

## ORIGINAL CONTRIBUTION

# *In Vitro* Screening to Identify Anti-*Toxoplasma* Compounds and *In Silico* Modeling for Bioactivities and Toxicity

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Toxoplasmosis, which affects more than a billion people worldwide, is a common parasitic infection caused by the obligate intracellular parasite, *Toxoplasma gondii*. Current treatment strategies have several limitations, including unwanted side effects and poor efficacy. Therefore, newer therapies are needed for toxoplasmosis. Drug repurposing and screening of a vast array of natural and/or synthetic compounds is a viable option for antiparasitic drug discovery. In this study, we screened 62 compounds comprising natural products (NPs†) and FDA-approved (FDA) drugs, to identify the hit compounds that suppress the growth of *T. gondii*. To determine the parasite inhibitory potential of the compounds, host mammalian cells were infected with a transgenic *T. gondii* strain, and the viability of the parasite was evaluated by luminescence. Of the 62 compounds, tubercidin, sulfuretin, peruvoside, resveratrol, narasin and diacetoxyscirpenol of the natural product isolates, as well as bortezoneb, 10-Hydroxycamptothecin, mebendazole, niflumic acid, clindamycin HCl, mecamylamine, chloroquine, mitomycin C, fenbendazole, daunorubicin, atropine, and cerivastatin of FDA molecules were identified as “hits” with  $\geq 40$  percent anti-parasite action. Additionally, mitomycin C, radicicol, naringenin, gitoxigenin, menadione, botulin, genistin, homobutein, and gelsemin HCl of the natural product isolates, as well as lomofungin, cycloctidine, prazosin HCl, cerivastatin, camptothecin, flufenamic acid, atropine, daunorubicin, and fenbendazole of the FDA compounds exhibited cytotoxic activity, reducing the host viability by  $\geq 30$  percent. Our findings not only support the prospects of drug repurposing, but also indicate that screening a vast array of molecules may provide viable sources of alternative therapies for parasitic infection.

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†Abbreviations: NPs, natural products; MOI, multiplicity of infection; HFF, Human fibroblast foreskin.

Keywords: Antiparasite, Drug discovery, Library of chemical compounds, Medicinal biochemistry, Parasitic infection

Conflicts of Interest: The authors have no competing interests.

## INTRODUCTION

Toxoplasmosis, which is caused by the obligate intracellular parasite *Toxoplasma gondii* [1-3], is a common parasitic infection affecting more than a billion people worldwide. The parasite exists in three forms, tachyzoites, bradyzoites, and sporozoites, found in definitive and non-definitive hosts. However, only two forms, tachyzoites and bradyzoites of the parasite exist in human and non-definitive hosts. In healthy individuals, a *T. gondii* infection can occur without symptoms, but in immune-compromised or pregnant individuals, *T. gondii* infection often results in serious illness or death [4,5]. For example, *T. gondii* infection in HIV-AIDS patients is associated with encephalitis, and in pregnant women, numerous cases of miscarriages and fetal deformities have been linked with the *Toxoplasma* infection [4,5]. Infection of a mammalian host usually comes about by contact with the parasite, either by the consumption of the bradyzoite form in undercooked meat or from the sporozoite form in felid feces, which can contaminate drinking water or occur elsewhere in the environment, such as on unwashed vegetables or in cat litter.

Currently, antimalarials, such as pyrimethamine, and/or antibiotics, such as clindamycin HCl, are among the therapies of choice for active toxoplasmosis [6]. However, poor tolerance and limited efficacy are major challenges of these therapeutic options. Additionally, the available treatments are not effective against latent infections. Thus, toxoplasmosis remains a growing health burden globally, necessitating the identification of newer therapies. Recent investigations have shown that both drug repurposing and screening a vast array of molecules for antiparasite potential are viable approaches to drug discovery [7-14]. Screening a vast array of chemical compounds, including the repurposing of already approved drugs, may allow the discovery of lead antiparasitic infection candidates in early drug development. For example, testing well-characterized drugs and other types of compounds, which are currently approved to treat other conditions, for potential off-label indications could not only allow the identification of lead targets but also boost efforts toward developing lead candidates. Indeed, emerging reports have identified several potent, new antiparasitic agents by screening well-characterized drugs or compounds that inhibit specific pathways or processes [6,9,10]. In this study, we screened both natural product isolates and FDA-approved compounds, to identify those that possess suppressive action against the *in vitro* growth of *T. gondii*.

## MATERIALS AND METHODS

### Natural Product Isolates and FDA-approved Drugs

The natural products and FDA-approved compounds in stock solutions of DMSO were provided without charge from the Kanazawa University Cancer Institute, Japan. Pyrimethamine (Sigma Aldrich, St. Louis, MO, USA) was included in all the experiments as the reference drug.

### Parasite

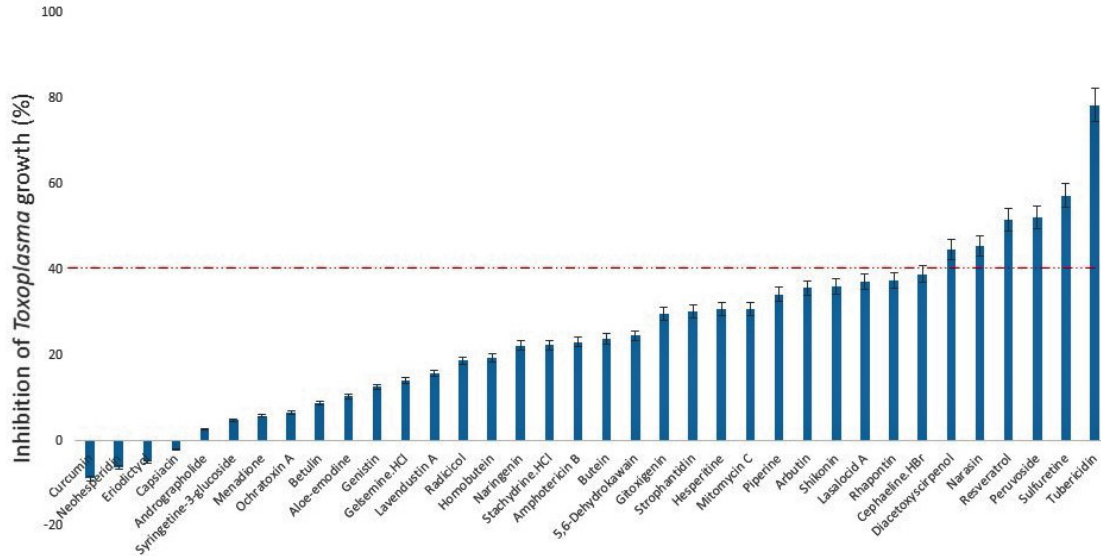
In this study, the transgenic *T. gondii* RH strain 2F (ATCC® 50839), which expresses  $\beta$ -galactosidase, was used, unless indicated otherwise. To sustain the parasite, *T. gondii* was repeatedly passaged in cultures of human foreskin fibroblast (HFF; ATCC®) monolayers in a medium composed of Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan), 1% L-glutamine (200 mM Gibco, Fisher, UK), fetal calf serum (10% v/v; Gibco, Fisher, UK), and 1% penicillin and streptomycin (100 U/mL Biowhittaker, UK). To obtain a purified suspension of *T. gondii*, the infected host cells were lysed, filtered, and washed several times with fresh culture medium, as described elsewhere [8].

### Assay to Determine Parasite Growth Inhibition

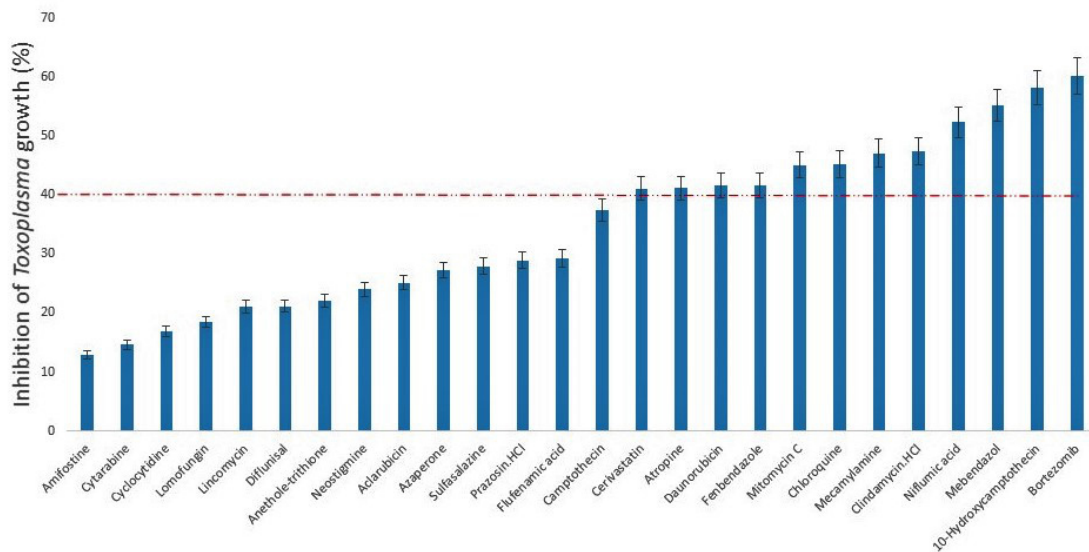
An assay to determine whether the compounds affect parasite growth was performed as described elsewhere [8]. The multiplicity of infection (MOI) was 1:5 (parasite/host cell ratio). In brief, the test compounds were reconstituted in culture medium at five concentrations (between 0 – 1.5  $\mu$ g/mL), and incubated with newly purified parasites in monolayers of HFF cells grown in 96-well optical bottom plates (Nunc; Fisher Scientific, Pittsburgh, USA). The cells treated with medium only served as the negative drug control, while the medium-only well was used for background signal correction. To validate the assay, pyrimethamine was included as a positive drug control. After a 72-h incubation at 37°C in a 5% CO<sub>2</sub> atmosphere, the parasite viability was determined by luminescence using a Beta-Glo assay kit (Promega, Madison, USA). Three independent biological experiments were performed, and each of which had three tests.

### Assay to Determine Host Cell Viability

The host cell viability assay was performed as previously reported elsewhere [8]. Cultures of HFF cells were grown to confluence in medium as described above in an atmosphere of 5% CO<sub>2</sub> and 37°C. The confluent cells were sub-cultured and seeded in 96-well plates (Nunc; Fisher Scientific, Pittsburgh, USA) at a density of  $1 \times 10^5$  cells per well, followed by 72-h incubation at 37°C and 5% CO<sub>2</sub> atmosphere. Subsequently, the test compounds were added at five concentrations (between 0 – 3  $\mu$ g/mL) to the culture medium. The negative drug control contained only the culture medium, whereas the medium-on-



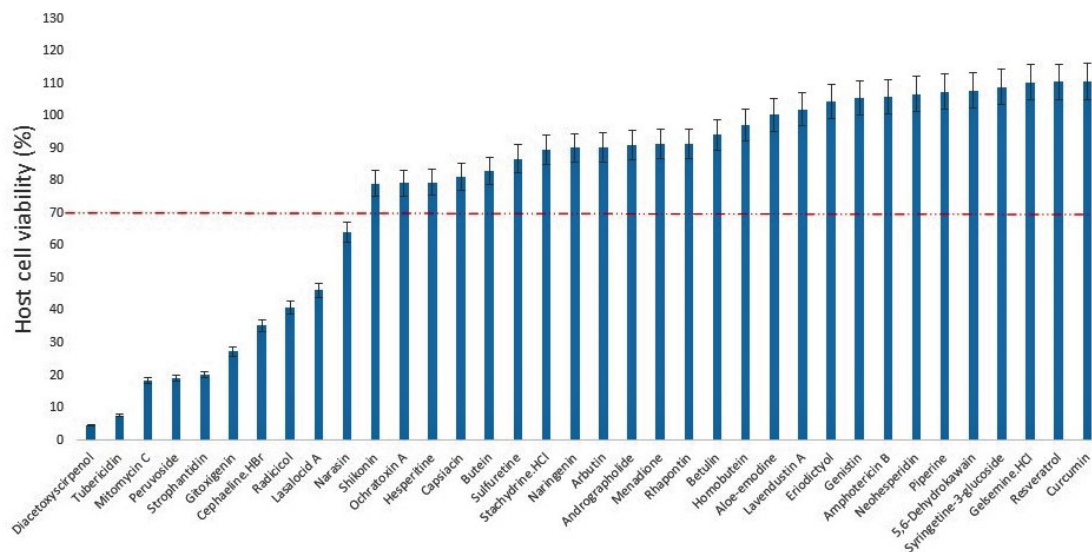
**Figure 1.** Growth inhibition of *Toxoplasma gondii* RH-2F strain by natural product isolates at a concentration of 1 µg/mL. DMSO (0.5%) control was calculated as 0% inhibition, and 200 µg/mL pyrimethamine positive drug control was calculated as 100% growth inhibition. Data are presented as mean values of three replicates ± standard errors of the mean.



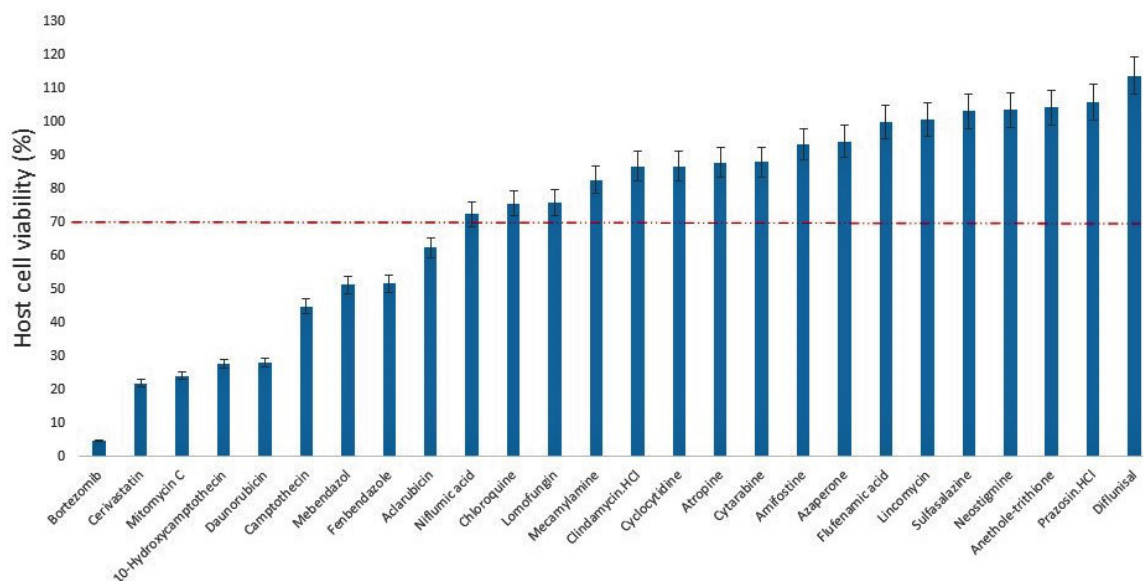
**Figure 2.** Growth inhibition of *Toxoplasma gondii* RH-2F strain by FDA-approved compounds at a concentration of 1.5 µg/mL. DMSO (0.5%) control was calculated as 0% inhibition, and 200 µg/mL pyrimethamine positive drug control was calculated as 100% growth inhibition. Data are presented as mean values of three replicates ± standard errors of the mean.

ly well was used for background noise correction. To validate the assay, a positive control drug, staurosporine (1 µM final concentration) was used [15]. After a 72-h incubation under the tissue culture conditions indicated

above, the viability of cells was determined by colorimetric measurement (CellTiter-Aqueous One kit, Promega, Madison, USA) at 490 nm on a microplate reader (MTP 500; Corona Electric, Hitachinaka, Japan). Three inde-



**Figure 3.** Viability of host cell (human foreskin fibroblast) by natural product isolates at 2 µg/mL. DMSO (0.5%) control was calculated as 100% cell viability. Data are presented as mean values of three replicates ± standard errors of the mean.



**Figure 4.** Viability of host cell (human foreskin fibroblast) by FDA-approved compounds at 3 µg/mL. DMSO (0.5%) control was calculated as 100% cell viability. Data are presented as mean values of three replicates ± standard errors of the mean.

pendent biological experiments were performed, each of which had three tests. Meanwhile, selectivity index (SI) for the compounds was estimated so as to identify promising anti-*Toxoplasma* compounds with low or no toxicity to host cells. The SI is the ratio of the  $IC_{50}$  concentration in host cells (HFF) to that in parasite (*T. gondii*). The  $IC_{50}$  is the concentration of the compound that either reduces

host cell viability or inhibited *Toxoplasma* growth by 50 percent.

**Molecular and In Silico Studies: ADME (Absorption, Distribution, Metabolism, Excretion), Bioactivity, and Toxicity**

**Table 1. Inhibition of *Toxoplasma gondii* growth and host cell viability by natural product isolates.**

Compounds	<sup>a</sup> Inhibition of <i>Toxoplasma gondii</i> (%)	<sup>b</sup> Host (HFF) cell viability (%)
Tuberacidin	78.45±1.23	≥40 % anti- <i>T. gondii</i> activity
Sulfuretine	57.31±2.58	≥40 % anti- <i>T. gondii</i> activity
Peruvoside	52.09±0.93	≥40 % anti- <i>T. gondii</i> activity
Resveratrol	51.60±1.66	≥40 % anti- <i>T. gondii</i> activity
Narasin	45.56±0.43	≥40 % anti- <i>T. gondii</i> activity
Diacetoxyscirpenol	44.67±2.98	≥40 % anti- <i>T. gondii</i> activity
Cephaeline.HBr	38.92±0.14	
Rhapontin	37.46±2.09	
Lasalocid A	37.10±2.64	
Shikonin	36.05±3.10	
Arbutin	35.64±1.22	
Piperine	34.16±1.14	
Mitomycin C	30.83±1.53	
Hesperitine	30.69±1.17	
Strophantidin	30.23±1.41	
Gitoxigenin	29.57±1.59	
5,6-Dehydrokawain	24.52±2.59	
Butein	23.79±0.93	
Amphotericin B	23.09±1.21	
Stachydrine.HCl	22.39±2.68	
Naringenin	22.20±0.73	
Homobutein	19.48±1.45	
Radicicol	18.68±1.86	
Lavendustin A	15.78±0.92	
Gelsemine.HCl	14.10±0.42	
Genistin	12.55±1.22	
Aloe-emodine	10.39±0.18	
Betulin	8.87±0.66	
Ochratoxin A	6.59±0.84	
Menadione	5.76±0.18	
Syringetine-3-glucoside	4.78±0.53	
Andrographolide	2.60±0.86	
Capsiacin	-2.14±1.30	
Eriodictyol	-4.93±1.12	
Neohesperidin	-6.24±1.71	
Curcumin	-8.89±0.28	

<sup>a</sup>Parasite growth inhibition values from preliminary screening with RH-2F at 1 µg/mL concentration. DMSO (0.5%) control was calculated as 0% inhibition, and 200 µg/mL pyrimethamine control was calculated as 100% growth inhibition. <sup>b</sup>Host cell viability values from preliminary screening with HFF cells at 2 µg/mL concentration. DMSO control was calculated as 100% cell viability.

**Table 2. Estimate of selectivity index for natural product isolates.**

Compounds	IC <sub>50</sub> <i>Toxoplasma gondii</i> (µg/mL)	IC <sub>50</sub> HFF cells (µg/mL)	Selectivity Index (SI)	
Rhapontin	1.22	20.05	16.38	SI >1
Homobutein	4.43	68.28	15.41	SI >1
Sulfuretine	0.68	8.20	12.10	SI >1
Arbutin	17.20	84.23	4.90	SI >1
Butein	2.12	7.92	3.74	SI >1
Naringenin	3.03	9.20	3.03	SI >1
Narasin	0.74	2.09	2.84	SI >1
Stachydrine.HCl	4.40	10.41	2.36	SI >1
Shikonin	3.96	5.81	1.47	SI >1
Hesperitine	13.66	12.42	0.91	
Lasalocid A	1.61	1.46	0.90	
Ochratoxin A	11.33	9.63	0.85	
Radicicol	4.90	2.58	0.53	
Cephaeline.HBr	0.95	0.45	0.47	
Menadione	44.70	17.93	0.40	
Peruvoside	0.37	0.11	0.30	
Sulfadiazine	185.20	53.35	0.29	
Mitomycin C	1.87	0.44	0.24	
Gitoxigenin	2.59	0.62	0.24	
Pyrimethamine	205.60	42.16	0.21	
Strophantidin	1.30	0.19	0.15	
Diacetoxyscirpenol	0.62	0.05	0.08	
Tuberacidin	0.46	0.02	0.04	
Capsiacin	ND	9.69	ND	
Curcumin	ND	ND	ND	
Resveratrol	1.03	ND	ND	
Genistin	5.46	ND	ND	
Eriodictyol	ND	ND	ND	
Andrographolide	388.10	ND	ND	
Syringetine-3-glucoside	16.64	ND	ND	
Piperine	1.71	ND	ND	
Aloe-emodine	8.14	ND	ND	
Neohesperidin	ND	ND	ND	
5,6-Dehydrokawain	4.22	ND	ND	
Betulin	224.40	ND	ND	
Lavendustin A	37.45	ND	ND	
Gelsemine.HCl	89.29	ND	ND	

\*ND – Not determined.

**Table 3. Inhibition of *Toxoplasma gondii* growth and host cell viability by FDA-approved chemical compounds.**

Compounds	<sup>a</sup> Inhibition of <i>Toxoplasma gondii</i> (%)		<sup>b</sup> Host (HFF) cell viability (%)
Bortezomib	60.13±1.78	≥40 % anti- <i>T. gondii</i> activity	86.64±6.65
10-Hydroxycamptothecin	58.16±2.01	≥40 % anti- <i>T. gondii</i> activity	100.55±1.58
Mebendazole	55.14±0.98	≥40 % anti- <i>T. gondii</i> activity	75.86±3.31
Niflumic acid	52.31±1.83	≥40 % anti- <i>T. gondii</i> activity	99.96±4.02
Clindamycin HCl	47.39±1.31	≥40 % anti- <i>T. gondii</i> activity	105.77±2.43
Mecamylamine	47.06±2.02	≥40 % anti- <i>T. gondii</i> activity	87.76±4.92
Chloroquine	45.14±2.62	≥40 % anti- <i>T. gondii</i> activity	82.54±4.10
Mitomycin C	45.06±1.87	≥40 % anti- <i>T. gondii</i> activity	103.37±3.84
Fenbendazole	41.62±1.70	≥40 % anti- <i>T. gondii</i> activity	21.82±1.24
Daunorubicin	41.60±1.19	≥40 % anti- <i>T. gondii</i> activity	62.27±0.72
Atropine	41.10±1.07	≥40 % anti- <i>T. gondii</i> activity	44.73±1.30
Cerivastatin	41.07±1.44	≥40 % anti- <i>T. gondii</i> activity	24.12±2.47
Camptothecin	37.38±1.32		27.68±2.54
Flufenamic acid	29.21±0.16		28.01±2.95
Prazosin.HCl	28.91±1.82		4.70±1.05
Sulfasalazine	27.93±1.74		104.16±5.22
Azaperone	27.24±2.00		93.09±3.22
Aclarubicin	25.13±0.42		94.05±4.80
Neostigmine	24.01±1.05		75.51±13.61
Anethole-trithione	21.98±1.00		86.65±4.93
Diflunisal	21.13±1.69		87.93±8.43
Lincomycin	21.04±1.32		113.67±8.48
Lomofungin	18.43±1.92		51.65±6.83
Cyclocytidine	16.93±1.05		51.24±2.09
Cytarabine	14.59±1.18		72.36±9.24
Amifostine	12.94±1.65		102.99±5.23

<sup>a</sup>Parasite growth inhibition values from preliminary screening with RH-2F at 1.5 µg/mL concentration. DMSO (0.5%) control was calculated as 0% inhibition, and 200 µg/mL pyrimethamine control was calculated as 100% growth inhibition. <sup>b</sup>Host cell viability values from preliminary screening with HFF cells at 3 µg/mL concentration. DMSO control was calculated as 100% cell viability.

The modeling for physicochemical, pharmacokinetic, and bioactivity properties of some selected compounds were performed using the Molinspiration Cheminformatics server (<http://www.molinspiration.com>). Compounds were randomly selected from among those with SI > 1. The SMILE formats of the compounds (with their corresponding identities) were obtained from <https://pubchem.ncbi.nlm.nih.gov/>. Features, including partition coefficient, total polar surface area, molecular weight, molecular volume, number of hydrogen bond acceptors/donors, mean number of rotatable bonds, and degree of violation of Lipinski's rule, were obtained following the reported standard procedure. The bioactivity score (based on Lipinski's "Rule of five"), which evaluates the ion channel

modulation, GPCR ligand, kinase inhibition, nuclear receptor interaction, protease inhibition, and enzyme inhibition activities, was also determined [16].

The *in silico* toxicity was determined using ProTox-II [17,18], a web-based computational modeling tool comprising about 33 models that can predict various toxicity endpoints, which include hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity, adverse outcomes pathways, toxicity targets, and acute toxicity.

#### Toxicity Potential and Classes

For the *in silico* toxicity evaluation, the toxic doses were given as LD<sub>50</sub> values in mg/kg body weight. The

**Table 4. Estimate of selectivity index for FDA-approved chemical compounds.**

Compounds	IC <sub>50</sub> <i>Toxoplasma gondii</i> (µg/mL)	IC <sub>50</sub> HFF cells (µg/mL)	Selectivity Index (SI)	
Atropine	2.20	61.35	27.85	SI >1
Niflumic acid	1.07	26.06	24.47	SI >1
Clindamycin HCl	1.59	25.79	16.22	SI >1
Azaperone	6.40	82.00	12.82	SI >1
Chloroquine	1.97	17.54	8.90	SI >1
Mecamylamine	2.65	21.30	8.04	SI >1
Mebendazol	2.05	2.03	0.99	
Lomofungin	31.64	18.02	0.57	
10-Hydroxycamptothecin	1.50	0.79	0.53	
Fenbendazole	5.42	2.69	0.50	
Camptothecin	4.48	1.77	0.39	
Mitomycin C	2.47	0.95	0.38	
Sulfadiazine	185.20	53.35	0.29	
Pyrimethamine	205.60	42.16	0.21	
Cerivastatin	3.29	0.60	0.18	
Bortezomib	1.82	0.12	0.07	
Daunorubicin	6.39	0.35	0.05	
Aclarubicin	110.70	3.52	0.03	
Lincomycin	9.39	ND	ND	
Flufenamic acid	4.32	ND	ND	
Prazosin.HCl	5.87	ND	ND	
Neostigmine	17.11	ND	ND	
Anethole-trithione	ND	ND	ND	
Amifostine	ND	59.81	ND	
Cyclocytidine	ND	27.52	ND	
Cytarabine	ND	42.01	ND	
Diflunisal	34.38	ND	ND	
Sulfasalazine	4.88	ND	ND	

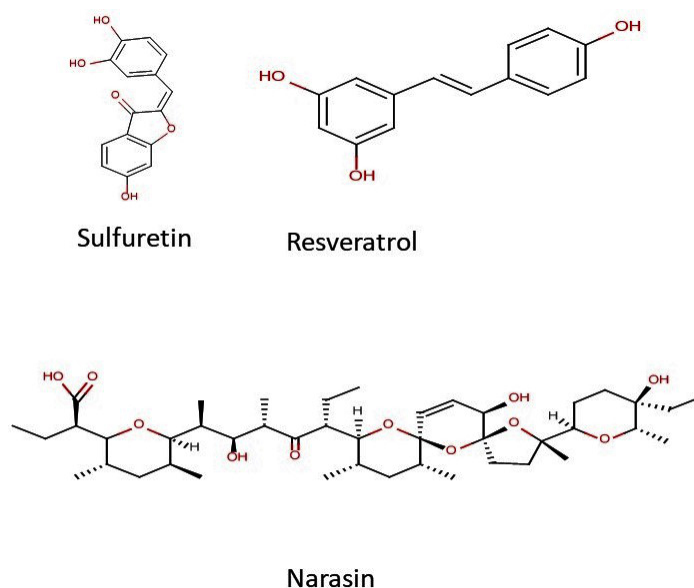
\*ND – Not determined.

model takes LD<sub>50</sub> as the median lethal dose, indicating the dose at which 50 % of the test subjects die upon exposure to the test compound. The toxicity classes were defined according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS). Within the group of substances that showed acute oral toxicity, the GHS classification, class 1 is designated as fatal if swallowed at LD<sub>50</sub> ≤ 5; class 2: fatal if swallowed at 5 < LD<sub>50</sub> ≤ 50; class 3: toxic if swallowed at 50 < LD<sub>50</sub> ≤ 300; class 4 harmful if swallowed at 300 < LD<sub>50</sub> ≤ 2000; class 5: may be harmful if swallowed at 2000 < LD<sub>50</sub> ≤ 5000, and class 6: non-toxic at LD<sub>50</sub> > 5000 [18,19].

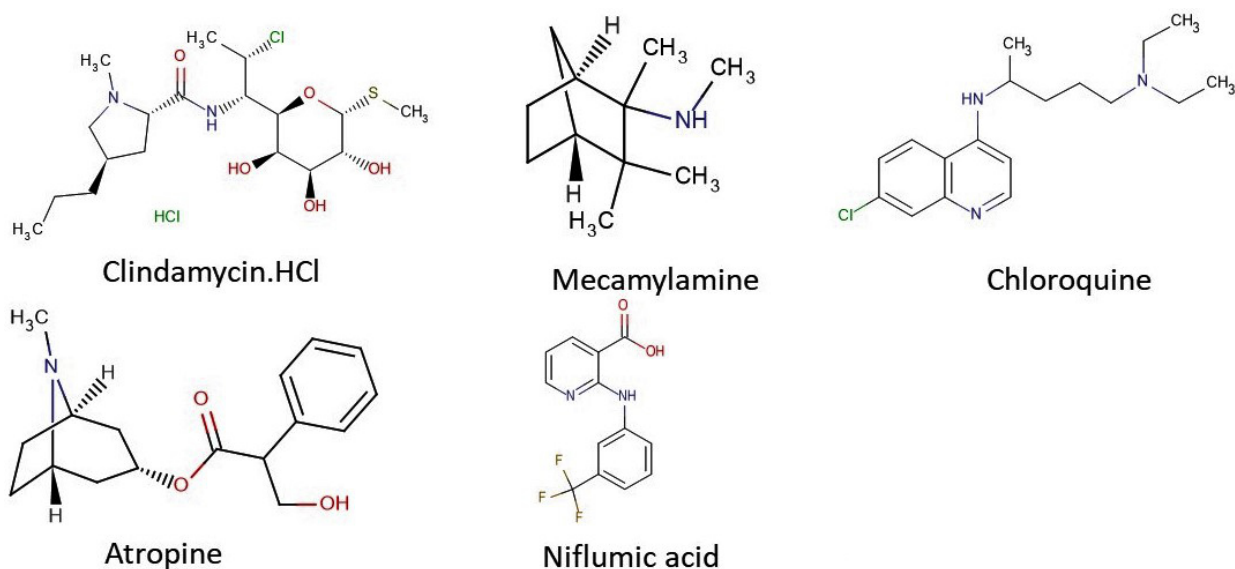
### Statistical Analysis

A one-way ANOVA was used to analyze the data utilizing GraphPad Software (San Diego, CA, USA). The results are presented as the mean of three replicates ± standard error of mean (SEM). The Dunnett post-hoc test was used to compare the groups, and the mean values were considered significant at  $p < .05$ . A non-linear regression analysis (curve fit) was used to estimate the concentration that caused inhibition of *T. gondii* growth or cellular toxicity by 50% (IC<sub>50</sub>).





**Figure 5.** Chemical structures of hit compounds from the natural product isolates.



**Figure 6.** Chemical structures of hit compounds from the FDA-approved drugs.

## RESULTS

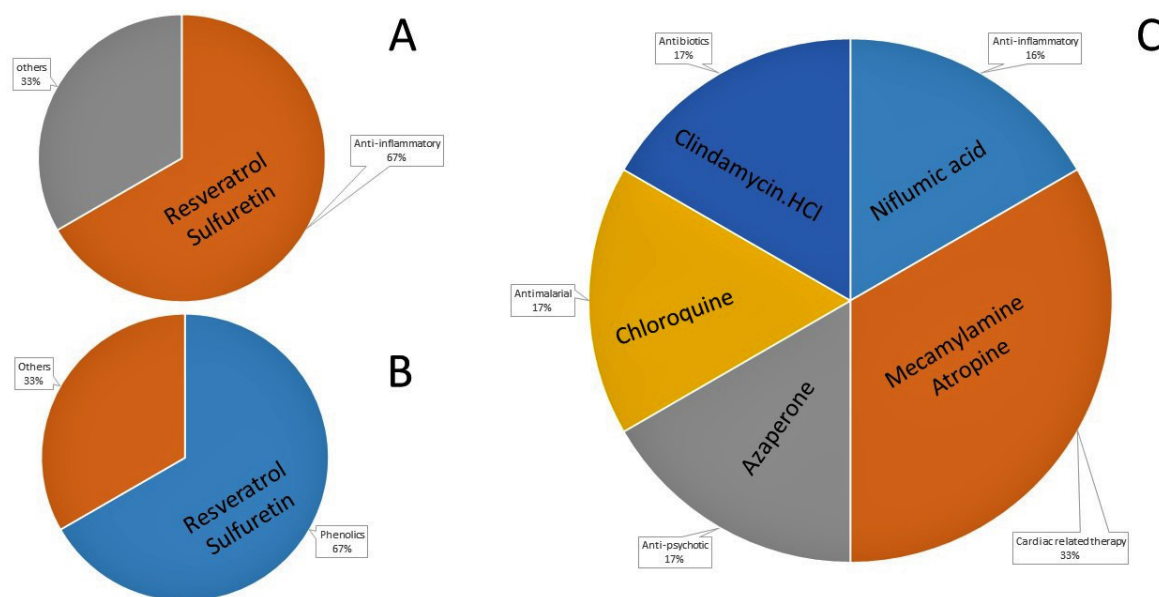
In this study, we screened a series of chemical entities comprising both natural product isolates (36) and FDA-approved drugs (26), to identify those with suppressive action or blocking capacity against the *in vitro* growth of *T. gondii*. Of the 62 compounds evaluated for dose-response antiparasite activity, the results showed that 18 compounds representing, 16.17% and 46.15%, respectively, of the natural product isolates and FDA-ap-

proved compounds inhibited ( $p < .05$ ) parasite growth by  $\geq 40$  percent (Figures 1 and 2). Meanwhile, determination of host cell viability in the presence of the same compounds showed that a total of 19 compounds representing, 27.77% and 34.62%, respectively, of the natural product isolates and FDA-approved drugs were cytotoxic ( $p < .05$ ), causing a reduction in host cell viability by  $\geq 30$  percent (Figures 3 and 4). Meanwhile,  $\geq 65$  percent of the total compounds from both the natural product isolates and the FDA-approved drugs, showed neither

**Table 5. ADME properties of the selected compounds/drugs.**

Compound or Drug PubChem ID	MiLogP	TPSA (Å <sup>2</sup> )	MW (g/mol)	nON	nOHNH	NoV	nRotB	MV (Å <sup>3</sup> )
Clindamycin hydrochloride	2.06	102.25	424.99	7	4	0	7	384.72
Mecamylamine	2.96	12.03	167.30	1	1	0	1	186.67
Chloroquine	5.00	28.16	319.88	3	1	1	8	313.12
Azaperone	2.88	36.44	327.40	4	0	0	6	307.55
Atropine	1.77	49.77	289.38	4	1	0	5	279.01
Niflumic acid	3.42	62.22	282.22	4	2	0	4	222.00
Sulfuretin	1.76	90.89	270.24	5	3	0	1	224.05
Homobutein	2.59	86.99	286.28	5	3	0	4	251.45
Arbutin	-0.81	119.61	272.25	7	5	0	3	232.20
Rhapontin	1.02	149.07	420.41	9	6	1	6	364.59
Pyrimethamine	2.84	77.83	248.72	4	4	0	2	216.62
Sulfadiazine	-0.04	97.98	250.28	6	3	0	3	202.26

miLogP - partition coefficient; TPSA - total polar surface area; MW - molecular weight; nON - number of hydrogen bond acceptors; nOHNH - number of hydrogen bond donors; noV - number of violations; nrotb - number of rotatable bonds; MV - molecular volume.



**Figure 7.** Distribution of active natural product compounds based on (A) bioactivity, (B) functional groups, and (C) treatment indications for FDA-approved drugs.

antiparasite activity nor any detectable host cytotoxicity. Furthermore, some of the natural compounds, including tubericidin, peruvoside, narasin, and diacetoxyscirpenol, that strongly blocked the *in vitro* growth of *T. gondii* at sub-microgram doses, lacked antiparasite specificity ( $p < .05$ ), as these compounds were also cytotoxic to the host cells at the same doses (Tables 1 and 2). Similarly, some of the FDA-approved drugs including 10-hydroxycamptothecin and bortezomib showed excellent anti-*T.*

*gondii* potential ( $p < .05$ ), but poor selectivity toward the parasite versus the host cells (Tables 3 and 4). Together, the findings suggest general cellular toxicity of these compounds. To validate our assay, pyrimethamine was included as a reference drug and, as expected, this drug blocked parasite growth.

Additionally, a few of the compounds that blocked the *in vitro* growth of *T. gondii*, also exhibited multiple-fold specificities (selectivity index (SI)  $\geq 3$ ) toward

**Table 6. *In silico* bioactivities for the selected compounds/drugs.**

Compound or Drug Name	PubChem CID	GPCR ligand	ICM	KI	NRL	PI	EI
<b>FDA Approved drugs</b>							
Clindamycin hydrochloride	16051951	0.33	-0.11	-0.54	-0.27	0.47	0.23
Mecamylamine	4032	-0.70	0.03	-1.47	-1.18	-0.80	-0.66
Chloroquine	2719	0.32	0.32	0.38	-0.19	0.05	0.11
Azaperone	15443	0.26	0.09	0.15	-0.17	-0.02	0.12
Atropine	174174	0.44	0.26	-0.09	-0.06	0.13	0.21
Niflumic acid	4488	0.04	0.04	0.29	-0.03	-0.11	0.11
<b>Natural Products</b>							
Sulfuretin	5281295	-1.18	-1.26	-0.52	-0.56	-1.21	0.00
Homobutein	6438092	-0.10	-0.19	-0.24	0.02	-0.31	0.06
Arbutin	440936	0.05	0.12	-0.13	0.04	-0.09	0.46
Rhapontin	637213	0.10	0.02	0.01	0.14	-0.02	0.30
<b>Standard anti-toxoplasma drugs</b>							
Pyrimethamine	4993	0.31	0.07	0.38	-0.61	-0.14	0.66
Sulfadiazine	5215	-0.29	-0.39	-0.14	-0.92	-0.47	-0.01

GPCR ligand, ICM - ion channel modulation; KI - kinase inhibition; NRL - nuclear receptor ligand; PI - protease inhibition; EI - enzyme inhibition

the parasite versus the host cells with  $EC_{50}$  ranging in the sub-microgram concentration. A selectivity index  $\geq 1$  is considered promising for early drug development against parasitic infection [20]. Some of the natural product isolates with promising antiparasite specificities included sulfuretin, resveratrol, and narasin (Figure 5), while those of the FDA-approved drugs were mecamylamine, atropine, niflumic acid, clindamycin HCl, and chloroquine (Figure 6). Taken together, the findings indicate not only specific antiparasite action by these compounds but also that the antiparasite action of these compounds was not a result of toxicity to the host cells. Given these results, the hit compounds could be considered as early drug development candidates for toxoplasmosis therapy.

Furthermore, of the identified antiparasitic compounds from both the natural product isolates and the FDA-approved drugs, the majority were effective and/or used against inflammatory related conditions (Figure 7a-c). Similarly, two of the effective natural anti-*T. gondii* isolates were phenolics. Considered together, the findings reinforce existing investigations that demonstrated that drugs and/or compounds used in inflammatory therapy blocked parasite growth *in vitro* [6,10,21].

Moreover, a few of the natural product isolates in this study showed mild activity against the growth of *T. gondii* and the same compounds also demonstrated a promising selectivity index (Tables 1 and 2). Among these are butein, homobutein, rhapontin, arbutin, and piperine. While butein and homobutein are flavonoids,

rhapontin and arbutin are glycosides and piperine is an alkaloid. Interestingly, our study is the first report showing a mild suppressive action of homobutein, butein, rhapontin, and arbutin against the *in vitro* growth of *T. gondii*. However, homobutein has been reported as having antimalarial activity [22,23] and anti-inflammatory, antioxidant, and antitumor potential [24-26]. In separate investigations, the anti-inflammatory, antioxidant, and antibacterial potential of rhapontin and arbutin have each been documented [27-29]. Additionally, piperine has been implicated as having anti-inflammatory properties [30] as well as anti-*T. gondii* activity [31].

Furthermore, the ADME properties and drug likeness (Lipinski's rule of five) of the standard anti-*Toxoplasma* drugs were compared with those of a select few of the FDA-approved drugs and natural product compounds that showed  $SI > 1$  (Tables 2, 4, and 5). While only chloroquine and rhapontin showed one violation each of the Lipinski's rule, mecamylamine had the lowest total polar surface area ( $12.03 \text{ \AA}^2$ ) and molecular volume ( $186.67 \text{ \AA}^3$ ) as well as molecular weight ( $167.30 \text{ g/mol}$ ). The majority of the compounds had molecular weights and molecular volumes below 500. While all the compounds showed prospects for application as anti-*Toxoplasma* agents, mecamylamine (an FDA-approved drug) and sulfuretin (a natural product) seemed to show greater potential based on their ADME properties.

The result of the bioactivity and drug-likeness measures are presented in Table 6. Comparatively, chloroquine

**Table 7. *In silico* toxicity model result.**

Compound or Drug Name	Potential Effects	Classification	Probability	Possible toxicity targets	Average Pharmacophore Fit (%)	Predicted LD <sub>50</sub> (mg/kg)	Predicted Toxicity Class
<b>FDA Approved drugs</b>							
Clindamycin hydrochloride	Immuno	TEP	0.93	Amine Oxidase A	70.29	1095	4
Mecamylamine	ND	NA	NA	Dopamine receptor D3	0	90	3
Chloroquine	Immuno Mutag	TEP TEP	0.99 0.94	ND	NA	311	4
Azaperone	Immuno	TEP	0.54	Histamine Receptor H1	56.12	63	3
Atropine	ND	NA	NA	PGH Synthase 1	48.05	75	3
Niflumic acid	MMP	TSRP	1.0	Amine Oxidase A	58.28	250	3
<b>Natural Products</b>							
Sulfuretin	Carcino	TEP	0.71	PGH Synthase 1	50.32	500	4
	Immuno	TEP	0.85				
	MMP	TSRP	0.76				
Homobutein	Immuno	TEP	0.99	Amine Oxidase A	63.3	2100	5
	MMP	TSRP	0.93				
Arbutin	ND	NA	NA	PGH Synthase 1	66.5	2420	5
Rhapontin	Immuno	Toxicity end points	0.99	PGH Synthase 1	0	1380	4
<b>Standard anti-Toxoplasma drugs</b>							
Pyrimethamine	ND	NA	NA	Amine Oxidase A	64.07	92	3
Sulfadiazine	Carcino	Toxicity end points	0.76	PGH Synthase 1	58.4	1500	4

Carcino - carcinogenicity; Mutag - mutagenicity; Immuno- immunotoxicity; MMP- mitochondrial membrane potential; TSRP - Tox21-stress response pathways; TEP - toxicity end points; ND - none detected; NA - not applicable; PGH - prostaglandin G/H

exhibited the highest potentials, in terms of its GPCR ligand, ion channel modulation, and kinase inhibition. In the natural products group, arbutin and rhapontin showed parameters comparable with the standard drug, pyrimethamine. All the selected FDA-approved drugs and natural products exhibited potential for further *in vivo* evaluation. The result of the *in silico* toxicity evaluation (Table 7) revealed that all the tested drugs and compounds had toxicities in classes 3 and 5. Interestingly, only homobutein and arbutin (natural product compounds) belong to toxicity class 5 (may be harmful if swallowed (2000<

LD<sub>50</sub> ≤ 5000) indicating potential relative safety on usage. Clindamycin hydrochloride, chloroquine, azaperone, homobutein, and rhapontin showed mild immunotoxic potential, and sulfuretin and sulfadiazine exhibited potential carcinogenicity. No specific toxicity effect was reported for mecamylamine, atropine, arbutin, or the standard, pyrimethamine in the model used. Like the standard (pyrimethamine), all the natural products showed average potential toxicity to prostaglandin synthase 1 and amine oxidase A.

## DISCUSSION

The screening of a vast array of chemical entities, including drug repurposing, is considered reasonable for identifying new therapeutic targets for the treatment of parasitic infection. For example, drug repurposing, in which well-characterized drugs and other types of chemical entities are tested for potential off-label indications, can not only identify lead targets but can also be useful for drug development for parasitic infections [7].

In this study, we screened 62 chemical entities, to identify those that suppress the growth of *T. gondii*. Altogether, a total of eight compounds from both the natural product isolates and the FDA-approved drugs showed antiparasitic activity at sub-microgram doses. Additionally, some of these same compounds demonstrated promising selectivity with a selectivity index (SI) of a  $\geq 3$ -fold activity against the parasite versus the host cells. Together, these findings not only underscore the specific antiparasite action shown by the compounds but also indicate that these compounds may be viable prospects in early drug development for toxoplasmosis. In comparison, pyrimethamine, which is one of the current treatment options for toxoplasmosis, exhibited a  $\leq 1$ -fold activity against the parasite versus the host cell.

Furthermore, most of the “hit” compounds (compounds that blocked parasite growth with promising SIs) among the FDA-approved drugs had not been reported as having anti-*T. gondii* activity; the exceptions were clindamycin HCl and chloroquine. Respectively, clindamycin HCl and chloroquine are therapeutic options for toxoplasmosis and malaria. Interestingly, the causative agents, *T. gondii* and *Plasmodium spp.* for toxoplasmosis and malaria, respectively, are intracellular apicomplexans. This may indicate that most of the FDA-approved drugs that blocked *T. gondii* growth in the present study have similar clinical indications. For example, two of the hit compounds, mecamlamine and atropine, are used as therapeutic options for cardiac-related conditions, whereas niflumic acid is used to treat inflammatory conditions. Previously, we showed that FDA-approved drugs used as anti-inflammatory therapies potently suppressed the *in vitro* growth of *T. gondii* [10]. Taken together, these findings may underscore the prospects of anti-inflammatory agents as a source of alternative anti-*T. gondii* therapy. However, while the FDA-approved compounds are mostly well-characterized, their mechanisms of action against the growth of *T. gondii* are yet to be revealed and thus warrant further studies.

Among the hits from the natural product isolates, resveratrol has been previously reported as having anti-*T. gondii* properties [32], while narasin, a derivative of salinomycin (an ionophoric anticoccidial compound), is a known antiprotozoan agent [33]. Additionally, two of the

hit compounds (resveratrol and sulfuretin) from the natural product isolates are phenolics. Interestingly, our study is the first report to show a suppressive action of sulfuretin against the *in vitro* growth of *T. gondii*. Nevertheless, some of the hit compounds have been reported as having other bioactive properties. For example, resveratrol has been implicated as having anti-inflammatory properties [34] as well as anti-*T. gondii* activity [32]. Also, the anti-inflammatory and antiparasitic actions of narasin have been previously documented [35]. It is worth noting that the majority of the hit compounds among the natural product isolates in this study have anti-inflammatory properties. This is consistent with our findings reported elsewhere [10], which revealed not only that the natural compounds active against *T. gondii* are mostly phenolics but also that the same compounds have anti-inflammatory activity. This same study also revealed that  $\geq 75$  percent of the FDA-approved drugs with activity against *T. gondii* are therapeutic options for inflammatory-related conditions. Given these findings, it is plausible to rationalize that the hit compounds identified in the study might have somewhat similar antiparasite mechanisms. Moreover, in the present study, the fact that a majority of the hit compounds possess anti-inflammatory potential may indicate a way to identify new targets that could be further explored for development of more effective therapeutic options for toxoplasmosis. Taken together, the findings in this study not only support our earlier report [10] but also are consistent with those from other studies [6,21] that showed that drugs with anti-inflammatory indications suppressed the *in vitro* growth of *T. gondii*. Additionally, the *in silico* model supported the prospects of the identified anti-*Toxoplasma*-active compounds from both the FDA-approved and natural products for early drug discovery against toxoplasmosis.

In conclusion, our study identified new chemical compounds from a group of natural product isolates and FDA-approved drugs that suppressed the *in vitro* growth of *T. gondii*. The findings are not only new and promising but also further reinforce the concept of screening a vast array of chemical entities, including drug repurposing, as a logical option in early drug development efforts for parasitic infections. Ongoing investigations aim to evaluate these compounds for *in vivo* efficacy against acute and latent toxoplasmosis in a mouse model.

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## REFERENCES

- Black MW, Boothroyd JC. Lytic Cycle of *Toxoplasma gondii*. *Microbiol Mol Biol Rev*. 2000;64:607–23.
- Beck HP, Blake D, Darde ML, et al. Molecular Approaches to Diversity of Populations of Apicomplexan Parasites. *Int J Parasitol*. 2009;39:175–89.
- Hill DE, Chirukandoth S, Dubey JP. Biology and Epidemiology of *Toxoplasma gondii* in Man and Animals. *Anim Health Res Rev*. 2005;6:41–61.
- Kamau ET, Srinivasan AR, Brown MJ, et al. A Focused Small-Molecule Screen Identifies 14 Compounds with Distinct Effects on *Toxoplasma gondii*. *Antimicrob Agents Chemother*. 2012;56(11):5581–90.
- Wang ZD, Liu HH, Ma ZX, et al. *Toxoplasma gondii* Infection in Immunocompromised Patients: A Systematic Review and Meta-Analysis. *Front Microbiol*. 2017;8:389.
- Dittmar AJ, Drozda AA, Blader IJ. Drug Repurposing Screening Identifies Novel Compounds that Effectively Inhibit *Toxoplasma gondii* Growth. *MSphere*. 2016;1(2):e00042–15.
- Adeyemi OS, Molina MT, Eseola AO, Fonseca-Berzal C, Gómez-Barrio A. New imidazole compounds active against *Trypanosoma cruzi*. *Comb Chem High Throughput Screen*. 2017a;20(1):20–4.
- Adeyemi OS, Murata Y, Sugi T, Kato K. Inorganic Nanoparticles Caused Death of *Toxoplasma gondii* through Alteration of Redox Status and Mitochondrial Membrane Potential. *Int J Nanomedicine*. 2017b;12:1647–61.
- Adeyemi OS, Murata Y, Sugi T, Han Y, Kato K. Modulation of Host HIF-1 $\alpha$  Activity and the Tryptophan Pathway Contributes to the Anti-*Toxoplasma gondii* Potential of Nanoparticles. *Biochem Biophys Rep*. 2017c;11:84–92.
- Adeyemi OS, Sugi T, Han Y, Kato K. Screening of Chemical Compound Libraries Identified New Anti-*Toxoplasma gondii* Agents. *Parasitol Res*. 2018a;117(2):355–63.
- Adeyemi OS, Murata Y, Sugi T, Han Y, Kato K. Exploring Amino Acid-Capped Nanoparticles for Selective Anti-Parasitic Action and Improved Host Biocompatibility. *J Biomed Nanotechnol*. 2018b;14(5):847–67.
- Adeyemi OS, Molefe NI, Awakan OJ, Nwonuma CO, Alejowolo O, Olaolu T, et al. Metal Nanoparticles Show Potential to Restrict *Trypanosoma* Growth. *Artif Cells Nanomed Biotechnol*. 2018c;46 S3:S86–94.
- Adeyemi OS, Otohinyi DA, Awakan OJ, Adeyanju AA. Cellular Apoptosis of HFF Cells by Inorganic Nanoparticles Not Susceptible to Modulation by *Toxoplasma gondii* Infection *In Vitro*. *Toxicol In Vitro*. 2019;54:280–5.
- Fonseca-Berzal C, Ruiz FA, Escario JA, Kouznetsov VV, Gomez-Barrio A. In vitro Phenotypic Screening of 7-Chloro-4-Amino(Oxy) Quinoline Derivatives as Putative Anti-*Trypanosoma Cruzi* Agents. *Bioorg Med Chem Lett*. 2014;24(4):1209–13.
- Šimenc J, Lipnik-Štangelj M. Staurosporine induces apoptosis and necroptosis in cultured rat astrocytes. *Drug Chem Toxicol*. 2012;35(4):399–405.
- Mishra SS, Kumar N, Singh HP, Ranjan S, Sharma CS. *In silico* Pharmacokinetic, Bioactivity and Toxicity Study of some Selected Anti-Asthmatic Agents. *Int J Pharm Sci Drug Res*. 2018;10(4):278–82.
- Banerjee P, Eckert OA, Schrey AK, Preissner R. ProTox-II: a webserver for the prediction of toxicity of chemicals. *Nucleic Acids Res*. 2018;46 W1:W257–63.
- Drwal MN, Banerjee P, Dunkel M, Wettig MR, Preissner R. ProTox: a web server for the in silico prediction of rodent oral toxicity. *Nucleic Acids Res*. 2014;(Web Server issue):W53–8.
- Globally Harmonized System of Classification and Labeling Of Chemicals GHS. [www.osha.gov](http://www.osha.gov). Archived from the original on 2007-07-02. [Retrieved May 2, 2019].
- Molefe NI, Yamasaki S, Macalanda AM, et al. Oral Administration of Azithromycin Ameliorates Trypanosomiasis in *Trypanosoma Congolense*-Infected Mice. *Parasitol Res*. 2017;116(9):2407–15.
- Murata Y, Sugi T, Weiss LM, Kato K. Identification of Compounds That Suppress *Toxoplasma gondii* Tachyzoites and Bradyzoites. *PLoS One*. 2017;12(6):e0178203.
- Tomar V, Bhattacharjee G, Rajakumar S, Srivastava K, Puri SK. Synthesis of New Chalcone Derivatives Containing Acridinyl Moiety with Potential Antimalarial Activity. *Eur J Med Chem*. 2010;45(2):745–51.
- Yenesew A, Induli M, Derese S, Midiwo JO, Heydenreich M, Peter MG, et al. Anti-Plasmodial Flavonoids from the Stem Bark of *Erythrina Abyssinica*. *Phytochemistry*. 2004;65(22):3029–32.
- Sung J, Lee J. Anti-Inflammatory Activity of Butein and Luteolin Through Suppression of NF  $\kappa$ B Activation and Induction of Heme Oxygenase-1. *J Med Food*. 2015;18(5):557–64.
- Cheng ZJ, Kuo SC, Chan SC, Ko FN, Teng CM. Antioxidant Properties of Butein Isolated from *Dalbergia Odorifera*. *Biochimica Et Biophysica Acta (BBA)- Lipids and Lipid Metabolism*. 1998;1392(2-3):291–9.
- Sivakumar PM, Prabhakar PK, Doble M. Synthesis, Antioxidant Evaluation, and Quantitative Structure–Activity Relationship Studies of Chalcones. *Med Chem Res*. 2011;20(4):482–92.
- Busetto GM, Giovannone R, Ferro M, Tricarico S, Del Giudice F, Matei DV, et al. Chronic Bacterial Prostatitis: Efficacy of Short-Lasting Antibiotic Therapy with Prulifloxacin (Unidrox®) In Association with Saw Palmetto Extract, *Lactobacillus Sporogens* and Arbutin (Lactorepens®). *BMC Urol*. 2014;14(1):53.
- Kajiwara R, Seto A, Kofujita H, Shiba Y, Oishi Y, Shibasaki Y. Enhanced Antimicrobial Activities of Polymerized Arbutin and its Derivatives Prepared by Oxidative Polymerization of Arbutin. *React Funct Polym*. 2019.
- Zhao W, Wang S, Qin T, Wang W. Arbutin Attenuates Hydrogen Peroxide-Induced Oxidative Injury Through Regulation of Microrna-29a in Retinal Ganglion Cells. *Biomed Pharmacother*. 2019;112:108729.
- Bang JS, Oh DH, Choi HM, et al. Anti-Inflammatory and Antiarthritic Effects of Piperine in Human Interleukin 1beta-Stimulated Fibroblast-Like Synoviocytes and in Rat Arthritis Models. *Arthritis Res Ther*. 2009;11(2):R49.
- Al-Zanbagi NA. In vivo Effect of some Home Spices Extracts on the *Toxoplasma gondii* Tachyzoites. *J Family Community Med*. 2009;16(2):59–65.
- Bottari NB, Baldissera MD, Tonin AA, Rech VC, Alves CB, D'Avila F, et al. Synergistic Effects of Resveratrol

- (Free and Inclusion Complex) And Sulfamethoxazole-Trimetopim Treatment on Pathology, Oxidant/Antioxidant Status and Behavior of Mice Infected with *Toxoplasma gondii*. *Microb Pathog*. 2016;95:166–74.
33. Gerhold RW, Fuller AL, Lollis L, Parr C, McDougald LR. The Efficacy of Anticoccidial Products against *Eimeria* Spp. In Northern Bobwhites. *Avian Dis*. 2011;55(1):59–64.
34. de Sá Coutinho D, Pacheco MT, Frozza RL, Bernardi A. Anti-Inflammatory Effects of Resveratrol: mechanistic Insights. *Int J Mol Sci*. 2018;19(6):1812.
35. Kevin Ii DA, Meujo DA, Hamann MT. Polyether Ionophores: Broad-Spectrum and Promising Biologically Active Molecules for the Control of Drug-Resistant Bacteria and Parasites. *Expert Opin Drug Discov*. 2009;4(2):109–46.