



Anti-*Toxoplasma In Vitro* and *In Vivo* Activity of *Pyrus boissieriana* Arbutin-Rich Fraction

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Abstract

Purpose *Pyrus boissieriana* is a rich source of arbutin and has been used in herbal medicine to treat infectious diseases. This study aimed to investigate the effect of the arbutin-rich fraction of *Pyrus boissieriana* aerial parts on *Toxoplasma gondii* *In Vitro* and *In Vivo*.

Methods An arbutin-rich fraction of *P. boissieriana* was prepared beforehand. Flow cytometry was used to evaluate the effect of different concentrations (1–512 µg/ml) of the *P. boissieriana* arbutin-rich fraction on *Toxoplasma* tachyzoites (RH strain). The cytotoxicity of the concentrations on the macrophage J774 cell line was also investigated by MTT assay. For *In Vivo* investigation, 4–6-week-old female mice infected with the RH strain of *T. gondii* were treated with different doses (16, 32, 64, 256, and 512 mg/kg) of the fraction using gavage.

Results The highest and lowest lethality of the tachyzoites were 89.6% and 25.9% related to the concentrations of 512 µg/ml and 1 µg/ml, respectively, with an IC₅₀ value of 18.1 µg/ml ± 0.37. The cytotoxicity test showed an IC₅₀ value of 984.3 µg/ml ± 0.76 after 48 h incubation. The mean survival of mice at the lowest treated dose (16 mg/kg) was 6.6 days, and it was 15 days at the highest dose (512 mg/kg). The concentrations of 512, 256, 128, and 64 mg/kg of the fraction compared to the negative control (6.2 days mean survival) significantly increased the survival time of mice ($P < 0.001$, $P = 0.009$, $P = 0.018$, and $P = 0.021$, respectively).

Conclusion The results showed that the arbutin-rich fraction of *P. boissieriana* is effective against *T. gondii* *In Vitro* and *In Vivo* and may be a reliable alternative to conventional treatment for toxoplasmosis, although further studies are necessary.

Keywords *Toxoplasma gondii* · *Pyrus boissieriana* · Arbutin · *In Vitro* · *In Vivo*

Introduction

Toxoplasma gondii (*T. gondii*) is an Apicomplexa protozoan that causes zoonotic toxoplasmosis. Humans and other warm-blooded animals can be infected as intermediate hosts,

and approximately 30% of all people around the world are infected with *Toxoplasma* [1, 2]. *Toxoplasma* infection in immunocompetent individuals causes asymptomatic or mild disease symptoms. However, in immunocompromised patients or during pregnancy, it may cause a serious disease. If left untreated, it can lead to the death of the host [3]. Sulfonamide, pyrimethamine, and spiramycin are the routine drugs to treat acute toxoplasmosis [4]. These drugs, in addition to increasing the risk of bone marrow system suppressing, cause various complications such as hematologic toxicity, thrombocytopenia, leukopenia, renal complications, and skin rashes [5]. Recently, many studies on herbal medicine have been underway for the non-toxic and effective treatment of toxoplasmosis. The native plants of each region have been the focus of researchers to conduct experiments [6, 7].

The *Pyrus boissieriana* (*P. boissieriana*) from the Rosaceae family is widely distributed in the forests of

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northern Iran [8]. Additionally, the *Pyrus* genus is found in other regions of the world, including Central, Southern, and Western Europe, Northwest and Southwest Africa, Asia from Anatolia to Turkmenistan, southwestern and northern Afghanistan through northern Pakistan and the Himalayas to China and Japan, and Armenia [9].

The petioles, leaves, bark, and fruits of some *Pyrus* species contain large amounts of a phenolic glycoside called arbutin [10]. The extract of this plant is used as a disinfectant for the urinary tract in moderate infection and inflammation of the urinary tract and bile, such as cystitis and dysuria [11]. Its bacteriostatic action in alkaline urine is related to hydroquinone glucuronide and hydroquinone sulfate formed after arbutin liver metabolism in the body. The maximum disinfection effect is 3–4 h after consumption [12, 13]. Due to the outstanding properties of arbutin and its applications in medicine and pharmacy, *P. boissieriana* can be used as a valuable source of arbutin. Also, the antifungal effects (anti-*Candida* and *Cladosporium*), antibacterial, and anti-larval effects of this plant have been proven [14, 15]. Furthermore, the phenolic compounds in *P. boissieriana* cause strong antioxidant as well as antimicrobial activity by releasing free radicals [16]. In this study, considering the outstanding properties of phenol glycoside (arbutin) and other effective compounds in *Pyrus boissieriana*, we evaluated the biological effects of the arbutin-rich fraction of *P. boissieriana* aerial parts on *Toxoplasma gondii* *In Vitro* and *In Vivo*.

Materials and Methods

Ethics Approval

The study protocol has been approved by the ethics committee of Shiraz University of Medical Sciences (Ethical code: IR.SUMS.MED.REC.1400.038). All experiments conform to institutional guidelines for the care and use of laboratory animals of the Institutional Animal and Ethics Committee, Shiraz University of Medical Sciences.

Plant Materials

Pyrus boissieriana, which grows in the Hyrcanian region of the forests of northern Iran, was obtained from there. The plant was identified by the Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Iran, and a voucher specimen was deposited with herbarium number: MPPRC-01-01. The aerial parts of *P. boissieriana* were dried away from direct sunlight in normal air flow and heat.

Preparation of Arbutin-Rich Fraction

The dried plant was pulverized and then extracted by percolation with hydroalcoholic solution (ratio 4:1). This extract was subjected to column chromatography on silica gel 60 (Merck, Germany) with the methanol: chloroform (3:7) %, v/v, as mobile phase. This system was used previously for arbutin extraction [17]. Then this arbutin-rich fraction was concentrated by a rotary evaporator and then dried and finally powdered.

Tachyzoites Preparation

The virulent RH strain of *T. gondii* for use in this study was maintained by serial intraperitoneal passages in female BALB/c mice (4–5-day intervals). Purification of tachyzoites was based on a previous study [18]. Briefly, after 4–5 days, the infected mice were killed following ethical guidelines. Then, peritoneal fluid was aspirated from the infected mice in phosphate-buffered saline (PBS), and the tachyzoites were purified by centrifugation. Initially, it was centrifuged at 200g for 10 min to remove host cells and debris. Then, the supernatant containing parasites was collected and centrifuged at 1000g for 10 min. The pellet containing tachyzoites was washed three times with PBS at pH 7.2.

Animal Model

Inbred female BALB/c mice (4–6 weeks old) were obtained from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. The weight of the animals was between 20 and 22 g. The animals were kept in cages at a temperature of 22 ± 2 °C and a 12-h light and 12-h dark cycle with 40–50% relative humidity. All animals had convenient access to drinking water and standard food during the experiment.

Cell Culture

J774 murine macrophage cell lines were obtained from the Department of Immunology, Shiraz University of Medical Sciences. DMEM medium (Sigma Company, USA) containing 10% heat-inactivated fetal calf serum (FCS; Gibco Company, USA) and 100 IU/mL penicillin–100 µg/mL streptomycin (Roche Company) was used to maintain and culture the cells at 37 °C with 5% CO₂ (Corning Costar UK, UK). The cells were routinely subcultured every 3 days by trypsinization and washing with PBS (pH 7.2).

Cytotoxicity Using MTT Assay

For the MTT assay, 100 μL of the culture media in each well of a 96-well plate (containing 3×10^4 macrophage cells) were incubated at 37 °C with 5% CO_2 for 24 h. Then, the cells were exposed to different concentrations of the *P. boissieriana* fraction (1–512 $\mu\text{g}/\text{mL}$ in PBS) for 48 h. All experiments were performed in triplicate. After this incubation, 20 μL of the MTT-PBS solution (5 mg/mL) was added to each well. The plate was then covered with aluminum foil (dark condition) and incubated at 5% CO_2 at 37 °C for 4 h. Positive controls were 400 $\mu\text{g}/\text{mL}$ of sulfadiazine (a conventional drug for toxoplasmosis), and DMEM was used as a control buffer. Then, the supernatant was removed, and 100 μL of acidic isopropanol was added to each well and shaken for 15 min. The optical density was measured at an absorbance of 570 nm using an ELISA reader (Bio-Rad). The cell viability percentage was calculated using the following formula [19]:

$$\text{Cell viability (\%)} = \frac{\text{OD}_{\text{Test}} - \text{OD}_{\text{Blank}}}{\text{OD}_{\text{Control}} - \text{OD}_{\text{Blank}}} \times 100$$

$$\text{Cytotoxicity (\%)} = 100 - \text{Viability (\%)}$$

In Vitro Anti-Toxoplasma Activity Using Flow Cytometry

For the flow cytometry assay, 15×10^4 tachyzoites/500 μL were deposited in each well and mixed with different concentrations (1–512 $\mu\text{g}/\text{mL}$ in PBS) of the fraction at a final volume of 1000 μL . After a 3-h incubation at room temperature, to stain apoptotic tachyzoites, propidium iodide (PI) was added to each microtube at a final concentration of 50 $\mu\text{g}/\text{mL}$. The microtubes were incubated for half an hour in the dark. Saponin 0.2% and a tube without treatment were used as positive and negative controls, respectively. Parasite death based on staining and fluorescence of PI was estimated by flow cytometry.

In Vivo Anti-Toxoplasma Activity in Mice

For the *In Vivo* evaluation of anti-*Toxoplasma* activity, forty female BALB/c mice (4–6 weeks old) were divided into 8 groups of 5 and kept in cages. All mice were infected with 10^5 tachyzoites by subcutaneous injection. After one day, different concentrations (16, 32, 64, 128, 256, and 512 mg/kg -1/day-1) of the fraction were orally gavaged for 7 days. One group was not given any medication or the fraction as a negative control, and a group was given sulfadiazine (400 mg/L /day of sulfadiazine in drinking water) as a

positive control. Also, a group of healthy mice received the highest concentration of the fraction (512 mg/kg) to evaluate the possible side effects of the fraction used. All groups were monitored every 24 h until the death of the last mouse, a maximum of 30 days, and daily deaths were recorded.

Statistical Analysis

Statistical analysis and graphs were performed using SPSS Software version 16 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA). All values were expressed as the mean \pm SD. For multiple comparisons *In Vitro*, the Kruskal–Wallis test or one-way ANOVA was performed. The survival time of the *In Vivo* studied groups was compared using the Kaplan–Meier method, and statistically significant was considered $P < 0.05$.

Results

Cytotoxicity Activity of *P. boissieriana*

The viability of J774 cells in the presence of different concentrations of the fraction after 48 h shows that 512 $\mu\text{g}/\text{mL}$ and 1 $\mu\text{g}/\text{mL}$ with 61.3% and 98.1% viability had the highest and lowest toxicity, respectively, with an IC_{50} value of 984.3 $\mu\text{g}/\text{mL} \pm 0.76$ (Fig. 1). It was also found that none of the concentrations was above 50% toxicity, and no significant difference was observed with the negative control group ($P > 0.5$).

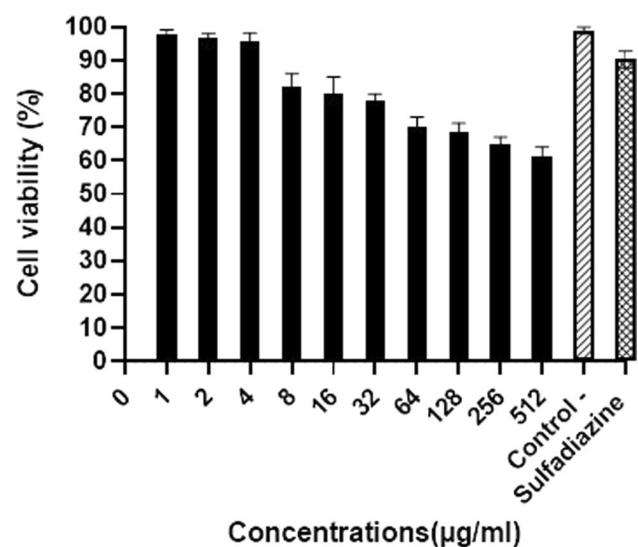


Fig. 1 Cytotoxicity of different concentrations of arbutin-rich fraction of *P. boissieriana* aerial parts on macrophage cells after 48 h

In Vitro Effect of *P. boissieriana* on *T. gondii* Tachyzoites

Flow cytometry results showed that the effect of the arbutin-rich fraction of the *P. boissieriana* aerial parts was directly related to the dose of the fraction and the mortality of tachyzoites. The concentration of 512 µg/mL with 89.6% and 1 µg/mL with 25.9% showed the highest and lowest lethality, respectively. The calculated IC₅₀ value was 18.32 µg/mL ± 0.37. Also, in the unexposed group, the viability of the *Toxoplasma* tachyzoites was 88.31%, and in the positive control (Tachyzoites that were exposed to 0.2% saponin), the mortality rate was 92.08% (Fig. 2).

In Vivo Effect of *P. boissieriana* on *T. gondii* Infection

The survival time of the mice in different groups was recorded (Fig. 3). In the negative control group, the death

rate of mice was from day 5 to day 8 (mean survival rate: 6.2 days). In the positive control group (sulfadiazine), the mean survival rate was 25.8 days, and in the toxicity assessment group, the mice did not die until the end of the experiments. In treated groups with different concentrations of the fraction, mortality was observed from day 5 to day 16. The mean survival rate of mice in the groups treated with concentrations of 16, 32, 64, 125, 256, and 512 mg/kg were 6.6 days, 7.4 days, 10.4 days, 12.8 days, 13.6 days, and 15 days, respectively.

There was no significant difference between the survival time of the treatment groups and the positive control group ($P > 0.05$), although between the negative control group and the concentration of 512 mg/kg ($P < 0.001$), 256 mg/kg ($P = 0.009$), 128 mg/kg ($P = 0.018$), and 64 mg/kg ($P = 0.021$) of the fraction were significant differences. The comparison between other treatment groups and the control group did not show a significant difference ($P > 0.05$).

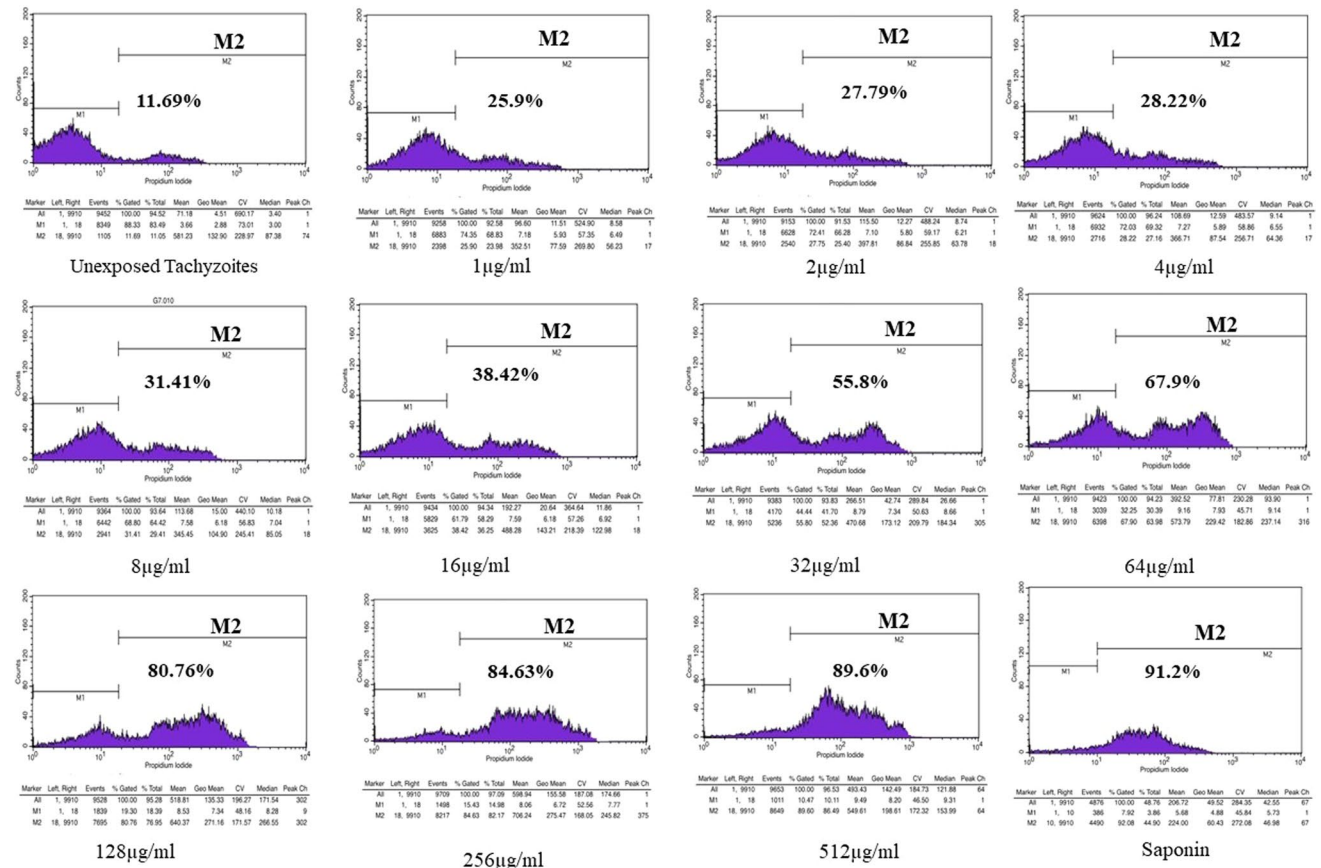
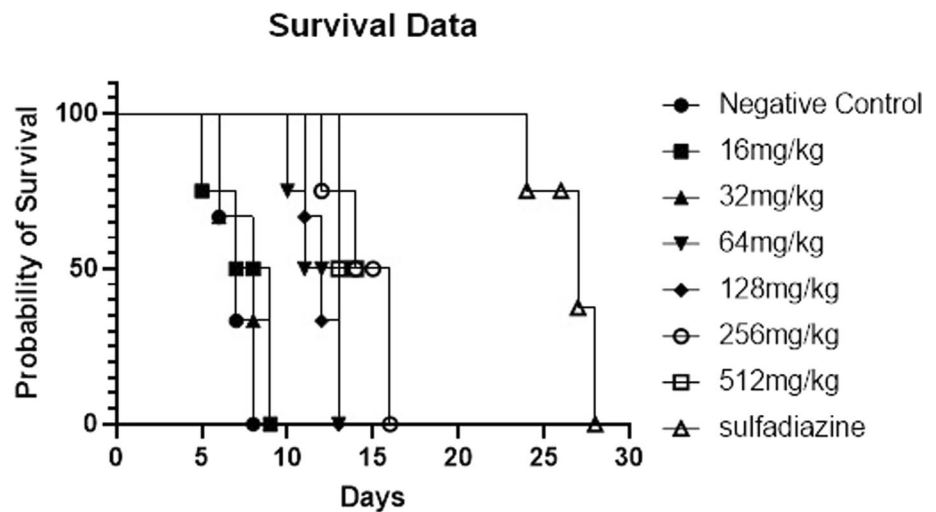


Fig. 2 The flow cytometry results of *In Vitro* effect of *P. boissieriana* aerial parts arbutin rich fraction on *T. gondii* tachyzoites. saponin and unexposed tachyzoites was positive and negative controls. The PI dye

stains the DNA of dead parasites. The M2 area indicates the percentage of dead tachyzoites

Fig. 3 Survival time of the mice treated with different doses of *P. boissieriana* aerial parts arbutin-rich fraction in different groups and control groups



Discussion

P. boissieriana is one of the effective herbal medicines that have been used in traditional medicine; however, there is limited available research on its antiparasitic activity [20]. In the present study, the lethality of *Toxoplasma* tachyzoites after exposure to different concentrations of *P. boissieriana* arbutin-rich fraction was between 25.9 to 89.6%. The concentrations of 512, 256, 128, and 64 mg/kg of the fraction significantly increased the survival rate of *Toxoplasma*-infected mice. Also, our study showed that *P. boissieriana* arbutin-rich fraction can kill *Toxoplasma* tachyzoites at a much lower concentration than the toxic concentration for the cell, which can be a very important and good advantage of this fraction (IC₅₀ values 18.32 µg/mL vs. 984.3 µg/mL, respectively).

Various studies have examined the antimicrobial aspects of *Pyrus boissieriana*. Azadbakht *et al.* [21], showed that methanolic and dichloromethane extracts of *P. boissieriana* leaves have antifungal (anti-*Candida* and *Cladosporium*), antibacterial (*Bacillus subtilis*), antioxidant, and anti-larval effects. Guven *et al.* [14], described the antimicrobial activity of ethyl acetate extract from the fruits of several species of *Pyrus* (Rosaceae). Jin *et al.* [22], confirmed the strong antibacterial activity of *P. boissieriana* extract against *Erwinia amylovora*. The results of these studies, in line with our study, confirm the antimicrobial activity of *Pyrus boissieriana*. The presence of compounds such as phenol, arbutin, and benzoquinones (a metabolic product of arbutin) in *P. boissieriana* can be the reason for this [22]. Arbutin, as a very useful biological substance, is present in large quantities in *Pyrus boissieriana*, which has proven its antioxidant and antimicrobial activity [8]. The lack of significant adverse effects of arbutin and plant extracts containing it makes them a valuable medical agent [23]. In some investigations, the

anti-inflammatory, antioxidant, and antibacterial potential of arbutin have each been documented [24, 25]. Also, Adeyemi *et al.* [26], showed a mild suppressive action of arbutin against the *In Vitro* growth of *T. gondii* (IC₅₀: 17.20 µg/mL). Arbutin has been shown to inhibit tyrosinase activity through different mechanisms of action [27]. Tyrosinase is an important and vital enzyme of *Toxoplasma* that is essential in tyrosine metabolism. Inhibition of this enzyme by disturbing tyrosine metabolism has been shown to reduce parasite growth as well as its pathogenicity [28]. Phenolic compounds in *P. boissieriana* have also been shown to be involved in its antioxidant and antimicrobial activity by releasing free radicals [16].

Conclusion

The results of this study showed that an arbutin-rich fraction of *P. boissieriana* aerial parts dose-dependently increases the mortality of *Toxoplasma* tachyzoites and significantly increases survival time in mice infected with acute toxoplasmosis.

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Author contributions QA, AH, and APL Conceived and designed the experiments. MSB, SFP, MGh, and MH Performed the experiments. MSB and QA Analyzed and interpreted the data. QA Contributed reagents, materials, analysis tools, or data. The first draft of the manuscript was written by MSB and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data sharing statement The authors confirmed that all the data for this manuscript are available; if someone wants to request the data, they can contact the corresponding author.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics and consent statement The current study was approved by the Ethical Committee of Shiraz University of Medical Sciences, Shiraz, Iran (ethical code: IR.SUMS.MED.REC.1400.038).

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