Ethanol Extracts of *Achillea millefolium* and *Hypericum perforatum* Low Anti-Toxoplasma Activity

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**Abstract**

**Objectives:** This study was performed to determine the lethal and the inhibitory effects of ethanol extracts of *Achillea millefolium* (*A. millefolium*) and *Hypericum perforatum* (*H. perforatum*) on *Toxoplasma gondii* (*T. gondii*) RH strain tachyzoites in vitro.

**Methods:** The tachyzoites were treated with concentrations of 10, 50, and 100 mg/mL of *A. millefolium* and *H. perforatum* extracts within 10, 30, and 45 minutes in the wells. The mortality rates of tachyzoites treated with extracts were determined by using alkaline methylene blue staining. Also, the tachyzoites in cell cultures were treated with concentrations of 50, 100, and 200 mg/mL of these extracts. The cell viability, inhibition concentration (IC⁵₀), and selectivity were determined from MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays.

**Results:** In the cell-free *in vitro* study, all of the tachyzoites were killed at concentrations of 100 mg/mL of both extracts while at concentration 10 mg/mL, the mortality was 4.53% − 5.31%. In the cell culture study, the values of the effective concentration (EC⁵₀) were 215 and 153 μg/mL and the selectivities were 0.73 and 0.69 for the *A. millefolium* and the *H. perforatum* extracts, respectively.

**Conclusion:** We conclude that neither extracts has any significant effect on the tachyzoites of *T. gondii* in cell cultures.

1. **Introduction**

*Toxoplasma gondii* (*T. gondii*) is one of the most common parasitic zoonosis. It can be found worldwide [1]. Treatment of toxoplasmosis is commonly performed with the synergistic drugs pyrimethamine and sul-fadiazine. Although, synthetic drugs have acceptable anti-*Toxoplasma* activities, their adverse effects are limiting factors, especially in pregnant women with acute toxoplasmosis. For example, pyrimethamine causes suppression of hematopoiesis in patients [2]. Therefore, production of an effective anti-*Toxoplasma* drug with low side effects is a priority in *Toxoplasma* research.

Based on a PubMed search, some *in vitro* studies have done on the effectiveness of herbal products in treating *T. gondii* [3-5]. However, only one report, a report from Iran, was found on the anti-*Toxoplasma* effect of herbal extracts; that research studied the effect of garlic extract on acute toxoplasmosis in mice [6]. This...
study was performed in order to determine the lethal and
the inhibitory effects of ethanol extracts of Achillea mille-
folium (A. millefolium) and Hypericum perforatum (H. perfo-
ratum) on T. gondii RH strain tachyzoites in vitro.

2. Materials and Methods

A. millefolium and H. perforatum were obtained from
herbal marketing Jahad Daneshgahi Karaj (Tehran, Iran)
and were confirmed by a botanist. Voucher specimens of
the herbal plants were saved at the Herbarium Center of
Medicinal Plants of the Academic Center for Education,
Culture and Research (ACECR), Karaj, Iran. Aerial parts
of plants were dried at room temperature and were pow-
dered. Fifty grams of each herb were extracted by using
the percolation method with 80% ethanol at room tem-
perature. The samples were stored at 4ºC until use (3 gr,
6% yield). The dried plant extracts were dissolved in 1%
dimethyl sulfoxide (DMSO) and diluted with phosphate
buffer solution (PBS) to different concentrations. Also, py-
rimethamine (Sigma, USA) dissolved in methanol-acetone
(50% v/v) and diluted with Roswell Park Memorial Insti-
tute (RPMI) 1640 medium was used as a positive control.

We used T. gondii RH strain tachyzoites. HeLa cells were
cultured and used to assay the anti-Toxoplasma effects of
the herbal extracts. The assays were performed by treat-
ing a 50-μL tachyzoites suspensions containing nearly 5
× 10⁵ tachyzoites with 50 μL of different concentrations
of the extracts (10, 50, and 100 mg/mL) within 10, 30, and 45
minutes at room temperature. All treatments were assayed
in triplicate.

The tachyzoites treated with both extracts that showed
100% mortality based on methylene-blue staining were
bioassayed in mice. Fifty microliters of suspensions con-
taining nearly 5 × 10⁵ tachyzoites were inoculated intra-
peritoneally into 3 mice. All of the mice were monitored
for up to one month after inoculation in terms of activity
and mortality.

HeLa cell suspensions were cultured and within 24 hours
after incubation, 100 μL of a suspension containing 3 × 10⁶
fresh tachyzoites were added to each well. Six hours after
the inoculation of the tachyzoites to wells, the cultures
were washed twice with RPMI 1640 medium without fe-
tal bovine serum (FBS) in order to remove non-adherent
tachyzoites. Eighteen hours after incubation, the herbal
extracts were individually added to the wells at a concen-
tration of 50, 100, or 200 μg/mL. Twenty four hours after
the addition of the herbal extracts, the anti-Toxoplasma
activity and the cytotoxicity of those traditional medi-
cines were examined using MTT (3-(4,5-dimethylthia-
zol-2-yl)-2,5-diphenyltetrazolium bromide) assay kits
(Bio Idea Company, Tehran, Iran). Optical densities (ODs)
were read using an enzyme-linked immunosorbent assay
(ELISA) microplate reader (Epoch, USA) at a wavelength
of 570 nm. All experiments were performed in triplicate. The
results were expressed as percent cell viability, half maxi-
mal effective concentration (EC₅₀), and selectivity.

3. Results

Both herbal extracts showed toxoplasmacidal effects. The
results for the mortality rates (%) of the tachyzoites are
shown in Table 1. The mortality rate at a 100-mg/mL con-
centration of A. millefolium was significantly higher than
that at a 50-mg/mL concentration (P < 0.001), but the dif-
ference in the mortality rates between of 10- and 50-mg/
ml concentrations of the extract were not significant. The
toxoplasmacidal effects of the H. perforatum extract were
similar at concentrations of 50 and 100 mg/mL; however,
compared to the concentration of 10 mg/mL, both the 50-
and the 100-mg/mL concentrations of the extract showed
significant increases in those effects. Also, a 100% morta-
listy rate of the tachyzoites in mice treated with the extracts
was confirmed by using bioassays, and all of the mice in-
oculated with the tachyzoites were alive and active one
month after the inoculation. Both herbal extracts showed
anti-Toxoplasma activity in the cell culture; however, the
EC₅₀ of H. perforatum extract (153 μg/mL) was lower than
the EC₅₀ of A. millefolium extract (215 μg/mL), (Table 2).

After T. gondii-infected HeLa cells had been incubated
with different concentrations of the extracts, their viabi-
ility decreased in a dose-dependent manner (Figs. 1, 2). The
viability showed significant decreases, compared with the
control, at all concentrations of the extracts (P < 0.05). On
the other hand, the inhibitory effect of pyrimethamine on
cell proliferation was significantly higher than the inhibi-
tory effects of the two herbal extracts (Figs. 1, 2).

| Incubation time (minutes) | Herbal extract type | Concentration (mg/mL) | 10  | 50  | 100 |
|---------------------------|---------------------|-----------------------|-----|-----|-----|
| 10 | H. perforatum | 5.01 ± 1.09 | 100 | 100 | 100 |
|  | A. millefolium | 4.53 ± 0.91 | 5.97 ± 2.43 | 100 | 100 | 100 |
| 30 | H. perforatum | 5.22 ± 0.77 | 100 | 100 | 100 |
|  | A. millefolium | 4.63 ± 0.75 | 6.11 ± 2.17 | 100 | 100 | 100 |
| 45 | H. perforatum | 5.31 ± 0.89 | 100 | 100 | 100 |
|  | A. millefolium | 5.11 ± 1.12 | 6.26 ± 2.70 | 100 | 100 | 100 |

T. gondii, Toxoplasma gondii, A. millefolium, Achillea millefolium; H. perforatum, Hypericum perforatum.
Table 2  *In vitro* anti-*Toxoplasma* activity and selectivity of *A. millefolium* and *H. perforatum* extracts and pyrimethamine

| Herbal extract/ drug (μg/mL) | EC<sub>50</sub> HeLa | EC<sub>50</sub> HeLa+ T. gondii | Selectivity<sup>*</sup> |
|-----------------------------|----------------------|-------------------------------|------------------------|
| *A. millefolium*             | 158                  | 215                           | 0.73                   |
| *H. perforatum*             | 105.9                | 153                           | 0.69                   |
| Pyrimethamine               | 0.6                  | 0.176                         | 3.40                   |

<sup>*</sup>Ratio of the EC<sub>50</sub> value for HeLa cells to the EC<sub>50</sub> value for *T. gondii* RH strain.

*A. millefolium, Achillea millefolium; H. perforatum, Hypericum perforatum; EC<sub>50</sub>, effective concentration; T. gondii, Toxoplasma gondii.*

4. Discussion

In the present *in vitro* study, ethanol extracts of *A. millefolium* and *H. perforatum* showed toxoplasmacidal activities in RH strain tachyzoites exposed to those extracts and had inhibitory effects on the parasite in cell cultures. However, the anti-*Toxoplasma* activity of the *H. perforatum* extract was stronger than that of the *A. millefolium* extract, but their selective toxicities were low. Other studies on herbal extracts have also shown that *Glycyrrhiza glabra* L., *Acorus gramineus* Soland and *Dryopteris crassirhiza* have anti-*Toxoplasma* activities [7, 8].

Previous studies have been shown that some extracts/fractions of medicinal plants have remarkable anti-*Toxoplasma* activities. The extracts of *Sophora flavescens* Aiton and *Zingiber officinale* have high anti-*Toxoplasma* activities (EC<sub>50</sub> = 0.20 and 0.18) with high selectivities (selectivity = 4.6% and 10.1%, respectively) [8]. Furthermore, in cell cultures, the inhibitory effects of *Torilis japonica* and *Sophora flavescens* on *T. gondii* and *Neospora caninum* tachyzoites have been shown to be higher than those of *Pulsatilla koreana*, *Ulmus macrocarpa*, and *Sinomenium acutum* [3]. Furthermore, the study of Youn *et al* [4] also showed that some high-performance liquid chromatography (HPLC) fractions of those plants have more efficient on tachyzoites in cell cultures [4].

5. Conclusion

Our study showed that the anti-*Toxoplasma* activities of *A. millefolium* and *H. perforatum* extracts were significantly lower than that of pyrimethamine which is a common synthetic drug for the treatment of toxoplasmosis. Therefore, these plants do not seem to be good candidates for continuance of anti-*Toxoplasma* studies. However, further studies to clarify the anti-*Toxoplasma* activities of other herbs are recommended.

The authors declare that there are no conflict of interest.

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