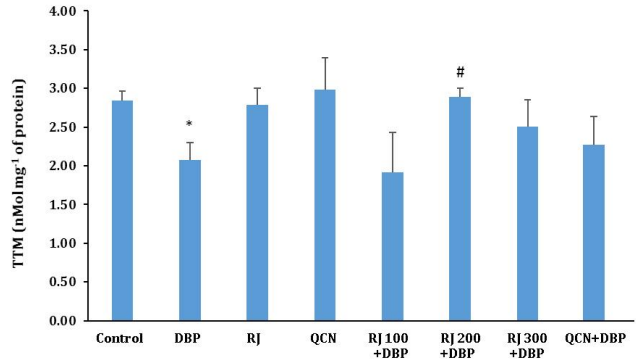

Fig. 5. Effect of royal jelly (RJ) and quercetin (QCN) on dibutyl phthalate (DBP)-reduced total thiol molecules (TTM) level in liver.
* indicates significant differences ($p < 0.05$) between the control and DBP-treated animals; # represents remarkable difference between DBP-treated animals and groups received combination of DBP with RJ or QCN ($p < 0.05$).

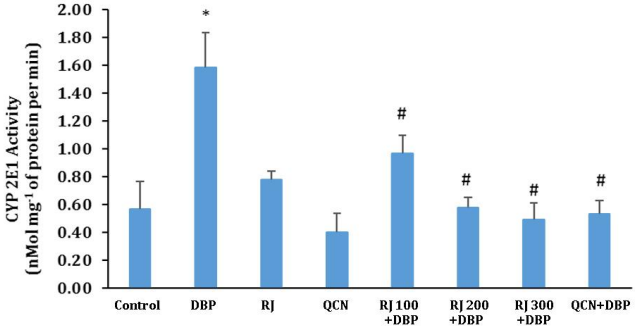

Fig. 6. Effect of royal jelly (RJ) and quercetin (QCN) on dibutyl phthalate (DBP)-induced hepatic cytochrome P450 2E1 (CYP 2E1) activity.
* indicates significant differences ($p < 0.05$) between the control and DBP-treated animals; # represents remarkable difference between DBP-treated animals and groups received combination of DBP with RJ or QCN ($p < 0.05$).

Discussion

Results of the current study revealed that DBP-induced hepatotoxicity characterized by functional and structural changes including imbalanced oxidative status and inflammation. Moreover, a marked CYP 2E1 induction following DBP exposure was also shown. Histopathological alterations due to DBP exposure confirmed the DBP-induced hepatotoxicity. On the other hand, administration of RJ as test compound and also QCN as a reference agent could ameliorate the DBP-induced hepatic injuries, both functionally and structurally.

Data obtained in this study demonstrated the induction of oxidative and nitrosative stress in liver and serum by DBP, based on considerable alteration in nitrosative and oxidative stress biomarkers, which was in accordance with previous research results. Zhang *et al*. argued that DBP exposure dramatically increase MDA content and decrease superoxide dismutase level in liver following the exposure of rats to 5.00 mg kg^{-1} *per* day DBP for 6 weeks.[25]

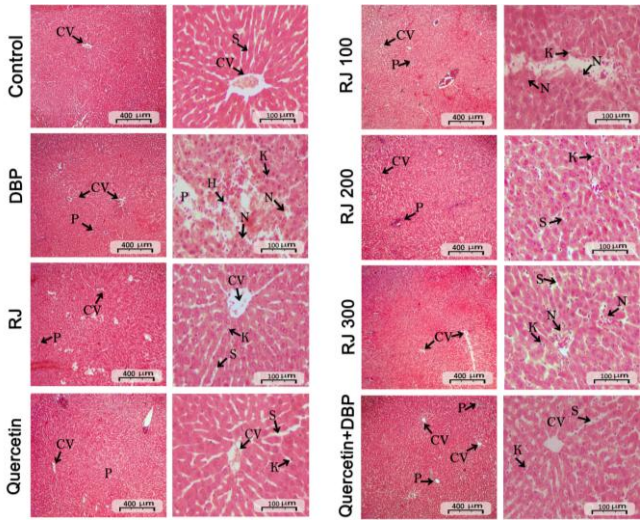

Fig. 7. Photomicrographs from the liver using Hematoxylin and Eosin staining. CV: Central vein, S: Sinusoid, N: Necrosis, H: Hemorrhage K: Kupffer cell, P: Portal region.

Moreover, current data on DBP-induced oxidative and nitrosative stress is similar to those reported by Ore *et al*.[8] and Shen *et al*.[26] There are increasing data indicating that DBP could induce oxidative and nitrosative stress injuries. On the other hand, the alterations of hepatic MDA, NO, TTM and serum TAC level were reversed in concurrently received DBP and RJ groups, indicating that RJ could protect from oxidative and nitrosative damages induced by DBP. Previous studies demonstrated that RJ could mitigate oxidative and nitrosative stress, as it has been reported that RJ reduced the taxol-induced NO and MDA levels and enhanced the taxol-reduced TTM level in the testis of rats.[27] Tohamy *et al*. also showed that RJ could reduce oxidative stress induced by hydroxyurea in liver.[28]

Another finding of this study demonstrates a remarkable elevation in plasma levels of insulin and glucose as well as insulin resistance in rats exposed to DBP. This finding is supported by previous reports of Ore *et al*.,[8] and Mondal and Mukherjee.[29] In the Ore *et al.* study exposure of BALB/c mice against diisononyl phthalate at 20.00 and 200 mg kg^{-1} BW dose levels resulted in remarkable oxidative stress and a significant increase in insulin resistance.[8] On the other hand, alterations in insulin, glucose, and insulin resistance index were reversed in DBP-combined RJ groups, demonstrating that RJ could reduce insulin resistance induced by DBP. The reducing effect of RJ on glucose, insulin and insulin resistance index is in accordance with the previous reports of Zamami *et al*.[14] and Metwally Ibrahim *et al*.[16] Although the precise mechanism by which phthalate induces insulin resistance has not been elucidated, previous studies have explained the involvement of oxidative stress in insulin resistance and inflammation.[8] Previous studies have identified the molecular pathway through which oxidative stress leads to insulin resistance.[30] Activated nitrogen and

oxygen molecules, by activating the serine-threonine kinase pathway, including c-Jun N-terminal kinase and nuclear factor-κB prevent the phosphorylation of the tyrosine amino acid of IRS and thus the progression of the IRS-dependent insulin pathway.[30] Thus, it is probable that RJ improve insulin resistance through its antioxidant effect. Investigation of lipid profile in rats exposed to DBP showed increased levels of cholesterol, TG, and LDL, as well as decreased levels of HDL. These findings were similar to those reported by Ore *et al*.[8] and Zhang *et al*.[25] showed that DBP leads to hyperlipidemia by stimulating PPARα receptors. Increasing PPARα activity leads in elevated expression of sterol regulatory element binding proteins, fatty acid synthase, and glycerol-3-phosphate acyltransferase proteins and reduced expression of phosphorylated AMP-activated protein kinase protein in liver, which ultimately causes hyperlipidemia.[25] Elevated influx of fatty acids into the liver results in the incomplete β-oxidation of fatty acids, leading to the generation of free oxygen radicals. These radicals induce lipid peroxidation of cell membranes and damage hepatocytes, as indicated in this study by elevated serum levels of liver enzymes.[9] In this study, the increased serum levels of LDH, ALT, and AST showed that DBP exposure caused hepatotoxicity in rats. Interestingly, our results showed that the groups of animals, which received combination of RJ and DBP, improved lipid profile and reduced the level of liver enzyme. The findings are supported by the published studies, which argued that RJ administration reduced the serum level of liver enzyme and improved the lipid profile in ovariectomized rats.[31] It has been also reported that RJ improved lipid profile in aged and obese rat that fed with high fat diet.[16] Royal jelly has been demonstrated to have estrogenic properties in both *in vivo* and *in vitro* studies.[32,33] It is well known that estrogen lowers the serum cholesterol levels.[34,35] Another study showed that the major RJ protein 1 interacts with bile acids in the intestine, preventing their absorption. As a result, the excretion of bile acid and cholesterol is increased. Additionally, major RJ protein 1 enhances cholesterol catabolism by stimulating the mRNA and protein expression of cholesterol 7α-hydroxylase in liver.[36] Previous studies indicated that the toxicity of phthalate may be related to the disruption of cytochrome P450 homeostasis.[37] Shi *et al.* indicated that Di-(2-ethylhexyl)-phthalate exposure enhanced constitutive androstane receptor and their downstream gene level, including CYP 2E1, in lung tissue and as result induced lung injury.[38] It is well documented that increasing CYP 2E1 activity, lead to increasing generation of reactive oxygen species and oxidative stress.[39] Consistent with the research mentioned, our findings indicated that DBP exposure induces CYP 2E1 activity in liver tissue. Interestingly RJ declined CYP 2E1 activity in groups received combination of RJ and DBP, suggesting that the hepatoprotective effects of RJ may partly related to its capability in the reduction of oxidative stress and inhibition of CYP 2E1 activity.

Histopathological analysis in the present research showed that DBP-induced hypertrophy and necrosis of hepatocyte and inflammatory cells infiltration, and scatter bleeding were obvious, these results are in consistent with another researcher.[8,25] However, RJ uptake significantly alleviated the histopathologic and structural changes by DBP-induced.

In this study, QCN was determined as a positive control due to its anti-inflammatory, antioxidant, anti-diabetic,[40] and hepatoprotective properties in liver steatosis, fatty hepatitis, liver fibrosis, and liver cancer.[41] Previous studies showed that QCN was able to ameliorate the phthalates-induced hepatotoxicity, which were characterized with increased serum concentration of ALT, AST, LDH, and hepatic levels of MDA in phthalates-received rats.[42] Our study results were also consistent with previous studies.

Our data suggest that RJ with anti-inflammatory and antioxidant effect could be novel compound to reduce the phthalate induced hepatotoxicity and in particular phthalate related insulin resistance.

Conflict of interest

The authors confirm that they have no conflicts of interest related to the work described in this manuscript.

References

1. Hannon PR, Flaws JA. The effects of phthalates on the ovary. Front Endocrinol (Lausanne) 2015; 6: 8. doi: 10.3389/fendo.2015.00008.
2. Radha MJ, Basha PM. Hepatotoxic evaluation of Di-n-butyl phthalate in Wistar rats upon sub-chronic exposure: a multigenerational assessment. Toxicol Rep 2020; 7: 772-778.
3. Chen X, Xu S, Tan T, et al. Toxicity and estrogenic endocrine disrupting activity of phthalates and their mixtures. Int J Environ Res Public Health 2014; 11(3): 3156-3168.
4. Gao H, Cheng Q-Y, Zhang C, et al. Association between phthalate exposure and insulin resistance: a review. Appl Ecol Environ Res 2022; 20(2): 1189-1197.
5. James-Todd T, Stahlhut R, Meeker JD, et al. Urinary phthalate metabolite concentrations and diabetes among women in the National Health and Nutrition Examination Survey (NHANES) 2001-2008. Environ Health Perspect 2012; 120(9): 1307-1313.
6. Sun Q, Cornelis MC, Townsend MK, et al. Association of urinary concentrations of bisphenol A and phthalate metabolites with risk of type 2 diabetes: a prospective investigation in the Nurses' Health Study (NHS) and NHSII cohorts. Environ Health Perspect 2014; 122(6): 616-623.

7. Stahlhut RW, van Wijngaarden E, Dye TD, et al. Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult US males. Environ Health Perspect 2007; 115(6): 876-882.
8. Ore A, Adewale AA, Kehinde SA, et al. Potential roles of oxidative stress and insulin resistance in diisononyl phthalate induced dyslipidemia and hepatosteatosis in BALB/c mice. Adv Redox Res 2022; 5: 100038. doi: 10.1016/j.arres.2022.100038.
9. Khan RS, Bril F, Cusi K, et al. Modulation of insulin resistance in nonalcoholic fatty liver disease. Hepatology 2019; 70(2): 711-724.
10. Zhang W, Shen X, Zhang WW, et al. Di-(2-ethylhexyl) phthalate could disrupt the insulin signaling pathway in liver of SD rats and L02 cells via PPARγ. Toxicol Appl Pharmacol 2017; 316: 17-26.
11. Yang R, Zheng J, Qin J, et al. Dibutyl phthalate affects insulin synthesis and secretion by regulating the mitochondrial apoptotic pathway and oxidative stress in rat insulinoma cells. Ecotoxicol Environ Saf 2023; 249: 114396. doi: 10.1016/j.ecoenv.2022.114396.
12. Bugianesi E, Moscatiello S, Ciaravella MF, et al. Insulin resistance in nonalcoholic fatty liver disease. Curr Pharm Des 2010; 16(17): 1941-1951.
13. Ahmad S, Campos MG, Fratini F, et al. New insights into the biological and pharmaceutical properties of royal jelly. Int J Mol Sci 2020; 21(2): 382. doi: 10.3390/ijms21020382.
14. Zamami Y, Takatori S, Goda M, et al. Royal jelly ameliorates insulin resistance in fructose-drinking rats. Biol Pharm Bull 2008; 31(11): 2103-2107.
15. Yoshida M, Hayashi K, Watadani R, et al. Royal jelly improves hyperglycemia in obese/diabetic KK-Ay mice. J Vet Med Sci 2017; 79(2): 299-307.
16. Metwally Ibrahim SEL, Kosba AA. Royal jelly supplementation reduces skeletal muscle lipotoxicity and insulin resistance in aged obese rats. Pathophysiology 2018; 25(4): 307-315.
17. Abdelsalam HM, Diab AA, Hussien AG, et al. Ginseng and royal jelly diminish diabetic hepato-and nephron-toxicity in STZ-induced diabetic male rats. Nutrire 2023; 48: 8. doi: 10.1186/s41110-023-00191-1.
18. Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28(7): 412-419.
19. Benzie IF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol 1999; 299: 15-27.
20. Niehaus WG Jr, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem 1968; 6(1): 126-130.
21. Green LC, Wagner DA, Glogowski J, et al. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem 1982; 126(1): 131-138.
22. Hu ML. Measurement of protein thiol groups and glutathione in plasma. Methods Enzymol 1994; 233: 380-385.
23. Koop DR. Hydroxylation of p-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isozyme 3a. Mol Pharmacol 1986; 29(4): 399-404.
24. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. J biol Chem 1951; 193(1): 265-275.
25. Zhang W, Li JY, Wei XC, et al. Effects of dibutyl phthalate on lipid metabolism in liver and hepatocytes based on PPARα/SREBP-1c/FAS/GPAT/AMPK signal pathway. Food Chem Toxicol 2021; 149: 112029. doi: 10.1016/j.fct.2021.112029.
26. Shen C, Wei J, Wang T, et al. Acute toxicity and responses of antioxidant systems to dibutyl phthalate in neonate and adult *Daphnia magna*. PeerJ 2019; 7: e6584. doi: 10.7717/peerj.6584.
27. Delkhoshe-Kasmaie F, Malekinejad H, Khoramjouy M, et al. Royal jelly protects from taxol-induced testicular damages via improvement of antioxidant status and up-regulation of E2f1. Syst Biol Reprod Med 2014; 60(2): 80-88.
28. Tohamy HG, El-Neweshy MS, Soliman MM, et al. Protective potential of royal jelly against hydroxyurea-induced hepatic injury in rats via antioxidant, anti-inflammatory, and anti-apoptosis properties. PLoS One 2022; 17(3): e0265261. doi: 10.1371/journal.pone.0265261.
29. Mondal S, Mukherjee S. Long-term dietary administration of diethyl phthalate triggers loss of insulin sensitivity in two key insulin target tissues of mice. Hum Exp Toxicol 2020; 39(7): 984-993.
30. Evans JL, Maddux BA, Goldfine ID. The molecular basis for oxidative stress-induced insulin resistance. Antioxid Redox Signal 2005; 7(7-8): 1040-1052.
31. You MM, Liu YC, Chen YF, et al. Royal jelly attenuates nonalcoholic fatty liver disease by inhibiting oxidative stress and regulating the expression of circadian genes in ovariectomized rats. J Food Biochem 2020; 44(3): e13138. doi: 10.1111/jfbc.13138.
32. Moutsatsou P, Papoutsi Z, Kassi E, et al. Fatty acids derived from royal jelly are modulators of estrogen receptor functions. PLoS One 2010; 5(12): e15594. doi: 10.1371/journal.pone.0015594.
33. Mishima S, Suzuki KM, Isohama Y, et al. Royal jelly has estrogenic effects *in vitro* and *in vivo*. J Ethnopharmacol 2005; 101(1-3): 215-220.
34. Nuttall ME, Bradbeer JN, Stroup GB, et al. Idoxifene: a novel selective estrogen receptor modulator prevents

bone loss and lowers cholesterol levels in ovariectomized rats and decreases uterine weight in intact rats. Endocrinology 1998; 139(12): 5224-5234.
35. Ke HZ, Chen HK, Simmons HA, et al. Comparative effects of droloxifene, tamoxifen, and estrogen on bone, serum cholesterol, and uterine histology in the ovariectomized rat model. Bone 1997; 20(1): 31-39.
36. Kashima Y, Kanematsu S, Asai S, et al. Identification of a novel hypocholesterolemic protein, major royal jelly protein 1, derived from royal jelly. PLoS One 2014; 9(8): e105073. doi: 10.1371/journal.pone.0105073.
37. Wang S, Cao Y, Wang S, et al. DEHP induces immunosuppression through disturbing inflammatory factors and CYPs system homeostasis in common carp neutrophils. Fish Shellfish Immunol 2020; 96: 26-31.
38. Shi YS, Zhao Y, Li XN, et al. Xenobiotic-sensing nuclear receptors as targets for phthalates-induced lung injury and antagonism of lycopene. Chemosphere 2023; 312(Pt 1): 137265. doi: 10.1016/j.chemosphere. 2022.137265.
39. Leung TM, Nieto N. CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. J Hepatol 2013; 58(2): 395-398.
40. Kawabata K, Mukai R, Ishisaka A. Quercetin and related polyphenols: new insights and implications for their bioactivity and bioavailability. Food Funct 2015; 6(5): 1399-1417.
41. Zhao X, Wang J, Deng Y, et al. Quercetin as a protective agent for liver diseases: a comprehensive descriptive review of the molecular mechanism. Phytother Res 2021; 35(9): 4727-4747.
42. Xia LZ, Jiang MZ, Liu LL, et al. Protective effect of quercetin against phthalates induced hepatotoxicity in rats. Toxicol Res (Camb) 2022; 11(5): 863-871.