

Activity of Green Algae Extracts against *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is a zoonotic protozoa of economic significance in livestock. Infected livestock meat and products act as a source of *T. gondii* infection in humans. Current drugs against *T. gondii* are limited by hypersensitivity and toxicity, and are not effective against the encysted bradyzoite stage of *T. gondii*. Thus, there is urgent need for safe and effective therapeutic agents against *T. gondii*. Marine algae possess potent antifungal and antibacterial properties, but there are no reports on its anti-protozoal activity. Therefore, in this study we obtained n-hexane and methanol extracts of green algae (*Chlorophyceae*) and analyzed their content by high performance liquid chromatography coupled with mass spectrometry, as well as tested their *in vitro* anti-*Toxoplasma* activities. Compared to the n-hexane extract of *Chlorophyceae*, the methanol extract contained higher content of flavonoids/polyphenols, alkaloids (elaecarpidine and auramine), and artemisic acid. Importantly, the methanol extract had more potent anti-*Toxoplasma* activity ($IC_{50}=4.43 \pm 1.26 \mu\text{g/mL}$) than the n-hexane extract ($IC_{50}=23.32 \pm 3.97 \mu\text{g/mL}$), corroborating the higher content of flavonoids, alkaloids, and artemisic acid in methanol extracts than in n-hexane extract. The anti-*Toxoplasma* IC_{50} values of the methanol and n-hexane extracts were 34-fold and 7-fold lower than their respective cytotoxic IC_{50} values in human fibroblast cell line. Consistent with our findings, flavonoids, alkaloids and artemisic acid have previously been shown to have potent anti-*Toxoplasma* activity. Together, our results show that *Chlorophyceae* contains significant amounts of bioactive compounds with potent anti-*Toxoplasma* activity.

Keywords: Green algae chlorophyceae; *Toxoplasma gondii*; Antiparasitic; Bioactive compounds

Short Communication

Toxoplasma gondii is zoonotic protozoa that is very prevalent worldwide and a major cause of abortions and neonatal deaths in sheep and other livestock, resulting in significant economic losses [1]. Infected livestock meat and products act as a source of *T. gondii* infection in humans [2]. Despite these challenges, there is currently no medicine that can eliminate *T. gondii* infection, particularly the encysted stage. Thus, there is urgent need to develop a generation of safe and efficacious drugs for use in controlling *T. gondii* infections. Marine algae contain significant amounts of bioactive compounds [3] and as a result has been shown to possess potent antifungal and antibacterial properties [4]. However, there has been no reports on its anti-protozoal properties. Therefore, in this study we endeavored to evaluate the *in vitro* anti-*Toxoplasma* activity of green algae extracts.

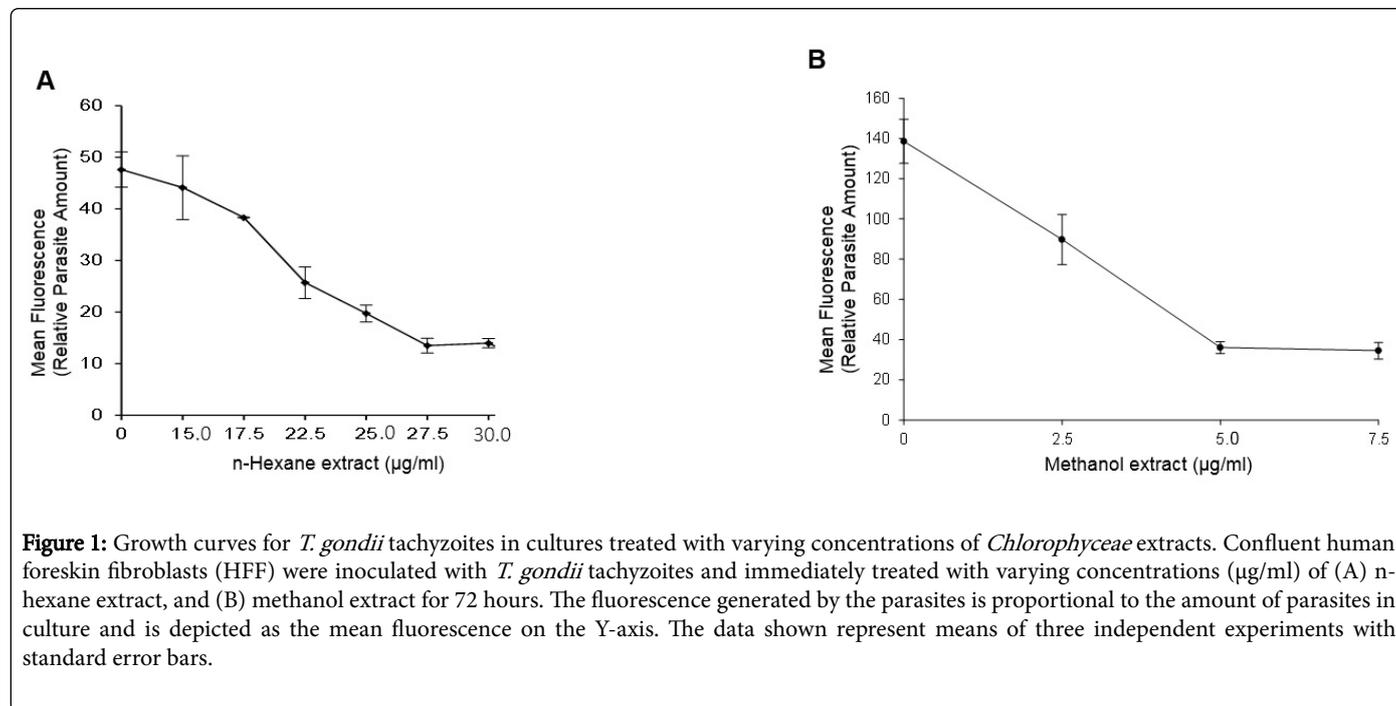
Dried, pure cultured green algae, *Chlorophyceae* was generously provided by Dr. Wei Liao of the Anaerobic Digestion Research and Education Center, Michigan State University. Dried algae samples were finely milled and soaked in methanol or n-hexane for 24 hours with agitation. The extraction solution was filtered, evaporated to dryness and the extracts reconstituted in dimethyl sulfoxide (DMSO) to desired concentration. *T. gondii* RH strain, engineered to constitutively express cytosolic yellow fluorescent protein (RH-YFP) [5] was cultured in human foreskin fibroblasts (HFF) maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) heat-

inactivated fetal bovine serum (Life Technologies), 1% (v/v) Glutamax and 1% (v/v) penicillin-streptomycin-fungizone (Life Technologies) at 37°C with 5% CO₂. *T. gondii* tachyzoites were extracted from HFF cells by passing the cell suspension twice through a 25-gauge needle, and extruded parasites isolated from cell debris by passing through a 3 μM filter, followed by washing the filtered parasites in PBS.

To determine the effect of the algae extracts on the growth of *T. gondii*, HFF cells were grown to confluence in 96-well plates. Prior to infection, old medium was replaced with fresh medium, and freshly extracted *T. gondii* added to the HFF cultures at 1000 tachyzoites per well. Test algae extracts at increasing concentrations were added immediately after parasite inoculation. Cultures without compounds (but with similar volume of DMSO) were maintained as controls. At, 24, 48 and 72 hour post-infection, parasite proliferation was determined using an image Xpress micro automated fluorescence microscope by measuring the parasite YFP fluorescence followed by quantification of YFP intensity using ImageJ version 1.37v software (NIH). We found that at 72 hours of culture, the methanol and n-hexane extracts of *Chlorophyceae* algae exhibited dose-dependent inhibitory effect on the intracellular growth of *T. gondii* tachyzoites (Figure 1A and 1B). However, at earlier times of 24 and 48 hours post-infection, there was no significant difference in parasite growth between the algae extract-treated and the DMSO-treated parasites. This suggested that the bioactive compounds from algae could have acted by inhibiting the invasion of host cells by egressed parasites, assuming that during the first 48 hours post-infection, the parasites would mostly be growing only in cells infected initially. Between 48 and 72 hours post-infection, it is expected that the first egress would

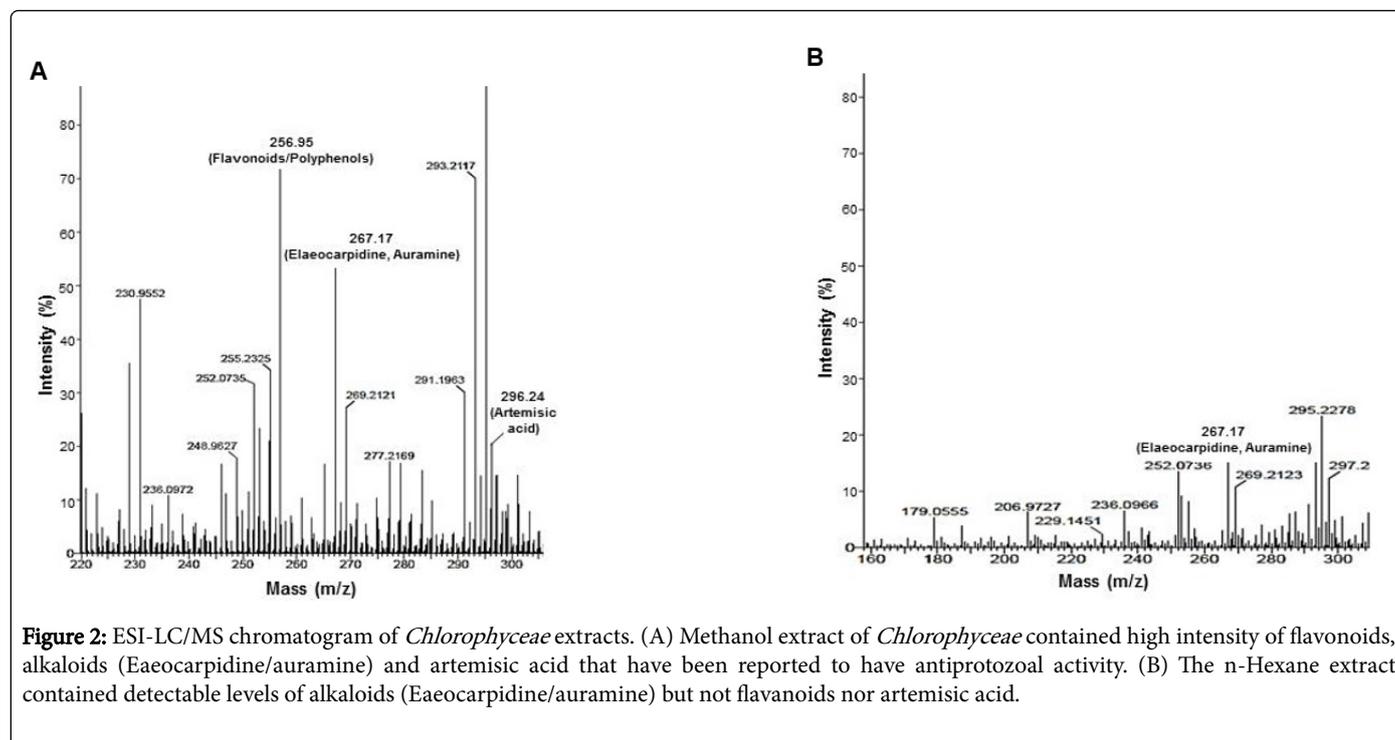
have occurred and the egressed parasites would have infected new host cells and started growing intracellularly [6], thereby increasing the number of parasites in the culture substantially. However, in the presence of the algae bioactive compound, the egressed parasites would

be directly exposed and internalize considerable amounts of the compounds, which would then compromise the parasites' infectivity and ability to proliferate in host cells.



By nonlinear regression analysis, using Graph Pad Prism software version 6.0 program (CA, USA), we found that the *T. gondii* growth inhibitory concentration (IC₅₀) values for the methanol and n-hexane *Chlorophyceae* extracts were, 4.43 ± 1.26 µg/mL and 23.32 ± 3.97 µg/mL, respectively. This indicated that, the methanol extract had more potent activity than the n-hexane extract, suggesting that the methanol extract was more enriched in bioactive compounds content than the n-hexane extract. We, therefore, performed mass-spectrometry analysis to determine the major constituents in the n-hexane and methanol. The extracts were analyzed by High Performance Liquid Chromatography (HPLC) and Electrospray

Ionization-Mass spectrometry (ESI-MS) at the Mass Spectrometry facility, University of Illinois at Urbana-Champaign. Briefly, equal amounts of the extracts were run on HPLC using a 2.1 mm ID reverse phase C-18 column with the mobile phase composed of 5% acetonitrile/95% water/0.1% formic acid, followed by ESI-MS analysis in negative mode, operated by Masslynx software v.4.1. Based on the elemental composition of the extracts, as determined by ESI-MS analysis, the methanol extract of *Chlorophyceae* contained higher content of flavonoids/polyphenols, alkaloids (elaecarpidine and auramine), and artemisic acid with mass numbers of 256.95, 2667.17 and 296.24, respectively, than the n-hexane extract (Figure 2A and 2B).



Consistent with our findings in the present study, in our previous study, we have found that Sorghum bicolor red leaf extract fraction that was rich in flavonoids content, had potent *in vitro* activity against *T. gondii* at dose levels that were non-toxic to mammalian cells [7]. Corroborating our observations in the present study, alkaloids have also been previously shown to have anti-*Toxoplasma* activity *in vitro* [8]. Additionally, artemisic acid has been documented to have potent activity against a broad range of protozoan parasites including *Plasmodium*, *Leishmania*, *Trypanosoma*, *Toxoplasma*, *Neospora*, *Eimeria*, *Acanthamoeba*, *Naegleria*, *Cryptosporidium*, *Giardia* and *Babesia* [9]. Therefore, the anti-*Toxoplasma* activities of the methanol *Chlorophyceae* extracts can be attributed to the high content of flavonoids, alkaloids and artemisic acid.

To determine the cytotoxic IC₅₀ values of the *Chlorophyceae* extracts in mammalian cells, HFF cells were cultured in supplemented IMDM medium (without red phenol) in 96-well plates and varying concentrations of the extracts added. Wells to which equivalent volumes of DMSO only were added were included as negative control. At 72 hours of culture, a colorimetric assay using the cell proliferation reagent WST-1 (Roche) for the quantification of cell viability was performed on the cultures by adding 20 µL of the WST-1 reagent to each well. After mixing, the plates were wrapped in aluminum foil and incubated for 1 hour at 37 °C with 5% CO₂. After 1 hour of incubation, 150 µL of the medium from each well was transferred to a new 96-well plate and quantification of the formazan dye produced by metabolically active cells was read as absorbance at a wavelength of 420 nm using a scanning multi-well spectrophotometer (Spectra Max 250; Molecular Devices). We generated dose-response curves using GraphPad PRISM software and found that the HFF cytotoxicity values for the methanol and n-hexane *Chlorophyceae* extracts were 150.83 ± 12.94 and 159.52 ± 15.71, respectively. Thus, the n-hexane and methanol *Chlorophyceae* extracts had *T. gondii* IC₅₀ values that were 34-fold and 7-fold lower than their respective cytotoxic IC₅₀ values in HFF host cells. This indicated that both extracts have potent *in vitro*

inhibitory activity against *T. gondii* parasites at concentrations that are non-toxic to mammalian cells suggesting that they may not have adverse side-effects *in vivo*. Together, our findings demonstrate that green algae, *Chlorophyceae*, contains significant amounts of bioactive compounds that possess potent anti-*Toxoplasma* activity.

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