ORIGINAL ARTICLE

Veterinary Research Forum. 2026; 17 (10): 775 - 784
doi: 10.30466/vrf.2026.2082953.5048
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Protective effects of royal jelly on dibutyl phthalate-induced oxidative and inflammatory ovarian damage in female Wistar rats

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Article Info

Article history:

Received: 04 January 2026
Accepted: 31 August 2026
Available online: 15 October 2026

Keywords:

Dehydroepiandrosterone
Dibutyl phthalate
Ovarian toxicity
Oxidative stress
Royal jelly

Abstract

Phthalates are endocrine-disrupting chemicals widely used in numerous consumer products, making human and animal exposure a significant public health concern. This study evaluated the protective effects of royal jelly (RJ) against dibutyl phthalate (DBP)-induced ovarian damage. Forty female Wistar albino rats were randomly allocated into eight groups (n = 5 *per* group): Control (corn oil), DBP (500 mg kg^{-1}), RJ (200 mg kg^{-1}), Quercetin (QCN; 50.00 mg kg^{-1}), DBP + RJ (100, 200 and 300 mg kg^{-1}) and DBP + QCN (50.00 mg kg^{-1}) for 28 consecutive days by gavage. QCN was used as a positive antioxidant control. The RJ was dissolved in normal saline, whereas, QCN and DBP were dissolved in corn oil. The levels of testosterone, dehydroepiandrosterone and tumor necrosis factor alpha in the serum were measured. Oxidative and nitrosative stress biomarkers (malondialdehyde, nitric oxide, total thiol molecules) were assessed in the ovarian tissue. The histopathological alterations of ovarian tissue and ovarian index were also evaluated. The RJ significantly reduced DBP-induced elevations in serum testosterone and dehydroepiandrosterone levels. Compared to the DBP group, RJ treatment significantly restored the ovarian index and reduced serum tumor necrosis factor alpha level. The RJ treatment significantly attenuated DBP-induced oxidative and nitrosative stress in ovarian tissue. The DBP disrupted normal ovarian architecture, characterized by increased atretic and cystic follicles and reduced corpora lutea formation. These alterations were markedly ameliorated by RJ. In conclusion, RJ attenuated DBP-induced ovarian toxicity as evidenced by reduced oxidative/ nitrosative stress, decreased serum tumor necrosis factor alpha and lowered serum testosterone as well as dehydroepiandrosterone levels.

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Introduction

Phthalates are recognized as reproductive toxicants and endocrine disruptors that interfere with normal reproductive functioning and hormonal signaling. Consequently, exposure to phthalates has become a significant public health concern.[1] Dibutyl phthalate (DBP), an important member of phthalates, is widely utilized as a plasticizer in medical devices, cosmetics and various industries. It creates a significant risk to human health as it readily migrates from plastic materials into the environment where individuals are exposed via food, drink, personal care products or directly through dermal absorption or inhalation.[2] Phthalates induce damage to various organs, including the liver, immune system, reproductive, respiratory and nervous systems.[3] Previous studies have shown that the reproductive system is more susceptible to phthalate toxicity compared to other organs.[4] The effects of phthalates on the male reproductive system have been investigated more than the female reproductive system, although women are exposed to phthalates more than men due to the widespread use of cosmetic and health products.

Phthalates affect both folliculogenesis and steroidogenesis, which are two important processes in the ovary.[1] Phthalates inhibit oocyte maturation and development, reduce the number of primordial follicles, accelerate primordial follicle recruitment, cause atresia in mature follicles and suppress ovulation.[1] By depleting or accelerating the development of primordial follicles, they

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will cause irreversible infertility as the primordial follicle pool is formed at birth and is a non-renewable asset.[5] The mechanisms by which phthalates disrupt folliculogenesis processes include oxidative stress, increased proapoptotic factors, decreased antiapoptotic factors and cell cycle disruption.[1] Wei Wang *et al*., indicated that di (2-ethylhexyl) phthalate inhibits the growth of ovarian antral follicles by increasing the levels of reactive oxygen species and reducing the expression and activity of Cu/Zn superoxide dismutase.[6] Previous studies indicated that phthalate exposure inhibits the production of estradiol, which in turn prevents the luteinizing hormone surge required for ovulation. Studies using cultured ovarian granulosa cells demonstrated that this action is due to the inhibition of the aromatase enzyme activity. This enzyme is responsible for converting testosterone to estradiol, which contributes to a hyperandrogenic state and to the development of polycystic ovaries in women.[7]

Polycystic ovary syndrome (PCOS), which is the most prevalent endocrine disorder, affects 8.00 – 13.00% of women of reproductive age and is determined by polycystic ovaries, elevated androgen levels, insulin resistance and chronic oligo-anovulation.[8] Hyper-androgenism is the most important clinical manifestation of PCOS.[8] According to the Rotterdam criteria, a diagnosis of PCOS can be made if two out of three of the following criteria are positive: An irregular or absent ovulation, the evidence of hyperandrogenism that can be either clinical or biochemical and the presence of polycystic ovarian structure observed through ultrasound examination.[8]

Royal jelly (RJ) is a yellowish-white acidic substance with a slightly pungent odor and taste, which is secreted from worker honeybee hypopharyngeal and mandibular glands. It is the main source of nutrition for the queen bee and larvae during their initial three days of life.[9] The major components of RJ are water (60.00 - 70.00%), proteins (9.00 - 18.00%), sugars (7.00 - 18.00%) and lipids (3.00 - 8.00%). The RJ also contains some minor components such as enzymes, hormones, minerals, amino acids, vitamins, nucleotides, polyphenols and minor heterocyclic compounds.[10] Royal jelly exhibits various pharmacological properties, including antioxidant, anti-inflammatory, estrogenic, and anti-androgenic effects. It can alleviate menopausal symptoms, enhance fertility and improve reproductive performance.[11] Previous studies showed that the use of RJ in a PCOS model led to improved levels of reproductive hormones, including decreased testosterone and increased follicle-stimulating hormone levels, and improved estrous cycles along with improved ovarian histopathology such as lowered number of cystic follicles and the presence of various stages of follicular development as well as corpora lutea.[11] Furthermore, a previous study indicated that RJ reduced oxidative stress in ovarian tissue by decreasing malondialdehyde (MDA) levels and enhancing total antioxidant capacity.[11]

Quercetin (QCN) is a polyphenolic flavonoid compound which is found in various plants and fruits including onion, grapes, cherries, apples, mangoes, citrus fruits, buckwheat, plums, tomatoes and tea.[12] The QCN has numerous pharmacological properties, including antioxidant, anti-inflammatory, anti-androgenic, and estrogenic-like effects.[12] Recent research indicated that QCN can promote the development of healthy ovarian follicles, restore healthy ovarian anatomy and improve uterus histology.[13] It has been shown that QCN prevents the rise in oxygen free radicals, MDA and glutathione depletion induced by diethylhexyl phthalate in the kidney.[14] Quercetin prevented the increase in lipid peroxidation and the decrease in superoxide dismutase, catalase and glutathione induced by diethylhexyl phthalate in testicular tissue.[15] In our previous study, QCN prevented the increased serum lactate dehydrogenase levels induced by DBP.[16] Previous studies have suggested that one mechanism by which QCN inhibits oxidative stress and inflammation is the inhibition of the nuclear factor kappa B pathway.[14]

So far, there has been no research examining the protective role of RJ against ovarian toxicity caused by phthalates. Therefore, we aimed to assess the impacts of RJ and QCN on oxidative/antioxidant balance, inflammation, reproductive hormone levels and ovarian tissue pathology resulting from DBP exposure.

Materials and Methods

Chemicals and assay kits. Chemicals utilized in this research comprised DBP, thiobarbituric acid, potassium chloride, phosphoric acid (85.00%), n-(1-naphthyl) ethylene diamine -2HCl, tris-base, ethylene diamine tetra acetic acid, sulphanilamide, folin reagent, sodium hydroxide, sodium carbonate, potassium sodium tartrate, copper (II) sulfate, nitrous acid were purchased from Merck (Darmstadt, Germany). Royal Jelly was bought from a local beekeeper (Hamedan, Iran). Corn oil was obtained from a local grocery (Ladan Inc., Tehran, Iran). Quercetin (Q4951) and 5,5′-dithiobis-(2-nitrobenzoic acid; D8130) were purchased from Sigma-Aldrich (Sigma-Aldrich., St. Louis, USA). Assay kits including testosterone and dehydroepiandrosterone were purchased from Siemens (Munich, Germany). Hematoxylin and Eosin staining kits were provided by Asia Pajohesh, Tehran, Iran.

Animal grouping and study design. Forty healthy and adult albino Wistar rats (8 weeks old, 200 - 250 g) were purchased from the Animal House, Urmia University of Medical Sciences, Urmia, Iran. All rats were kept in the laboratory for 1 week before the experiment as an adaptation period under standard laboratory conditions, with a 27.00 ± 2.00 ˚C temperature and a 12:12 hr light/dark cycle. Tap water was provided *ad libitum* and animals were fed a standard pellet diet. Then, the rats were randomly divided into eight groups (n = 5 *per* group)

and treated daily for 4 weeks 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 substances were administered orally by gavage. Royal jelly was dissolved in normal saline and both QCN and DBP were dissolved in corn oil. 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. At the end of the experimental period, all animals were anesthetized between 08:00 and 11:00 AM to minimize the effect of diurnal hormonal fluctuations via intraperitoneal injection of ketamine (90.00 mg kg^{-1}; Rotex Medica GmbH, Aachen, Germany) and xylazine (5.00 mg kg^{-1}; Alfasan, Woerden, The Netherlands). Following the induction of deep general anesthesia, blood samples were obtained through direct cardiac puncture and subsequently centrifuged at 1500 *g* for 5 min to isolate the serum. The serum samples were stored at – 20.00 ˚C until further analyses. The ovarian tissues were quickly collected and rinsed with a cold normal saline solution. Then, one portion of the ovarian tissue was separated for routine histological analysis and was kept in 10.00% formalin, while another portion was collected for molecular investigations and was stored at – 80.00 ˚C. The final body weight of each rat and the weight of their ovaries were measured in grams. The ovarian index was calculated as the ratio of the total ovarian weight to the body weight.[17]

Analysis of tumor necrosis factor alpha (TNF-α) in serum. The levels of TNF-α in serum samples were assessed using a Mouse TNF-α DuoSet kit (R&D Systems™, Minneapolis, USA) according to the manufacturer's guidelines.

Hormonal assay. The concentration of testosterone and dehydroepiandrosterone (DHEA) hormones in serum samples was measured using assay kits (Siemens) according to the manufacturer's guidelines.

Evaluation of MDA levels in the ovarian tissues. In ovarian samples, the levels of MDA, an indicator of lipid peroxidation, were measured using the thio-barbituric acid assay according to the previously described method.[18] The MDA content was stated as nmol *per* mg of protein in samples.

Nitric oxide (NO) assay. To evaluate nitrosative stress in the ovary homogenates, the concentration of NO was measured using the Griess reaction.[19] In this reaction, NO is transformed into nitrite, a compound that is more stable than NO. In an acidic environment, produced by phosphoric acid, it is then transformed into nitrous acid, which reacts with sulfanilamide to create the diazonium salt. Ultimately, the azo dye formed from the reaction between the diazonium salt and N-(1-naphthyl) ethylenediamine 2HCl (NED) was detected at a wave-length of 540 nm. The NO content was stated as nmol *per* mg of protein in samples.

Total thiol molecules (TTM) assessment. The content of TTM was determined using previously established method.[20] The ovary homogenized supernatant was reacted with 5,5′ - dithiobis 2-nitrobenzoic acid (10.00 mM in pure methanol) and incubated for 15 min at room temperature (25.00 ˚C). Eventually, the absorbance of supernatants was measured at 412 nm. The concentration of TTM was expressed as nmol *per* mg of protein.

Evaluation of protein content. Total protein concentration of the samples was evaluated according to the Lowry's method.[21]

Histopathological examination. Ovarian tissues were fixed in 10.00% neutral buffered formalin, dehydrated in ethanol, and embedded in paraffin. Sections (5.00 μm) were stained with Hematoxylin and Eosin and examined using a light microscope (Nikon, Tokyo, Japan). To ensure representative findings, semi-serial sections were analyzed. All slides were evaluated by a blinded pathologist unaware of the group assignments. The histological assessment was qualitative and descriptive, focusing on morphological trends and the presence of specific structures (e.g., atretic/cystic follicles and corpora lutea) rather than numerical quantification.

Statistical analysis. The statistical analysis of the test groups was performed using One-way ANOVA, followed by Tukey's post hoc test in GraphPad Prism (version 9.0; GraphPad Software Inc., San Diego, USA). The results were expressed as Mean ± SD. A *p*-value of less than 0.05 was considered significant.

Results

Body weight and ovary index. As illustrated in Table 1, exposure to DBP resulted in a statistically significant increase in ovarian weight and ovarian index compared to the control group ($p < 0.05$). Compared to the DBP group, the ovarian index was significantly reduced in the group receiving a medium dose (200 mg kg^{-1}) of RJ and QCN, whereas, the low and high doses of RJ also decreased both ovarian weight and index, however, these changes were not statistically significant.

Effect of RJ and QCN on changes of serum TNF-α levels following DBP treatment. As presented in Figure 1, DBP exposure increased the serum level of TNF-α ($p < 0.05$) compared to the control group. In contrast to the DBP group, the medium (200 mg kg^{-1}) and high (300 mg kg^{-1}) doses of RJ + DBP groups and QCN+DBP group demonstrated a significant reduction in serum level of TNF-α ($p < 0.05$). Although the low dose of RJ also led to a decrease in TNF-α, this change was not statistically significant. Additionally, RJ or QCN alone showed a non-significant reduction in TNF-α levels compared to the control group.

Table 1. Effects of royal jelly and quercetin on alterations in body weight, ovary weight and ovary index induced by dibutyl phthalate

Parameters	Control	DBP	RJ	QCN	RJ100 + DBP	RJ200 + DBP	RJ300 + 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
Ovary weight (g)	1.23 ± 0.00	1.66 ± 0.12*	1.70 ± 0.38	1.24 ± 0.30	1.45 ± 0.24	1.25 ± 0.37	1.41 ± 0.32	1.11 ± 0.35#
Ovary index	0.0057 ± 0.00056	0.0078 ± 0.00051*	0.0068 ± 0.00129	0.0056 ± 0.00109	0.0068 ± 0.00072	0.0057 ± 0.00139#	0.0066 ± 0.00122	0.0054 ± 0.00072#

DBP: dibutyl phthalate, RJ: royal jelly and QCN: quercetin.
* Asterisk indicates significant differences ($p < 0.05$) between the control and DBP-treated animals, and # hash symbol indicates significant differences between DBP-treated animals and groups that received a combination of DBP with RJ or QCN ($p < 0.05$).

Table 2. Effects of royal jelly and quercetin on dibutyl phthalate induced alterations in reproductive hormones.

Parameters	Control	DBP	RJ	QCN	RJ 100 + DBP	RJ 200 + DBP	RJ 300 + DBP	QCN+ DBP
Testostrone (ng dL^{-1})	17.40 ± 0.57	20.50 ± 0.71*	18.00 ± 0.00	19.00 ± 1.41	18.00 ± 1.41	19.00 ± 0.00#	19.00 ± 1.41	18.25 ± 0.35#
Dehydroepiandrosterone (ng mL^{-1})	1.45 ± 0.01	2.68 ± 0.63*	1.17 ± 0.00	1.46 ± 0.28	1.095 ± 0.01#	1.60 ± 0.54#	1.58 ± 0.42#	1.34 ± 0.08#

DBP: dibutyl phthalate, RJ: royal jelly and QCN: quercetin.
* Asterisk indicates significant differences ($p < 0.05$) between the control and DBP-treated animals, and # hash symbol indicates significant differences between DBP-treated animals and groups that received a combination of DBP with RJ or QCN ($p < 0.05$).

Fig. 1. Effect of royal jelly (RJ) and quercetin (QCN) on serum tumor necrosis factor alpha (TNF-α) levels elevated by dibutyl phthalate (DBP) exposure.
* Asterisk indicates significant differences ($p < 0.05$) between the control and DBP-treated animals, and # hash symbol indicates significant differences between DBP-treated animals and groups that received a combination of DBP with RJ or QCN ($p < 0.05$).

Effect of RJ and QCN on MDA level following DBP treatment. As shown in Figure 2, DBP exposure significantly enhanced ovarian MDA levels compared to the control group (15.20 ± 1.09 *vs.* 9.89 ± 1.06; $p < 0.05$). In contrast, the MDA levels in the medium and high RJ + DBP groups (13.20 ± 0.71 and 12.77 ± 0.51, respectively), as well as the QCN + DBP group (10.82 ± 0.59), were significantly decreased compared to the DBP group ($p < 0.05$). Furthermore, although the low dose of RJ led to a reduction in MDA levels compared to the DBP group (14.30 ± 0.41), this change was not statistically significant.

Fig. 2. Impact of royal jelly (RJ) and quercetin (QCN) on changes in malondialdehyde (MDA) levels caused by dibutyl phthalate (DBP) in ovarian tissue.
* Asterisk indicates significant differences ($p < 0.05$) between the control and DBP-treated animals, and # hash symbol (#) indicates significant differences between DBP-treated animals and groups that received a combination of DBP with RJ or QCN ($p < 0.05$).

Effect of RJ and QCN on NO content following DBP treatment. The NO content of the ovarian tissue of the DBP-exposed animals was significantly enhanced compared to the control group ($p < 0.05$). The RJ+DBP groups at medium and high doses as well as the QCN + DBP group showed a significant decrease in NO content in the

ovary compared with DBP group (Fig. 3). Royal jelly and QCN alone did not change the NO level significantly (p > 0.05) when compared to the control animals.

Effect of RJ and QCN on TTM level following DBP treatment. Compared to the control group, in the groups exposed to DBP, the total thiol molecule content in ovarian tissues did not show a significant difference (p > 0.05), while QCN individually significantly enhanced the thiol molecules in the ovary compared to the control group (Fig. 4).

Effect of RJ and QCN on testosterone and DHEA level following DBP treatment. As shown in Table 2, compared to the control group, testosterone and DHEA levels were significantly elevated in the DBP-treated groups (p < 0.05). At the same time, testosterone levels were significantly decreased in the groups receiving the medium dose of RJ + DBP and QCN + DBP, and DHEA levels were significantly decreased in all groups receiving the RJ + DBP or QCN + DBP (p < 0.05).

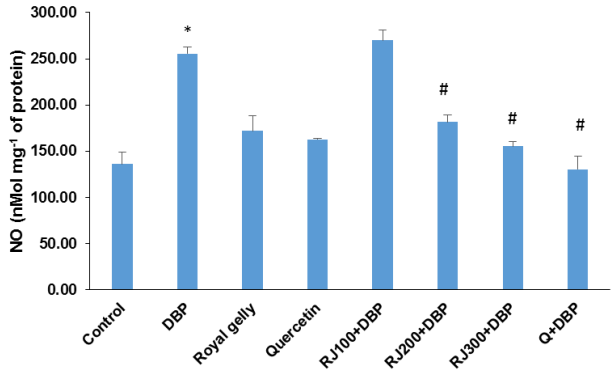


Fig. 3. Impact of royal jelly (RJ) and quercetin (QCN) on changes in nitric oxide (NO) levels in the ovarian tissue caused by dibutyl phthalate (DBP). * Asterisk indicates significant differences (p < 0.05) between the control and DBP-treated animals, and # hash symbol indicates significant differences between DBP-treated animals and groups that received a combination of DBP with RJ or QCN (p < 0.05).

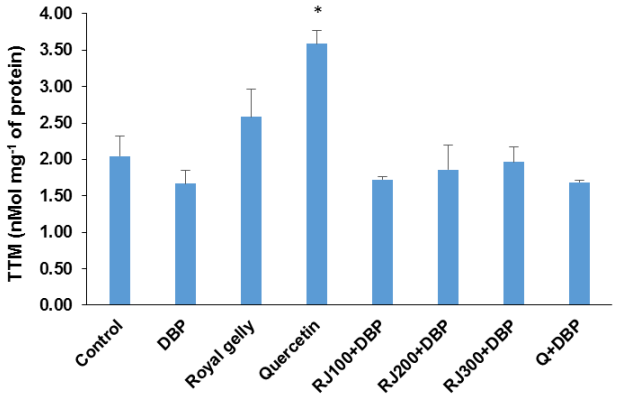


Fig. 4. Impact of royal jelly (RJ) and quercetin (QCN) on changes in total thiol molecules (TTM) levels caused by dibutyl phthalate (DBP) in the ovary. * Asterisk indicates significant differences (p < 0.05) between the control and quercetin groups.

Histopathological findings. The control group exhibited normal ovary structure, with the presence of primary and secondary follicles, and corpora lutea. In contrast, DBP-treated animals showed a marked increase in the presence of atretic and cystic follicles, along with a visible reduction in corpora lutea compared to the control group. No distinct histopathological alterations were observed in the groups that received either RJ or QCN individually. Compared to the DBP group, the groups that received a combination of RJ and DBP exhibited a noticeable increase in corpora lutea and a reduction in atretic follicles. Similarly, the group that received a combination of QCN and DBP showed a prominent decrease in atretic follicles and an increase in corpora lutea compared to the DBP group (Fig. 5).

Discussion

To the best of our knowledge, this was the first study to investigate the protective effects of RJ and QCN against DBP-induced ovarian toxicity in rats. In the present study, the ovarian index was significantly increased in the DBP-treated group, however, the medium dose of RJ and QCN significantly reduced this index compared to DBP group. It has been shown that prenatal exposure to phthalate mixtures results in higher ovarian weight in the third generation compared to control groups. Although the mechanistic pathways of prenatal transgenerational exposure often involving epigenetic reprogramming differ from the direct toxicological impacts observed in adult models, these findings underscored the persistent vulnerability of ovarian tissues to phthalate-induced disruptions across different life stages.[22] In previous studies, the increase in ovarian weight due to phthalate compounds was attributed to the development of ovarian cysts and an increase in the size of both follicles and corpora lutea.[22-24]

Oxidative stress is a condition in which the balance between the production of reactive oxygen species and the antioxidant defense system is disrupted.[25] Oxidative stress contributes to the pathophysiology of some female reproductive diseases including endometriosis and PCOS.[25-26] Reportedly, DBP reduced the level of antioxidant enzymes in the uterus and ovaries, including catalase, superoxide dismutase and glutathione peroxidase.[26-27] Chen *et al*., showed that diisononyl phthalate leads to an increase in MDA levels while causing a reduction in glutathione, glutathione peroxidase and superoxide dismutase levels in ovarian tissue.[27-28] The results of this study indicated that exposure to DBP led to an increase in MDA and NO levels in ovarian tissue. As previously reported in our related study on liver tissue using the same animal model and treatment protocol,[16] DBP exposure significantly reduced total antioxidant capacity in the serum. This systemic oxidative stress was consistent

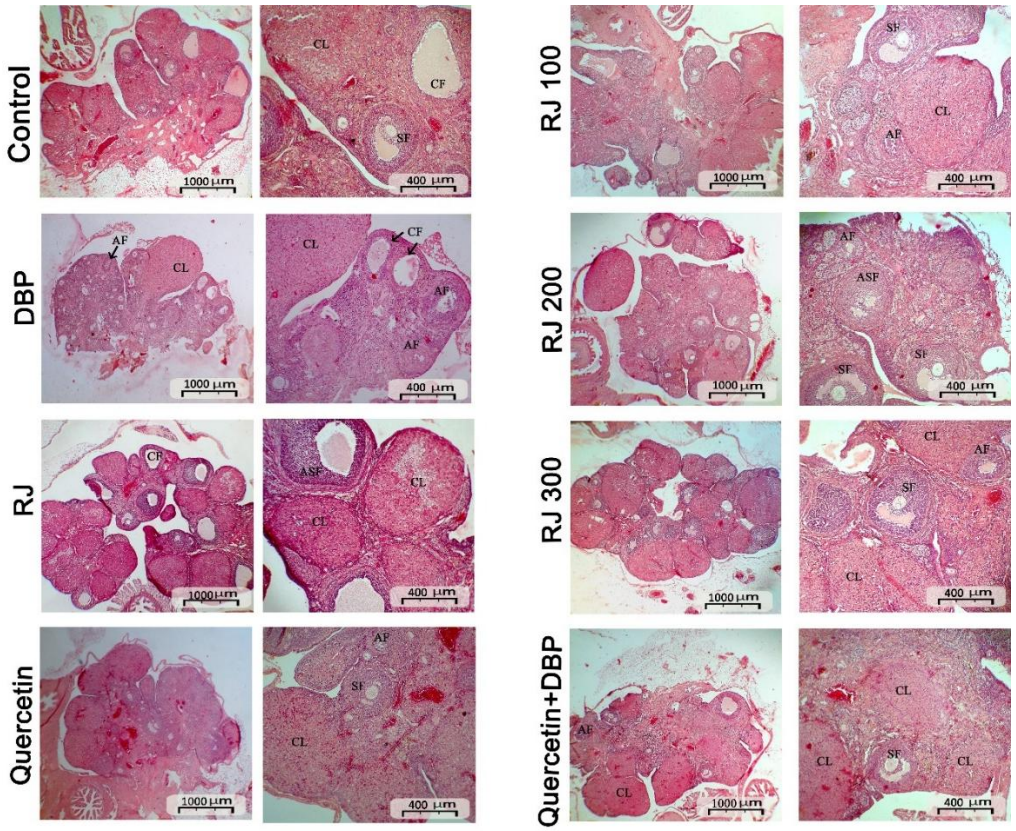


Fig. 5. Photomicrographs of ovarian tissue stained with Hematoxylin and Eosin. CF: cystic follicle, CL: corpus luteum, AF: atretic follicle, ASF: atretic secondary follicle and SF: secondary follicle; DBP: dibutyl phthalate; RJ: royal jelly.

with the increased MDA and NO levels observed in the ovarian tissue in the present study. Additionally, the lack of a significant reduction in TTM levels in the DBP group may reflect the involvement of compensatory antioxidant mechanisms, although this interpretation remains speculative.[29] Conversely, the elevation in MDA and NO levels induced by DBP was attenuated in the groups that received RJ indicating that RJ could prevent oxidative and nitrosative stress induced by DBP. These results were consistent with previous studies which indicated that RJ has antioxidant properties.[28] It has been demonstrated that RJ decreased NO and increased ferric-reducing antioxidant power levels in a rat model of PCOS.[30] The RJ components that contribute to its antioxidant properties include phenolic and flavonoid compounds, free amino acids, free fatty acids and their esters, including octanoic acid, benzoic acid, 2-hexenedioic acid, dodecanoic acid and vitamins C and E.[31] Based on previous studies, RJ improves the antioxidant system by upregulating the nuclear factor erythroid 2-related factor 2 expression and increasing the transcription of nuclear factor erythroid 2–related factor 2target genes including heme oxygenase-1.[32]

In this study, a significant increase in the serum concentration of TNF-α was shown in rats exposed to DBP, which is similar to that observed previously.[3,33] The pathophysiology of oxidative stress and inflammation is closely related in a wat that one can be easily triggered by the other. Previous studies have indicated that oxidative stress can activate the nuclear factor kappa B pathway, resulting in the production of cytokines and ultimately leading to inflammation.[34] In this study, oxidative stress induced by DBP might resulted in elevated levels of TNF-α. In the present study, as shown in Fig.1, these results were attenuated by pretreatment with the medium and high doses of RJ, which was supported by a previous report that RJ reduced TNF-α receptor 1 concentration in serum and adipose tissue of aged obese rats.[35] Anti-inflammatory effects of RJ are attributed to phenolic and flavonoid compounds, major RJ protein 1, major RJ protein 3, fatty acids including 10-hydroxy-2-decenoic acid, 10-hydroxydecanoic acid, sebacic acid and 2-Decenedioic.[31] According to previous studiesRJ anti-inflammatory mechanism is attributed to a reduction in the production of inflammatory cytokines, regulation of immune cell function and inhibition of the nuclear factor kappa B signaling pathway.[31]

Previous studies have shown that mono(2-ethylhexyl) phthalate reduces transcription of the *CYP19A1* gene via activation of PPAR (Peroxisome proliferator-activated receptor) α/γ signaling in granulosa cells. This down-regulates mRNA and protein levels of the aromatase enzyme, thereby, limiting the conversion of androgens

(testosterone and androstenedione) into estrogens (estradiol and estrone).[36-38] In a cross-sectional study of 116 mothers and 129 female children, the levels of phthalate metabolites in the urine of mothers during their third trimester were evaluated as a proxy for fetal in utero exposure. In that study, higher prenatal exposure to di(2-ethylhexyl) phthalate metabolites were associated with increased DHEA levels in daughters aged 8 - 13 years. Prenatal exposure to monobenzyl and monoethyl phthalate was similarly associated with elevated testosterone levels.[39] Consistent with these findings, DBP exposure in our model significantly increased serum testosterone and DHEA levels. It has been reported that RJ lowered testosterone levels in a rat model of PCOS induced by testosterone administration.[11] In agreement with previous findings, RJ administration significantly attenuated DBP-induced elevations in serum testosterone and DHEA.

Histopathological analysis in the present research showed that DBP increased cystic and atretic follicles and decreased corpora lutea compared to the control group. In agreement with our findings, Nivedita Sen *et al*. demonstrated that DBP exposure reduced secondary and antral follicle numbers as well as corpora lutea.[40] On the other hand, RJ supplementation reduced the pathological alteration induced by DBP.

Notably, an apparent non-monotonic response pattern was observed in the present study for both the ovarian index and serum testosterone levels, where, only the medium dose (200 mg kg^{-1}) exerted a significant effect, while the higher dose (300 mg kg^{-1}) failed to show additional or comparable improvement. A similar pattern was reported by Ab Hamid *et al*., in which the medium dose of RJ (200 mg kg^{-1}) was identified as the most effective dose for improving ovarian histology, estrus cycle regularity and hormonal balance, despite the higher dose (400 mg kg^{-1}) being more effective in reducing certain oxidative markers such as MDA. Although these findings may suggest a non-monotonic response to RJ, further studies are needed to better characterize the dose-response relationship. This observation may also reflect differences in the sensitivity of biochemical, structural and hormonal endpoints to RJ treatment.[11] In this experiment, QCN was used as a positive control, as previous studies have indicated that QCN mitigated phthalate-induced toxicity due to its antioxidant, anti-inflammatory and antiapoptotic effects.[15,41] Quercetin significantly reduced the elevated levels of MDA, NO and TNF-α induced by DBP which was consistent with its known pharmacological properties. Furthermore, QCN reduced the elevated levels of testosterone triggered by DBP. This finding was supported by a previous report which indicated that QCN reduced testosterone level in a rat model of PCOS.[42] Despite the significant findings, several limitations should be acknowledged. First, estrous-cycle stages were not determined at the time of sacrifice, which may have influenced endocrine and histopathological outcomes. Second, the use of different vehicles (saline for RJ and corn oil for other groups) may have introduced a confounding factor. Third, the sample size was relatively small (n = 5 *per* group) without a priori power analysis, which may limit the statistical generalizability. Additionally, our assessment was restricted to serum testosterone and DHEA levels, and we did not evaluate molecular mediators such as aromatase activity or androgen receptor expression. Consequently, the observed reductions in serum androgens should be interpreted as correlative rather than mechanistic. Future studies with uniform vehicle administration, larger sample sizes and comprehensive mechanistic assays are warranted to further clarify the pathways underlying these hormonal alterations.

In conclusion, the results of this study showed that RJ, due to its anti-inflammatory, antioxidant properties, along with its ability to attenuate elevated androgen levels, protected against DBP-induced ovarian dysfunction. More detailed studies on RJ at the molecular level as well as clinical trials are needed before it can be suggested as a supplement for the prevention and treatment of PCOS.

Acknowledgments

Not applicable.

Conflict of interest

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

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