Ascorbic acid potentiates the *Giardia duodenalis* growth inhibitory activity of pure *Terminalia ferdinandiana* Exell compounds

Running title: Inhibition of giardiasis by combinations of Kakadu plum compounds and ascorbic acid

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Abstract

Giardiasis, one of the most common causes of diarrhoeal disease, is caused by gastrointestinal protozoal parasites of the genus Giardia. Metronidazole is the most commonly used drug to treat giardiasis. However, metronidazole resistance is increasingly common, making the development of new anti-giardial drugs a high priority. A panel of 11 compounds previously identified in *T. ferdinandiana* fruit extracts with potent *G. duodenalis* growth inhibitory activity were investigated for the ability to inhibit *G. duodenalis* proliferation. Eight of the 11 compounds inhibited the growth of all three *G. duodenalis* strains. 2,3-Dihydroxyphenyl B-D-glucopyranosiduronic acid (DPGA)DPGA was the most potent anti-giardial compound, with IC_{50} values as low as 126μM (38μg/mL). Notably, DPGA inhibited a metronidazole resistant *G. duodenalis* strain with similar potency as determined for the metronidazole sensitive strains. Furthermore, the potency of DPGA was greatly potentiated when it was tested in combination with ascorbic acid, to approximately 17μM (5μg/mL) for the metronidazole sensitive *G. duodenalis* strains and 40μM (12mg/mL) for the resistant strain. The *T. ferdinandiana* tannins (gallic acid and chebulic acid) were also moderate inhibitors of *G. duodenalis* growth when tested in combination with ascorbic acid, although they had only low levels of activity when tested alone. All of the tested compounds (and their combinations with ascorbic acid) displayed low toxic and all compounds conformed to Lipinski's rules of 5 with few violations, indicating their potential as drug leads and chemotherapies for the treatment and prevention of giardiasis.

Keywords Gastrointestinal parasite, combinational therapies, synergy, gallic acid, ascorbic acid, anti-giardial activity.

Introduction

Giardiasis is a major cause of infectious diarrhoea in humans and livestock worldwide. It is caused by gastrointestinal infections of protozoal parasites of the genus Giardia. There is a limited range of drugs available for chemotherapeutic treatment of this disease and they are only used after clinical diagnosis and not for prophylaxis. The majority of these drugs are ineffective against some life stages of the protozoa, are toxic, have unpleasant side effects and may have limited availability in developing countries. Frequent reports of treatment failure and parasite resistance (Tejman-Yarden et al. 2011; Escobedo and Cimerman 2007) also highlight the importance to develop new chemotherapeutic treatments for giardiasis with greater efficacy and less severe side effects.

A re-examination of traditional herbal medicines for the treatment of giardiasis is an attractive prospect as the therapeutic qualities of medicinal plants have been long recognised and recorded. A recent study from our group reported potent anti-giardial activity of extracts prepared from the fruit of *Terminalia ferdiandiana* Exell and linked the activity to the high antioxidant capacity of the extracts (Rayan et al. 2015). That study used high accuracy QTOF HPLC-MS to examine the metabolomic profiles of the various *T. ferdianadiana* fruit extracts and thereby narrow the focus of compounds likely to contribute to the anti-giardial activity. Several compounds with properties consistent with anti-giardial activity were highlighted in that study. A purine analogue (Figure 1a) was identified in all extracts with growth inhibitory activity. Interestingly, numerous studies have reported that *Giardia duodenalis* are unable to synthesise their own purine or pyrimidine nucleotides and are reliant on salvage pathways to supply them with nucleotides for nucleic acid synthesis (Baum et al. 1989; Jarrol et al. 1989). Furthermore, *G. duodenalis* are incapable of interconversion between purine nucleotides and therefore require the correct purine nucleotides for replication. Indeed, purine analogues inhibit

the growth of *G. duodenalis* and have been highlighted as potential chemotherapeutic agents for giardiasis (Berens and Marr 1986). Our earlier study (Rayan et al. 2015) also noted a relative abundance of tannins in the bioactive extracts, with particularly high levels of gallic acid (Figure 1b) and chebulic acid (Figure 1c) reported. Gallotannins inhibit the growth of multiple microbial species (Buzzini et al. 2008) via binding cell surface lipotoichoic acid and proline-rich membrane proteins (Wolinsky and Sote 1984; Hogg and Embery 1982), and by inhibiting glucosyltransferase enzymes (Wu-Yuan et al. 1988).

The majority of the other compounds highlighted in our previous study (Rayan et al. 2015) contain lactone moieties. These include ribonolactone (Figure 1d), ascorbic acid (Figure 1e), gluconolactone (Figure 1f) and glucohepatonic acid-1,4-lactone (Figure 1g). The presence of lactone moieties is interesting as many of the current anti-giardial chemotherapeutic drugs used are lactone containing compounds, particularly lactone substituted nitroimidazoles (e.g. metronidazole, secnidazole, tinidazole, ornidazole and albendazole). Compounds containing a lactone moiety may block the giardial lipid deacylation/reacylation pathways, thereby inhibiting proliferation (Das et al. 2001). As *Giardia* spp. are unable to synthesise lipids by de novo pathways, they must use host gastrointestinal precursor lipids for the synthesis of membrane and cellular lipids by deacylation/reacylation reactions (Das et al. 2001). Thus, the lactone containing compounds in the *T. ferdinandiana* extracts may contribute to *G. duodenalis* growth inhibition via inhibition of lipid metabolism pathways.

Our previous study also highlighted quinic acid (Figure 1h) in the *T. ferdinandiana* extracts (Rayan et al. 2015). Substituted quinic acid compounds can block leucyl-tRNA synthase activity in *G. duodenalis* cells (Zhang et al. 2012). As aminoacyl-tRNA synthases are essential for translation of the genetic code by attaching the correct amino acid to each tRNA, blockage of leucyl-tRNA synthase activity results in ineffective Leu-tRNA production and thus

the inhibition of protein synthesis. Therefore, quinic acid may also contribute to the antigiradial activity of the *T. ferdianadiana* fruit extracts. The previous metabolomics study also highlighted eujavonic acid (Figure 1i), 5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-pentanoic acid (HMDP) (Figure 1j) and 2,3-dihydroxyphenyl B-D-glucopyranosiduronic acid (DPGA) (Figure 1k) as further potential anti-giardial compounds. Despite highlighting this panel of compounds, the previous study (Rayan et al. 2015) did not verify the activity of these compounds using anti-proliferative assays. Our current study was undertaken to extend that study and examine the *G. duodenalis* growth inhibitory activity of pure *T. ferdinandiana* compounds. Furthermore, as *T. ferdinandiana* fruit are known for their extremely high ascorbic acid content, ascorbic acid may alter/enhance the growth inhibitory activity of the individual components. Therefore, all compounds were also tested in combination with ascorbic acid to quantify its effects on the activity of those components.

Materials and methods

Materials

The purine, gallic acid, chebulic acid, ribonolactone, ascorbic acid, gluconolactone, glucohepatonic acid-1,4-lactone, quinic acid, eujavonic acid, 5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-pentanoic acid (HDMP), and 2,3-dihydroxyphenyl β-D-glucopyranosiduronic acid (DPGA) used in this study were all obtained from Sigma Chemicals, Australia and were all AR grade. All compounds were prepared as 1mg/mL solutions and aliquots were stored at -30^{o}C until use. All solvents were supplied by Ajax Fine Chemicals, Australia and were AR grade.

T. ferdinandiana fruit pulp samples

T. ferdinandiana fruit pulp was supplied and verified/quality assured by David Boehme of Wild Harvest, Northern Territory, Australia. The pulp was frozen for transport and stored at -10ºC until processing. A voucher sample (KP2014GD) is stored at Griffith University.

Preparation of extracts

T. ferdinandiana fruit pulp was thawed at room temperature and dried in a Sunbeam food dehydrator. The dried pulp material was subsequently ground to a coarse powder. A mass of 1g of ground dried pulp was extracted extensively in 50mL of either methanol or deionised water for 24 hours at 4ºC with gentle shaking The extracts were subsequently filtered through filter paper (Whatman No. 54). The methanolic extract was air dried at room temperature. The aqueous extract was lyophylised by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in 10mL deionised water (containing 0.5% DMSO). The extract was passed through 0.22µm filter (Sarstedt) and stored at 4ºC until use.

Inhibitory bioactivity against *Giardia duodenalis* trophozoites

Parasite culture

The *Giardia duodenalis* S-2 (sheep strain 2) trophozoite strain used in this study for the initial anti-proliferative screening studies was previously supplied by Professor Ann MacDonnell of Griffith University. The reference metronidazole sensitive (ATCC203333) and resistant strains (ATCC PRA-251) were obtained from the American Type Culture Collection. All *G. duodenalis* tropozoites were maintained and subcultured anaerobically at 37ºC in TYI-S-33

growth media supplemented with 1% bovine bile (Sigma), 10% Serum Supreme (Cambrex Bioproducts) and 200 IU/mL penicillin/200µg/ml streptomycin (Invitrogen, USA). Confluent mid log phase cultures were passaged every 2 days by chilling the cultures on ice for a minimum of 10 min, followed by vortexing to dislodge the adherent trophozoites from the walls of the culture vessel. Fresh culture media (5mL) was seeded with approximately 1 x 10^5 trophozoites for each passage.

Evaluation of anti-giardial activity by direct parasite enumeration

Anti-giardial activity of the extracts was assessed by direct enumeration of parasite numbers in the presence or absence of extracts (Hart et al. 2014). For each test, aliquots of the trophozoite suspension (70µL) containing approximately 1 x 10^5 trophozoites were added to the wells of a 96 well plate. A volume of 30µL of the test extracts, compounds, combinations or the vehicle solvent or culture media (for the negative controls) was added to individual wells and the plates were incubated anaerobically at 37ºC for 8 hours in a humidified anaerobic atmosphere. Following the 8h incubation, the plates were placed on ice for a minimum of 10min, followed by shaking to dislodge the adherent trophozoites from the walls of the wells. The suspensions were mounted onto a Neubauer haemocytometer (Weber, UK) and the total trophozoites per mL were determined. The anti-proliferative activity of the extracts, compounds and combinations was determined and expressed as a % of the untreated control trophozoites per mL.

Determination of IC_{50} values against giardial trophozoites

For IC_{50} determinations, the extracts and compounds were tested by the direct enumeration method across a range of concentrations. The assays were performed as outlined above and

graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the IC_{50} values.

Inhibitory effects of compound combinations

Fractional inhibitory concentration (FIC) assessment

Interactions between the pure compounds were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC_{50}) for each combination (Hübsch et al. 2014). The FIC values for each component (a and b) were calculated using the following equations where a represents the plant extract sample and b represents the conventional antibiotic:

$$\mathrm{FIC50(a)} = \left(\frac{\mathrm{FIC50[a\ in\ combination\ with\ b]}}{\mathrm{FIC50[a\ independently]}}\right)$$

$$\mathrm{FIC50(b)} = \left(\frac{\mathrm{FIC50[b\ in\ combination\ with\ a]}}{\mathrm{MIC50[b\ independently]}}\right)$$

The ΣFIC_{50} was then calculated using the formula $\Sigma FIC_{50} = FIC_{50}(a) + FIC_{50}(b)$. The interactions were classified as synergistic ($\Sigma FIC_{50} \leq 0.5$), additive ($\Sigma FIC_{50} > 0.5\text{-}1.0$), indifferent ($\Sigma FIC_{50} > 1.0\text{-}4.0$) or antagonistic ($\Sigma FIC_{50} > 4.0$) (Hübsch et al. 2014).

Varied ratio combination studies (isobolograms)

For each combination producing synergistic interactions, nine different ratios spanning the range 10:90 (extract:antibiotic) to 90:10 (extract:antibiotic) were tested. All combinations were tested in triplicate in three independent experiments, providing nine replicates for each

combination ratio. The data is presented as the mean of the nine replicates. Data points for each ratio examined were plotted on an isobologram and this was used to determine optimal combination ratios to obtain synergy. Data points on or below the 0.5:0.5 line indicated synergy; those above the 0.5:0.5 line, up to and including the 1.0:1.0 line indicated an additive interaction; data points above the 1.0:1.0 line indicated indifferent interaction.

Toxicity screening

Two assay methods were used to assess the toxicity of the individual samples. The *Artemia* lethality assay (ALA) was utilised for rapid preliminary toxicity screening, whereas an MTS cellular viability assay was used as a cellular evaluation of toxicity.

***Artemia franciscana* Kellogg nauplii toxicity screening**

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6mg/mL solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay. Toxicity was tested using a modified *A. franciscana* nauplii lethality assay (ALA) (Ruebhart et al. 2009; Cock and Ruebhart 2009). Briefly, 400µL of seawater containing approximately 46 (mean 45.7, n = 125, SD 10.3) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400µL of diluted plant extracts or the reference toxin were transferred to the wells and incubated at 25 ± 1ºC under artificial light (1000 Lux). A negative control (400µL seawater) was included on each plate. All treatments and controls were performed three times in triplicate. The wells were checked at regular intervals and the number of dead were counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 24h, all nauplii

were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Cellular viability assay

The *T. ferdinandiana* extracts were also screened against human primary dermal fibroblasts (HDF). The HDF cells were obtained from American Type Culture Collection (ATCC PCS-201-012) by standard methods (Shalom and Cock 2018). Briefly, the cells were cultured and maintained in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific, Australia), supplemented with 10% foetal calf serum (Invitrogen), 50µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigma Aldrich, Australia). The cells were maintained as monolayers in 75mL flasks at 37ºC, 5% CO_2 in a humidified atmosphere until approximately 80% confluent. Once confluency was achieved, 1 mL of trypsin (Sigma, Australia) was added to the culture flasks and incubated at 37ºC, 5 % CO_2 for 15 min to dislodge the HDF cells. The cell suspensions were then transferred to a 10mL centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9mL of fresh media. Aliquots of the resuspended cells (70µL, containing approximately 5000 cells) were added to individual wells of a 96 well plate. A volume of 30µL of the test extracts or cell media (for the negative control) was added to individual wells and the plates were incubated at 37ºC, 5% CO_2 for 24 hours in a humidified atmosphere. All test compounds were screened at 300 µg/mL. The cells were then washed in PBS (pH 7.2) to remove interference due to sample colour. A volume of 20 µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3 hours. Absorbances were recorded at a test wavelength of 540nm and a blank wavelength of 690nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were

performed three times in triplicate and triplicate controls were included on each plate. The % cellular viability of each test was calculated using the following formula:

$$\% \text{ cellular viability} = \frac{\text{Abs test sample} - (\text{mean Abs control} - \text{mean Abs blank})}{(\text{mean Abs control} - \text{mean Abs blank})}$$

Cellular viability ≤50% of the untreated control indicated toxicity, whereas extracts or controls with >50% untreated control viability were deemed to be nontoxic.

Therapeutic Index Evaluation

To determine the suitability of the compounds as potential therapeutic agents, their therapeutic index was calculated using the formula:

Therapeutic index = (HDF LC_{50})/(S2 IC_{50})

Statistical analysis

Data were expressed as the mean ± SEM of at least three independent experiments. One way ANOVA was used to calculate statistical significance between the negative control and treated groups with a P value <0.01 considered to be statistically significant.

Results

***T. ferdinandiana* fruit extraction yields and qualitative phytochemical screening**

Extraction of 1g of dried *T. ferdinandiana* fruit with methanol and deionised water yielded relatively high masses of dried extracted material (370 and 290µg/mL for the methanolic and

aqueous extracts respectively). The dried extracts were resuspended in 10 mL of deionised water (containing 0.5% DMSO) resulting in the extract concentrations shown in Table 1. Qualitative phytochemical studies (Table 1) showed that both extracts contained high levels of phenolics and flavonoids, as well as moderate to high levels of tannins. Saponins were also present in low to moderate levels. Triterpenes and alkaloids were present in low levels.

Inhibition of *Giardia duodenalis* proliferation by *T. ferdinandiana* extracts and pure compounds

The methanol and water *T. ferdinandiana* fruit extracts displayed potent inhibitory activity, each inhibiting 100% of the giardial growth (compared to the untreated control). The efficacy of the extracts were further evaluated by determination of the concentration required to inhibit *G. duodenalis* growth by 50% (IC_{50}). The water extract was a particularly good inhibitor of *G. duodenalis* proliferation, with an IC_{50} of 143 µg/mL. The methanol extract, whilst less potent, also displayed good anti-giardial activity (704 µg/mL).

Several of the pure *T. ferdinandiana* fruit compounds also significantly inhibited *G. duodenalis* trophozoite proliferation when tested at 300µg/mL (Figure 2). DPGA was a particularly good growth inhibitor, blocking 100% of trophozoite growth. Interestingly, DPGA was as effective against the metronidazole resistant *G. duodenalis* strain as it was against the sensitive strains, indicating that DPGA may block giardial growth by different mechanisms than metronidazole. Several of the other compounds also significantly inhibited *G. duodenalis* trophozoite proliferation, albeit with lower efficacy. Gallic acid (~50% inhibition of proliferation), chebulic acid (~40% inhibition), quinic acid (~30% inhibition), eujavonic acid (~20% inhibition) and 5-(4-hydroxy-2,5-dimethylphenoxy) -2,2- dimethylpentanoic acid (~20% inhibition) each inhibited all three *G. duodenalis* strain, including the metronidazole

resistant strain. Two of the other *T. ferdinandiana* fruit compounds (ascorbic acid, ~15% inhibition; glucohelapatonic acid lactone, ~30% inhibition) also significantly inhibited the metronidazole sensitive *G. duodenalis* strain, yet were ineffective inhibitors of the metronidazole resistant *G. duodenalis* strain. Purine, ribolactone and gluconolactone did not significantly affect the growth of any of the *G. duodenalis* strains.

Quantification of IC_{50} for the pure *T. ferdinandiana* compounds

The anti-proliferative activity of the pure *T. ferdinandiana* compounds was further tested over a range of concentrations to determine the IC_{50} values against *G. duodenalis* trophozoites (Table 2). Interestingly, most of the compounds produced only moderate to low *G. duodenalis* inhibitory activity, with IC_{50} values >1000µg/mL. DPGA was a substantially more potent inhibitor of *G. duodenalis* proliferation than the other compounds, with IC_{50} values for the different strains ranging from 38-72µg/mL (126-238µM). Interestingly, DPGA was a relatively minor component of the aqueous and methanolic *T. ferdinandiana* extracts, accounting for substantially less than 0.1% of the total extract's mass (results not shown) and it is therefore unlikely that DPGA alone would account for the strong activity of the crude aqueous extract (143 µg/mL). Instead, it is likely that other compounds in the extract synergise the activity of one or more of the anti-proliferative *T. ferdinandiana* compounds. As *T. ferdinandiana* fruit has a very high ascorbic acid content (Cock 2015), it is possible that ascorbic acid may have synergistic interactions with one or more of the the *T. ferdinandiana* compounds. Therefore, these compounds were further investigated in combination with ascorbic acid to identify any interactions which may occur.

Combinational effects of the *T. ferdinandiana* compounds and ascorbic acid on *G. duodenalis* proliferation

A range of combinational effects were observed between *T. ferdinandiana* extract components and ascorbic acid (Table 2). Of particular note, 2 combinations produced synergistic interactions (gallic acid + ascorbic acid; DPGA + ascorbic acid). Indeed, some combinations produced approximately 10 fold increases in activity compared to the activity of either compound alone. The increase in activity for DPGA in combination with ascorbic acid was particularly noteworthy against the sheep S2 *G. duodenalis* strain, with IC_{50} values decreasing from 47µg/mL (156µM) alone, to 5µg/mL (17µM) in combination with ascorbic acid. A similar increase in potency was recorded against the metronidazole sensitive reference *G. duodenalis* strain (ATCC203333), with a decrease of IC_{50} from 38µg/mL (126µM) alone, to 6µg/mL (20µM) in combination with ascorbic acid. Whilst slightly less potent against the metronidazole resistant *G. duodenalis* strain (ATCC PRA-251), the DPGA + ascorbic acid combination also produced clinically relevant IC_{50} values of 12µg/mL (40µM). Substantial increases in potency were also recorded for the gallic acid + ascorbic acid combinations against all *G. duodenalis* strains. The decrease in IC_{50} against the sheep S2 strain from 1150µg/mL (6795µM) alone, to 146µg/mL (858µM) in combination with ascorbic acid was notable. This combination was also synergistic against the other *G. duodenalis* strains. Interestingly, the IC_{50} values were similar between both the metronidazole sensitive and resistant *G. duodenalis* strains, both with IC_{50} values of approximately 250µg/mL (1469µM).

The majority of the other combinations produced additive effects. These combinations may therefore also be beneficial in the treatment of giardiasis, as they produce enhanced efficacy over either component when used separately. A further combination (glucohepatonic acid lactone) was non-interactive. Whilst this combination does not provide any significant therapeutic benefit above that of either compound alone, the components also do not antagonise

each other's effects and therefore it would not be detrimental if the two components were administered concurrently. Notably, none of the combinations produced antagonistic effects.

Varied ratio combination studies (isobolograms)

Synergistic interactions between gallic acid and ascorbic acid

As the gallic acid/ascorbic acid combination induced a synergistic interaction (Table 2), the combination was further examined using isobologram analysis across a range of gallic acid:ascorbic acid ratios to identify the ideal ratios to obtain synergy. Similar susceptibility profiles were evident against all three *G. duodenalis* strains. In all cases, the data correlated more closely with the gallic acid axis than with the ascorbic acid axis, indicating that the anti-proliferative activity is most reliant on the gallic acid. However, whilst ratios containing between 30-60% gallic acid induced synergistic responses, the lower (≤20%) and higher ratios (≤70%) generally produced additive effects. As these responses are greater than either of the individual components alone, they would therefore be beneficial for the treatment of giardiasis. As synergy was determined using the ΣFIC_{50} formula, synergy is defined in this study as a response at least 4 times greater than that of sum of the individual components alone. Thus the ratios which induce synergistic responses are far preferable as anti-giardial therapies compared to the other ratios. Therefore, the ideal gallic acid/ascorbic acid ratios for the treatment of giardiasis are the combinations containing 30-60% gallic acid.

Synergistic interactions between 2,3-dihydroxyphenyl-B-gloucopyranosiduronic acid and ascorbic acid

DPGA also induced synergistic *G. duodenalis* growth inhibition when tested in combination with ascorbic acid (Table 2). The association between the growth inhibitory activity and the DPGA axis was even more pronounced than for gallic acid (Figure 3), indicating that this compound is more important than ascorbic acid for the anti-proliferative activity of this combination. This is consistent with the IC_{50} data for the compounds which reports the IC_{50} of DPGA as approximately 5% of the IC_{50} of ascorbic acid (Table 2). Thus, DPGA is approximately a 20 times more potent *G. duodenalis* growth inhibitor than ascorbic acid when the components were tested separately. Interestingly, all combinations containing ≤60% DPGA produced synergistic inhibition of the growth for the metronidazole sensitive *G. duodenalis* strains (Figure 4a and 4b). Therefore, ≥40% ascorbic acid is required to effectively synergise the effects of DPGA.

The growth inhibition isobologram against the metronidaxole resistant *G. duodenalis* strain displays a different trend (Figure 4c). The majority of the combination ratios produced additive interactions against this strain. These ratios would still be beneficial for treating giardiasis as the growth inhibitory activity of the combination is greater than that of either component alone. However, whilst the treatment efficacy is increased for these ratios, the increase in relatively minor. In contrast, combinations containing 30-50% DPGA had substantially increased efficacy (≥4 fold increases in potency compared to the sum of the compounds tested alone). Therefore, the ideal synergistic ratio for the treatment and prevention of giardiasis against the metronidazole resistant *G. duodenalis* strain was identified to be 30-50% DPGA in combination with ascorbic acid.

Quantification of toxicity

All extracts were screened across a range of concentrations using both the *Artemia* nauplii lethality assay (ALA) and a human dermal fibroblast assay (HDF) (Table 3). For comparison, the reference toxin potassium dichromate (1000 µg/mL) was also tested. No LC_{50} values are reported for purine, ribolactone, gluconolactone, glucohepatonic acid lactone, quinic acid, eujavonic acid, HMDP, or DPGA as less than 50 % mortality was seen for all concentrations of these compounds tested in both assays. All of these compounds were therefore deemed to be nontoxic. In contrast, gallic acid, chebulic acid and ascorbic acid displayed apparent toxicity in both assays following 24 hours exposure. However, it is noteworthy that the toxicity detected in our study generally correlated with acidic components. Acidic pH can suppress the rate of mitochondrial protein synthesis and potentially be fatal to the growth and development of both *Artemia* nauplii and HDF cells. Indeed, previous studies have reported that extracts high in ascorbic acid can provide fallacious toxicity determinations (Murhekar et al. 2017). Thus, this assay may have overestimated the toxicity of these compounds.

Therapeutic index and drug like properties

To determine the suitability of the *T. ferdinandiana* compounds as therapeutic agents, their drug-like properties were examined with reference to Lipinsky's rules of five (Lipinski 2004). All of the compounds had ≤10 H bond acceptors, molecular weights <500 Da and octanol-water coefficients ≤5. The majority of the compounds also had ≤5 H bond donors. Interestingly, the only compounds that violated this rule (chebulic acid and DPGA) included the compound with the greatest *G. duodenalis* anti-proliferative activity (DPGA), both alone and in combination with ascorbic acid. Both DPGA and chebulic acid have 6 H bond donors and therefore exceed Lipinsky's rules of five by one H bond donor. However, given their

conformity in all other categories, these compounds were deemed to have good drug-like properties.

The therapeutic index (TI) was also calculated for the pure compounds and combinations. We were unable to calculate TI's for purine, ribolactone, gluconolactone, glucohepatonic acid lactone, quinic acid, eujavonic acid, HMDP and DPGA as none of these compounds displayed toxicity at any concentration tested. However, with the exception of DPGA, these compounds generally displayed only low *G. duodenalis* anti-proliferative activity and were therefore of little use therapeutically. For DPGA, this lack of apparent toxicity indicates that the compound may have a high TI and therefore be a promising drug-lead. If the dose range that DPGA was tested over was extended to test higher concentrations to determine an LC_{50}, the TI would be relatively high.

An interesting trend was noted for the TI of gallic acid. The TI of this compound alone was relatively low (0.3) due to its apparent toxicity, indicating that it may have limited therapeutic potential. However, when the TI of gallic acid was determined in combination with ascorbic acid, it had increased substantially to 2.3. Thus, it is likely that ascorbic acid may also provide dual benefits in combination with DPGA: it may synergise the anti-proliferative activity of DPGA, as well as protecting the cells against its toxicity.

DISCUSSION

A number of chemotherapeutic options are currently available for the treatment of giardiasis. Of these, nitroimidazole compounds (e.g. metronidazole, timidazole, secnidazole, ornidazole) are the most widely used and metronidazole is the current "gold standard" drug to treat giardiasis in humans. Whilst these drugs have proven effective in the past, they are also

associated with toxicities and multiple unpleasant side effects including nausea, vertigo, anorexia, vomiting, diarrhoea, and hallucinations (Upcroft et al. 2006). Indeed, the side effects of the drug may be worse than the symptoms of giardiasis and the disease often goes untreated. Of further concern, there have been numerous recent reports of nitroimidazole treatment failure and drug resistance by *G. duodenalis* is now a major concern. There is an urgent need to develop better chemotherapeutic treatments for giardiasis with greater efficacy (especially against nitroimidazole resistant strains) and less adverse side effects. Despite this, *G. duodenalis* is still one of the most neglected gastrointestinal diseases in humans, with over 280 million cases of giardiasis reported worldwide per annum (Rahman et al. 2014; Esch and Petersen 2003).

This study examined the *G. duodenalis* growth inhibitory properties of a panel of compounds previously detected in *T. ferdinandiana* fruit extracts and evaluated their efficacy against medtronidazole sensitive and metronidazole resistant strains. Despite the relatively potent activity of the methanolic and aqueous *T. ferdinandiana* fruit extracts (740 and 143µg/mL respectively) and the known bioactivities of the highlighted compounds, approximately 30% were devoid of *G. duodenalis* growth inhibitory activity and the majority of the others had only low levels of activity. Surprisingly, purine had no detectable effects on *G. duodenalis* proliferation despite the organism's reliance on nucleotide salvage pathways and previous reports that purine analogues are good inhibitors of *G. duodenalis* growth (Berens and Marr 1986). Quinic acid also failed to inhibit *G. duodenalis* growth despite reports of leucyl-tRNA synthase inhibitory activity for substituted quinic acid derivatives in *G. duodenalis* cells (Zhang et al. 2012). As functional aminoacyl-tRNA synthase is necessary for protein synthesis, exposure to quinic acid was expected to inhibit *G. duodenalis* protein synthesis, thereby inhibiting growth of the protozoa. However, the previous studies examined substituted quinic acid derivatives rather than the parent compound and it is likely that the substituted groups are

ultimately responsible for this activity. The lack of inhibitory activity of several of the other compounds was less surprising as there are no reports of any of these compounds (nor their derivatives) having anti-giardial activity.

Strong *G. duodenalis* growth inhibitory activity was detected for only a single compound (DPGA) when tested alone. We were unable to find any reports of any therapeutic bioactive properties for DPGA. However, it is likely that the glycosidic linkage in DPGA may be hydrolysed in aqueous solutions, releasing either glucuronic acid and pyrogallol, or gluconic acid and catechol. Both pyrogallol and catechol are have been reported to be cytotoxic to a wide variety of prokaryotic microorganisms and the strength of their toxicity has been linked to the number of hydroxyl groups (Jeong et al. 2009; Han et al. 2009). Thus, pyrogallol is generally a better antimicrobial agent than catechol against most microorganisms. Pyrogallol and catechol bind cell surface lipotoichoic acid and proline-rich membrane proteins, thereby altering their signal transduction pathways (Wolinsky and Sote 1984; Hogg and Embery 1982). They can also bind and inactivate glucosyltransferase enzymes, inhibiting proliferation (Wu-Yuan et al. 1988). Whilst we were unable to find reports of pyrogallol or catechol inhibiting the growth of *Giardia* spp., both have profound effects on other eukaryotic cells. Pyrogallol is cytotoxic to Calu-6 lung cancer cells via the induction of apoptosis (Han et al. 2009). It also has cytostatic effects via inhibition of the G2-M transition phase of the cell cycle in human lung tumour cells (Yang et al. 2009). It is possible that pyrogallol and catechol may have similar cytotoxic and cytostatic effects on *G. duodenalis* although this is yet to be verified.

Interestingly, the inhibitory activity of both DPGA and gallic acid was substantially more potent when tested in combination with ascorbic acid. This is consistent with previous studies which have reported that ascorbic acid potentiates the activity of other cytotoxic compounds, whilst having minimal effect alone. A recent study reported that ascorbic acid

substantially increased the antimicrobial efficacy of isoniazid and rifampin in *Mycobacterium tuberculosis* infected mice (Vilchéze et al. 2018). Similarly, ascorbic acid potentiates the cytotoxic mechanism of arsenic trioxide in promyelocytic leukemia (Yedjou et al. 2009). It is possible that ascorbic acid functions in multiple ways. The cellular redox environment has profound effects on cell function and survival. Interestingly, ascorbic acid can function as either a antioxidant or as a pro-oxidant depending on its concentration and the relative reducing power of the other molecules present in its environment. In the presence of metallic ions (as are present in the *G. duodenalis* growth media), the relatively high reducing power of ascorbic acid may induce it to function as a pro-oxidant (Joel 1995). Furthermore, ascorbic acid is readily oxidised by dissolved oxygen to produce the pro-oxidant dehydroascorbic acid in neutral or alkaline solutions. Both dehydroascorbic acid and ascorbic acid can induce apoptosis in eukaryotic cells in a pro-oxidant environment (Ha et al. 2009; Hong et al. 2007). It is therefore possible that ascorbic acid plays a direct role in inhibiting *G. duodenalis* proliferation. However, similar effects would have been expected for ascorbic acid in the absence DPGA if this was the sole inhibitory mechanism. Furthermore, the close association of the inhibitory ratios with the DPGA axis in the isobologram analysis (Figure 4) is more consistent with DPGA having a more important role in inhibiting *G. duodenalis* growth.

Instead, it is more likely that ascorbic acid plays a support role in inhibiting *G. duodenalis* growth as proposed in Figure 6. DPGA has 6 H bond donors and 9 H bond acceptors. Thus, it could readily alter the redox state of multiple *G. duodenalis* components including membrane lipids, DNA and proteins. These alterations could potentially have profound effects if high enough concentrations of the effector compound DPGA are present. However, the level of DPGA present in the *T. ferdinandiana* extracts is relatively low and would be rapidly oxidised and thereby inactivated. In combinations containing ascorbic acid (or in extracts), the DPGA could be rapidly re-reduced, thereby allowing it to again alter the

redox status of the *G. duodenalis* cells. If enough ascorbic acid is present in the combination, reduced DPGA will be recycled multiple times, accounting for the highly potentiated activity reported here. However, this mechanism is yet to be evaluated.

Of further note, the structural features of DPGA are consistent with the drug like properties defined by Lipinski. Only chebulic acid and DPGA had any violations of the rules of 5, and in both cases this was due to each compound having 6 H bond donors. However, the rules of 5 are best considered as only a set of guidelines for drug discovery, based on broad chemical similarities to other drugs. These rules do not predict whether a compound will have therapeutic properties and they are meant as a means of narrowing the focus for screening large compound libraries. These selection criteria are based on characteristics which enhance oral bioavailability of the drugs. There are numerous examples of front line drugs which have poor compliance with the rules of 5 and only 51% of orally administered FDA-approved drugs are compliant with the rules of 5 (Zhang et al. 2007). The other 49% of approved drugs have multiple violations of Lipinski's rules. Indeed, if Pfizer had followed Lipinski's rules of 5 during the period that he was employed by them, their bestselling drug atorvastatin (marketed as Lipitor) would not have been developed as it violates 2 of the 4 rules (it has a molecular weight of 558.65Da and an log P octonal-water coefficient of 5.7). Generally, a compound with >1 violation is unlikely to be pursued as a potential drug as it is deemed to lack the properties required for absorption when administered orally. DPGA had only a single violation of the rules and is therefore still deemed to have drug-like qualities under these guidelines. However, even if it was not compliant with these guidelines, it would not preclude its potential for the treatment of giardiasis. Indeed, a lower gastrointestinal absorption rate would be beneficial for the treatment of giardiasis as it would provide a longer time for the parasite to be exposed to the therapeutic, potentially increasing its efficacy.

Conclusion

The potent *G. duodenalis* growth inhibitory activity of the combination of DPGA with ascorbic acid demonstrates its potential for therapeutic use to treat giardiasis. Furthermore, this combination was equally as effective against a metronidazole resistant *G. duodenalis* strain, indicating its potential for treating metronidazole resistant giardiasis.

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Conflicts of interest

Both authors declare that they have no conflicts of interest.

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Table 1. The mass of material extracted from 1g of dried *T. ferdinandiana* fruit, qualitative phytochemical screenings, IC_{50} values in the *G. duodenalis* growth inhibition assay and toxicity of the *T. ferdinandiana* extracts.

Extract	Mass of extracted material (mg)	Polyphenolics	Flavonoids	Phytosterols	Saponins	Triterpenoids	Tannins	Alkaloids	Anti-giardial IC_{50} (ug/mL)	Toxicity	
										LC_{50} in ALA (ug/mL)	LC_{50} in the HDF assay (ug/ML)
M	370	+++	+++	-	++	+	++	+	740	1150	1450
W	290	+++	+++	-	+	-	++	+	143	1093	1540

+++ indicates a large response; ++ indicated a moderate response; + indicates a low response; - indicates no response. Values indicate the mean IC_{50} or LC_{50} values of three experiments each with triplicate determinations. M = methanolic extract; W = aqueous extract.

Table 2: IC_{50} values of *T. ferdinandiana* compounds against *G. duodenalis* trophozoites tested alone and in combination with ascorbic acid.

Compound alone or in combination with ascorbic acid	IC_{50} (µg/mL) Values and Class of Combination											
	Sheep S2 Strain				ATCC203333				ATCC PRA-251			
	Single Compound	Combination	ΣFIC_{50}	Class of Interaction	Single Compound	Combination	ΣFIC_{50}	Class of Interaction	Single Compound	Combination	ΣFIC_{50}	Class of Interaction
Gallic acid	1156 (6795µM)	146 (858µM)	0.15	**Synergy**	1368 (8041µM)	228 (1340µM)	0.38	**Synergy**	1255 (7377µM)	276 (1622µM)	0.32	**Synergy**
Chebulic acid	1283 (3602µM)	427 (1199µM)	0.56	Additive	985 (2765µM)	320 (898µM)	0.6	Additive	1220 (3425µM)	446 (1252µM)	0.62	Additive
Glucohepatonic acid lactone	1746 (9914µM)	1330 (7552µM)	1.19	Independent	2255 (12804µM)	1685 (9567µM)	1.57	Independent	3538 (20089µM)	3207 (18209µM)	1.25	Independent
Quinic acid	1172 (6099µM)	418 (2175µM)	0.51	Additive	1428 (7431µM)	632 (3289µM)	0.73	Additive	1755 (9133µM)	882 (4590µM)	0.95	Additive

Eujavonic acid	1438 (6038µM)	525 (2204µM)	0.58	Additive	1683 (7067µM)	695 (2918µM)	0.64	Additive	1850 (7768µM)	878 (3687µM)	0.72	Additive
HMDP	1835 (6895µM)	725 (2724µM)	0.69	Additive	1585 (5955µM)	655 (2461µM)	0.81	Additive	2032 (7635µM)	1058 (3975µM)	1.1	Independent
DPGA	47 (156µM)	5 (17µM)	0.11	**Synergy**	38 (126µM)	6 (20µM)	0.18	**Synergy**	72 (238µM)	12 (40µM)	0.18	**Synergy**
Ascorbic acid	1869 (10618µM)	NA	NA	NA	1906 (10828µM)	NA	NA	NA	2850 (16190µM)	NA	NA	NA

Results are expressed as mean of three independent experiments with internal triplicate determinations (n = 9). Interactions classes are synergistic ($\Sigma FIC_{50} \leq 0.5$), additive ($\Sigma FIC_{50} > 0.5\text{-}1.0$), independent ($\Sigma FIC_{50} > 1.0\text{-}4.0$) or antagonistic ($\Sigma FIC_{50} > 4.0$). NA = results not available.

Table 3: Toxicity of the *T. ferdinandiana* compounds alone and in combination with ascorbic acid determined by *Artemia* lethality assay (ALA) and human dermal fibroblast (HDF) cytotoxicity assay.

Compound	Toxicity (µg/mL)			
	Compound Alone		Compound/ascorbic acid combination	
	ALA	HDF	ALA	HDF
Purine	CND	CND	CND	CND
Gallic acid	132	320	147	336
Chebulic acid	165	380	224	375
Ribolactone	CND	CND	CND	CND
Ascorbic acid	203	358	CND	CND
Gluconolactone	CND	CND	CND	CND
Glucohepatonic acid lactone	CND	CND	>500	>500
Quinic acid	CND	CND	>500	>500
Eujavonic acid	CND	CND	>500	>500
HMDP	CND	CND	>500	>500
DPGA	CND	CND	>500	>500
PC	37	42	NT	NT

PC = potassium dichromate; CND = could not determine as the mortality or % inhibition did not exceed 50% at any concentration tested; NT = not tested.

Table 4: Drug like properties and therapeutic index of the *T. ferdinandiana* compounds alone and in combination with ascorbic acid.

Compound	≤5 H bond donors	≤10 H bond acceptors	MW ≤500Da	Octanol-water coefficient ≤5	Therapeutic Index of Compound	Therapeutic Index in combination with AA
Purine	Yes	Yes	Yes	Yes	TBT	NT
Gallic acid	Yes	Yes	Yes	Yes	0.3	2.3
Chebulic acid	No	Yes	Yes	Yes	0.3	0.9
Ribolactone	Yes	Yes	Yes	Yes	TBT	NT
Ascorbic acid	Yes	Yes	Yes	Yes	0.2	NA
Gluconolactone	Yes	Yes	Yes	Yes	TBT	NT
Glucohepatonic acid lactone	Yes	Yes	Yes	Yes	TBT	TBT
quinic acid	Yes	Yes	Yes	Yes	TBT	TBT
eujavonic acid	Yes	Yes	Yes	Yes	TBT	TBT
HMDP	Yes	Yes	Yes	Yes	TBT	TBT
DPGA	No	Yes	Yes	Yes	TBT	TBT

TBT = the therapeutic index could not be determined as the toxicity was too low to determine an LC_{50}; NT = not tested in combination as the TI of the compound alone was inactive.

Fig. 1 Chemical structures of the compounds reported in anti-proliferative methanolic and aqueous *T. ferdinandiana* fruit extracts (3): (a) purine; (b) gallic acid; (c) chebulic acid; (d) ribonolactone; (e) ascorbic acid; (f) gluconolactone; (g) glucohepatonic acid-1,4-lactone; (h) quinic acid, (i) eujavonic acid; (j) 5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-pentanoic acid (HMDP); (k) 2,3-dihydroxyphenyl B-D-glucopyranosiduronic acid (DPGA).

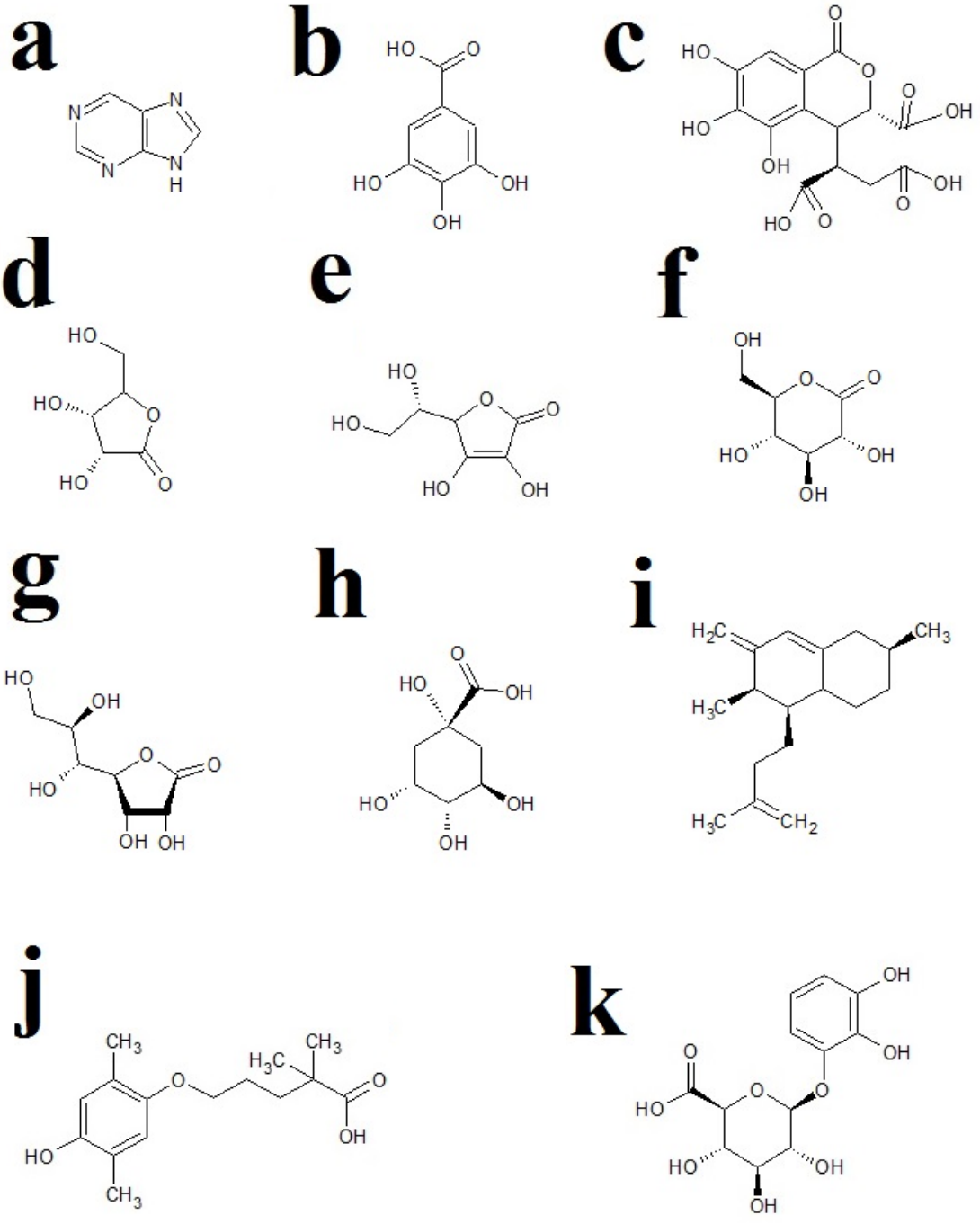

Fig. 2 Inhibitory activity of the *T. ferdinandiana* extracts and pure compounds against three strains of *Giardia duodenalis* trophozoites measured as a percentage the untreated control. NC = negative control; M = methanolic extract; W = water extract; 1 = purine; 2 = gallic acid; 3 = chebulic acid; 4 = ribolactone; 5 = ascorbic acid; 6 = gluconolactone; 7 = glucohelapatonic acid lactone; 8 = quinic acid; 9 = eujavonic acid; 10 = HMDP; 11 = DPGA; PC = metronidazole control (50 µg/ml). Results are expressed as the mean ± SEM of three independent experiments with internal triplicate determinations (n = 9). *, # and ^ indicate results that are significantly different to the untreated controls for the sheep S2, ATCC 203333 and ATCC PRA-251 *G. duodenalis* strains respectively ($p<0.01$).

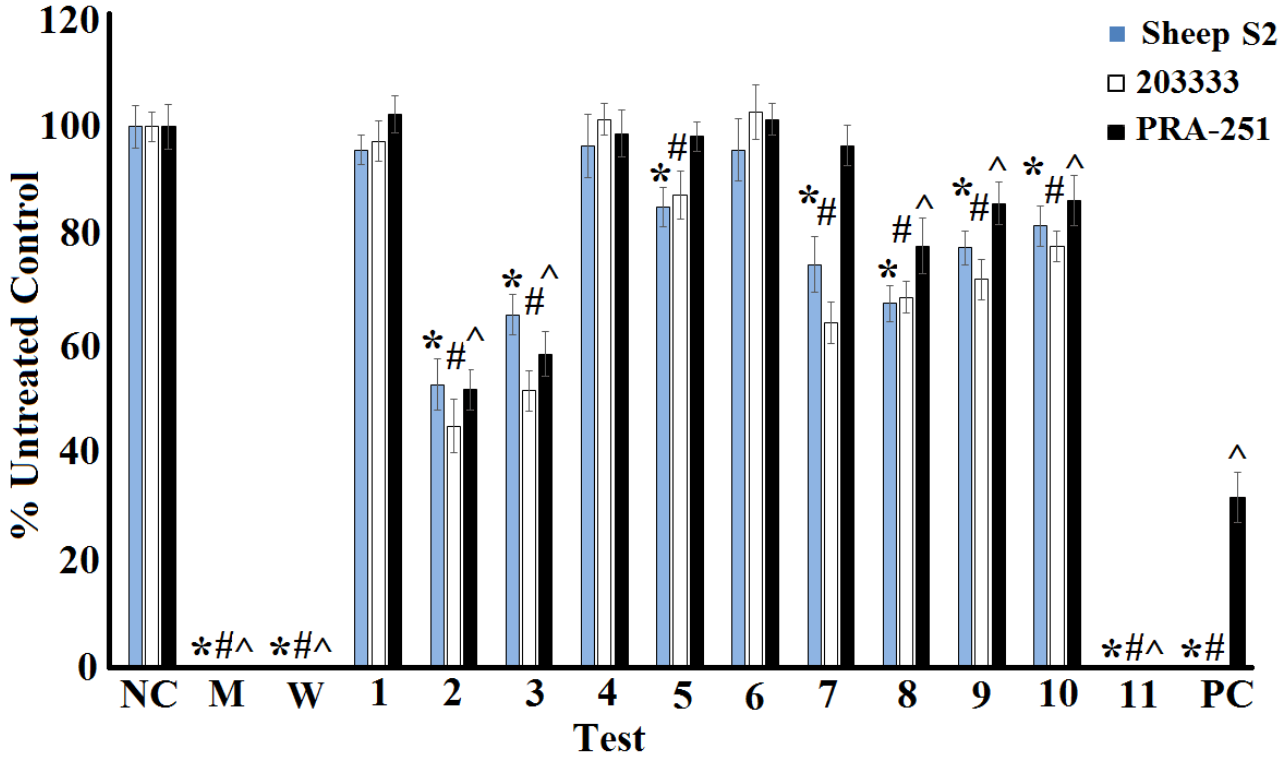

Fig. 3 Isobolograms for combinations of gallic acid and ascorbic acid tested at various ratios against (a) the sheep S2, (b) reference metronidazole sensitive (ATCC203333) and (c) reference metronidazole resistant (ATCC PRA-251) *G. duodenalis* strains. GA = gallic acid; AA = ascorbic acid. Results represent mean FIC_{50} values of three independent experiments, each consisting of 3 replicates (i.e. 9 data points for each ratio). Ratios lying on or underneath the 0.5/0.5 line are considered to be synergistic ($\Sigma FIC_{50} \leq 0.5$). Any points between the 0.5/0.5 and 1.0/1.0 lines are deemed to be additive ($\Sigma FIC_{50} > 0.5\text{-}1.0$).

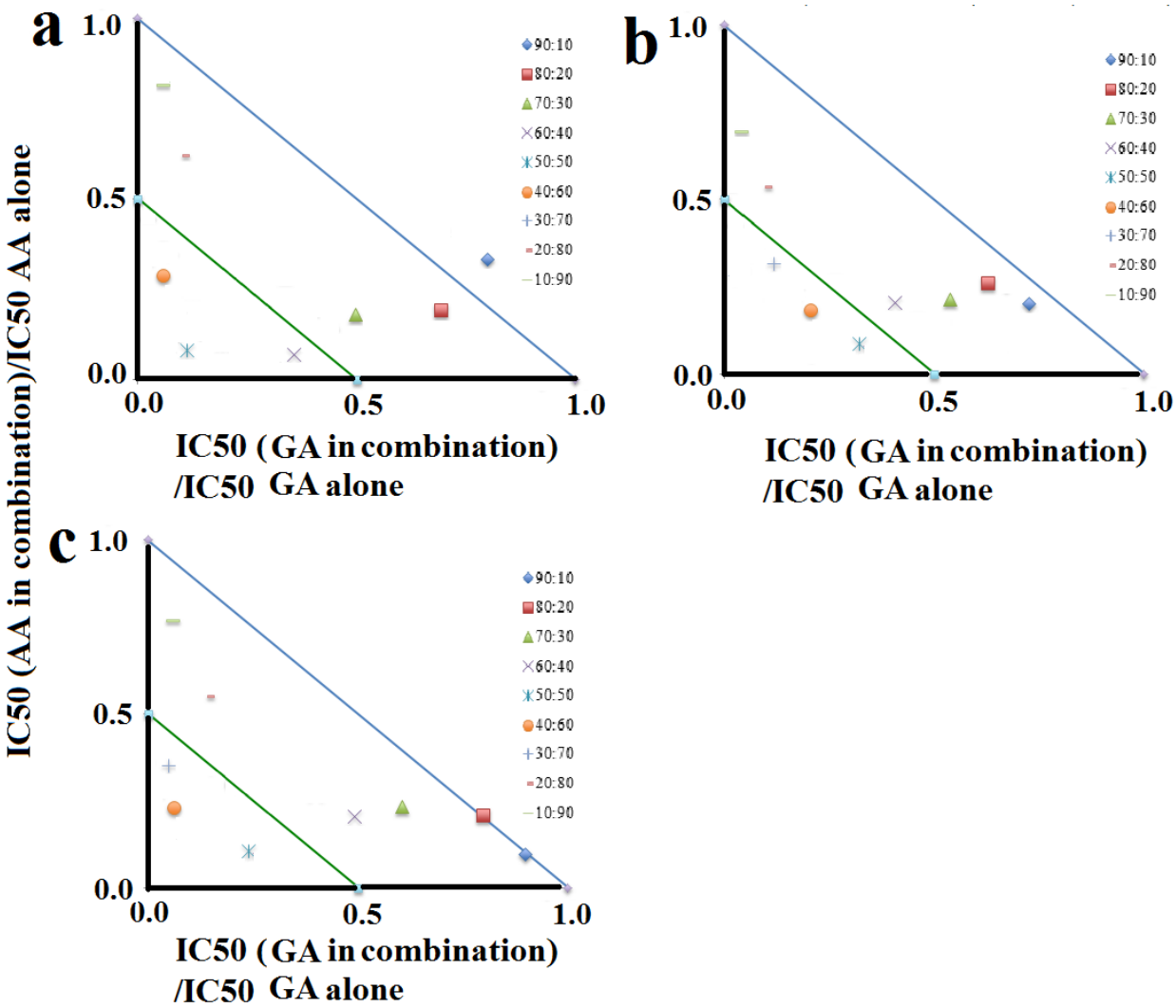

Fig. 4 Isobolograms for combinations of DPGA and ascorbic acid tested at various ratios against (a) the sheep S2, (b) reference metronidazole sensitive (ATCC203333) and (c) reference metronidazole resistant (ATCC PRA-251) *G. duodenalis* strains. DPGA = 2,3-dihydroxyphenyl-B-gloucopyranosiduronic acid; AA = ascorbic acid. Results represent mean FIC_{50} values of three independent experiments, each consisting of 3 replicates (i.e. 9 data points for each ratio). Ratios lying on or underneath the 0.5/0.5 line are considered to be synergistic ($\Sigma FIC_{50} \leq 0.5$). Any points between the 0.5/0.5 and 1.0/1.0 lines are deemed to be additive ($\Sigma FIC_{50} > 0.5\text{-}1.0$).

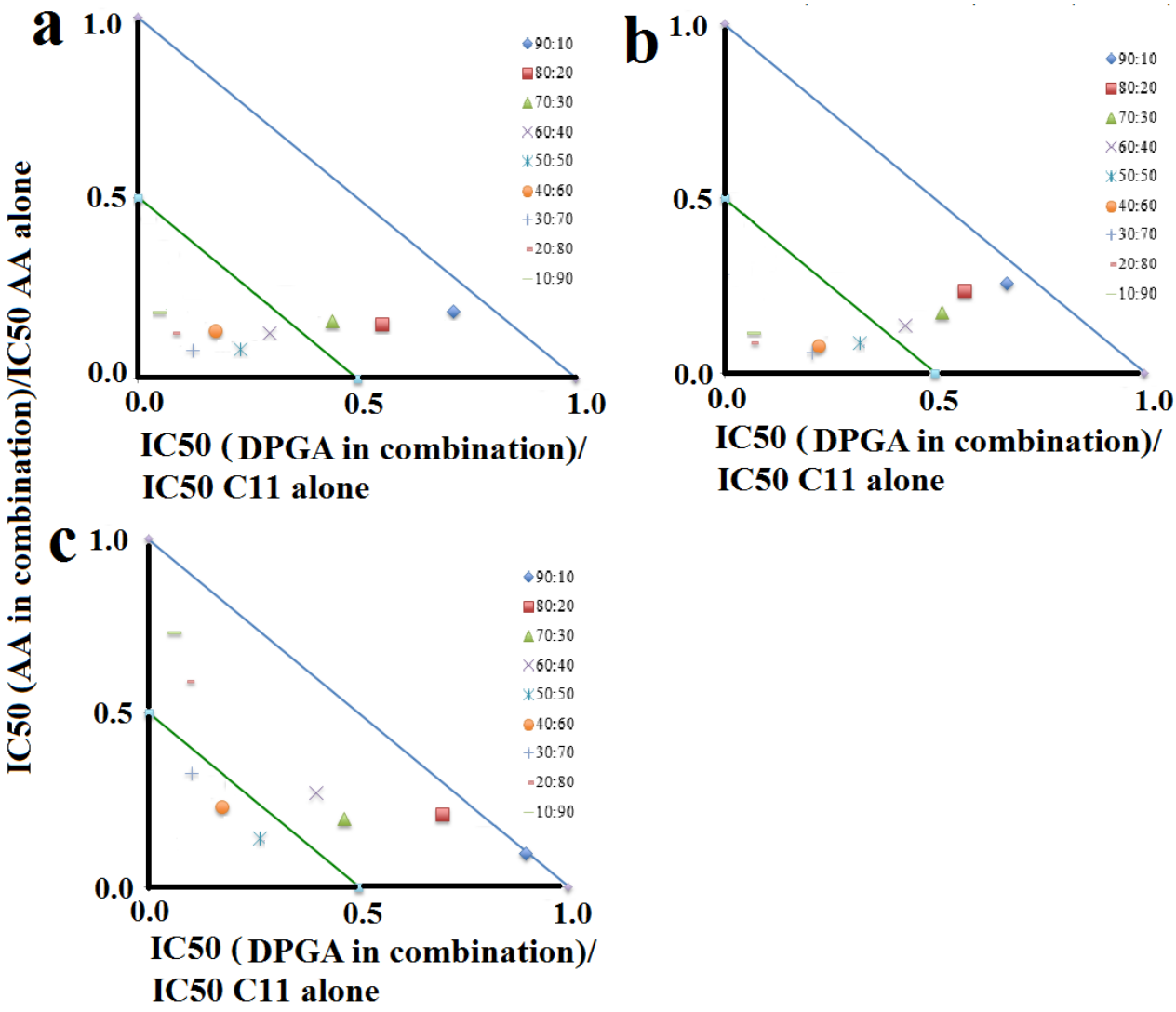

Fig. 5 A proposed synergistic mechanism for the interaction between DPGA and ascorbic acid. Relatively small quantities of DPGA exhibit anti-giardial activities via redox related mechanisms and in the process become oxidised. Ascorbic acid rapidly reduces the oxidised DPGA, returning it to an active form.