



RESEARCH ARTICLE

Therapeutic potential of royal jelly to control *Toxoplasma gondii* infection in mice

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ABSTRACT

At present, there are several synthetic medications for toxoplasmosis therapy; however, these agents cannot be permanently applied because of adverse side effects or therapeutic failures and drug resistance in parasites. The present experimental investigation was aimed to study the effects of royal jelly (RJ) obtained from *Apis mellifera* in comparison with atovaquone against *Toxoplasma gondii* infection in mice. After treatment of infected mice with RJ at the doses of 200, 400, and 600 mg/kg for 14 consecutive days, we evaluated the therapeutic activity of RJ by measuring the mean number and the mean size of *T. gondii* tissue cysts, oxidant-antioxidant enzymes, pro-inflammatory cytokines, the mRNA expression levels of bradyzoite surface antigen 1 (BAG1), as well as the toxic effect on liver and kidney function. Treatment of the infected mice with RJ significantly ($p < 0.001$) decreased the mean number and the mean diameter of *T. gondii* tissue cysts and downregulated BAG1 in a dose-dependent response. After treatment of infected mice with RJ, the level of oxidative stress markers was significantly diminished, but a significant increase ($p < 0.05$) in the level of antioxidant markers such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) enzymes was observed. Treatment of the infected mice with RJ significantly enhanced the level of pro-inflammatory cytokines IFN- γ and IL-1 β , whereas it caused no substantial change in the serum levels of liver and kidney enzymes. The findings of this *in vivo* study revealed the favorable therapeutic effect of RJ on latent *T. gondii* infection in mice. It was found that RJ considerably inhibited the infection by decreasing the number and size of tissue cysts, reducing oxidative stress, and boosting the level of pro-inflammatory cytokines, but had no significant toxic impact on the function of vital organs such as liver and kidney. However, additional surveys are required to confirm these findings and clarify the exact mechanisms and their efficiency in clinical subjects.

Keywords: Toxoplasmosis; royal Jelly; oxidative stress; antioxidant; cytokines.

INTRODUCTION

Toxoplasma gondii infection or toxoplasmosis is a zoonotic disease among humans and animals with global distribution (Hill *et al.*, 2005). Intermediate hosts such as humans can be infected by eating *T. gondii* cysts in raw or uncooked meat, fruits, and vegetables or by drinking water contaminated with oocysts (Tenter *et al.*, 2000). The transmission of the infection might also occur congenitally or through blood and organ transplants (Saadatnia & Golkar, 2012). Acquired toxoplasmosis in immunocompetent or healthy individuals is generally reported to cause mild symptoms with lymphadenopathy and rarely leads to brain-eye involvement (Saadatnia & Golkar, 2012). Nevertheless, the disease in immunocompromised individuals is an opportunistic infection which results in serious complications such as encephalitis (Wang *et al.*, 2017). Congenital toxoplasmosis also can lead to various clinical symptoms ranging from mild signs to serious and fatal complications, e.g., abortion, hydrocephalus, microcephaly, and microcephaly (Torgerson & Mastroiacovo, 2013).

Nowadays, synthetic chemical drugs such as pyrimethamine, sulfadiazine, atovaquone, spiramycin, azithromycin, and

cotrimoxazole are used for the treatment of toxoplasmosis (Harrell & Carvounis, 2014). Although these synthetic agents have strong inhibitory effects on toxoplasmosis, recent investigations have reported that they are associated with various complications, e.g., impaired bone marrow function, toxicity on hematological parameters, teratogenic effects, and renal problems (Iaccheri *et al.*, 2008; Ben-Harari *et al.*, 2017). On the other hand, there is no effective vaccine that can be relied on to prevent toxoplasmosis in humans (Munoz *et al.*, 2011). Therefore, finding a new drug with unique features such as high efficiency in the prevention, control, and treatment of toxoplasmosis infection seems essential.

Natural products have long been a valuable resource for the production of medicinal compounds around the world (Thillaivanan & Samraj, 2014). Recently, it has reported growing attention on the portion of consumers and the food industry into beneficial food resources and the traditions in which it may assist keep human health while the important role of diet has been extensively described in controlling and treatment of various diseases such as infectious ones (Craig, 1999; Cheraghpour *et al.*, 2021).

One of the most complete dietary sources with health-promoting applications is products originating from the beehive, e.g., honey, royal jelly (RJ), and propolis (Pasupuleti *et al.*, 2017). RJ is an acidic substance with a yellowish-white color, a fruity taste, and high nutritional value, which is secreted from the submandibular glands of nursing bees (Pavel *et al.*, 2011). Researchers have shown that this substance has strong antioxidant, neuroprotective, anticancer, anti-inflammatory, antidiabetic, and anti-lipidemic effects, especially due to the presence of proteins, phenolic compounds, and lipids (Ramadan & Al-Ghamdi, 2012). Moreover, experimental and clinical investigations have reported the antimicrobial activities of RJ and its main compounds against a broad spectrum of bacterial, viral, fungal, and parasitic pathogenic strains (Ahmad *et al.*, 2020). The present experimental investigation was aimed to study the effects of RJ obtained from *Apis mellifera* in comparison with atovaquone against *T. gondii* infection in mice.

MATERIAL AND METHODS

Royal jelly material

In order to confirm the qualified purity and validity of sources, RJ materials were acquired in June 2021 from Langstroth hives with colonies of *A. mellifera* grown at Shaqra University in Saudi Arabia. To prepare the required doses of RJ, after dissolving the materials in normal saline, they were filtered (Whatman membrane, England) under vacuum conditions to obtain the doses of 200, 400, and 600 mg/kg for toxoplasmosis assessment and 50 mg/mL for the evaluation of secondary metabolite contents (Kanbur *et al.*, 2009; Ghanbari *et al.*, 2018).

Secondary metabolite contents

Total phenol content

Briefly, the RJ sample was mixed with the Folin-Ciocalteu's reagent (0.2 N) and sodium carbonate (7.5%) in a test tube. After 2 h of incubation at 21°C, the absorbance of the mixture was measured at 760 nm (Singleton *et al.*, 1999).

Total flavonoid content

To determine the total flavonoid content, the RJ sample was mixed with aluminum chloride (20%) and incubated for 1 h at 21°C. The optical density of the mixture was recorded at 420 nm (El-Guendouz *et al.*, 2016).

Total protein content

The protein content was measured based on the Bio-Rad assay. Briefly, the RJ sample was diluted in methanol/water (50/50; v/v) and the mixture was sonicated for 60 min. After adjusting the pH by 2.5, the solution was ten times diluted and mixed with the Bio-Rad reagent. Finally, the optical density of the mixture was recorded at 595 nm and the protein content was indicated as percentage (%) using the standard curve (Hartfelder *et al.*, 2013).

Parasite

We used the ME49 *T. gondii* avirulent strain, which was kindly provided by Shaqra University in Saudi Arabia and was intraperitoneally injected into BALB/c mice.

Animals

In total, 92 male BALB/c mice aged 4–6 weeks (20–25 g) were kept at a temperature of 24 ± 1°C, a 12-h cycle of light/dark, and humidity of 40–70% and were fed sufficient water and food ad libitum.

Induction of animal model of *T. gondii* infection

We established the chronic toxoplasmosis mice model according to a previous method by intraperitoneal inoculation of 0.5 mL of suspension (20–25 *T. gondii* tissue cysts) supplemented

with antibiotics, namely streptomycin and penicillin. Chronic toxoplasmosis was finally confirmed using the modified agglutination test kit (Toxo screen DA, Biomérieux, Lyon, France) based on the detection of anti-*T. gondii* IgG antibody in mice sera according to the producer's instructions.

Therapeutic effects on infected mice with toxoplasmosis

To study the therapeutic efficacy of RJ in the *T. gondii* infected mice, 24 h after the infection, 60 infected mice were randomly allocated to five groups with 12 mice in each group:

- Group 1: Infected mice were treated with normal saline for two weeks.
- Group 2: Infected mice were treated with atovaquone at the dose of 50 mg/kg/day for two weeks.
- Group 3: Infected mice were treated with RJ at the dose of 200 mg/kg/day for two weeks.
- Group 4: Infected mice were treated with RJ at the dose of 400 mg/kg/day for two weeks.
- Group 5: Infected mice were treated with RJ at the dose of 60 mg/kg/day for two weeks.

Assessment of oxidative stress factors

One day after the two-week treatment, six mice from each tested group were euthanized by intraperitoneal injection of sodium pentobarbital to investigate the levels of liver lipid peroxidation (LPO), nitric oxide (NO), and several proinflammatory cytokines.

After preparing the liver homogenates, the tissue level of LPO and NO were studied using the malondialdehyde (MDA) colorimetric assay (abcam, USA) and NO colorimetric kit (ab65328, Abcam), respectively (Albalawi *et al.*, 2022).

Assessment of antioxidant enzymes

The level of glutathione peroxidase (GPx) and superoxide dismutase (SOD) enzyme activities, as the main antioxidant markers, was examined using the commercial kits (Abcam, USA) based on the producer's protocols as described by Sun *et al.* (1988).

Measuring proinflammatory cytokines

The serum level of interferon gamma (IFN-γ) and interleukin 1-beta (IL-β), as the main proinflammatory cytokines, was calculated according to the commercial kits (Abcam, USA) based on the manufacturer's guidelines.

Collection of brain samples

In the 8th week post-infection, mice were deep anesthetized, and the whole brain of the tested mice was aseptically collected. The right and left brain hemispheres were utilized for parasitological and molecular tests, respectively.

Parasitological examination by assessing the *T. gondii* tissue cysts

In order to evaluate the anti-parasitic activity of RJ, unstained smears were first prepared from the right hemispheres of the tested mice, and the diameter and number of *T. gondii* tissue cysts were recorded using light microscopy (Saadatmand *et al.*, 2021).

Effects on bradyzoite surface antigen 1 (BAG1)

Here, we evaluated the efficacy of RJ treatment on BAG1 mRNA expression level in the tested mice using quantitative real-time PCR. Initially, total RNA was extracted from the left brain hemispheres according to the commercial kits' protocol (Qiagen, Hilden, Germany). The extracted RNA was reverse transcribed using the commercial kits (Qiagen, Hilden, Germany), and the obtained complementary DNA (cDNA) was utilized for real-time PCR by SYBR Green. Briefly, the temperature steps were primary denaturation at 95°C for 10 min, 40 cycles of amplification (denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and elongation at 73°C for 30 s),

and then one cycle at 73°C for 5 min. In the final step, the acquired data were examined by measuring $2^{-\Delta\Delta CT}$. Sequences of the used oligonucleotides in the real-time PCR are exhibited in Table 1 (Azami et al., 2018).

Toxic effects of RJ treatment on liver and kidney function

Toxic effects of the two-week treatment with RJ on the function of liver and kidney were measured. Briefly, three groups of healthy BALB/c mice (eight mice per group) received RJ at the doses of 200, 400, or 600 mg/kg/day for two weeks. The fourth group of healthy mice received normal saline as the control group. After the duration of treatment (on the 15th day), mice were deep anesthetized and blood samples were obtained through cardiac puncture. After centrifuging the blood samples and the obtained sera, the serum levels of liver functional enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)], as well as kidney function biomarkers [creatinine (Cr) and blood urea nitrogen (BUN)], were determined by commercial kits (Roche, Germany) (Albalawi et al., 2021).

Statistical analysis

Using SPSS software (version, 22.0), the study findings were analyzed. The changes of outcomes between the tested groups were studied by one-way analysis of variance (ANOVA) with Tukey's post hoc test. $P < 0.05$ was statistically significant.

RESULTS

Analysis of secondary metabolites

The analysis of secondary metabolites of RJ is presented in Table 2. The total phenolic, flavonoid, and protein contents of the obtained RJ were 84.2 mg GEA/g DW, 14.36 mg QE/g DW, and 12.5%, respectively.

Evaluating the oxidant/antioxidant markers

As shown in Figure 1, in the untreated infected mice (receiving normal saline), the level of MDA and NO was increased, but the level of GPx and SOD, as antioxidant markers, was reduced. The findings also revealed that the treatment of infected mice with RJ at the doses of 200, 400, and 600 mg/kg results in a significant reduction in the level of LPO and NO ($p < 0.01$), whereas a significant rise ($p < 0.05$) in the amount of GPx and SOD was observed. Statistical analysis also showed that RJ treatment, especially at the doses of

Table 1. The oligonucleotide primers applied for real-time PCR

Amplicon	Primers	Sequence (5'–3')	Size (bp)
BAG1	F	AGTCGACAACGGAG CCATCGTTATC	182
	R	ACCTTGATCGTGACACGTAGAACGA	
β-actin	F	GTGACGTTGACATCCGTAAAGA	245
	R	GCCGGACTCATCGTACTCC	

Table 2. The findings of total amount of the secondary metabolites of RJ

Compound	Test	Amount
Phenolic	Folin–Ciocalteu's reagent colorimetric	84.2 ± 1.62 mg GEA/g DW
Flavonoids	Aluminum chloride (AlCl ₃ 2%) colorimetric	14.36 ± 1.12 mg QE/g DW
Protein	Bradford method	12.5%

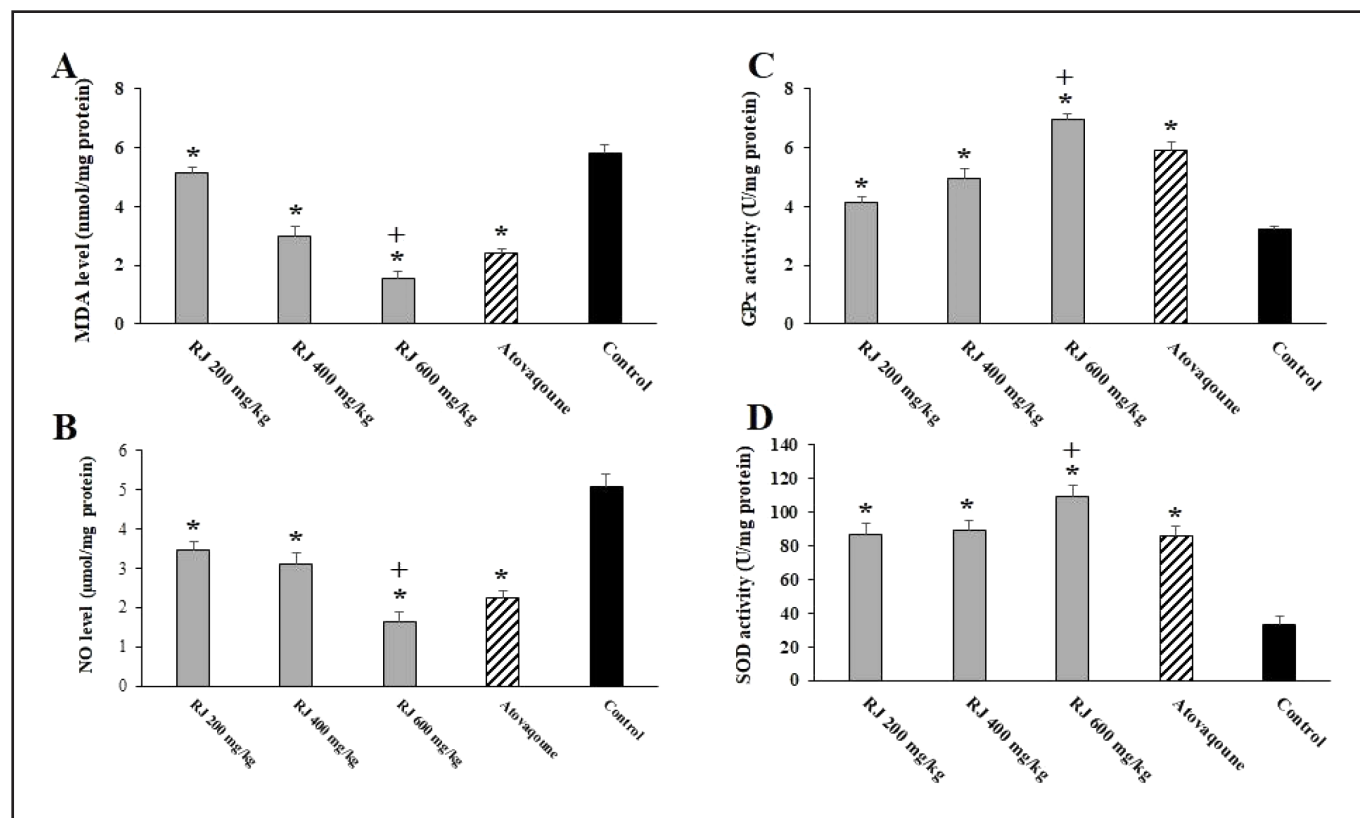


Figure 1. Effect of different concentration of royal jelly (RJ) on the level of A: malondialdehyde (MDA); B: nitric oxide (NO); C: glutathione peroxidase (GPx); D: superoxide dismutase enzyme activity (SOD) in the infected mice when compared with the control groups. Results are represented as Mean ± SD. * $p < 0.001$ significant difference with control. + $p < 0.01$ significant difference with atovaquone.

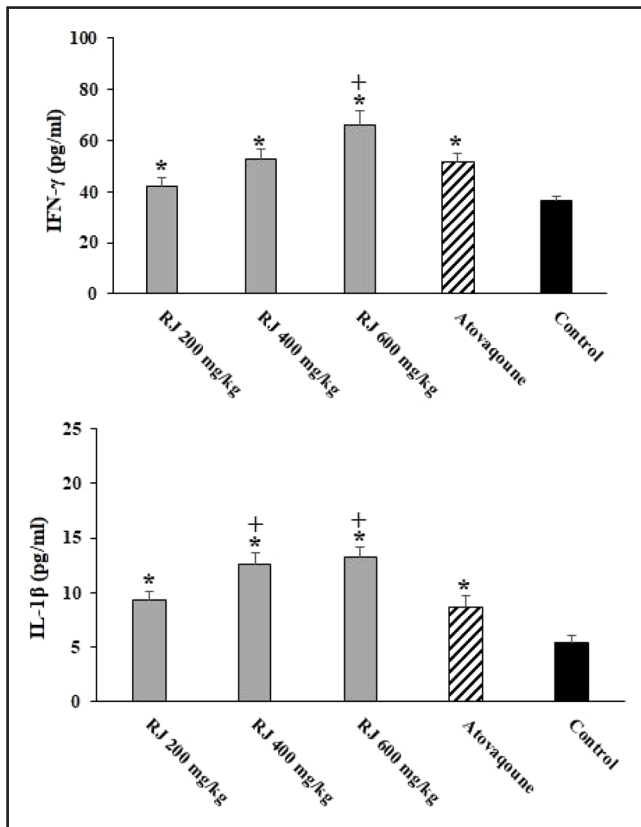


Figure 2. Effect of different concentration of royal jelly (RJ) on the level of IFN- γ and IL-1 β on the infected mice in comparison with the control groups. Results are represented as Mean \pm SD. * $p < 0.001$ significant difference with control. + $p < 0.001$ significant difference with atovaquone.

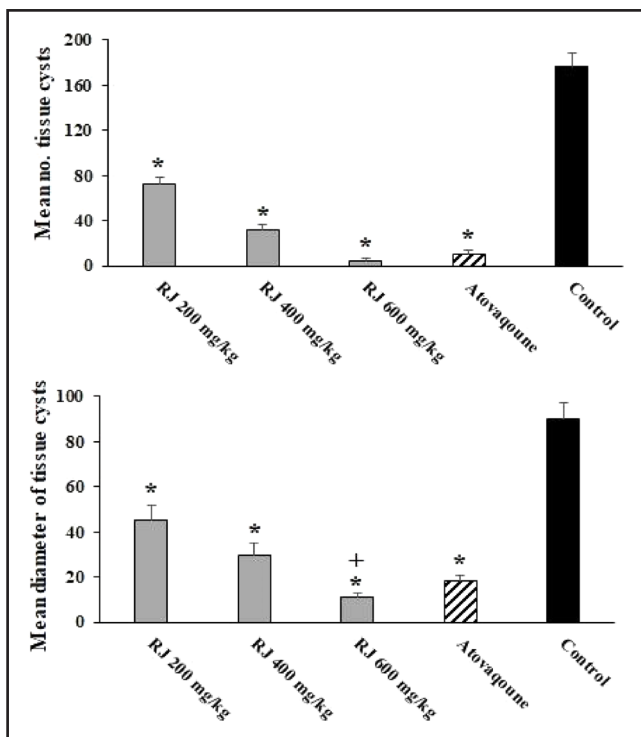


Figure 3. The mean number and the mean diameter of the brain tissue cysts followed by treatment of infected mice with various concentrations of royal jelly (RJ) for two weeks. Results are represented as Mean \pm SD. * $p < 0.001$ significant difference with control. + $p < 0.001$ significant difference with atovaquone.

400 and 600 mg/kg, improved the oxidative and antioxidant markers in comparison with atovaquone as the control drug ($p < 0.001$).

Evaluating the proinflammatory cytokines

Based on the obtained results, the level of proinflammatory cytokines such as IFN- γ and IL-1 β was significantly ($p < 0.001$) increased after the treatment of infected mice with RJ at the doses of 200, 400, and 600 mg/kg in a dose-dependent response (Figure 2). The statistical analysis showed that RJ treatment at the doses of 400 and 600 mg/kg led to stronger effects on the level of IFN- γ and IL-1 β when compared with atovaquone ($p < 0.001$).

Therapeutic effects on infected mice with toxoplasmosis

Treatment of the infected mice with RJ at the doses of 200, 400, and 600 mg/kg for two weeks significantly decreased ($p < 0.001$) the mean number of tissue cysts in a dose-dependent manner when compared with the control mice receiving normal saline (Figure 3). The highest efficacy was observed in the tested mice receiving RJ at the dose of 600 mg/kg, where only 4.3 *T. gondii* tissue cysts were recorded. Consequently, the mean diameter of *T. gondii* tissue cysts was also considerably smaller ($p < 0.001$) by 49.3%, 67.0%, and 87.4% after the treatment of infected mice with RJ at the doses of 200, 400, and 600 mg/kg, respectively. The statistical analysis revealed that RJ at the dose of 600 mg/kg displayed stronger anti-parasitic effects on the mean number and the mean diameter ($p < 0.001$) of *T. gondii* tissue cysts compared with the control mice receiving atovaquone.

Effects of RJ treatment on the expression level of BAG1

As depicted in Figure 4, after the induction of chronic toxoplasmosis in mice, the expression level of BAG1 was considerably upregulated in infected mice; however, the treatment of infected mice with various doses of RJ resulted in a significant ($p < 0.001$) downregulation of BAG1 in a dose-dependent response ($p < 0.001$). Based on the statistical analysis, RJ at the dose of 600 mg/kg led to a significant ($p < 0.01$) downregulation in BAG1 compared with atovaquone.

Toxic effects of RJ treatment on liver and kidney function

Evaluation of serum levels of liver and kidney functional enzymes demonstrated that the treatment of healthy mice with RJ at the doses of 200, 400, and 600 mg/kg for 14 consecutive days resulted in no significant abnormality in the serum amounts of ALT, AST, BUN, and Cr in comparison with the control mice receiving normal saline (Figure 5).

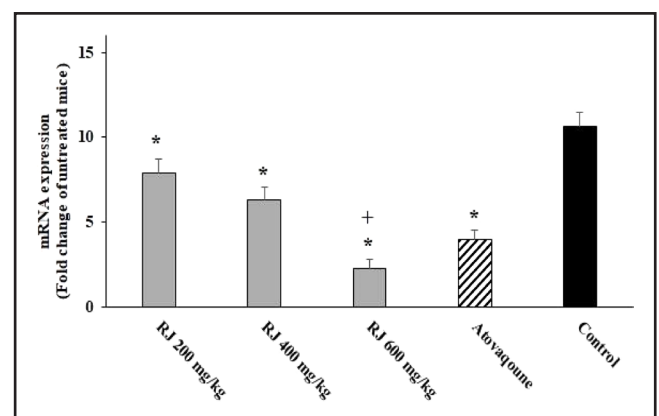


Figure 4. Effect of different concentration of royal jelly (RJ) on the expression level of bradyzoite surface antigen 1(BAG1) in infected mice in comparison with the control groups. Results are represented as Mean \pm SD. * $p < 0.001$ significant difference with control. + $p < 0.01$ significant difference with atovaquone.

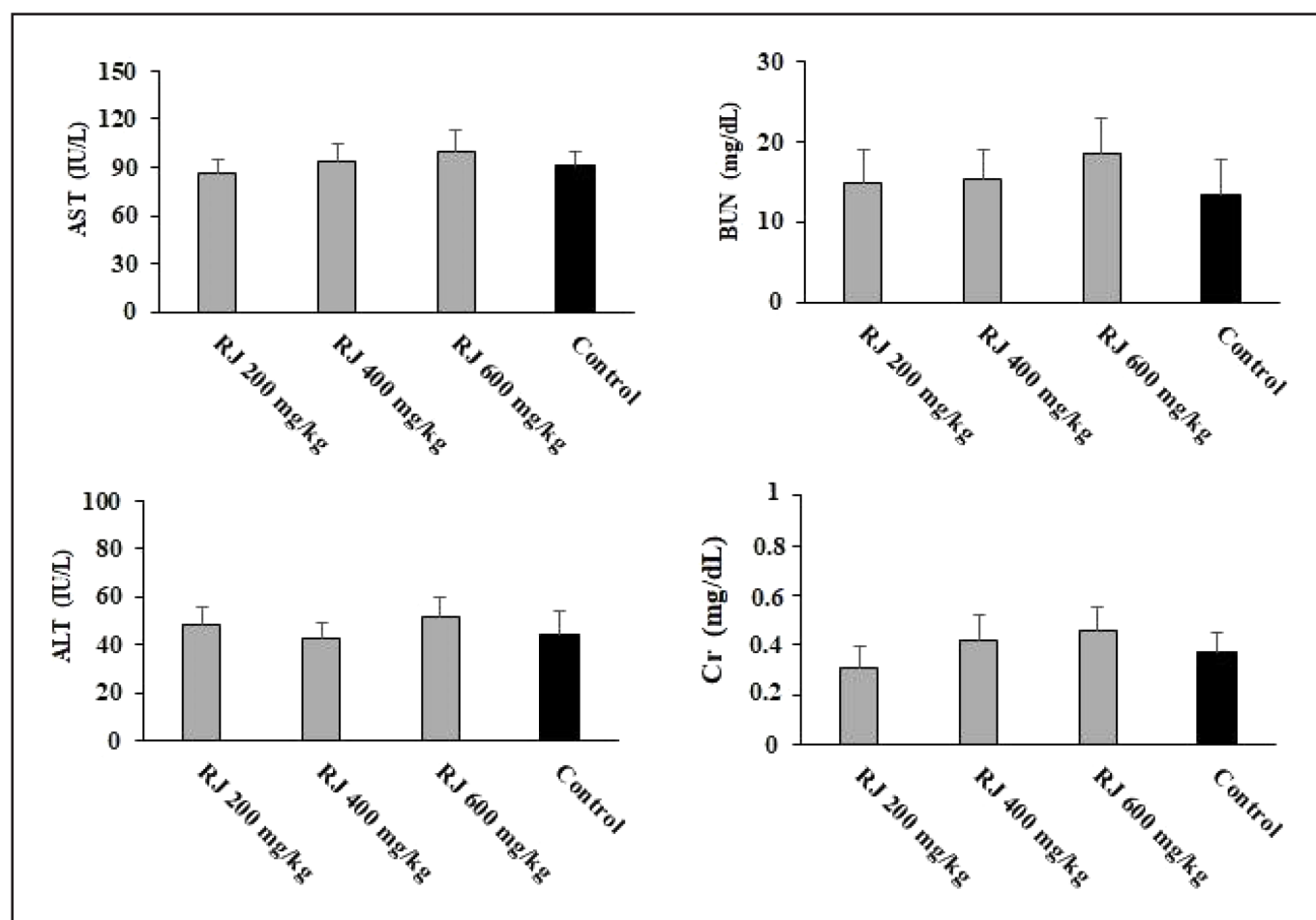


Figure 5. Effect of different concentration of royal jelly (RJ) on the level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), Blood Urea Nitrogen (BUN) and Creatinine (Cr) in the healthy mice when compared with the control mice receiving normal saline. Mean \pm SD.

DISCUSSION

At present, there are several synthetic medications for toxoplasmosis therapy; however, these agents cannot be permanently applied because of side effects or therapeutic failures correlated to drug intolerance or malabsorption and drug resistance in parasites (Iaccheri *et al.*, 2008; Ben-Harari *et al.*, 2017). The search for new antimicrobials derived from natural products has grown recently, since a significant number of the recommended synthetic drugs are derived from some natural sources, indicating that natural products play a key role in the development and discovery of new drugs (Sepulveda-Arias *et al.*, 2014). The present experimental investigation intended to evaluate the effects of RJ, as a valuable and beneficial natural source, in comparison with the effect of atovaquone against *T. gondii* infection in mice.

In this study, the analysis of the secondary metabolites of RJ revealed that the total phenolic, flavonoid, and protein contents of the obtained RJ were 84.2 mg GEA/g DW, 14.36 mg QE/g DW, and 12.5%, respectively. Alkhaibari and Alanazi (2022) reported that the phenolic, flavonoid, and protein contents of RJ in Saudi Arabia were 83.6 mg GEA/g DW, 1.78 mg QE/g DW, and 11.3%, respectively, indicating a higher phenolic content in RJ than the previous study. Another study conducted by Albalawi *et al.* (2021) showed that the total phenolic, flavonoid, and protein contents were 96.3 mg GEA/g DW, 2.85 mg QE/g DW, and 11.3%, respectively. According to the literature, RJ has some biological metabolites, such as polyphenols, flavonoids (e.g., quercetin), and fatty acids (e.g., trans-10-hydroxy-2-decanoic acid (10-H2DA) and 10-hydroxydecanoic acid (HDAA)), (Fratini *et al.*, 2016; Ahmad *et al.*, 2020). However, the difference in

the presence of these compounds in the RJ may be due to factors such as biodiversity of species existing in various ecosystems (Ahmad *et al.*, 2020).

As mentioned, RJ contains phenolic and flavonoid compounds and in other studies, it has been proven that these metabolites exert their antimicrobial impacts through several possible mechanisms, e.g., altering the cell wall permeability, suppressing the synthesis of DNA, RNA, and critical proteins, inducing energy metabolism dysfunction, declining the pathogenicity, and other similar mechanisms (Cushnie & Lamb, 2005; Nohynek *et al.*, 2006; Ahmad *et al.*, 2020). In addition, in a study conducted by Alkhaibari and Alanazi (2022) on the anti-parasitic mechanisms of RJ, it was revealed that RJ by inducing apoptosis and negative effects on the permeability of the plasma membrane may inhibit the growth of *Leishmania* and *Plasmodium* parasites.

We found that the treatment of the infected mice with RJ at the doses of 200, 400, and 600 mg/kg for two weeks significantly decreased the mean number and the mean diameter of *T. gondii* tissue when compared with the control mice receiving normal saline. The statistical analysis indicated that RJ at the dose of 600 mg/kg displayed stronger anti-parasitic effects on the mean number and the mean diameter ($p < 0.001$) of *T. gondii* tissue cysts compared with the control mice receiving atovaquone. In addition, we reported that after the induction of chronic toxoplasmosis in mice, the expression level of BAG1 was considerably upregulated in infected mice; however, the treatment of infected mice with various doses of RJ resulted in the significant downregulation of BAG1 in a dose-dependent response ($p < 0.001$).

Regarding the antimicrobial properties of RJ, previous *in vitro* and *in vivo* investigations have reported the promising effects of RJ against a wide range of bacterial (e.g., *Bacillus* spp., *Bacteroides* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Lactobacillus* spp., *Salmonella* spp., *Staphylococcus* spp., *Escherichia coli*, and *Klebsiella pneumoniae*), fungal (e.g., *Aspergillus* spp., *Candida* spp., and *Syncephalastrum racemosum*), and viral pathogenic strains (e.g., heart virus coxsackie B3, herpes simplex virus type 1 (HSV-1), herpes 2 virus, and influenza virus) (Alreshoodi & Sultanbawa, 2015; Fratini et al., 2016; Ahmad et al., 2020). Regarding the anti-parasitic activities of RJ, Alkhaibari and Alanazi (2022) reported the antiparasitic and antileishmanial activity of RJ and its main metabolites, such as 10-H2DA, 10-HDAA, and sebacic acid against chloroquine-resistant *Plasmodium falciparum* K1-strain with the IC₅₀ values of 7.6, 2.4, 2.6, and 3.1 µg/mL, respectively. In addition, they found the promising *in vitro* antileishmanial effects of RJ, 10-H2DA, HDAA, and sebacic acid against amastigote forms of *Leishmania major* with the IC₅₀ values of 8.1, 3.8, 3.7, and 4.1 µg/mL, respectively (Alkhaibari & Alanazi, 2022).

LPO is recognized as an oxidative stress marker that, when elevated, can cause some biological damage such as destruction of cell membranes and release of hepatotoxicity indicator enzymes (Niki et al., 2005). During the *T. gondii* infection, mainly in the primary phase of diseases, tissue damage can occur due to the rise in the production of free radicals (Szewczyk-Golec et al., 2021). Here, we reported that the treatment of infected mice with RJ at the doses of 200, 400, and 600 mg/kg resulted in a significant reduction in the levels of LPO and NO ($p < 0.01$) whereas a significant increase ($p < 0.05$) in the amount of GPx and SOD was observed. In agreement with our results, Çavuşoğlu et al. (2009) demonstrated that RJ improved the cadmium-induced genotoxicity and oxidative stress in mice through refining the antioxidant factors (e.g., glutathione) and declining the production of MDA. Moreover, Gu et al. (2018) reported that RJ displayed its antioxidant properties through the suppression of NO and boosting antioxidant factors, e.g., glutathione and SOD enzymes.

Today, it has been proven in various studies that improving the body's immune system, mainly cellular immunity through the stimulation of the release of inflammatory factors, is one of the most important factors in controlling and preventing toxoplasmosis (Yarovinsky, 2014). Our findings showed that the level of proinflammatory cytokines such as IFN-γ and IL-1β was significantly ($p < 0.001$) increased after the treatment of infected mice with RJ at the doses of 200, 400, and 600 mg/kg in a dose-dependent response, which indicated that RJ therapy probably improved the host survival and controlled toxoplasmosis by improving the immune system.

Concerning the toxicity of RJ therapy, we found that treatment of healthy mice with RJ at the doses of 200, 400, and 600 mg/kg for 14 consecutive days resulted in no significant abnormality in the serum levels of ALT, AST, BUN, and Cr in comparison with the control mice receiving normal saline. This finding indicated that although RJ has a promising effect in controlling toxoplasmosis, it has no significant toxic effect on liver and kidney function at the tested doses.

CONCLUSION

The obtained findings of this *in vivo* study revealed that favorable therapeutic activity of RJ on latent *T. gondii* infection in mice; so that it considerably inhibited infection through decreasing the number and size of tissue cysts, decreasing oxidative stress, and improving pro-inflammatory cytokines, where had no considerable toxicity on function of vital organs such as liver and kidney; however, additional surveys are required to approve these findings, clarify the exact mechanisms, their efficiency and toxicity in higher doses.

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Conflict of Interest

The authors declare no conflict of interest.

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