Original article

# Anti-*Toxoplasma* Effects of Methanol Extracts of *Feijoa sellowiana*, *Quercus castaneifolia*, and *Allium paradoxum*

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## Abstract

**Objectives:** The currently available agents for use against toxoplasmosis have serious limitations. Thus, the aim of the present study was to investigate the anti-*Toxoplasma gondii* (*T. gondii*) activities of methanol extracts of *Feijoa sellowiana* (*F. sellowiana*) (leaves and fruits), *Quercus castaneifolia* (*Q. castaneifolia*) (fruits), and *Allium paradoxum* (*A. paradoxum*) (leaves) *in vitro* and *in vivo*.

**Methods:** Vero cells were treated with different concentrations (from 0 to 400 µg/mL) of the above extracts or with pyrimethamine at a dose of 50 mg/mL (positive control). Then, the viabilities of the *T. gondii*-infected cells were measured by using colorimetric MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide) assays. In addition, the survival rates of mice acutely infected with $2 \times 10^4$ RH strain tachyzoites of *T. gondii* were examined *in vivo* after intraperitoneal injection of the extracts at doses of 100 and 200 mg/kg/ day for 5 days.

**Results:** In the *in vitro* anti- *T. gondii* assay, the $IC_{50}$ values were 12.77, 180.2, 74.73, 213.2 and 163.8 µg/mL, and the selectivity indices were 6.05, 1.31, 0.35, 0.69 and 1.30 for the *F. sellowiana* (leaves and fruits), *Q. castaneifolia*, and *A. paradoxum* extracts and pyrimethamine, respectively. Moreover, the mice treated with *F. sellowiana* (leaves and fruits) achieved better results in terms of survival than the others ($P < 0.05$).

**Conclusion:** The results of the current study indicate that methanol extract of *F. sellowiana* has significant anti-*Toxoplasma* activity. Further study should be conducted to investigate the potential bioactivity of this extract through bioactivity-guided fractionation.

## 1. Introduction

*Toxoplasma gondii* (*T. gondii*) is a ubiquitous intracellular protozoan parasite that causes toxoplasmosis in humans and other warm-blooded animals [1]. Globally, over 1 billion people are estimated to be infected with *Toxoplasma* [2]. Acute toxoplasmosis in immunocompetent people is usually subclinical and asymptomatic, but may lead to chronic infection. However, toxoplasmosis can lead to serious health problems and

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mortality among immunocompromised individuals, such as human immunodeficiency virus (HIV) positive people, cancer patients, and organ transplant recipients, and congenitally infected neonates [3-6].

The recommended drugs for treatment of toxoplasmosis are pyrimethamine and sulfadiazine [7]. In addition, azithromycin, clarithromycin, spiramycin, dapsone, atovaquone, and cotrimoxazole have been used to treat clinical toxoplasmosis [8-10]. Unfortunately, these atovaquone drugs are poorly tolerated and have no effect on the bradyzoite form [7, 11]. Moreover, the recommended drugs have side effects, such as neutropenia, severe drops in the platelet count, thrombocytopenia, leukopenia, abnormalities, and hypersensitivity reactions [7, 12]. Furthermore, resistance to anti-*T. gondii* drugs has been reported among acquired immunodeficiency syndrome patients [13]. Accordingly, new drugs with low toxicity and teratogenicity, effective penetration through and concentration in the blood-brain barrier and placenta, parasiticidal effect against the different stage of *T. gondii*, particularly the cystic form, are urgently needed [14].

In recent years, studies on the therapeutic potential of natural or herbal products have been increasing. Medicinal plants are generally considered to be safe and to have low toxicity compared with synthetic drugs [15]. According to our literature review, the extracts of *Artemisia annua L.*, *Glycyrrhiza glabra L.*, *Eurycoma longifolia*, *Vernonia colorata*, *Ginkgo biloba*, *Allium cepa*, *Zingiber officinale*, *Myristica fragrans*, *Astragalus membranaceus Scutellaria baicalensis*, *Myrrh*, *Nigella sativa*, *Sambucus nigra*, etc. have been tested for the treatment of toxoplasmosis [16].

*Feijoa sellowiana* (*F. sellowiana*) (Myrtaceae) is a small ever green shrub native to the southern areas of South America, where it is extensively distributed. It is widely cultivated in many countries, including Iran [17, 18]. *Quercus castaneifolia* (*Q. castaneifolia*) is a plant native to the forests of north, northwest, and west Iran [19]. *Allium paradoxum* (*A. paradoxum*), *Alezi*, is a perennial herbaceous species cultivated in the northern area of Iran, especially in Mazandaran [20]. To the best of our knowledge, we have evaluated *in vitro* and *in vivo* for the first time the effects of methanolic extracts from *F. sellowiana* (leaves and fruits), *Q. castaneifolia* (fruits), and *A. paradoxum* (leaves) on *T. gondii* infection.

## 2. Materials and Methods

This study was performed according to institutional animal ethics guidelines of the Animal Research Center, Mazandaran University of Medical Sciences (ARCMUMS). The animal protocols used in this research were approved by the Mazandaran University of Medical Sciences Ethics Committee (MUMSEC) (Permit number 492).

RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin solution, and trypsin-EDTA (Sigma, USA) were used for the cell cultures. Dimethyl sulfoxide (DMSO, 99% purity) and MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide) were purchased from Merck Chemical Company, Germany. Six-week-old, female, inbred Balb/c mice weighing 18 - 20 g were used

in this study. The mice were housed in cages (n = 5) under standard laboratory conditions with an average temperature of 20 - 25°C and were given drinking water and a regular mouse diet [21].

Three types of plants were used in this study: *F. sellowiana* leaves and fruits, *Q. castaneifolia* fruits, and *A. paradoxum aerial* parts were collected from different areas of Mazandaran, Iran, in autumn 2015 and were confirmed by Dr. B. Eslami, Assistant Professor of Plant Systematic and Ecology (Department of Biology, Islamic Azad University of Qhaemshahr, Iran). Voucher specimens were deposited in the Sari School of Pharmacy herbarium (Nos. 194, 195, 991 and A4411). Parts were dried completely in the shade and were triturated in a mechanical mill. The powders were kept in tightened protected containers before use.

To obtain the methanolic extract, we added 150 g of dry powder to 350 mL of pure methanol and used a magnetic stirrer to mix the solution gradually for 1 hour. After the extraction procedure had been completed, the solution was left at room temperature overnight. It was then stirred again and filtered through Whatman No. 1 filter paper, after which the solvent was removed at 40°C by using a rotary evaporator. The remaining semisolid material was freeze-dried at -50°C for 24 h. The obtained filtrate (14.5 g) was placed into a sterile glass container and was stored at 4°C for future use [22]. In order to prepare stock solution (the most concentrated solution), we dissolved 0.02 grams of the extract powders in a minimum amount of RPMI with 10% FBS. A filtration was done using a 0.22-micron filter. Then, the extract was diluted with complete culture medium to a 5-mL volume. Other concentrations (200, 100, 50, 20, 10, and 5 µg/mL) were obtained by dilution of the stock. The solvent was complete culture medium. All processes were performed under sterile conditions. In order to increase the preciseness during this process, we obtained the concentrations in greater volumes than usual.

The RH strain of *T. gondii* was provided by the Toxoplasmosis Research Center (TRC) in Mazandaran University of Medical Sciences, Sari, Iran. *T. gondii* tachyzoites were harvested from the peritoneal cavity of Balb/c mice 3 - 4 day after intraperitoneal injection with $1 \times 10^5$ of the parasite. The tachyzoites were suspended in sterile phosphate-buffered saline (PBS; pH = 7.4) containing 100 IU/mL of penicillin and 100 µg/mL of streptomycin [23]. The number of tachyzoites was determined by counting them in a hemacytometer under light microscopy. Vero cells (ATCC No. CCL-81) were used for *in vitro* assays. Cells were cultivated in RPMI-1640 medium with the addition of 10% inactivated FBS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. Cultures were kept in disposable cell culture flasks and incubated at 37°C with 5% $CO_2$.

The cytotoxicity analysis was done on Vero cells. Cells were seeded in 96-well plates at a density of $1 \times 10^5$ cells/mL suspended in RPMI supplemented with 10% FBS. After 24 h, the cells were exposed to methanol extracts of *F. sellowiana* leaves, *F. sellowiana* fruits, *Q. castaneifolia* fruits, *A. paradoxum* leaves, and pyrimethamine (positive control) at final concentrations of 5, 10, 20, 50, 100, 200 and 400 µg/mL; the culture medium was used as a negative control. The next day, the viabilities of the Vero cells were evaluated by using a colorimetric MTT assay [24, 25]. Then
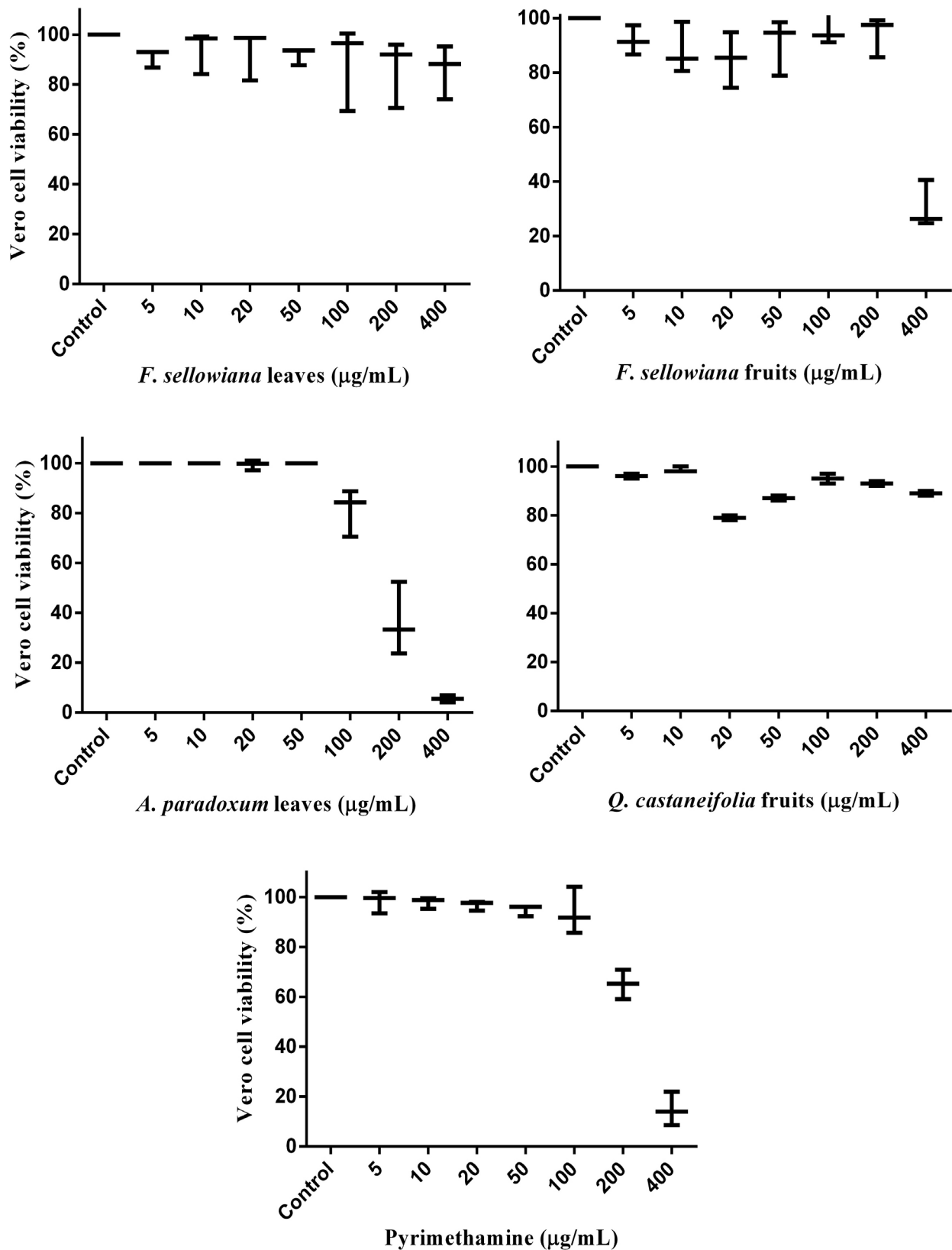
**Figure 1** Cell viability verified by MTT assay in Vero cells treated with *F. sellowiana* (fruits and leaves), *Q. castaneifolia* (fruits), *A. paradoxum* (leaf) extracts and pyrimethamine.
MTT, (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide); *F. sellowiana, Feijoa sellowiana*; *Q. castaneifolia, Quercus castaneifolia*; *A. paradoxum, Allium paradoxum.*

**Table 1** Values of $CC_{50}$, $IC_{50}$ and SI in uninfected Vero cells and infected cells

| Extract/drug (µg/mL) | $CC_{50}$ (µg/mL) | $IC_{50}$ (µg/mL) | SI* |
|---|---|---|---|
| *F. sellowiana* leaves | 77.36 | 12.77 | 6.05 |
| *F. sellowiana* fruits | 236.5 | 180.2 | 1.31 |
| *Q. castaneifolia* fruits | 26.81 | 74.73 | 0.35 |
| *A. paradoxum* leaves | 148.5 | 213.2 | 0.69 |
| Pyrimethamine | 218.7 | 163.8 | 1.30 |

*SI = $CC_{50}/IC_{50}$.

$CC_{50}$, half cytotoxic concentration; $IC_{50}$, half maximal inhibitory concentration; SI, selectivity index; *F. sellowiana*, *Feijoa sellowiana*; *Q. castaneifolia*, *Quercus castaneifolia*; *A. paradoxum*, *Allium paradoxum*.

the 50% cytotoxic concentrations ($CC_{50s}$) were calculated by using the Graph Pad Prism 6.0 software (Graph Pad Software, Inc., San Diego, USA).

Vero cells at concentrations of $1 \times 10^5$ Vero cells/mL, suspended in RPMI supplemented with 10% FBS, were seeded in 96-well plates. After 8 h of seeding, the cells were infected with the RH strain of *T. gondii* ($1 \times 10^6$ tachyzoites/mL) and placed in a 37°C incubator maintained at 5% $CO_2$ for 24 h. After that, the cells were exposed to the extracts and to pyrimethamine at final concentrations from 0 to 400 µg/mL; the culture medium was used as a negative control.

The next day, the viabilities of the *T. gondii*-infected Vero cells were evaluated by using MTT assays [24, 25]. Then, the 50% inhibitory concentrations ($IC_{50s}$) were calculated by using the Graph Pad Prism 6.0 software. In addition, selectivity indices (SIs) of the drugs were calculated using the $IC_{50}$ and the host-cell cytotoxicity profiles (SI = $CC_{50}/IC_{50}$).

Initially, for control of the drugs' side effects, a preliminary experiment was done on Balb/c mice receiving the same doses of drugs, and the maximum dose at which no mortality or clinically significant toxicity was observed was measured. Then, 50 female Balb/c mice were intraperito-
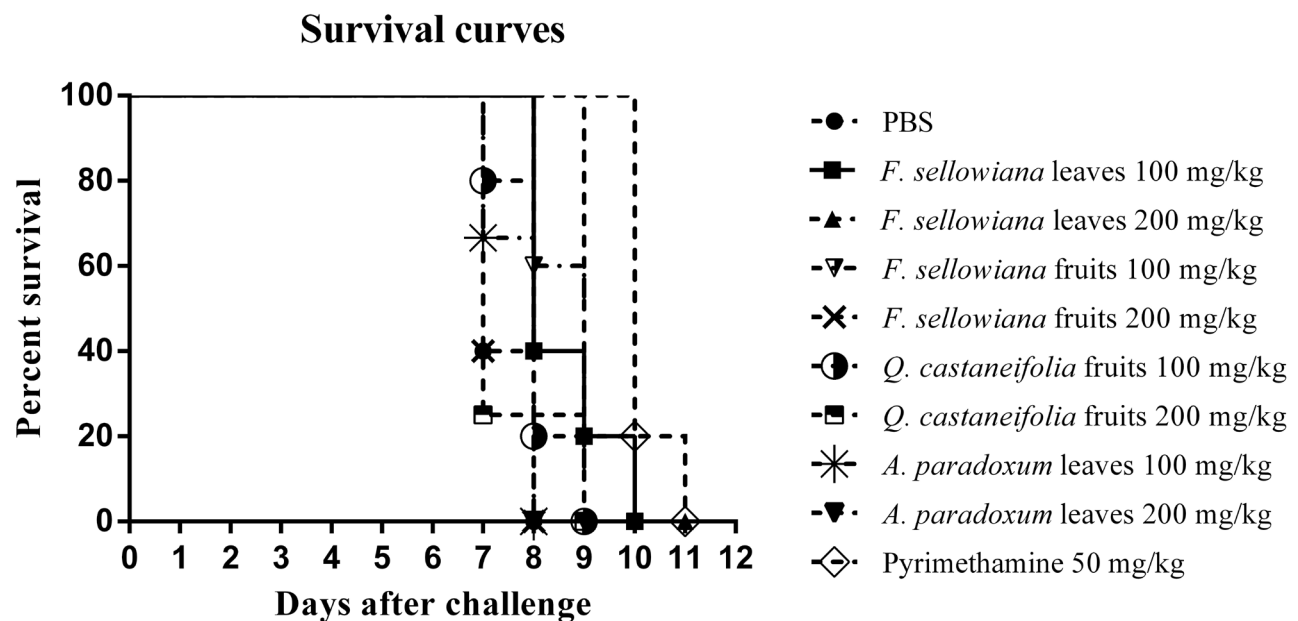


**Figure 2** Survival curves for mice following acute toxoplasmosis. Balb/c mice (n = 5) infected with $2 \times 10^4$ tachyzoites of the *T. gondii* RH strain were treated for 5 days *via* the intraperitoneal route with methanolic extracts of *F. sellowiana* (fruits and leaves), *Q. castaneifolia* (fruits), and *A. paradoxum* (leaves) at doses of 100 and 200 mg/kg/day, pyrimethamine at a dose of 50 mg/kg/day (positive control), and PBS (negative control).

*T. gondii*, *Toxoplasma gondii*; *F. sellowian*a, *Feijoa sellowiana*; *Q. castaneifolia*, *Quercus castaneifolia*; *A. paradoxum*, *Allium paradoxum*. PBS, phosphate buffer saline.

neally inoculated with $2 \times 10^4$ tachyzoites of *T. gondii* RH strain and distributed into 10 groups with 5 animals in each group. On the same day, the mice were intraperito- neally injected with the following extracts and the controls, after which injections were done at regular 24-h intervals for 5 days: *F. sellowiana* leaves and fruits, *Q. castaneifolia* fruits, and *A. paradoxum* leaves extracts at doses of 100 and 200 mg/kg/day, pyrimethamine at a dose of 50 mg/ kg/day (positive control), and PBS (negative control). The survival times were recorded daily until all mice had died.

All tests in this study were performed in triplicate. Statisti- cal analyses were performed using SPSS-14 and Graph Pad Prism 6.0 software. Also, the Kaplan-Meier curve was used to show the survival times, and differences were compared using the log-rank test. Differences were considered statis- tically significant when $P < 0.05$.

## 3. Results

Treatments of Vero cells with different concentrations of the extracts and pyrimethamine were not toxic compared to the negative control (Fig. 1). However, treatment with *F. sellowiana* fruit extract and pyrimethamine at a concen- tration of 400 µg/mL reduced the viability of Vero cells compared to the control while treatments at concentra- tions from 5 to 200 µg/mL did not affect cell viability. Nei- ther did treatments with *F. sellowiana* leaf and *Q. castanei- folia* extracts at doses from 5 to 400 µg/mL. Moreover, cells treated with *A. paradoxum* extract at concentrations of 200 and 400 µg/mL showed reduced cell viabilities compared to the control while those treated at concentrations from 5 to 100 µg/mL showed no effect on cell viability.

In this study, the growth inhibitions of *T. gondii* in infect- ed cells were measured at different concentrations of the *F. sellowiana*, *Q. castaneifolia*, and *A. paradoxum* extracts and pyrimethamine. *F. sellowiana* leaves showed anti-*T. gondii* activity with an $IC_{50}$ of 12.77 and SI of 6.05. In addi- tion, pyrimethamine's activity was characterized by an $IC_{50}$ of 163.8 and SI of 1.33 (Table 1).

Clinically, the numbers of mice in the untreated infected groups (negative control) started to decrease on the sev- enth day of the study, and all mice had died by the end of the eighth day. Mice in the other treatment groups started to die on the eighth day, and all mice had died by the end of the eleventh day. The treatment with *F. sellowiana* leaves achieved better survival results in the mice than treat- ments with *F. sellowiana* fruits, *Q. castaneifolia*, and *A. paradoxum* did (Fig. 2). Mice in the groups treated with *F. sellowiana* fruits at a dose of 100 mg/kg/day and with *F. sel- lowiana* leaves and *A. paradoxum* at doses of 100 and 200 mg/kg showed statistically significant higher survival rates than the mice in the untreated infected control group ($P < 0.05$). Statistically significant differences were observed among the group treated with F. sellowiana fruit at a dose of 100 mg/kg/day), the groups treated with *F. sellowiana* leaves, *Q. castaneifolia*, and *A. paradoxum* at a dose of 200 mg/kg/day), and the positive control ($P < 0.05$).

## 4. Discussion

The standard anti-*T. gondii* chemotherapy is still limited as it is not well-tolerated by immunocompromised patients and cannot completely eradicate tissue cysts produced by the parasite [14]. Thus, new, less toxic compounds for use against *Toxoplasma* are critically needed [12, 26]. Natural compounds and traditional herbal medicine have high availability and lower side effects compared with current chemical drugs [16]. This study was, therefore, performed to evaluate *in vitro* and *in vivo* the activities of methanol extracts of *F. sellowiana*, *Q. castaneifolia*, and *A. paradox- um* against *T. gondii*. To the best of our knowledge, such a study has not been previously reported in the literature.

In our *in vitro* results, the anti-*T. gondii* activity of the agents was calculated as the selectivity index. The selectiv- ity index was indirectly applied to the cell viability of Vero cells to represent the parasite infection ratio. According to the results, the SIs of the different compounds were ob- tained in the following order: *F. sellowiana* leaves > *F. sel- lowiana* fruits > pyrimethamine > *A. paradoxum* leaves > *Q. castaneifolia* fruits. Thus, *F. sellowiana*, especially *F. sel- lowiana* leaves, showed an anti-*T. gondii* activity against *T. gondii*-infected Vero cells higher than that of the standard treatment.

*F. sellowiana* has multi-pharmacological effects, includ- ing antimicrobial, antifungal, antioxidation, antidepres- sant and anti-cancer activities, which has been confirmed by several researchers [17, 18, 27, 28]. The leaves and the fruits of *F. sellowiana* contain many basic components, such as flavonoids, tannins, terpenes, and steroidal sa- ponins [29]. Similarly, Zhang *et al* showed that the activi- ties and SIs of oxymatrine and matrine, the main alkaloids in sophora leguminous plants, were higher than that of spiramycin against the RH strain of *T. gondii*-infected HeLa cells [30].

In our study, the effects of *F. sellowiana*, *Q. castaneifolia*, and *A. paradoxum* were analyzed in acute infections with *T. gondii* RH strain in Balb/c mice *in vivo*. Given that a con- centration of 400 µg/mL was found to be toxic in *in vitro* studies on some extracts, we selected concentrations of 100 and 200 mg/kg/day for our *in vivo* study. Treatment of experimental mice with *F. sellowiana* and pyrimethamine for 5 days after infection with $2 \times 10^4$ tachyzoites of the *T. gondii* RH strain statistically increased their survival rates when compared to mice in the untreated infected control. Although, the mice treated with methanol extract of *F. sel- lowiana* leaves (200 mg/kg/day) exhibited better survival than the mice treated with the other methanol extracts, the differences in survival between those mice and the mice in either the positive or the negative control groups were not statistically significant, indicating that that the activity of *F. sellowiana* is probably due to its high antioxidation properties and its high phenolic and flavonoid contents [17]. Keles *et al* demonstrated that *F. sellowiana* extracts displayed remarkable antioxidant activity and decreased lipid peroxidation in rats [29].

Moreover, *Q. castaneifolia* increased the survival rate of the mice compared to that of the mice in the untreated infected control, and the differences compared to *F. sel- lowian*a leaves (200 mg/kg/day) and pyrimethamine

were statistically significant. *Q. castaneifolia* fruit extract contains many basic components and has antimicrobial effects on the *E. coli*, *salmonella typhimurium*, *Shigella dysenteriae* and *Yersinia enterocolitica* [31]. Also, a significant difference existed between *A. paradoxum* and the untreated group (negative control) and the group treated with *F. sellowiana* fruits (200 mg/kg/day). Interestingly, Raeisi and Rahimi Esboei showed that *A. paradoxum* was not significantly effective during hydatid cyst treatment at concentrations of 1, 10, 50 and 100 mg/mL [32].

A. *paradoxum* has been reported to be an Iranian native plant that exhibits antimicrobial, antifungal, and antiviral effects. Also, the aerial extracts of *A. paradoxum* have been reported to exhibit antioxidant activity [31]. B-Pinene, limonene, Z-nerolidol, spathulenol, alpha-bisabolol, phytol, n-docosane and n-tricosane are the most important reported chemical components of *A. paradoxum* [31]. In an *in vivo* study, Hezarjaribi *et al* found that an ethanol extract of *A. paradoxum* at a concentration of 100 mg/mL was more effective for treating *Giardia* cysts than the chloroform extract [33]. In addition, Elmi showed that the ethanol extract of *A. paradoxum* was significantly more effective than the chloroform extract in the treatment of mice infected with Giardia parasites [34]. Our present study showed that the methanol extract of *A. paradoxum* exhibited no significant anti-*Toxoplasma* activity *in vitro* and *in vivo*. Based on these results, we hypothesize that the ethanol, liquid, and chloroform extracts of *A. paradoxum* may be more active against toxoplasma infection than the methanol extract.

## 5. Conclusion

In conclusion, this is the first report on the anti-*Toxoplasma* activities of methanol extracts of *F. sellowiana*, *Q. castaneifolia*, and *A. paradoxum*. Given that the *Q. castaneifolia* extract at the concentration used in this study had no toxicity in the *in vitro* study, higher concentrations should be used in future studies to evaluate its therapeutic potential *in vitro* and *in vitro*. The results of the current study indicate that *F. sellowiana* promotes significant activity against *T. gondii*. Further study should be designed to investigate the potential bioactivity of this extract through bioactivity-guided fractionation and to identify the additional mechanisms involved in its effects.

## Acknowledgment

## Conflict of interest

The authors declare that they have no conflicts of interest.

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