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Study on anti-*Toxoplasma* effects of *Myrtus communis* and *Artemisia aucheri* Boiss extracts

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Abstract

Achieving an anti-*Toxoplasma* product with high efficacy and low side effect is a priority in research on *Toxoplasma gondii*. Herbal products may be a suitable candidate for this purpose. The present study was performed to evaluate anti-*Toxoplasma* effects of *Myrtus communis* and *Artemisia aucheri* Boiss extracts *in vitro*. *T. gondii* RH strain tachyzoites were exposed to extracts of *M. communis* and *A. aucheri* Boiss both in cell-free medium and cell culture. Mortality rate of tachyzoites and EC50 of the extracts were calculated. The results were analyzed with one way ANOVA, followed by Tukey's HSD using SPSS software. Both extracts demonstrated cidal effects on tachyzoites in cell-free medium. But, in cell culture, EC50 and selectivity of *M. communis* and *A. aucheri* Boiss were significantly lower than pyrimethamine. *M. communis* and *A. aucheri* Boiss extract failed to act as a good alternative to synthetic anti-*Toxoplasma* drugs.

Keywords: *Toxoplasma gondii*, herbal extract, *Myrtus communis*, *Artemisia aucheri* Boiss, Iran

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite with a wide variety of warm-blooded vertebrate hosts. It causes a benign and self-limited lymphadenopathy in immunocompetent patients, a life threatening disease in immunocompromised subjects, and also severe cerebral and orbital syndromes in newborns with congenital toxoplasmosis. Acquired toxoplasmosis occurs by eating raw or uncooked meat containing tissue cysts or raw vegetables and water contaminated with the oocysts excreted by feces of felids. Congenital toxoplasmosis occurs through transplacental transmission of tachyzoites in primary infections during pregnancy [1].

Treatment of toxoplasmosis is commonly achieved by administration of a combination of pyrimethamine and sulfadiazine which have synergistic efficacy on *T. gondii* [2]. Pyrimethamine inhibits dihydrofolate reductase which is a key enzyme in biosynthesis of purines. Sulfadiazine is a competitive inhibitor of dihydropteroate synthetase, an essential enzyme required for proper processing of para-aminobenzoic acid (PABA) which is a vital component for biosynthesis of folic acid. The spectrum of side effects reported for these drugs; include hypersensitivity, nervous system toxicity gastrointestinal, respiratory, cardiovascular, metabolic, hematologic, and oncologic complications [3]. These effects are considered as the most important limitation in the treatment of toxoplasmosis with synthetic drugs. Considering this limitation, it is a priority for *Toxoplasma* researchers to find a new product with high efficacy and low side effect. The use of herbal products may be a good alternative for these drugs.

Anti-*Toxoplasma* activity of some herbal extracts/ fractions has been reported by several authors in recent years [4-8]. In the present study, for the first time, the effect of *Myrtus communis* and *Artemisia aucheri* Boiss extracts on *T. gondii* is described.

M. communis is a species of the family Myrtaceae which was used for treatment of urinary infections, digestive problems, vaginal discharge, bronchial congestion, sinusitis, dry coughs, and also acne, wounds, gum infections, and haemorrhoids [9]. *A. aucheri* Boiss is a shrub from the Asteraceae family and used as astringent, disinfectant, anti-microbial and anti-parasitic agent in traditional medicine [10].

2. Materials and Methods**2.1 Preparation of herbal extracts and pyrimethamine**

The aerial parts of *A. aucheri* Boiss and leaves of *M. communis* were purchased from the Jahad Daneshgahi department in Karaj where the herbs were identified by a botanist.

A sample of each plant kept at the Institute of Medicinal Plants. Extraction was carried out by an expert Pharmacognosy. Briefly, extractions were started after drying and grinding of 500 gram of each herb using percolation method with ethanol 80% at room temperature. Solvent was completely removed by drying under reduced pressure at 40 °C in a rotary evaporator. The samples were stored at 4 °C until use (3 gr, 6% yield) [11]. Different concentrations of the extracts were prepared by dissolving the extracts in 1% dimethyl sulfoxide (DMSO), followed by diluting with phosphate buffered saline (PBS, PH 7.2, and 0.15 M). Also, pyrimethamine (Sigma, USA) was used as positive control. It was dissolved in methanol-acetone (50% v/v) and diluted with RPMI medium.

2.2 Preparation and propagation of *T. gondii* strain

We used RH strain tachyzoites of *T. gondii*, kindly provided by the Department of Parasitology and Mycology, School of Health, Tehran University of Medical Sciences. This strain was propagated by serial intraperitoneal passages in mice in our laboratory. The propagated tachyzoites were collected 72 hours after inoculation by rinsing peritoneal cavity with PBS. The fresh tachyzoites were then washed 3 times with PBS and finally, a suspension containing 10⁷ tachyzoites /ml was used in our experiments.

2.3 Preparation and propagation of Cell culture

Hela cells were purchased from the National Cell Bank of Iran, Pasteur Institute, Iran. Hela cells were maintained in RPMI 1640 (Gibco, U.S.A), containing 10% fetal bovine serum (FBS Gibco, U.S.A), glutamine 2Mm and pen/strep 1000 unit/ml (Gibco, U.S.A). Hela cells were incubated at 37 °C with humid air and 5% CO₂ for 2 days. The harvested cells were treated with trypsin and washed twice in PBS. Viability of cells was confirmed using trypan blue exclusion test [11].

2.4 Evaluation of toxoplasmaicidal effect of herbal extracts in cell-free medium

This assay was carried out in microtubes by treating 50 µl of suspension containing 5×10⁵ tachyzoites with the herbal extracts at concentrations 10, 50, and 100 mg/ml for 10, 30, and 45 min at room temperature. Later, the tachyzoites were stained with alkaline methylene blue. The results were expressed as mortality rate (%) of tachyzoites (unstained tachyzoites) under a light microscope at 400× magnification. All treatments were assayed in triplicate and replicated three times.

2.5 Bioassay in mice

A bioassay technique in mice was used to confirm complete mortality of the tachyzoites exposed to *M. communis* and *A. aucheri* Boiss extracts by staining method. For this purpose, 5×10⁵ of tachyzoites exposed to these extracts were inoculated to 3 mice in each experiment. Also, 5×10⁵ unexposed tachyzoites were inoculated to 3 control mice. All mice were monitored for reduced morbidity and mortality up to one month following.

2.6 Assay for inhibitory effect of herbal extracts on *T. gondii* in cell culture

This assay was carried out based on the procedure used by Kavita et al [7]. Briefly, 6×10⁴ Hela cells (100 µl) harvested during the exponential growth (day 2), were added to 96-well plates and incubated at 37 °C and 5% carbon dioxide for 24 hours. Later, 3×10⁵ tachyzoites (50 µl) were added to each well and incubated at 37 °C and 5% carbon dioxide for six

hours. Subsequently, the plates were washed twice with RPMI 1640 medium without FBS to remove non-adherent tachyzoites. After 24 hours following incubation, 100 µl RPMI 1640 medium supplemented with 2% FBS was added to each well plus 50 µl of different concentrations of the extracts (at final concentration of 50, 100, and 200 µg/ml) or pyrimethamine (at final concentration of 1, 0.1, and 0.01 µg/ml). After 24 h, the anti- *T. gondii* activity and cytotoxicity of these extracts were examined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit ((Bio Idea Company, Iran). This assay was performed according to the manufacturer's instructions and the optical density (OD) were measured with an ELISA microplate reader (Epoch, USA) using an initial wavelength of 570 nm followed by measuring the absorbance at 630 nm as reference. All experiments were performed in triplicate with three replications. The results were presented as cell viability (%), EC₅₀, and selectivity. Cell viability was calculated as follows: the OD obtained for the test samples (Hela cell+tachyzoites+extract) was divided by the OD obtained for the control (Hela cell+tachyzoites) and the result was multiplied by 100. The average effective concentration (EC₅₀) was determined by using Prism software (version 5.04). Selectivity was calculated as follows: the EC₅₀ of Hela cells was divided by the EC₅₀ of *T. gondii* [6].

2.7 Statistical analysis

All results were expressed as mean (SEM) of three measurements. Statistical analysis on data was carried out using ANOVA followed by Turkey's Honestly Significant Differences (HSD) by SPSS software (version 16.0). Data were normalized and transformed using Prism software (version 5.04). Later, the EC₅₀ values were calculated using nonlinear regression (curve fit). The significance level was *P*<0.05.

2.8 Ethics: All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

3. Results & Discussion

3.1 Toxoplasmaicidal effect of herbal extracts in cell-free medium

Both extracts of *M. communis* and *A. aucheri* Boiss showed toxoplasmaicidal effects. The mortality rate of tachyzoites exposed to *M. communis* at concentrations of 50 mg/ml was significantly higher than that found for *A. aucheri* Boiss (*P*<0.001), however, the difference was insignificant at 100 mg/ml concentration. At concentration of 10 mg/ml and treatment times of 10 and 30 min, the mortality rate of tachyzoites exposed to *M. communis* were significantly lower than that obtained for *A. aucheri* Boiss (*P*<0.001) (Table 1).

3.2 Bioassay in mice

All mice inoculated with the tachyzoites exposed to *M. communis* and *A. aucheri* Boiss extracts and showed 100% mortality with methylene blue stain survived up to 1 month after inoculation.

3.3 Inhibitory effects of the herbal extracts on *Toxoplasma gondii* in cell culture

Both extracts of *M. communis* and *A. aucheri* Boiss showed anti-*Toxoplasma* activity in cell culture. The EC₅₀ of *M. communis* extract (122µg/ml) was less than *A. aucheri* Boiss extract (215 µg/ml), however, there was no significant difference between the selectivity of these extracts. Pyrimethamine showed a high inhibitory effect on tachyzoites

compared to the extracts. Similarly, the selectivity of pyrimethamine was significantly higher than those found for our extracts ($P < 0.001$) (Table.2).

The viability percentage of the tachyzoites exposed to the extracts showed a significant decrease in a concentration-dependent manner. Viability of the tachyzoites exposed to pyrimethamine was significantly less than the two extracts (Fig. 1, 2).

We showed that *M. communis* and *A. aucheri Boiss* extracts have antiprotozoal activity which is in agreement with the results of Mahmoudvand et al (2015) and Mojarrab et al (2014) who reported anti-leishmanial and anti-malarial activities for *M. communis* and *A. aucheri Boiss* extracts, respectively [12,13]. There are reports that some herbal extracts/fractions have anti-*Toxoplasma* activities nearly similar to synthetic drugs. For examples, in the study of Jones-Brando et al (2006), 50% inhibitory dose (ID50) of artemisinin derivatives (0.5-2.7 µg/ml) was less than trimethoprim (5.2 µl) [4]. Chen et al (2008) showed the inhibitory effect of ginkgolic acids (GAs) on DNA and protein synthesis of *T. gondii* was higher than trimethoprim [5]. In a study by Choi et al (2013), the fraction 1 obtained from *Zingiber officinale* (ginger) root extract (GE/F1) strongly inhibited the proliferation of *T. gondii*-infected C6 cells and *T. gondii* compared with sulfadiazine [8].

It seems that components with anti-*Toxoplasma* properties make a small percentage of *M. communis* and *A. aucheri Boiss* extracts. In a study by Asghari et al (2012), the main components in the seed essential oils of *A. aucheri Boiss* were

decane, para-cymene, 1,8-cineole, linalool, menth-3-en, 8-ol, borneol, lavandulol, bornyl acetate, chrysanthenyl acetate, dehydroaromadendrene, and caryophyllene oxide [10]. Tuberoso et al (2006) reported that the major compounds in essential oils of *M. communis* L. were alpha-pinene, 1,8-cineole, and limonene found in leaves and berries [14]. Sfara et al (2009) showed that 1,8-cineole has insecticidal activity and repellent effect [15]

We used a bioassay technique in mice to confirm 100% mortality of tachyzoites exposed to *M. communis* and *A. aucheri Boiss* extracts. In our preliminary design, this assay was not considered as a substitute confirmatory test for alkaline methylene blue staining. In fact, in a separate experiment, we obtained an unexpected finding regarding the tachyzoites exposed to an herbal extract (except *M. communis* and *A. aucheri Boiss*) which remained unstained while; their microscopic structure appeared to be intact (unpublished data). We hypothesized that the herbal extract has probably an impact on staining properties of tachyzoites with alkaline methylene blue. Therefore, we used a bioassay procedure in mice as a confirmatory test for tachyzoites staining method. The parasites exposed to this extract were inoculated into 3 mice in whom the parasites caused the death of all animals within 4-5 days after inoculation. Based on this finding, we decided to use this bioassay method for all experiments in which the tachyzoites were exposed to different concentrations of extracts and showed 100% mortality by staining method.

Table 1: Mortality (%) of *T. gondii* RH strain tachyzoites after treatment with ethanolic extracts of *Myrtus communis* and *Artemisia aucheri Boiss* in cell-free medium

Treatment time	Mortality (%) of tachyzoites at three concentrations (mg/ml)					
	10		50		100	
	<i>M. communis</i>	<i>A. aucheri Boiss</i>	<i>M. communis</i>	<i>A. aucheri Boiss</i>	<i>M. communis</i>	<i>A. aucheri Boiss</i>
10	5.91 ± 1.5	9.0 ± 2.4	100	64.8 ± 12.2	100	97.6 ± 2.3
30	6.5 ± 0.9	11.4 ± 3.0	100	79.1 ± 7.0	100	98.9 ± 0.8
45	8.9 ± 1.6	13.2 ± 2.8	100	82.3 ± 4.0	100	99.4 ± 0.5

Table 2: Inhibitory effect of *Myrtus communis* and *Artemisia aucheri Boiss* extracts on *Toxoplasma gondii* tachyzoites in cell culture

Herbal extract/Drug	EC ₅₀ (µg/ml)		Selectivity ^a
	HeLa	HeLa+ <i>T. gondii</i>	
<i>Myrtus communis</i>	111.0	122.0	0.90
<i>Artemisia aucheri Boiss</i>	130.2	131	0.99
pyrimethamine	0.6	0.176	3.40

^a = Ratio of the EC50 value for HeLa cells to the EC50 value for *T. gondii* RH strain (12)

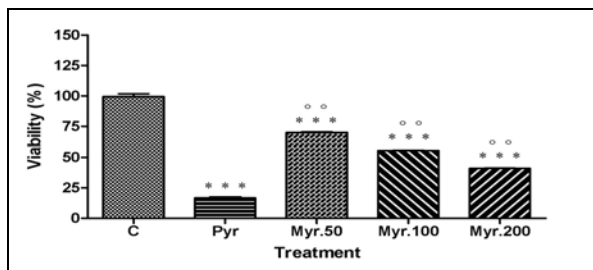


Fig 1: Inhibitory effect of *Myrtus communis* extract (Myr) at concentrations 50, 100, and 200 µg/ml and pyrimethamine at concentration 0.1 µg/ml on *T. gondii* RH strain tachyzoites in cell culture. C= control; Pyr = pyrimethamine; Myr = *Myrtus communis*. *** $P < 0.001$ compared to control, °° $P < 0.01$, compared to pyrimethamine, Tukey- Kramer test

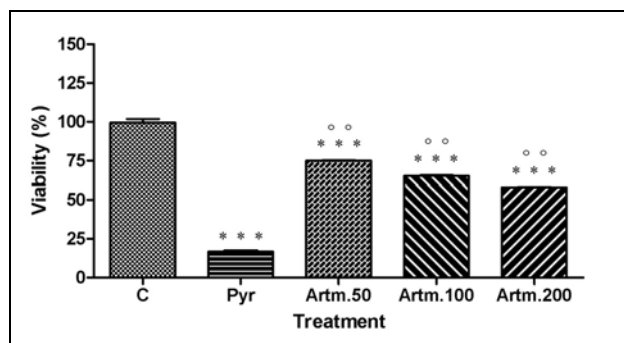


Fig 2: Inhibitory effect of *Artemisia aucheri Boiss* extract (Artem) at concentrations 50-200 µg/ml and pyrimethamine at concentration 0.1 µg/ml on *T. gondii* RH strain tachyzoites in cell culture. C= control; Pyr= pyrimethamine; Artem= *Artemisia aucheri Boiss*. *** $P < 0.001$ compared to control, °° $P < 0.01$, compared to pyrimethamine, Tukey- Kramer test

4. Conclusion

Both *M. communis* and *A. aucheri Boiss* demonstrated anti-*Toxoplasma* activity which was remarkably less than pyrimethamine. It seems these herbal plants are not good candidates for further studies to obtain a component with high anti-*Toxoplasma* property.

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