***In vitro* Activity of *Sorghum bicolor* Extracts, 3-deoxyanthocyanidins, Against
*Toxoplasma gondii***

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Abstract

We investigated dried red leaf extracts of *S. bicolor* for activity against *Toxoplasma gondii* tachyzoites. *S. bicolor* red leaf extracts were obtained by bioassay-guided fractionation using ethanol and ethyl acetate as solvents. Analysis of the crude and fractionated extracts from *S. bicolor* using electrospray ionization mass spectrometry (ESI-MS) showed that they contained significant amounts of apigeninidin, luteolinidin, 7-methoxyapigeninidin, 5-methoxyapigeninidin, 5-methoxyluteolinidin, 5-7-dimethoxyapigeninidin or 5-7-dimethoxyluteolinidin, based on mass per charge (m/z). When tested *in vitro*, the IC_{50} s for inhibitory activity against *T. gondii* tachyzoites' growth of the ethanol and ethyl acetate extracts were 2.3- and 4-fold, respectively, lower than their cytotoxic IC_{50} s in mammalian cells. Ethyl acetate extracts fractionated in chloroform-methanol and chloroform had IC_{50} s against *T. gondii* that were 56.1- and 3-fold lower than their respective cytotoxic IC_{50} s in mammalian cells. These antiparasitic activities were found to be consistent with those of the respective pure 3-deoxyanthocyanidin compounds identified to be contained in the fractions in significant amounts. Further, we observed that, the position and number of methoxy groups possessed by the 3-deoxyanthocyanidins influenced their antiparasitic activity. Together, our findings indicate that *S. bicolor* red-leaf 3-deoxyanthocyanidins-rich extracts have potent *in vitro* inhibitory activity against the proliferative stage of *T. gondii* parasites.

Keywords: *Sorghum bicolor*; 3-deoxyanthocyanidins extracts; *Toxoplasma gondii*; antiparasitic compounds

1. Introduction

Toxoplasmosis is a disease caused by *Toxoplasma gondii*, a zoonotic apicomplexan protozoa of medical and veterinary significance globally. Humans are infected post-natally through the ingestion of undercooked *T. gondii*-infected meat or through ingestion of parasite oocysts in contaminated water or plants (Dubey, 2009). Because of the multiple paths *T. gondii* uses to infect, one in three people globally are infected (Sullivan and Jeffers, 2012). Prevalence of *T. gondii* seropositive humans is markedly high in countries where raw or undercooked meat is consumed. Congenital infections are frequent and cause gross detriment to the health of infected children (Miller et al., 2009).

Several therapeutic agents are available for managing toxoplasmosis in humans, including azithromycin, roxithromycin, spiramycin, pyrimethamine, clindamycin, erythromycin and a combination of pyrimethamine-sulfadiazine (Tee et al., 1998; Chamberland et al., 1991; Alder et al., 1994; Van der Ven et al., 1996). However, these drugs have serious toxic, hypersensitivity and teratogenic side-effects and are not effective against the encysted bradyzoite stage of the parasite (McLead et al., 2006; Fomovska et al., 2012). Thus, there is urgent need to develop a new generation of safe and efficacious drugs for use in controlling *T. gondii* infections.

Plant extracts containing phenolic compounds and their derivatives, especially, flavonoids, tannins, flavonol, flavone, luteolin, quercetin have been shown to have growth inhibitory activity against apicomplexan protozoan parasites including *T. gondii* (Weiss et al 1998; Dobbin et al., 2002), *Plasmodium falciparum* (Lehane and Saliba, 2008), *Trypanosoma*

(Mamani-Matsuda et al., 2004), and *Leishmania* (Kirmizibekmez et al., 2004; Sen et al., 2005). *S. bicolor* contains abundant amounts of 3-deoxyanthocyanidins compounds (Oladiji et al., 2007). However, there is no available information regarding these compounds' ability to both inhibit and kill *T. gondii* tachyzoites. Thus, in this study we endeavored to extract, fractionate and identify compounds from *S. bicolor* red leaf with activity against *T. gondii* using *in vitro* bio-assays.

2. Materials and Methods

2.1. Plant material

Leaves of mature *S. bicolor* Red plants with grain that is ready for harvest were obtained from Northern Ghana, West Africa. The leaves were air-dried under mild sunlight and then packaged in amber-colored zip-lock bags until use. They were authentically identified as *S. bicolor* Red leaves. A voucher specimen of the leaves has been deposited in the Department of Chemistry, Tuskegee University, Alabama, USA.

2.2. General analytical and fractionation procedures

All solvents were of high performance liquid chromatography grade (HPLC grade) obtained from Sigma and Fisher Scientific, USA. The *S. bicolor* leaf extracts were obtained using procedures reported by Wang *et al.* (2013) and Abugri *et al.* (2013). Approximately 41.04 g of the dried and ground plant samples were mixed with 500 mL ethanol (99.9%) and incubated in a water bath with shaking at 100 rpm for 2 h at 30°C. The procedure was repeated three more times and the resultant extracted volumes pooled, filtered using Whatman number 2 filter paper and evaporated to dryness under reduced pressure at 60°C. The obtained crude extract was

washed with water, followed by successive treatment with n-hexane, ethyl acetate and n-butanol (water saturated) (Wang et al., 2013).

2.3. Fractionation and purification of compounds from crude ethyl acetate extracts

Size exclusion chromatography was carried out with Biogel-P60 (BioRad. Inc., USA) on ethyl acetate extracts using solvents of different polarities, namely pure chloroform, chloroform-methanol mixture (10:1), pure methanol or pure ethyl acetate to extract bioactive compounds (Figure 1). The various fractions were collected in 15 mL Pyrex culture test tubes, dried and analyzed by qualitative thin layer chromatography (TLC) on pre-coated plates (silica gel 60 F_{254nm}, 0.2 mm: Merck). Spots on the plates were visualized under UV light (254) and excised from the plates. The excised spots were eluted from silica gel into their respective solvents and analyzed using Electrospray Ionization-Mass spectrometry (ESI-MS) and High Performance Liquid Chromatography. Identified fractions that were obtained in significant amounts were reconstituted in dimethyl sulfoxide (DMSO) and tested *in vitro* for growth inhibitory activity against *Toxoplasma gondii* and for cytotoxicity against parasite host human foreskin fibroblasts as well as human aortic endothelial cells.

2.4. Characterization of fractionated compounds

Samples were analyzed using an Ultra Performance LC System (ACQUITY, Waters Corp., Milford, MA, USA) coupled with a quadrupole time-of-flight mass spectrometer (Q-TOF Premier, Waters) with electrospray ionization (ESI) in negative ESI-MS operated by Masslynx software (V4.1). Each sample, in acetonitrile, was directly injected into the ESI source at a flow rate of 50 µL/min with mobile phase of 100% or 50% acetonitrile (ACN) in water. The ion source voltages were set at 3 kV for positive and negative ion mode acquisitions. The sampling cone was set at 37 V and the extraction cone was at 3 V. In both negative and positive modes, the

source and desolvation temperatures were maintained at 120°C and 225°C, respectively, with the desolvation gas flow at 200 L/h. The TOF MS scanning was from 50 to 1500 m/z at 1s with 0.1 s inter-scan delay using extended dynamic range acquisition with centroid data format. For real time mass calibration, direct infusion of sodium formate solution (10% formic Acid/0.1 M NaOH/ACN at a ratio of 1:1:8) at 1 sec/10 sec to ion source at 2 µl/min was used for a single point mass calibration.

Ions of interest were analyzed for elemental composition using accurate mass (less than 5 ppm error) and isotope modeling to identify the chemical formulae. Collision-induced dissociation (CID) by argon on precursor ions resulted in structural fragmentation that further assisted the identification.

2.5. Maintenance of Parasites and Cell Culture

Confluent monolayers of human foreskin fibroblasts (HFF) and aortic endothelial cells (HAEC) were 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. *Toxoplasma gondii* RH strain parasites, constitutively expressing cytosolic yellow fluorescent protein (RH-YFP) (Gubbels et al., 2003) were maintained in HFF cells. For parasite growth inhibitory activity tests of the compounds, HFF cells were seeded into 96 well plates with a total volume of 200 µL of medium and allowed to proliferate to full confluency prior to use.

2.6. Preparation of compounds for toxicity Assays.

The dried initial crude ethanol extract, the ethyl acetate extract and fractionated compounds, as well as pure synthetic compounds, were reconstituted in dimethyl sulphoxide (DMSO). When compounds were added to cell cultures, the final concentration of DMSO did not exceed 1%.

2.7. Analysis of compounds' *in vitro* inhibitory activity against *T. gondii*

Confluent HFF cells grown in 96 well plates were inoculated with RH-YFP *T. gondii* tachyzoites at a concentration of 3,500 parasites/ml. Test compounds at increasing concentrations were added immediately after inoculation in triplicate. Cultures without compounds (but with similar volume of DMSO) were maintained as controls. At 24, 48 and 72 h 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). The IC₅₀ of the compounds against *T. gondii* were determined using nonlinear regression analysis by Graph pad Prism software version 6.0 program (CA, USA).

2.8. Cytotoxicity in HFF cells

HFF cells were seeded into 12 well plates at a concentration of 10⁵ cells/ml and a total volume of 1 mL medium per well. The cells were maintained until 100% confluency after which 1 mL of the old medium was aspirated out and replaced with an equal volume of fresh medium. Different concentrations of crude ethanol extract or fractionated or pure compounds were added to culture wells in triplicate for a total of three independent experiments. DMSO was used as a negative control. After 48 h of incubation at 37°C with an atmosphere of 5% CO₂, the medium was aspirated out from the wells and 250 µL 1x trypsin (Gibco) added followed by 2 minute

incubation at room temperature. Then, 1 mL of fresh medium containing 10% FBS was added and mixed by pipetting gently. Exactly 20 μ L of the cell suspension was transferred to a 1.5 mL microcentrifuge tube and an equal volume of trypan blue (BioRad) added and mixed gently. Exactly 10 μ L of the mixture was pipetted into a counting slide chamber and the number of live and dead cells counted using a TCA 20 automated cell counter (BioRad).

2.9. Cell viability assays

Confluent HFF and HAEC cells maintained in supplemented IMDM medium (without red phenol) in 96-well plates were used. Prior to addition of the compounds, 150 μ L of medium was aspirated out of each well and replaced with an equal volume of fresh medium. Varying concentrations of the ethanol extract, ethyl acetate extract and the two ethyl acetate fractions were added to the wells in triplicate. Triplicate wells to which an equivalent volume of DMSO only was added were included as negative control. At 48 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 μ L of the WST-1 reagent to each well. After mixing, the plates were wrapped in aluminum foil and incubated for 1 h at 37°C with 5% CO₂. After 1 hour of incubation, exactly 150 μ L 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). Three independent assays were performed and the dose-response curves of the means of triplicate assays for each compound were generated using GraphPad PRISM software and the IC₅₀ values derived.

2.10. Statistical analysis

The selectivity index (SI) was determined as the ratio of minimum cytotoxic concentration at 50% (IC_{50c}) to minimum parasite growth inhibitory concentration at 50% (IC_{50p}). The statistical difference of effect of test compounds and fractions for the inhibition of *T. gondii* replication and survival were compared with control group or compared between different extracts, fractions and pure compounds using Welch's t-test on non-homogeneous variances ($p < 0.05$). The dose-dependent effect of inhibitory activities against *T. gondii* of the crude extracts, fractions, apigeninidin and luteolinidin were determined by non-linear regression. The dose-response curves were derived using GraphPad PRISM software. Calculation of confidence intervals was done at the level of 95%. All data are reported as triplicate assays. Analyses of the differences between the compounds' effective IC₅₀ against the parasite compared to the cytotoxic IC₅₀ in mammalian cells were done using two-tailed Student's *t* test ($p < 0.05$).

3.0. Results

3.1. Fractionation and identification of Compounds

The anti-*Toxoplasma* activity of crude ethanol extracts, ethyl acetate, ESI-MS-identified ethyl acetate-fractionated compounds from *S. bicolor* red leaf, as well as that of the synthetic 3-deoxyanthocyanidins (apigeninidin, luteolinidin, 7-methoxyapigeninidin, 5, 7-dimethoxyluteolinidin), were tested (Table 1). The major 3-deoxyanthocyanidins found in the extracts and fractionated samples of *S. bicolor* leaf were apigeninidin and luteolinidin, that constituted more than 2% relative to all other 3-deoxyanthocyanidins derivatives (Figure 2-4). The different fractions analyzed depicted distinct ion counts for the major deoxyanthocyanidins present (Figure 2-4).

3.2. Testing of fractions' inhibitory activity against *T. gondii* and cytotoxicity

To determine the *S. bicolor* extract that had potent activity against *T. gondii*, but with less cytotoxicity to mammalian cells, we measured the IC_{50s} of the crude ethanol extract and the crude ethyl acetate extract against *T. gondii* tachyzoites and against human fibroblasts (HFF). The crude ethanol extract had an HFF cytotoxic IC₅₀ that was 6-fold higher than that of the crude ethyl acetate extract (Table 1). However, the ethyl acetate extract was found to have significant activity against *T. gondii* tachyzoites, with a mean IC₅₀ that was about 15-fold lower than that of the ethanol crude extract (Table 1), implying that the ethyl acetate extract had more potent activity against *T. gondii* than the ethanol extracts. Thus, the ethyl acetate crude extract was fractionated and the activity of its fractions against *T. gondii* and HFF cells tested. The two ethyl acetate fractions (EA F1-F4 and EA F2-5) had lower (though not significant) HFF cytotoxic IC_{50s}, but were potently active against *T. gondii* tachyzoites with IC_{50s} comparable to that of the crude extract (Table 1). The data from which the IC_{50s} of the extracts, fractions and pure compounds were derived by nonlinear regression analysis are presented in graphical form in Figure 5 and Figure 6 and depict compound concentration-dependent decline in parasite growth.

Because the ESI-MS analysis of the ethyl acetate fractions indicated that they contained significant amounts of 3-deoxyanthocyanidins, we tested the activity of commercially-obtained pure 3-deoxyanthocyanidins compounds (apigeninidin chloride, luteolinidin chloride, 7-methoxyapigeninidin and 5, 7-dimethoxyapigeninidin). Compared to the ethyl acetate fractions of *S. bicolor*, the pure 3-deoxyanthocyanidins compounds depicted cytotoxic IC_{50s} that were 3-4-fold lower, but had IC_{50s} for inhibition of *T. gondii* that were similar to those of ethyl acetate

fractions (Table 1). This implied that the ethyl acetate fractions from *S. bicolor* were as potent as the pure synthetic 3-deoxyanthocyanidins against *T. gondii* but had much lower cytotoxicity to mammalian cells than the pure synthetic 3-deoxyanthocyanidins compounds. Further, the synthetic 5,7-dimethoxyluteolinidin methoxy groups exhibited the highest inhibition against parasite growth at 48 h than the apigeninidin and luteolinidin without one or two methoxy groups at 95% confidence interval (Table 1).

Table 1. *T. gondii* growth inhibitory and cytotoxic concentrations (IC₅₀) for the *S. bicolor* red leaf crude extracts, fractionated compounds and 3-deoxyanthocyanidins pure synthetic compounds

Compound Tested	IC _{50c} [*] (µg/mL)	IC _{50p@48hrs} [*] (µg/mL)
Ethanol Extract	46.67(41.63-52.32)	20.38 (17.86-23.24)
Ethyl acetate Extract	7.56 (4.56-12.53)	1.39 (0.87-2.22)
EA F1-F4 (C/M 10:1)	20.20 (18.72-21.79)	0.36 (0.17-0.74)
EA F2-5 (chloroform)	20.39 (16.85-24.67)	7.00 (4.89-10.05)
Apigeninidin chloride	5.24 (4.73-5.81)	1.08 (1.03-1.13)
Luteolinidin chloride	6.54 (4.79-8.94)	0.66 (0.45-0.97)
7-methoxyapigeninidin	7.49 (5.025-11.18)	1.48 (1.24-1.77)
5,7-dimethoxyluteolinidin	2.62 (1.68- 4.08)	0.53 (0.18-1.57)

^{*} Results in the table are reported as means of three independent assays at 95% confidence interval; IC_{50c}: minimum inhibitory concentration required to kill 50% of HFF cells; IC_{50p}-minimum inhibitory concentration of crude, fractionated and pure compounds required to inhibit *T. gondii* tachyzoite growth by 50% in culture; EA F- ethyl acetate fraction; C/M is hexane-methanol.

Table 2. Selective index determination for *S. bicolor* red leaf crude extracts, fractionated compounds and 3-deoxyanthocyanidins pure synthetic compounds

Compound Tested	SI _{@48hrs}
Ethanol Extract	2.29
Ethyl acetate Extract	5.44
EA F1-F4 (C/M 10:1)	56.11
EA F2-5 (chloroform)	2.91
Apigeninidin chloride	4.85
Luteolinidin chloride	9.91
7-methoxyapigeninidin	5.06
5,7-dimethoxyluteolinidin	4.94

^{*} Results in the table are reported as means of three independent assays at 95% confidence interval. EA F- ethyl acetate fraction; C/M is hexane-methanol; SI-selectivity index (IC_{50cytotoxicity}/IC_{50T. gondii} inhibition).

Table 3. Cytotoxic concentrations (IC₅₀) for the *S. bicolor* red leaf crude extracts and fractionated compounds in HFF and HAEC cells derived by the *in vitro* cell viability assay

Compound	IC ₅₀ (µg/mL) In HFF Cells	IC ₅₀ (µg/mL) In HAEC Cells
Ethanol Extract	40.89 ± 3.51	39.62 ± 2.74
Ethyl acetate Extract	5.92 ± 1.63	4.97 ± 1.31
EA F1-F4 (C/M 10:1)	13.74 ± 2.15	11.82 ± 1.77
EA F2-5 (chloroform)	14.11 ± 2.36	15.26 ± 2.46

Results in the table are reported as means of three independent assays ± Standard Error of the Mean; HFF: human foreskin fibroblasts; HAEC: human aortic endothelial cells; IC₅₀: minimum inhibitory concentration required to decrease the metabolic activity of the cells by 50%; EA F- ethyl acetate fraction; C/M is hexane-methanol

Apigeninidin and luteolinidin were both found to have significant inhibitory activity against *T. gondii* at 48 h of culture (Table 1). However, the selectivity index (SI) calculated for luteolinidin was higher than that of apigeninidin (Table 2), indicating that luteolinidin had greater inhibitory activity against *T. gondii* than apigeninidin. The ethyl acetate extract as well as fractions derived from ethyl acetate extract depicted higher SI against *T. gondii* than the ethanol extract (Table 2). Overall, the EA F1-F4 fraction from the ethyl acetate crude extract (fractionated using chloroform-methanol) had the highest SI, which was at least 40-fold higher than that of all other extracts, fractions and pure compounds. All crude and fractionated compounds had cytotoxic IC_{50s} in human fibroblasts that were at least 2-fold higher than their respective IC_{50s} against *T. gondii* (Table 1), implying that their effective concentration against the parasite would not have detrimental side-effects in humans. Similarly, the pure, synthetic compounds had cytotoxic IC_{50s} in human fibroblasts that were at least 2-fold higher than their respective *T. gondii* IC_{50s}. However, the levels of concentrations for pure compounds were generally lower than those of the extracts, indicating that high level of purity of the compounds enhanced their activity. The above stated cytotoxic IC_{50s} of the compounds in HFF cells were derived using an assay based on distinguishing between dead and live cells. Thus, to determine the effect of the compounds on

the metabolic activity of the mammalian cells, we performed a cell proliferation assay for the quantification of cellular mitochondrial dehydrogenases activity of HFF and HAEC cells. As, shown in Table 3, cytotoxic IC_{50} s of the compounds based on cellular metabolic activity corroborated those obtained based on cell death (Table 1), indicating that the effective concentrations of the compounds against the parasites were not toxic to mammalian cells (human foreskin fibroblasts and human aortic endothelial cells) tested in this study.

To determine if the number and position of methoxy groups in the 3-deoxyanthocyanidin compounds' B-ring has effect on their inhibitory activity against *T. gondii*, we compared the activity of 7- methoxyapigeninidin and 5, 7-dimethoxyluteolinidin. The activity of 5, 7-dimethoxyluteolinidin ($IC_{50} = 0.53 \mu\text{g/mL}$) with two methoxy groups in position C-5 and C-7 was significantly higher than that of 7-methoxyapigeninidin ($IC_{50} = 1.48 \mu\text{g/mL}$) with one methoxy group in position C-7 at 48 h of parasite culture. Importantly, 7-methoxyapigeninidin had lower cytotoxicity ($IC_{50} = 7.49 \mu\text{g/mL}$) in human fibroblasts than 5,7-dimethoxyluteolinidin ($IC_{50} = 2.62 \mu\text{g/mL}$). The selectivity indexes (SI) for 7- methoxyapigeninidin and 5, 7-dimethoxyluteolinidin were 5.06 and 4.94, respectively, at 48 h of culture (Table 2). This suggested that 7- methoxyapigeninidin possesses higher potential activity against *T. gondii* than 5, 7-dimethoxyluteolinidin.

In comparison to existing drugs, the IC_{50} s obtained for the ethyl acetate crude extract and its two fractions (EA F1-F4 and EA F2-5) were all notably lower than those of roxithromycin, spiramycin and azithromycin that have IC_{50} s of 27.07, 20.16 and $8.61 \mu\text{g/mL}$, respectively (Chamberland et al., 1991). However, we observed that pyrimethamine has been reported to have

an IC₅₀ of 0.16 µg/mL against *T. gondii* that is lower than the IC_{50s} for all the extracts we obtained from *S. bicolor* (Cantin and Chamberland, 1993). A combination of sulfadiazine and pyrimethamine has an IC₅₀ that is significantly lower than the EA F2-5 fraction of the ethyl extract ((Derouin and Chastang, 1989). Nevertheless, it is noteworthy that these existing drugs have been reported to have severe toxic side effects in humans (McLead et al., 2006).

4.0. Discussion

Herein, we tested inhibitory activity of extracts from dried *S. bicolor* red leaf against *T. gondii* tachyzoites. The *S. bicolor* crude extracts, fractions and pure compounds were observed to have significant inhibitory activity against *T. gondii* tachyzoites at µg/ml level. Importantly, their cytotoxic concentrations were at least 2-fold higher than their respective effective concentrations against *T. gondii*, suggesting that they would not have adverse side-effects *in vivo*. *S. bicolor* has been shown to contain abundant amounts of 3-deoxyanthocyanidins compounds (Oladiji et al., 2007). Consistent with this report, the results of our ESI-MS chromatographic analysis of *S. bicolor* extracts and fractions revealed that they contained significantly relative high amounts of 3-deoxyanthocyanidins compounds. Importantly, when we tested pure, commercially obtained 3-deoxyanthocyanidins compounds, we observed that they had *T. gondii* inhibitory effects that were comparable to the *S. bicolor* extracts and fractions. Taken together, this indicated that the observed activity against *T. gondii* of the fractionated compounds from *S. bicolor* was attributable to the 3-deoxyanthocyanidins that they contained, implying that *S. bicolor* can be a source of bioactive compounds for the development of a new generation of effective and safe drugs against *T. gondii* infections.

Plant-derived natural products are now increasingly being found to have anti-*Toxoplasma* activity (Sepulveda-Arias et al., 2014). It is noteworthy that traditionally, in West Africa, *S. bicolor* grains, leaves, roots and stalks are considered to have medicinal properties and are thus used in the treatment of a range of ailments (Oladiji et al., 2007; Abugri et al., 2013). In this study, the 3-deoxyanthocyanidins compounds fractionated from *S. bicolor* were found to have more potent anti-parasitic activity than other flavonoid compounds reported in previous studies (Lehane and Saliba, 2008). In particular, the IC_{50s} against *T. gondii* for the ethyl acetate crude extract and its fractions, as well as the pure 3-deoxyanthocyanidins compounds tested in this study, were lower than those reported in different traditional medicinal plants against *T. gondii* tachyzoites (Choi et al., 2008). Additionally, the IC_{50s} against *T. gondii* for the *S. bicolor* extracts and fractions we tested were lower than those of the standard compounds/drugs such as roxithromycin, azithromycin, spiramycin, and pyrimethamine-sulfadiazine used for treating *T. gondii* infections (Derouin et al., 1989; Chamberland et al., 1991; Cantin and Chamberland, 1993).

Our observation that 7-dimethoxyluteolinidin possesses higher potential activity against *T. gondii* than 5,7- methoxyapigeninidin based on the position and number of the methoxy group was consistent with previous studies that showed that synthetic 7-methoxyapigeninidin exhibited higher activity against fungi (*G. sorgi*) than apigeninidin which lacks methoxy group at position C-7 (Aida et al., 1996).

Even though flavonoids have been shown to possess anti-parasitic activity, most of them have also been shown to be toxic to mammalian cells at their effective concentrations (Matsuo et al.,

2005). Importantly, the *S. bicolor* extracts and fractions tested in this study had cytotoxic IC_{50s} against human fibroblasts and human endothelial cells that were at least 2-fold higher than their respective IC_{50s} against *T. gondii*. Thus, *S. bicolor* extracts and fractions investigated in this study are promising in that their effective anti-parasitic IC_{50s} were significantly lower than their respective cytotoxic IC_{50s}. Studies are underway to determine the mechanism of action of 3-deoxyanthocyanidins in inhibiting growth and proliferation of *T. gondii*. Once determined, their modes of action will serve as a guide in establishing the combination types and concentrations of 3-deoxyanthocyanidins that would have synergistic or additive effects in order to improve their efficacy and reduce their cytotoxicity, thus making them safer and effective for use in treating *T. gondii* infections in humans.

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Competing interests: None.

Ethical approval: Not required.

Figure Legends

Figure 1: Flow-chart for fractionation of ethanol extracts from *Sorghum bicolor* using size exclusion chromatography. AcOET: ethyl acetate; MeOH: pure methanol.

Figure 2: ESI-MS chromatogram of compounds extracted from *S. bicolor*. The 3-deoxyathocyaninindins compounds were fractionated in chloroform-methanol (10:1 ratio) from the ethyl-acetate crude extract at a stock concentration of 1.398 mg/ml. The compounds, 7-methoxy-apigeninidin and luteolinidin accounted for 3.04% and 52.76%, respectively, of the total count ion content in the crude extract.

Figure 3: ESI-MS chromatogram of compounds extracted from *S. bicolor*. The 3-deoxyathocyaninindins were fractionated in chloroform from the ethyl-acetate crude extract at a stock concentration of 3.80 mg/ml. Apigeninidin, 7-methoxy-apigenidin, luteolinidin and 7-methoxy-luteolinidin accounted for 4.61%, 2.75%, 36.87% and 2.50%, respectively, of the total ion count in the crude extract.

Figure 4: ESI-MS chromatogram of compounds extracted from *S. bicolor*. The 3-deoxyathocyaninindins were fractionated in hexane-methanol from the ethyl-acetate crude extract at a stock concentration of 18.389 mg/mL. Apigeninidin, 7-methoxy-apigenidin, luteolinidin and 7-methoxy-luteolinidin accounted for 4.44%, 0.72%, 1.38% and 0.57%, respectively, of the total ion count in the crude extract.

Figure 5: Growth curves for *T. gondii* tachyzoites in cultures treated with varying concentrations of compounds. Confluent human foreskin fibroblasts (HFF) were inoculated with *T. gondii* tachyzoites and immediately treated with varying concentrations ($\mu\text{g/mL}$) of (A.) Ethanol crude

extract, (B.) Ethyl acetate crude extract, (C.) Ethyl acetate fraction F2-5 and (D.) Ethyl acetate fraction F1-F4, for 48 hours. The graphs depict compound dose-response patterns of decline in growth. The data are representative of means of three independent assays at 95% confidence limit (95% CL).

Figure 6: Growth curves for *T. gondii* tachyzoites in cultures treated with varying concentrations of pure compounds. Confluent human foreskin fibroblasts (HFF) were inoculated with *T. gondii* tachyzoites and immediately treated with varying concentrations ($\mu\text{g/mL}$) of (A.) Luteolinidin, (B.) 7-Methoxyapigeninidin, (C.) 5,7-Dimethoxyluteolinidin and (D.) Apigeninidin, for 48 hours. The graphs depict compound dose-response decline in growth. The data are representative of means of three independent assays at 95% confidence limit (95% CL).

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Figure 1: Flow-chart for fractionation of ethanol extracts from *Sorghum bicolor* using size exclusion chromatography. AcOET: ethyl acetate; MeOH: pure methanol.

Figure 2. ESI-MS chromatogram of compounds extracted from *S. bicolor*. The 3-deoxyathocyaninindins compounds were fractionated in chloroform-methanol (10:1 ratio) from the ethyl-acetate crude extract at a stock concentration of 1.398 mg/ml. The compounds, 7-methoxy-apigeninidin and luteolinidin accounted for 3.04% and 52.76%, respectively, of the total count ion content in the crude extract.

Figure 3. ESI-MS chromatogram of compounds extracted from *S. bicolor*. The 3-deoxyathocyaninidins were fractionated in chloroform from the ethyl-acetate crude extract at a stock concentration of 3.80 mg/ml. Apigeninidin, 7-methoxy-apigenidin, luteolinidin and 7-methoxy-luteolinidin accounted for 4.61%, 2.75%, 36.87% and 2.50%, respectively, of the total ion count in the crude extract.

Figure 4. ESI-MS chromatogram of compounds extracted from *S. bicolor*. The 3-deoxyathocyaninidins were fractionated in hexane-methanol from the ethyl-acetate crude extract at a stock concentration of 18.389 mg/ml. Apigenininidin, 7-methoxy-apigeninidin, luteolinidin and 7-methoxy-luteolinidin accounted for 4.44%, 0.72%, 1.38% and 0.57%, respectively, of the total ion count in the crude extract.

Figure 5. Growth curves for *T. gondii* tachyzoites in cultures treated with varying concentrations of compounds. Confluent human foreskin fibroblasts (HFF) were inoculated with *T. gondii* tachyzoites and immediately treated with varying concentrations (µg/ml) of (A.) Ethanol crude extract, (B.) Ethyl acetate crude extract, (C.) Ethyl acetate fraction F2-5 and (D.) Ethyl acetate fraction F1-F4, for 48 hours. The graphs depict compound dose-response patterns of decline in growth.. The data are representative of means of three independent assays at 95% confidence limit (95% CL).

A. **B.** **C.** **D.**

Figure 6. Growth curves for *T. gondii* tachyzoites in cultures treated with varying concentrations of pure compounds. Confluent human foreskin fibroblasts (HFF) were inoculated with *T. gondii* tachyzoites and immediately treated with varying concentrations (µg/ml) of (A.) Luteolinidin, (B.) 7-Methoxyapigeninidin, (C.) 5,7-Dimethoxyluteolinidin and (D.) Apigeninidin, for 48 hours. The graphs depict compound dose-response decline in growth. The data are representative of means of three independent assays at 95% confidence limit (95% CL).