



# Screening of a library of traditional Chinese medicines to identify compounds and extracts which inhibit *Toxoplasma gondii* growth

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**ABSTRACT.** *Toxoplasma gondii* can cause severe encephalitis in immunocompromised patients. Although pyrimethamine and sulphadiazine have been standard therapeutic agents for the treatment of acute toxoplasmosis, they have toxic side effects. Therefore, there is a need to identify new drugs that are less toxic. Some traditional Chinese medicines (TCMs) have shown good efficacy in controlling *T. gondii* replication in mouse models. Here, we screened a natural product library comprising TCMs with the aim of identifying compounds and extracts with anti-toxoplasmosis activities. We found several hit compounds and extracts that could be candidates for new drugs against *T. gondii* infection.

**KEY WORDS:** acute toxoplasmosis, drug screening, *Toxoplasma gondii*, traditional Chinese medicine

Toxoplasmosis is a disease caused by *Toxoplasma gondii*, an obligate intracellular protozoan parasite. This parasite causes congenital toxoplasmosis, which causes fetopathy in pregnant women who initially become infected during pregnancy, as well as acquired toxoplasmosis, which causes serious symptoms in immunocompromised patients when the latent parasites reactivate. Latent infection in animals used for meat is a major source for human infections [11].

The therapeutic agents for the treatment of toxoplasmosis are the combination of pyrimethamine and sulphadiazine, but they have toxic adverse effects such as bone marrow suppression. According to a systematic review, prevalence of hematologic adverse effects ranged from 2.8% to 10% [2]. Therefore, new drugs that are less toxic are needed. In China, traditional Chinese medicines (TCMs) have long been used to treat various diseases [3]. Both *Astragalus membranaceus* and *Scutellaria baicalensis* GEORGI, which are widely used for the treatment of various inflammatory diseases in Asia, have been shown to be effective in controlling *T. gondii* replication in the mouse model [12]. In addition, some TCMs have been reported to have growth inhibitory effects on *Plasmodium falciparum* [10] and *Trypanosoma cruzi* [7]. Therefore, other TCMs may also have anti-parasitic activities. Accordingly, the aim of this study was to determine whether these compounds have anti-toxoplasma activity.

## MATERIALS AND METHODS

### Compounds and extracts

The Institute of Natural Medicine, University of Toyama provided the natural drug library used in this study. Pyrimethamine (Wako, Osaka, Japan) and DS10 (dextran sulfate MW 10 kDa) (Sigma-Aldrich, St. Louis, MO, USA) [6], served as control drugs.

### *Toxoplasma gondii* in vitro culture

Vero cells (RIKEN BioResource Center: RCB0001) and human foreskin fibroblasts (HFF) (ATCC: SCRC-1041) were used as host cells for *T. gondii* culture. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5% FBS (Sigma-Aldrich), L-glutamine (Wako), penicillin, and streptomycin (Wako). HFF cells were maintained in DMEM supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin. *T. gondii*

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RH-2F (ATCC: 50839) strain was cultured in confluent Vero cells maintained in DMEM as described above.

#### Parasite growth assay

For the primary screening, we used the RH strain with a  $\beta$ -galactosidase reporter parasite termed RH-2F (a type I strain) [4]. RH-2F tachyzoites were purified by syringe lysis and passed through a 5- $\mu$ m filter; they were then passaged to the monolayer of confluent HFF cells cultured in 96-well plates with DMSO (negative control), 10  $\mu$ M pyrimethamine (positive control), 10  $\mu$ M test compounds or 100  $\mu$ g/ml test extracts. The plates were then incubated at 37°C for 48 hr in a CO<sub>2</sub> incubator. To evaluate parasite growth,  $\beta$ -Galactosidase activity was measured by using the Beta-Glo Assay System (Promega, Madison, WI, USA) following the manufacturer's instructions. Inhibitory effects of test compounds and extracts (%) were calculated by assigning the activity value of wells containing DMSO as 0% and that of wells that containing no parasites as 100%.

To calculate the IC<sub>50</sub> values of the hit compounds and extracts for parasite growth, the parasites were treated with various doses of compounds and extracts (0.04–50  $\mu$ M compounds; 0.4–500  $\mu$ g extracts). After 48 hr, the parasites number was measured by using the Beta-Glo Assay System (Promega). IC<sub>50</sub> values were calculated by using Graph Pad Prism 6.07 (MDF, Tokyo, Japan).

#### Host cell cytotoxicity assay

HFF cells were treated with compounds and extracts at the same concentration as that used for the primary screening. After a 48-hr incubation, cell viabilities were measured by using a Cell-Titer Glo (Promega) following the manufacturer's instructions. The control wells were treated with DMSO alone in culture medium to calculate the 100% viability.

To evaluate the IC<sub>50</sub> values of the hit compounds and extracts for host cells, HFF cells were treated with various doses of compounds and extracts (0.08–50  $\mu$ M compounds; 0.8–500  $\mu$ g of extracts). After 48 hr, cell viabilities were measured as described above. IC<sub>50</sub> values were calculated by using Graph Pad Prism 6.07.

#### Intracellular parasite growth assay

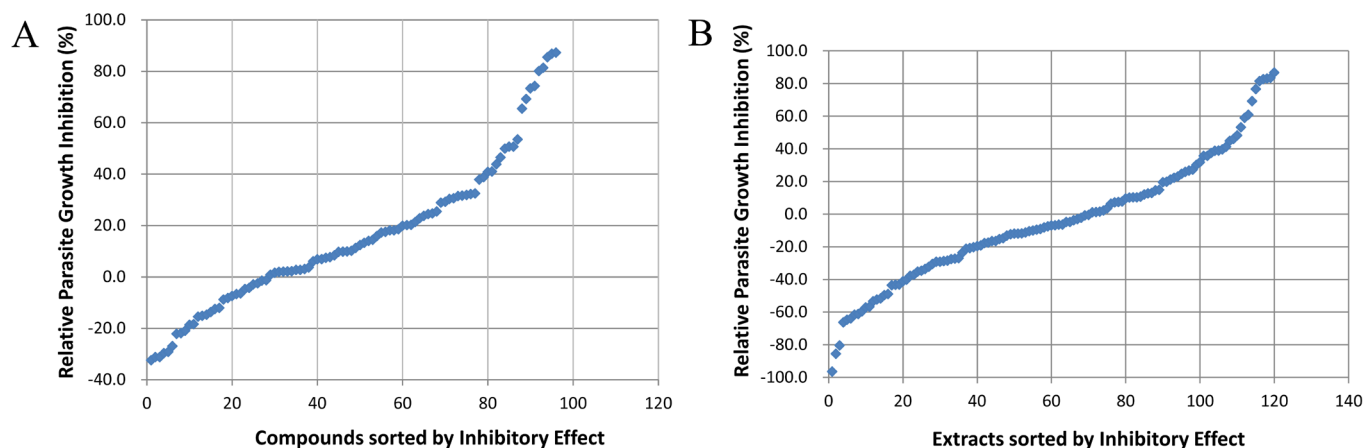
Tachyzoites were purified as described above and inoculated to HFF cells in 96-well plates. After a 2-hr incubation to allow parasites invasion, uninvaded parasites were washed away, and fresh medium containing the compounds or extracts was added. The concentrations of compounds and extracts were: 10  $\mu$ M pyrimethamine, 25 mg/ml dextran sulfate (10 kDa fraction), 10  $\mu$ M baicalein and luteolin, and 50  $\mu$ g/ml *Scutellariae radix*, *Vitidis fructus*, *Phellodendri cortex*, and *Coptidis rhizoma*. After 48 hr, parasite numbers were measured by using the Beta-Glo Assay System (Promega).

## RESULTS

#### Screening of the natural compound library for anti-Toxoplasma effects

A total of 96 compounds and 120 extracts of natural products were screened for *T. gondii* growth inhibitory effects at 10  $\mu$ M for the compounds and 100  $\mu$ g/ml for the extracts (Fig. 1a and 1b). Of these natural products, seven compounds and six extracts inhibited growth by >70%.

To exclude the possibility that host cell death causes the reduction in parasite number, we evaluated the host cell viabilities at the same doses of compounds and extracts. One of the test compounds (timosaponin A-III) killed almost all of the host cells, however, the other six compounds (shikonin, alkannin, berberine chloride, baicalein, luteolin, and coptisine chloride) did not kill the host cells (Table 1). Among the extracts, *Albizziae cortex* also killed host cells. In contrast, *Scutellariae radix*, *Vitidis fructus*,



**Fig. 1.** Natural compound screening for anti-toxoplasma activity using the RH-2F strain. *Toxoplasma gondii* RH-2F strain-infected human foreskin fibroblast cells were incubated with 10  $\mu$ M test compounds or 100  $\mu$ g/ml test extracts at 37°C for 48 hr. A. Seven compounds inhibited growth by more than 70%. B. Six extracts inhibited growth by more than 70%.

**Table 1.** Hit compounds from the first screening

Compound	Parasite inhibition (%)	Host cell inhibition (%)
Shikonin	87.3	43.4
Alkannin	86.9	-0.3
Timosaponin A-III	81.4	98.3
Berberine Chloride	85.5	-3.0
Baicalein	80.1	3.3
Luteolin	74.3	-7.9
Coptisine Chloride	73.3	6.4

**Table 2.** Hit extracts from the first screening

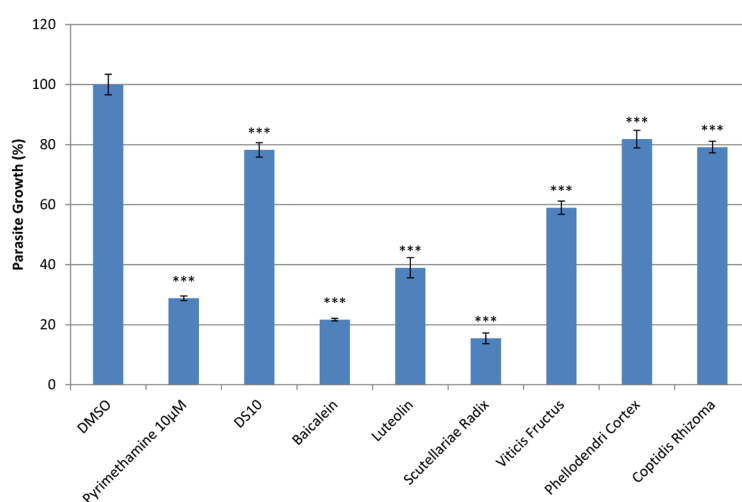
Extract	Parasite inhibition (%)	Host cell inhibition (%)
Scutellariae Radix	86.7	-4.4
Vitidis Fructus	83.5	22.7
Glycyrrhizae Radix	83.1	-3.4
Albiziae Cortex	82.6	97.6
Phellodendri Cortex	81.5	11.7
Coptidis Rhizoma	76.6	14.7

**Table 3.** Effects of hit compounds on RH-2F tachyzoites and human foreskin fibroblast cells

Compound	IC <sub>50</sub> for parasite growth (μM)	IC <sub>50</sub> for host cell (μM)
Shikonin	1.4	17.8
Alkannin	1.3	5.4
Berberine Chloride	2.2	2.8
Baicalein	5.0	>50
Luteolin	5.7	>50
Coptisine Chloride	2.1	>50

**Table 4.** Effects of hit extracts on RH-2F tachyzoites and human foreskin fibroblast cells

Extract	IC <sub>50</sub> for parasite growth (μg/ml)	IC <sub>50</sub> for host cell (μg/ml)
Scutellariae Radix	7.1	>500
Vitidis Fructus	14.9	>500
Glycyrrhizae Radix	96.9	>500
Phellodendri Cortex	14.6	>500
Coptidis Rhizoma	7.3	>500



**Fig. 2.** Growth inhibitory effects on intracellular parasites. Baicalein, luteolin, and *Scutellariae radix* effectively inhibited the intracellular parasite growth. \*\*\*:  $P < 0.001$ .

*Glycyrrhizae radix*, *Phellodendri cortex*, and *Coptidis rhizoma* caused less host cell death (Table 2). These data suggest that six compounds and five extracts might selectively inhibit parasite growth.

### Validation

Next, to evaluate how these hit compounds and extracts might exert selective inhibitory effects on parasites, we calculated IC<sub>50</sub> values for parasite growth and host cell viabilities.

Among the test compounds, shikonin, baicalein, luteolin, and coptisine chloride inhibited parasite growth by 50% at one-tenth of the concentration relative to their IC<sub>50</sub> values for host cell viability (Table 3). In particular, the IC<sub>50</sub> values for host cell viabilities of baicalein, luteolin, and coptisine chloride were greater than 50 μM, which was the highest concentration used in this assay, suggesting that these compounds have high selectivities for inhibiting parasite growth. However, shikonin killed some of the host cells (Table 1) and coptisine chloride did not inhibit parasite growth in a dose-dependent manner at the high concentrations (data not shown). These data indicate that baicalein and luteolin were the most potent compounds among the natural products for treating *T. gondii* infection.

None of the test extracts showed cytotoxicity at 500  $\mu\text{g/ml}$ , which was the highest concentration assayed (Table 4). The  $\text{IC}_{50}$  values for parasite growth of *Scutellariae radix*, *Vitidis fructus*, *Phellodendri cortex*, and *Coptidis rhizoma* were less than 50  $\mu\text{g/ml}$ , suggesting that these extracts also have high selectivity for inhibiting parasite growth and might control *T. gondii* infection at a safe concentration for human cells.

### Intracellular growth assay

To confirm that the hit compounds and extracts could inhibit intracellular parasite growth, we performed an intracellular growth assay. Pyrimethamine, which is an inhibitor of dihydrofolate reductase, inhibited the intracellular growth as previously reported [3]. In contrast, dextran sulfate, which mainly inhibits the invasion process, hardly affected the intracellular parasites. Both baicalein and luteolin effectively inhibited intracellular parasite growth. Among the natural extracts, only *Scutellariae radix* effectively inhibited intracellular growth; other extracts hardly inhibited growth, like dextran sulfate (Fig. 2).

## DISCUSSION

Parasites rarely exist outside their host cells, so it is important for anti-parasitic drugs to inhibit the growth of intracellular parasites. Accordingly, baicalein, luteolin, and *Scutellariae radix* may be promising new anti-toxoplasmosis drugs. Baicalein and luteolin have not previously been reported to have growth inhibitory effects on *T. gondii*. Both compounds have DNA topoisomerase I and II inhibitory effects. Baicalein is used to treat hypertension, atherosclerosis, dysentery, and inflammatory diseases [8]. Luteolin is used to treat *Leishmania donovani* infection [9]. In addition, both compounds have growth inhibitory effects on *Trypanosoma cruzi* [7]. Selectivity indexes (SI) of these compounds are higher than those of pyrimethamine and sulphadiazine, and those SI are 0.21 and 0.29, respectively [1]. Also toxic adverse effects of these compounds have not been reported, so they might be less toxic than pyrimethamine and sulphadiazine. In this study, we showed that baicalein and luteolin have potential as anti-toxoplasmosis drugs. As for *Scutellariae radix*, its growth inhibitory effects on *Toxoplasma gondii* have already been reported [12], and our results confirm these previous. In conclusion, here we identified two compounds with anti-toxoplasma activity that may be of value in the development of new anti-toxoplasmosis drugs.

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