Research Article

In Vitro and In Vivo Survey of Ethyl Acetate Extract of Acorus calamus (Sweet Flag) Rhizome on Toxoplasma gondii

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The current treatment in toxoplasmosis associated a range of side effects such as toxicity in pregnancy and bone marrow suppression, requirement of long courses, and lack of effect on the parasite cystic forms [5]. Due to the toxicity of chemical drugs and people’s tendency to use traditional drugs and routs, many studies have been done on medicinal herbs and traditional methods in the world [6]. Acorus calamus (sweet flag) has used as traditional medicinal herb in China and India. The American native people, the dried root and its powder, put into up the nose for inhibition of inflammation of the mucous membrane [5, 7]. The other pharmacological properties

1. Introduction

Toxoplasmosis is a zoonosis parasitic disease with worldwide prevalence which caused by an obligate intracellular protrozoan; Toxoplasma gondii. Clinical congenital manifestation of toxoplasmosis is abortion and premature birth, hydrocephalus, microcephaly, jaundice and also chorioretinitis, anemia, pneumonia, and intracranial calcification in infants. Toxoplasmosis can also cause severe disease in individuals with immunodeficiency such as HIV-positive and cancer and transplant patients due to treatment with immunosuppressive drugs [1–4].
of *A. calamus* contain anti-inflammatory, antipyretic, antidiar- 
heal, antimutagenic, anticeular and immunosuppressive, larvicald, antimicrobial, antienler, and cytotoxic [8]. The 
rhizomes and leaf oils of *A. calamus* have been reported from 
the lower Himalayan region of India, with the major com-
ounds in the rhizome oil, while β-arosane and inalool in the 
leaf oil [9]. Joshi et al. showed the essential oil of *A. calamus* 
and its major compound β-arosane that has a bactericidal 
property against pathogen bacteria and fungi [10]. Anthelmintic 
and antibacterial properties of rhizome are probably belonged to 
phenylpropanoid β-arosane [11]. This study was conducted to 
determine effects of ethyl acetate extract of subterranean parts 
of *Acorus calamus* on *Toxoplasma gondii* in vivo and in vitro.

2. Material and Methods

2.1. Ethics Approval. The present study is based on guidelines 
for the care and use of laboratory animals [12]. The Ethics 
Committee of Animal Experiments of the Shiraz University 
of Medical Sciences approved this research project (permit 
number IR.SUMS.REC.1398.355).

2.2. Collection of Samples. Several batches of rhizome of the 
fresh plant of *Acorus calamus* were prepared from regions of 
high altitude of forest in Sari city, northern Iran, in March 
2015. Identification was carried out in Faculty of Pharmacy, 
Shiraz University of Medical Sciences. The parts of the plant 
were dried and grinded to powder. The plant herbarium 
specimens are collected in Payam Noor University of Maz- 
daran, and the dried rhizomes samples (Number: MPRCM- 
94-87) are maintained in Medicinal Plants Processing 
Research Center, Shiraz University of Medical Sciences.

2.3. Extraction. Rhizomes of the plant were dried in shade 
(26°C, 2 weeks). To prepare the ethyl acetate extract, 30 g pow-
der of the dried parts was poured in dark sterilized kartush 
containing 600 ml of ethyl acetate. The extract was provided 
using Soxhlet extractor. Each step of Soxhletation elongated 
approximately 6 hours. The extraction was concentrated with 
rotary evaporator apparatus and then dried by speed vacuum 
during 48 hours. The extract was maintained in dark bottle at 
2-8°C condition.

2.4. Parasites. The virulent RH strain of *T. gondii* was obtained 
from Tehran University of Medical Sciences, Tehran, Iran. 
Tachyzoites of the RH strain of *T. gondii* were maintained by 
serial intraperitoneal passaging in BALB/c inbred mice. After 72 hours, 10⁶ parasite inoculation in the mice, the tachy-
zoites were collected after repeated flushing of the peritoneal 
cavity by phosphate buffered saline (PBS) at a pH of 7.2. Then, 
tachyzoites were harvested and centrifuged for 5 min at 200 g 
at room temperature to remove peritoneal cells and cellular 
debris. The supernatant was collected and centrifuged for 
10 min at 800 g [1]. The pellet, enriched with parasite tachy-
zoites, was recovered with PBS and used in the experiments.

2.5. Extracellular Viability Assay. We dissolved the extract in 
DMSO and then PBS to obtain a final concentration of 
10 mg/ml. The final concentration of DMSO should not 
exclude 1%. Various concentrations (25, 50, 100, 200, 400, 
800 μg/ml) of the extract were then prepared by the follow-
ing: 2.5–80 μl of the final concentration was added to 920-
997.5 μl of PBS that contained 2 × 10⁷ tachyzoites. Tachy-
zoites were incubated with either DMSO (as control) or the 
diluted compounds for 1.5 h at 4°C. Next, the tachyzoites 
were collected in Eppendorf tubes and incubated for 30 min 
at 4°C with 50 μg/ml propidium iodide (PI, Sigma Company, 
USA). After incubation, the parasites were kept on ice and in 
the dark until analysis. Positive controls for PI staining were 
acquired by incubating parasites in the presence of 0.2% 
saponin. The cell suspension was transferred into polystyrene 
flowcytometry tubes (BD Falcon Company, USA). We per-
duced data acquisition and analysis, with a FACS Calibur 
flow cytometer (Becton-Dickinson, San Jose, USA) and Cell 
Quest Pro software. A total of 1000-30000 events were 
acquired in the region that had been previously established 
as corresponding to the parasites [13]. All of the tests were 
undertaken in duplicate.

2.6. In Vivo Experiments. In this study, a total of 9 groups 
including 10 BALB/c inbred mice were considered. 2 × 10⁵ 
tachyzoites were intradermally inoculated into 8 groups 
including 10 mice. Based on results of in vitro experiments, 
6 groups, doses 32, 64, 128, and 256 mg/kg, were orally, and doses 128 and 256 mg/kg, intraperitoneally administered: 
VII—received sulphadiazine as positive control and VIII—r-
ceived PBS including DMSO 1%. These concentrations were 
daily administrated 24 hours after inoculation due 10 days 
continuously. Mice were followed for 15 days after inocula-
tion. If the mice died, their liver touch smears were stained 
with Giemsa stain and observed under light microscopy for 
parasite detection [13].

2.7. The Acute Toxicity Assay of the Extract. For toxicity eval-
uation of this extract, a group only received a maximum dose 
of (256 mg/kg). Then, the mice were followed for any manifes-
tation of this extract, a group only received sulphadiazine as standard. First, a 
dosages 128 and 256 mg/kg, intraperitoneally administered: 
—received sulphadiazine as positive control and VIII—r-
ceived PBS including DMSO 1%. These concentrations were 
daily administrated 24 hours after inoculation due 10 days 
continuously. Mice were followed for 15 days after inocula-
tion. If the mice died, their liver touch smears were stained 
with Giemsa stain and observed under light microscopy for 
parasite detection [13].

2.8. Assessment of the Total Phenolic Content in the Extract. 
The total phenolic content of the extract was measured by 
the Folin–Ciocalteu method and Folin’s phenol reagent. In 
this experiment, garlic acid was used as standard. First, a 
serial dilution of garlic acid (0.024, 0.03, 0.075, 0.105 mg/ml) 
was provided by methanol and then filtered by Whatman 
Grade 1 filter paper. 0.5 ml of the different concentrations 
was diluted by 2.5 ml of Folin–Ciocalteu reagent and 2 ml of 
7.5% (w/v) sodium carbonate in 20°C. Absorbance was 
measured at 765 nm. All of the tests were undertaken in trip-
licate, and calibration curve was drawn. 500 μl of the crude 
extract was diluted by 2.5 ml of Folin–Ciocalteu reagent and 
2 ml of 7.5% (w/v) sodium carbonate in 20°C. Absorbance 
was measured at 765 nm. The total phenolic content was 
calculated from the calibration curve.

2.9. Total Flavonoid Content. 250 mg of dried powder was 
diluted with 20 ml and sonicated for 15 minutes. The extract 
was filtered, and 5 ml of it was mixed with 5 ml of 2% (w/v) 
AlCl₃ solution for 15 minutes in dark condition. Absorbance
was measured at 415 nm. The total flavonoid content was calculated from a calibration curve obtained from quercetin.

2.10. Data Analysis. Data were gathered in SPSS software (version 16, Chicago, USA). In vitro results were analyzed by the Kuruskal–Wallis and Spearman correlation tests, whereas Kaplan–Meier and log rank (Mentel-Cox) were used in vivo. \( P < 0.05 \) was considered statistically significant.

3. Results

In this study, the mortality of *Toxoplasma* tachyzoite cells exposed to the different concentrations of ethyl acetate...
Acorus calamus was measured using the flowcytometry technique. Figures 1 and 2 show that there is an inverse relationship between the concentrations and mortality rate of the parasite tachyzoite. More than 62 percent of Toxoplasma tachyzoites were killed at maximum concentration (256 μg/ml). IC_{50} of the extract on the parasite was calculated 200.01 ± 7.74 (μg/ml).

In vivo results showed a significant difference at maximum concentration (256 mg/kg) with gavage administration (P < 0.031), whereas peritoneal inoculation of this concentration did not affect on longevity of the mice.

Any signs of physical changes that belonged to the toxicity of the extract in the tested animals were not detected, and the entire mice group was live (Table 1).

Figure 3 shows the phenolic compound content of the extract based on standard graph of gallic acid. The plant extract was rich of phenolic compounds (41.27 ± 0.21 mg/g).

Figure 4 shows the flavonoid compound content of the extract based on standard graph of quercetin. The flavonoid compound content of plant extract was 4.79 ± 0.01 mg/g.

### 4. Discussion

_Acorus calamus_ is consumed in traditional medicine, and its rhizomes are widely used to subside in clinical signs such as chronic diarrhea, dysentery, fever, and rheumatism [8]. Also, it is used traditionally in the treatment of various ailments including neuralgia, dyspepsia, kidney and liver troubles, eczema, sinusitis, asthma, bronchitis, hair loss, and other disorders [14].

In vivo results of our experiments on the extract toxicity showed that any clinical signs were not occurred in the tested animals. Similarly, Muthuraman et al. showed that the high doses of the hydroalcoholic extract of the plant rhizome

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Mean of mortality rate±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.08±0.11</td>
</tr>
<tr>
<td>2</td>
<td>0.325±0.46</td>
</tr>
<tr>
<td>4</td>
<td>0.34±0.48</td>
</tr>
<tr>
<td>8</td>
<td>2.39±0.29</td>
</tr>
<tr>
<td>16</td>
<td>7.23±6.31</td>
</tr>
<tr>
<td>32</td>
<td>12.8±1.32</td>
</tr>
<tr>
<td>64</td>
<td>21.29±4.58</td>
</tr>
<tr>
<td>128</td>
<td>35.88±3.48</td>
</tr>
<tr>
<td>256</td>
<td>62.12±0.81</td>
</tr>
</tbody>
</table>

**Table 1:** In vivo results of different administrations of ethyl acetate extract of _Acorus calamus_ in treatment of murine toxoplasmosis.

<table>
<thead>
<tr>
<th>Administration</th>
<th>Time of death (postinoculation day) 6 7 8 9 10 11</th>
<th>Mean of longevity</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 mg/kg (oral)</td>
<td>**       ** **     **     **       **       **       **       **</td>
<td>7.9</td>
</tr>
<tr>
<td>64 mg/kg (oral)</td>
<td>**       **       ***     **       **       **       **       **       **       **</td>
<td>7.3</td>
</tr>
<tr>
<td>128 mg/kg (oral)</td>
<td>**       **       **       **       **       **       **       **       **       **</td>
<td>7.2</td>
</tr>
<tr>
<td>Ethyl acetate extract of <em>Acorus calamus</em></td>
<td>128 mg/kg (intraperitoneal)</td>
<td>*       ****     ***     ***     **       **       **       **       **       **       **       **       **</td>
</tr>
<tr>
<td>256 mg/kg (oral)</td>
<td>***       **       ****     **       **       **       **       **       **       **       **       **       **</td>
<td>8.9</td>
</tr>
<tr>
<td>256 mg/kg (intraperitoneal)</td>
<td>****     ***       **       **       **       **       **       **       **       **       **       **       **</td>
<td>7</td>
</tr>
<tr>
<td>256 mg/kg (oral) without parasite</td>
<td>***       ***     ***       **       **       **       **       **       **       **       **       **       **</td>
<td></td>
</tr>
<tr>
<td>Sulfadiazine (30 mg/kg) as positive control</td>
<td>***       ***     ***       **       **       **       **       **       **       **       **       **       **</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* shows death event.
could not create any toxic effects in rats [15]. Another study has shown that the high dose of the extract is very well tolerated in rodents but it is associated with a mild elevation in levels of the liver enzymes [16].

In our study, the ethyl acetate extract of the rhizome was rich of phenolic compounds. It has commonly been assumed that the plant pharmacological fundamentals such as antidepressant, antianxiety, anti-Alzheimer’s, anti-Parkinson’s, antiepileptic, anticancer, antihyperlipidemic, antithrombotic, anticholestatic, and radioprotective activities were related to phenolic compounds such as α and β-asarone molecules. Isoeugenol, another phenolic compound, is found in A. calamus leave samples [19].

Oliveira et al. showed that the mortality in mice with toxoplasmosis can be inhibited by phenolic compounds such as vanillin [20]. Moreover, Choi et al. indicated that the phenolic compounds of ginger root extract can inactivate apoptotic proteins in host cells infected to Toxoplasma. Remarkably, the proteins inhibit secretion of inflammatory cytokines in vivo [21].

In this study, we measured a low content of the flavonoid compound in the plant extract, but in other studies, apigenin, luteolin, and diosmetin as flavonoid compounds were found in A. calamus [22]. Mac Laren et al. showed that some flavonoids, such as apigenin and genistein, can inhibit Toxoplasma growth due to inhibition of protein tyrosine kinase [23]. Similarly, other flavonoid compounds such as narigenin and genistein had the notable activities against Cryptosporidium in cell culture [24].

Quercetin as a flavonoid compound can inhibit synthesis protection factors such as Hsp90, Hsp70, and Hsp27, and consequently, Toxoplasma remains sensitive due to the effects of host immune responses [25].
Lehane and Saliba showed that certain common dietary flavonoids especially luteolin can inhibit the intraerythrocytic growth of the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* [26].

In our study, the direct effect of the different concentrations of ethyl acetate extract of *A. calamus* on *Toxoplasma* tachyzoite was measured using the flow cytometry technique. In this technique, the propidium iodide, a fluorescent DNA-binding dye, is used for the evaluation of dying cells. The results showed an inverse relationship between the concentrations and the mortality rate of the parasites.

In vivo results showed a significant difference at maximum concentration with gavage administration, whereas peritoneal inoculation of this concentration did not effect on longevity of the mice. However, the effect of peritoneal administration of concentration 128 µg/ml was better than oral concentration.

5. Conclusion

Our study demonstrated that *Acorus calamus* extract had significant activities against *T. gondii* in vivo and in vitro which may be connected to high amount of phenolic compounds. We suggest that the effects of the various fractions of this extract on the parasite are investigated. Alternatively, the administration types and dosage of the extract on the parasite must be evaluated.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Acknowledgments

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References


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