Original Article

In vitro anti-*Toxoplasma* effects and apoptotic induction of queen bee acid (10-hydroxy-2-decenoic acid) alone and in combination with atovaquone

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ABSTRACT

Toxoplasmosis, which is caused by the *Toxoplasma gondii* parasite, is a parasitic, infectious disease. 10-hydroxy-2-decenoic acid (10-H2DA, queen bee acid (QBA), is one of the most prevalent fatty acids (>40%) present in royal jelly. Studies have pointed to antitumor, anti-inflammatory, antiangiogenic, and antimicrobial effects of 10-H2DA, improving the immune system. This experimental survey aimed to assess the *in vitro* efficacy of QBA against tachyzoites and intracellular parasites of the *T. gondii* RH strain. Anti-*Toxoplasma* effects of QBA against tachyzoites were examined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay for 30, 60, 120, and 180 min. In addition, the effect of QBA on infection rate and intracellular parasites was studied. Real-time polymerase chain reaction (Real-Time PCR) was also applied to assess the expression level of the Caspase-3 gene. The best efficiency of QBA was obtained at 100 and 50 µg/mL, whereas all tachyzoites were diminished, followed by 120- and 180-min treatment, respectively. It was also found that the best repressing efficacy of QBA in the infection rate and the load of parasites into the Vero cells was indicated at 100 µg/mL (P<0.001). Nonetheless, the combination of QBA (12.5 µg/mL) along with atovaquone 30 µg/mL displayed the most marked effect on the infection rate and a load of parasites into the Vero cells in the infected Vero cells. The expression level of the Caspase-3 gene was dose-dependently increased after the exposure of tachyzoites to QBA, mainly at ½ IC_{50} and IC_{50} compared to normal saline. The obtained findings exhibited the high *in vitro* potency of QBA, especially in combination with atovaquone against *T. gondii* RH strain tachyzoites. Although apoptosis induction can be suggested as one of the principle mechanisms, more studies are required to elucidate its accurate mechanisms, as well as its efficacy and safety in animal models and clinical settings.

Keywords: Apoptosis, *In vitro*, Tachyzoites, Toxoplasmosis

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1. Introduction

Toxoplasmosis infection, which is caused by *Toxoplasma gondii* parasite, is a parasitic infectious disease (1) usually transmitted to humans through direct contact with cat feces containing oocysts, water, unpasteurized milk, vegetables, contaminated soil, or undercooked meat (2). Even though the infection is asymptomatic or mild in healthy people, transplant transmission during pregnancy in the first exposure can be very disastrous, leading to serious pathological complications, such as microcephaly, hydrocephaly, blindness, abortion, and even death (3, 4). In addition, *T. gondii*, as an opportunistic parasite in immunocompromised people, such as HIV patients, organ transplant patients, and cancer patients, demonstrated serious complications (5). Today, medicines, such as pyrimethamine, sulfadiazine, spiramycin, and atovatakone, are used as the first line of treatment for the prevention and treatment of toxoplasmosis (6, 7). Based on the reports, the serious side effects of these drugs are bone marrow suppression, blood toxicity, gastrointestinal ailments, and drug resistance to these agents (7, 8). Therefore, it is necessary to find a novel medication with such characteristics as high efficiency and minimum toxicity. In recent years, bee products(e.g., honey and royal jelly (RJ)) have been widely used for biological and pharmacological goals to enhance human health (9). Royal jelly is a valuable resource with various confirmed pharmacological properties, including anti-inflammatory, antitumor, immune system booster, and antimicrobial (10). 10-hydroxy-2-decenoic acid (10-H2DA, queen bee acid (QBA), is one of the most prevalent fatty acids (>40%) present in RJ (11). Studies have pointed to antitumor, anti-inflammatory, antiangiogenic, and antimicrobial effects of 10-H2DA, improving the immune system (12-16). This experimental survey aimed to evaluate the *in vitro* efficacy of the QBA against tachyzoites and intracellular parasites of the *T. gondii* RH strain.

2. Materials and Methods

2.1. Queen bee acid

10-HAD ($C_{10}H_{18}O_3$, > 97%, Figure 1) was procured from Sigma-Aldrich Company, Germany.

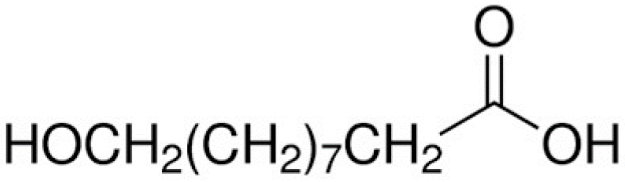

Figure 1. Compound structure of queen bee acid (10-HDA, $C_{10}H_{18}O_3$)

2.2. Parasites

Tachyzoites forms of the *T. gondii* RH strain obtained from Tehran University of Medical Sciences, Tehran, Iran, were preserved in BALB/c mice through intraperitoneal (IP) passages and were regulated using a hemocytometer slide to 1,000,000 tachyzoites per mL (Figure 2A).

2.3. Cell culture

The cell lines of Vero with ATCC CCL-81 (Figure 2B) were kept in the RPMI-1640 medium + fetal bovine serum (10%), strep/pen (100 µg/mL) and kept warm at 37°C with 5% CO_2.

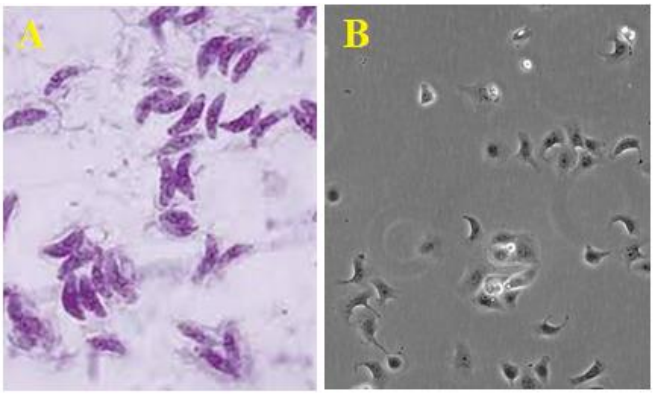

Figure 2. Tachyzoites forms of *T. gondii* (A) and cell line of Vero (B) used in this study

2.4. In vitro anti-*Toxoplasma* effects against tachyzoites

Anti-*Toxoplasma* effects of QBA against tachyzoites were examined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay for 30, 60, 120, and 180 min (17). In brief, 0.1 mL of QBA (25-100 µg/mL) was mixed with 0.1 mL of tachyzoites in a 96-well plate and kept for 30-180 min at 37°C. Following that, MTT solution (0.01 mL) was mixed and kept again at the same conditions for 4 h. Ultimately, dimethylsulfoxide (0.05 mL, Merck, Germany) was put in, and the optical density (OD) of the plate was calculated at 570 nm by an enzyme-linked immunosorbent assay (ELISA) reader (17). The mortality rate of parasites was determined.

2.5. Effect on infection rate and intracellular parasites

Firstly, 0.1 mL of the Vero cells (100,000 cells) was planted in a 24-well plate at 37°C for one day. Thereafter, parasites were added to the wells at 10 times the number of cells and incubated for another day. By discarding the supernatant and washing the wells with sterile phosphate-buffered saline (PBS), the infected cells were treated with QBA (25-100 µg/mL) for 180 min. Once washed and stained by Giemsa, the slides were ready and checked through a light microscope to clarify the rate of infection and parasite intracellular number in 100 infected cells,

whereas the 50% inhibitory concentrations (IC_{50}) were measured using the Probit test in SPSS software (version 22.0) (18).

2.6. Real-time polymerase chain reaction to assess the apoptosis gene

The expression level of the caspase-3 gene linked to apoptosis in parasites treated with the QBA was evaluated using real-time polymerase chain reaction (Real-time PCR). In this study, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control gene. The RNA was extracted from the treated cells using the Qiagen RNA extraction kit and based on the company's protocol. The quality and quantity of RNA were evaluated using Nanodrop. The synthesis of cDNA was made using the Qiagen commercial kit, and the obtained product was used in real-time PCR after equalizing the concentration. The materials used in real-time PCR and the sequence of primers are displayed in Table 1. The expression of gene was assessed by estimating the $2^{-\Delta\Delta CT}$ in Bio-Rad iQ5 Optical System Software, USA.

2.7. Statistical analysis

The data were analyzed in SPSS software (version 22.0) using ANOVA and Tukey's follow-up test. The significance level was considered less than 0.05.

3. Results

3.1. In vitro anti-*Toxoplasma* effects against tachyzoites

Various concentrations of QBA exhibited a considerable (P<0.001) anti-*Toxoplasma* action after 30-180 min compared to normal saline (Figure 3). The best efficiency of QBA was reported at 100 and 50 µg/mL, whereas all tachyzoites were diminished, followed by 120- and 180-min treatment, respectively. The findings also indicated that the combination of QBA (12.5 µg/mL) and atovaquone 30 µg/mL displayed a higher effect on the mortality of tachyzoites in comparison with atovaquone alone (P<0.01).

3.2. Effect of QBA on infection rate in Vero cells

The best repressing efficacy of QBA in infection rate was obtained at 100 µg/mL, with a 56.8% reduction in infectivity (P<0.001) (Figure 4). Moreover, QBA at 12.5, 50, and 75 µg/mL markedly diminished (P<0.05) the infection rate by 83.6%, 74.2%, and 59.6%, respectively. The combination of QBA (12.5 µg/mL) and atovaquone 30 µg/mL displayed the highest effect on the infection rate in the infected Vero cells.

Table 1. Primers sequence and the thermal condition of real-time polymerase chain reaction

Primers	Sequence	Conditions
Casepase-3 GAPDH	F: 5′ TTCATTATTCAGGCCTGCCGAGG-3′ R: 5′ TTCTGACAGGCCATGTCATCCTCA-3	95 ºC (5 min)-95 ºC (5 s)- 57ºC (40 s)- 74 ºC (40s)

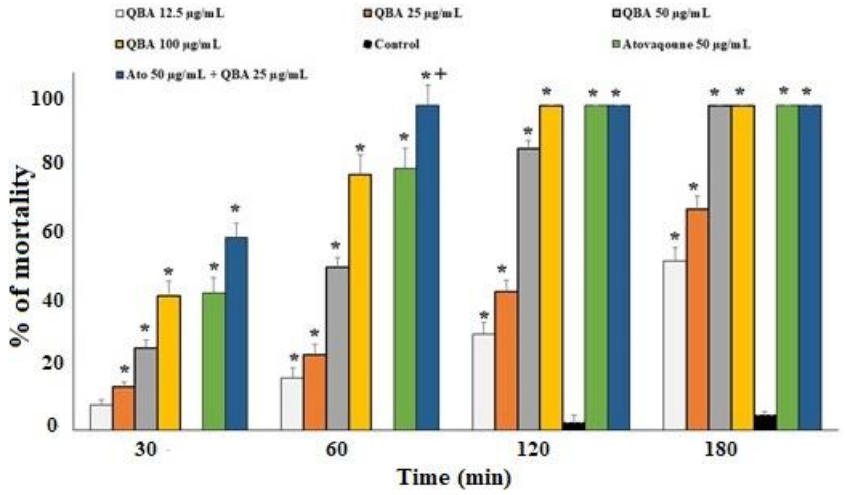

Figure 3. Anti-*Toxoplasma* efficiency of QBA, atovaquone, and their combination on tachyzoites at different times. * p<0.05 compared to normal saline. + p<0.05 compared to atovaqoune.

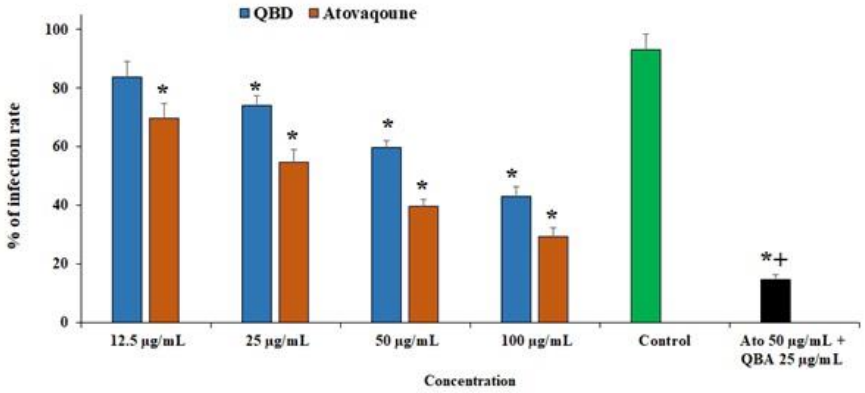

Figure 4. Effect of QBA, atovaquone, and their combination on infection rate in Vero cells. *p<0.05 compared to normal saline. + p<0.05 compared to atovaquone.

3.3. Effect of QBA on Intracellular Parasites

Figure 5 depicts the effect of QBA on the reproduction of parasites in the Vero cells. The QBA and atovaquone extensively reduced the load of parasites in the Vero cells with IC_{50} of 48.2 and 54.2 µg/mL, respectively. Nonetheless, the combination of QBA and atovaquone displayed better inhibitory effects on the reproduction of parasites in the Vero cells than each one alone (P<0.001).

3.4. Effects of QBA on Caspase-3 expression gene

We assessed the effects of QBA at 1/3 IC_{50}, ½ IC_{50}, and IC_{50} on the Caspase-3 expression gene in treated parasites. As indicated in Figure 6, the expression level of the Caspase-3 gene was dose-dependently increased after the exposure of tachyzoites to QBA, while a noticeable increase was observed at ½ IC_{50} and IC_{50} compared to normal saline.

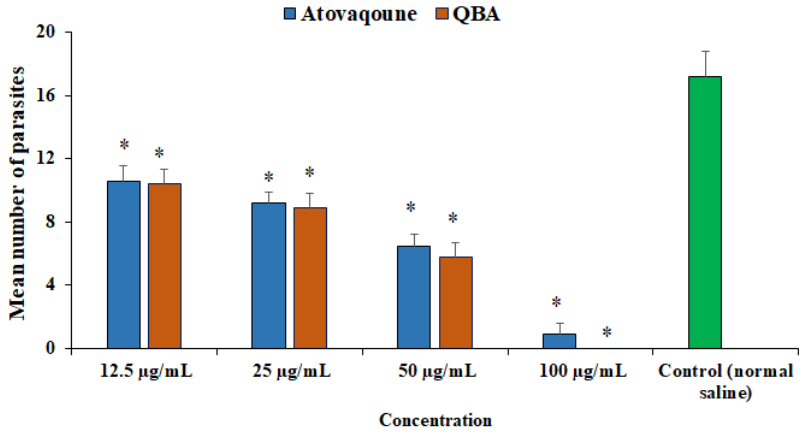

Figure 5. The effect of QBA, atovaquone, and their combination on the reproduction of parasites in the Vero cells. * p<0.05 compared to normal saline. + p<0.05 compared to atovaquone.

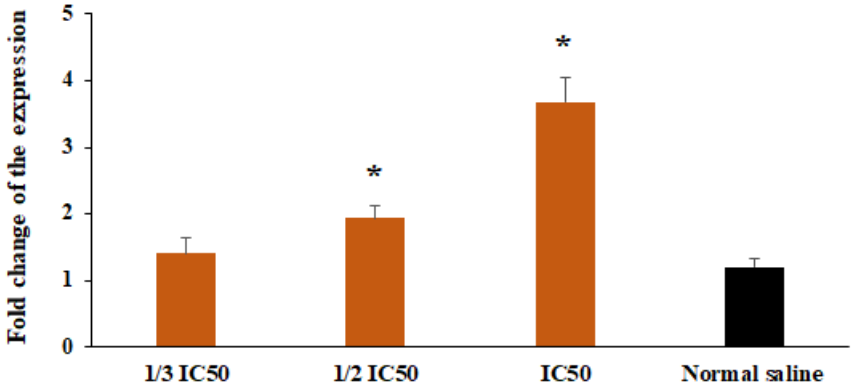

Figure 6. Effects of QBA on the Caspase-3 expression gene in treated parasites. * p<0.05 compared to normal saline.

4. Discussion

Here, we observed that the best efficiency of QBA was obtained at 100 and 50 µg/mL, whereas all tachyzoites were diminished, followed by 120- and 180-min treatment, respectively. The findings also indicated that the combination of QBA (12.5 µg/mL) and atovaquone 30 µg/mL displayed a more marked effect on the mortality of tachyzoites in comparison with atovaquone alone. It was so found that the best repressing efficacy of QBA on the infection rate and a load of parasites into the Vero cells was obtained at 100 µg/mL; nonetheless, the combination of QBA (12.5 µg/mL) along with atovaquone 30 µg/mL displayed the highest effect on the infection rate and a load of parasites into the Vero cells in the infected Vero cells. Previously, it has been proven that bee derivatives (e.g., RJ, honey, venom, and propolis) displayed promising efficacy in folk remedies for the treatment of several diseases, including infectious ones (19). Recently, investigations reported the high potency of bee derivatives in the control and treatment of bacterial, viral, and fungal diseases (19). In addition, several *in vitro* and *in vivo* studies highlighted the high efficiency of bee products against various pathogenic parasites (e.g., *Schistosoma* spp, *Trypanosoma* spp., *Leishmania* spp., *Plasmodium* spp., *Toxocara* spp., Entamoeba spp., and *Cryptosporidium* spp. (19). Although based on previous studies, the main antimicrobial mechanisms of bee products are cell proliferation inhibition, disruption of cell membranes and cytoplasm, inhibition of microbial motility, disruption of the activity of vital enzymes, interruption of protein synthesis, and H_2O_2 production (20), several investigations have reported that the principle antimicrobial mechanisms of action fatty acids, such as 10-HAD, are cell membrane destruction, disruption or the electron transport chain, apoptotic induction, and oxidative phosphorylation (21). Studies reported that apoptosis induction is a worthy goal for the discovery and recognition of new medicines to manage and control parasitic diseases (22). Caspases, mainly caspase-3, are the principal genes involved in modulating apoptosis (23-25). Here, we examined the effects of QBA at 1/3 IC_{50}, ½ IC_{50}, and IC_{50} on Caspase-3 gene expression levels in treated parasites. As illustrated in Figure 6, the expression level of the Caspase-3 gene was dose-dependently increased after the exposure of tachyzoites to QBA, while a noticeable increase was observed at ½ IC_{50} and IC_{50} compared to normal saline, indicating that apoptosis induction can be considered one of the main anti-*Toxoplasma* mechanisms of QBA. The obtained findings exhibited the high *in vitro* potency of QBA, especially in combination with atovaqoune against *T. gondii* RH strain tachyzoites. Although apoptosis induction can be suggested as one of the principle mechanisms, more studies are required to elucidate its accurate mechanisms, as well as efficacy and safety in animal models and clinical settings. Among the notable limitations of this study, we can refer to a failure to identify the mechanisms of action of QBA and assess its anti-*Toxoplasma* effects in animal models. If all aspects of the effectiveness and toxicity of this compound are clarified in clinical studies, it can be used as a new medicine in the treatment and prevention of toxoplasmosis.

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Authors' Contribution

SP designed the experiments; SP. and PA., performed experiments and collected data; SP Supervised, directed, and managed the study; all authors approved the final version to be published.

Ethics

This experimental study was approved by the ethical board of Faculty of Pharmacy, Eastern Mediterranean University, Famagusta, North Cyprus (Number. 15701813).

Conflict of Interest

The authors declare no conflict of interest.

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None.

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