Repurposing Common Food Additives (Benzo Derivatives) As New Anti-parasitic Agents

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Graphical Abstract
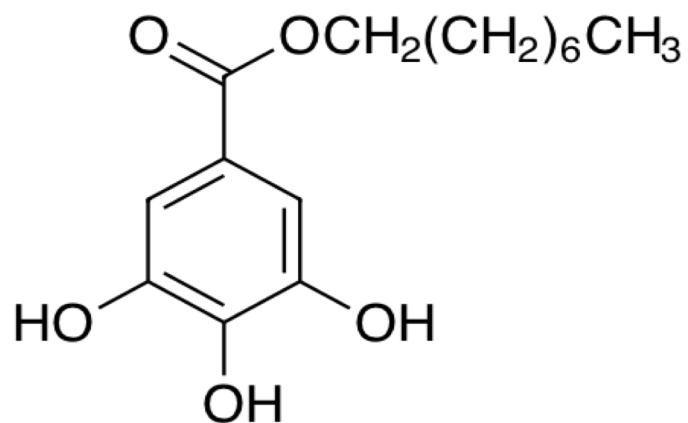
Abstract

This study examined the anti-protozoal effects of selected benzo derivatives, namely ten gallic acid (GA) alkyl esters (viz., benzoic acid analogs) and twenty-three benzaldehyde analogs, against six different anaerobic human protozoal pathogens- - *Trichomonas vaginalis, Tritrichomonas foetus*, *Tritrichomonas foetus*-like, *Giardia lamblia, Entamoeba histolytica*, and *Naegleria fowleri*. The efficacy of benzaldehyde and gallate (3,4,5-trihydroxybenzoic acid) analogs were investigated in two respects: (1) changing types of side chains and their positions on the benzaldehyde ring [structure–activity relationships (SAR)]; and, (2) changing lengths of alkyl chains esterified with the carboxyl group on gallate. Results of parasite growth inhibition assays indicated that benzo derivatives could be further developed as potent anti-protozoal drug candidates/leads, where GA having longer alkyl chains exhibited higher anti-protozoal activity than compounds with shorter alkyl chains or all benzaldehyde analogs tested. The chemical libraries were also screened against common human microbiome bacteria and no detectable inhibition was observed. Structure-activity relationships and their implications for new drug discovery against these sexually-transmitted, water-borne, and food-borne parasites are discussed.

Keywords: Benzaldehydes; Protozoal pathogens; Structure-activity relationship

Introduction

Gallic acid (GA; 3,4,5-trihydroxybenzoic acid) is a redox-active polyphenol, viz., possessing both antioxidant and prooxidant potential, which is abundantly found in many plants (Badhani et al., 2015). Redox-active phenolics can serve as potent redox-cyclers in pathogens, hence function as effective antimicrobial agents by disrupting redox homeostasis and/or redox-sensitive structures in the cell (Guillen and Evans, 1994; Jacob, 2006). In addition to its free acid form, plant hydrolyzable tannins are important source of GA, which could also function as a natural anti-mycotoxigenic agent (Mahoney and Molyneux, 2004). Since oxidative stress triggers aflatoxin production in *Aspergillus* sp. (Jayashree and Subramanyam, 2000), the anti-aflatoxigenic effect of GA could result from the antioxidant potential of the chemical (Kim et al., 2005). Besides, GA has also been documented for its pharmacological effects, such as anticancer (Kosuru et al., 2018), anti-inflammatory (Kroes et al., 1992) and anti-diabetic complication activity (Huang et al., 2018).

While GA has long been studied for control of bacterial pathogens (Kubo et al., 2002a; Wang et al., 2017), pathogen-inhibitory effect of GA has also been investigated in lower eukaryotes, such as yeast or filamentous fungal pathogens. For instance, administration of GA prevented the growth of *Candida albicans* (yeast) or *Trichophyton rubrum* (filamentous fungus), where GA disrupted ergosterol biosynthesis (thus interfering with the integrity of cellular membrane) by lowering the activity of sterol 14α-demethylase P450 (CYP51) and squalene epoxidase in the membrane (Li et al., 2017).

Introduction (Continued)

Of note, methods have been developed for the enhancement of bioavailability or biologically relevant application of GA. For example, to improve GA solubility for treating *C. albicans* biofilms, antifungal efficacy of GA/hydroxypropyl-β-cyclodextrin complex (HPβCD) has been examined (Teodoro et al., 2017). Results showed that GA/HPβCD complex was active against *C. albicans* biofilms, where the complex preserved the antifungal potency of the pure GA while it also exerted anti-inflammatory activity.

However, despite its potential as an effective antimicrobial agent, the level of antifungal activity of GA is still not comparable to conventional drugs or fungicides. One of the strategies for potency improvement of lead compounds is a structural modification of chemical backbones. To enhance the antifungal efficacy of GA, the effect of incorporating alkyl esters into GA backbone has been studied by changing the length of alkyl esters, as follows: (1) GA alkyl esters, such as octyl gallate (OG), reduced fluidity of the fungal membrane and/or inhibited the activity of H(+)-ATPase, thus prevented the growth of fungal growth with enhanced GA potency (Fujita and Kubo, 2002a), (2) Tests using mutants of *Saccharomyces cerevisiae* (model yeast) or *Aspergillus fumigatus* (causative agent of human invasive aspergillosis) indicated that GA alkyl derivatives also negatively affected fungal antioxidant systems, where *S. cerevisiae* mitochondrial superoxide dismutase mutant or *A. fumigatus* antioxidant mitogen-activated protein kinase (MAPK) mutant were highly susceptible to alkyl gallates (Kim et al., 2010a & 2010b), (3) In addition to inhibiting

Introduction (Continued)

antioxidant system, OG also negatively affected cell wall-integrity system of fungi (Kim et al., 2014), which was determined *via* the inhibition of *S. cerevisiae* cell wall-integrity MAPK pathway mutants, (4) Antimicrobial activity of alkyl gallates has been quantified against *Aspergillus brasiliensis* (mold) and also *Pseudomonas aeruginosa* (Gram negative bacterium) with a hazard assessment (Buckley et al., 2017). OG was determined to possess better antimicrobial activity with lower hazards, when compared to other alkyl derivatives or conventional preservatives (Buckley et al., 2017), (5) Meanwhile, in a separate study, dodecyl gallate (DG) was determined as the most effective alkyl gallate for control of the yeast pathogen *C. albicans* (Singulani et al., 2017), where GA and DG were the least toxic compounds comparing to other derivatives (Zebrafish embryo model).

Anti-pathogenic activity of alkyl gallates has been investigated in another eukaryote *Trypanosoma cruzi, a causative agent of Chagas disease (*Andréo et al., 2015). Results showed that longer-chain alkyl gallates, viz., nonyl, decyl, undecyl, dodecyl gallates, exhibited potent anti-trypanosomal activity, where loss of mitochondrial potential in *T. cruzi* could be one mechanism of anti-trypanosomal action of alkyl gallates *(*Andréo et al., 2015).

Similar to the benzoic acid (-COOH) analogs, as described above, certain benzaldehyde (-CHO) analogs could also be developed as redox-active antimicrobial agents. Benzaldehyde is also a natural product, which can be isolated from crops such as almond (Geng et al., 2016).

Introduction (Continued)

Prior study in fungal pathogens showed that benzaldehyde derivatives, such as cinnamaldehyde, could target cellular antioxidant system (viz., MAPK pathway, antioxidant enzymes including Cu,Zn-SOD, Mn-SOD, or glutathione reductase), where the growth of fungal mutants lacking genes in antioxidant system was greatly inhibited (Kim et al., 2011). Other benzaldehyde analogs, such as 2-hydroxy-4-methoxybenzaldehyde, further inhibited the growth of cell wall integrity MAPK mutants of the model yeast *S. cerevisiae* (Kim et al., 2015).

Anti-protozoal effect of benzaldehyde-containing drug candidates have been investigated, where 1,3,4-thiadiazole derivatives of *R*-(+)-limonene benzaldehyde-thiosemicarbazones triggered *T. cruzi* death by causing cellular oxidative stress (Martins et al., 2016). Study showed these compounds increased the level of mitochondrial-derived superoxide radicals, total reactive oxygen species, nitric oxides, and lipid peroxidation, while the same treatment reduced thiol (antioxidant) levels in *T. cruzi* (Martins et al., 2016).

Results and discussion

Anti-protozoal efficacy of benzaldehyde and gallate (3,4,5-trihydroxybenzoic acid) analogs were investigated in two respects: changing types of side chains and their positions on the benzaldehyde ring [structure–activity relationships (SAR)]; and, changing lengths of alkyl chains esterified with the carboxyl group on the gallate. In each modification, the effectiveness of structural analogs was tested in three different protozoa, viz., *T. vaginalis* trophozoites (G3, C1) and *T. foetus* bovine strain (D1).

Effect of alkyl chain length on gallate

The alkyl gallate analogs tested were methyl (C_1), ethyl (C_2), propyl (C_3), butyl (C_4), pentyl (C_5), octyl (C_8), nonyl (C_9), decyl (C_{10}) and lauryl (C_{12}) gallate. Alkyl gallates were categorized as "Group 1" to "Group 5" compounds, depending on the level of anti-protozoal activity tested at 100 μM (See **Table 1**). Esterification with a lauryl (C_{12}) group greatly enhanced the anti-protozoal activity of gallate against all pathogens tested (G3, C1, D1), which achieved 100% growth inhibition. Of note, differential susceptibility of protozoal strains could be found as follows:

Results and discussion

Table 1. Level of growth inhibition of G3, C1 and D1 by alkyl gallates at 100 µM. nd, not determined (low anti-protozoal activity).

Inhibition level	G3	% Inhibition	IC50 (µM)	C1	% Inhibition	IC50 (µM)	D1	% Inhibition	IC50 (µM)
Group 1 (n > 95%)	Octyl (C_8) Nonyl (C_9) Decyl (C_{10}) Lauryl (C_{12})	100.0 ± 0.0 100.0 ± 0.0 98.6 ± 2.4 100.0 ± 0.0	24.9 24.1 21.8 19.8	Decyl Lauryl	100.0 ± 0.0 100.0 ± 0.0	31.9 56.6	Lauryl	100.0 ± 0.0	54.9
Group 2 (60% < n ≤ 75%)							Octyl Decyl	65.1 ± 9.9 73.5 ± 5.7	nd nd
Group 3 (45% < n ≤ 60%)				Pentyl Octyl	49.3 ± 9.0 59.4 ± 2.9	nd nd			
Group 4 (30% < n ≤ 45%)	Propyl (C_3)	32.3 ± 4.6	nd[1]	Ethyl Nonyl	41.2 ± 4.4 41.2 ± 1.5	nd nd	Butyl Nonyl	38.4 ± 9.5 35.3 ± 4.0	nd nd
Group 5 (n ≤ 30%)	Gallate (C_0) Methyl (C_1) Ethyl (C_2) Butyl (C_4) Pentyl (C_5)	17.5 ± 3.1 26.9 ± 5.8 27.1 ± 3.4 19.9 ± 6.9 29.6 ± 5.5	nd nd nd nd nd	Gallate Methyl Propyl Butyl	1.0 ± 1.8 13.7 ± 0.6 20.5 ± 5.1 22.7 ± 9.6	nd nd nd nd	Gallate Methyl Ethyl Propyl Pentyl	19.7 ± 7.2 5.5 ± 8.1 5.5 ± 9.5 20.9 ± 6.0 18.9 ± 6.1	nd nd nd nd nd

Results and discussion (Continued)

T. vaginalis G3 (Most susceptible to alkyl gallates)
In G3, four gallates with longer alkyl chains (Group 1: octyl-, nonyl-, decyl- and lauryl gallate; C_8 to C_{12}) showed the highest activity, thus achieving 98.6% to 100% growth inhibition at 100 μM of each compound (**Table 1**). The IC50 values further confirmed that gallate with a longer alkyl chain possesses higher anti-protozoal activity, viz., lauryl- (C_{12}; 19.8 μM) > decyl- (C_{10}; 21.8 μM) > nonyl- (C_9; 24.1 μM) > octyl gallate (C_8; 24.9 μM) (high to low activity).

In comparison, gallates with shorter alkyl chain exhibited very low anti-protozoal activity, hence belong to Group 4 (30 < n ≤ 45%; n: growth inhibition rate; propyl gallate) or Group 5 (≤ 30% growth inhibition; free gallate, methyl-, ethyl-, butyl-, pentyl gallate) compounds (**Table 1**).

T. foetus-like C1 (Moderately susceptible to alkyl gallates)
In C1, treatments of the pathogen with decyl- or lauryl gallate at 100 μM achieved 100% growth inhibition (Group 1). However, in contrast to G3, the IC50 value of lauryl gallate (56.6 μM) was higher than that of decyl gallate (31.9 μM). Also, unlike G3, treatment of C1 with octyl- or nonyl gallate at 100 μM only reached 59.4% or 41.2%

Results and discussion (Continued)

growth inhibition, respectively, which is similar to that of pentyl- or ethyl gallate (Group 3 or 4, respectively). Therefore, results indicated differential susceptibility of test pathogens to these alkyl gallates. The remaining alkyl gallates achieved ≤ 30% of growth inhibition in C1 at 100 μM (Group 5).

T. foetus D1 (Least susceptible to alkyl gallates)

In D1, lauryl gallate at 100 mM achieved 100% growth inhibition (Group 1), where the IC50 value (54.9 μM) of the compound was very similar to that in C1 (56.6 μM). However, other longer alkyl chain gallates possessed relatively lower anti-protozoal activity, where octyl-, decyl- or nonyl gallate achieved 65.1% (Group 2), 73.5% (Group 2) or 35.3% (Group 4) growth inhibition, respectively, at 100 μM. The remaining alkyl gallates, namely Group 4 (butyl-) and Group 5 (free gallate, methyl-, ethyl-, propyl- or pentyl gallate) compounds, achieved 38.4% or ≤ 30% of growth inhibition, respectively, in D1.

Results and discussion (Continued)

The cutoff chain length of gallate analogs for achieving ~100% growth inhibition in G3, C1 or D1 pathogens was C_8, C_{10} or C_{12}, respectively. The order of susceptibility of pathogens to alkyl gallates tested was G3 > C1 > D1 (high to low), where four, two or one alkyl gallate(s) could be selected as Group 1 compound(s), respectively [Note: In the model yeast *Saccharomyces cerevisiae*, the cutoff for growth inhibitory and/or fungicidality was C_{10} (Fujita and Kubo, 2002b)].

In contrast to that observed in G3, nonyl gallate (longer alkyl chain analog) possessed very low anti-protozoal activity in both C1 (41.2% inhibition) and D1 (35.3% inhibition) (which is also lower than that of other longer chain alkyl gallates tested in C1 and D1; See **Table 1**).

Except Group 1 compounds selected in G3 (where the longer the alkyl chain, the higher the activity, based on IC50 values), there was no general relationship/trend between the length of alkyl chain and the incremental increase of anti-protozoal activity of compounds in test pathogens.

Results and discussion (Continued)

Effect of benzaldehyde analogs

Effect of changing types of side chains on the benzaldehyde ring was also investigated. Twenty-three benzaldehyde analogs were examined against G3, C1 and D1, where test compounds were categorized as Group 1 to 4 depending on the level of anti-protozoal activity (**Table 2**). Results showed that the level of activity of benzaldehyde analogs was much lower than that of alkyl gallates. For example, the highest growth inhibition rate, which was observed in G3 only, was 51.8% (achieved by 4-methoxy-2-methylbenzaldehyde), whereas up to 100% growth inhibition could be achieved in all protozoa tested (G3, C1, D1) by longer chain alkyl gallates (octyl-, nonyl-, decyl- or lauryl gallate; See **Table 1**). As observed in alkyl gallate testing, there was differential susceptibility of G3, C1 and D1 strains to benzaldehydes as follows:

Table 2. Level of growth inhibition of G3, C1 and D1 by benzaldehyde analogs at 100 μM. Note that % inhibition values were described only in Groups 1 and 2 compounds (Groups 3 and 4 compounds: See supplementary Table S1).

Pathogens Inhibition level	G3	C1	D1
Group 1 (n > 45%)	4-Methoxy-2-methylbenzaldehyde (51.8 ± 8.2)		
Group 2 (30% < n ≤ 45%)	Cinnamaldehyde (39.0 ± 9.1), 2-Methylbenzaldehyde (31.3 ± 9.8), 2-Methoxybenzaldehyde (36.8 ± 4.2), 3-Methoxybenzaldehyde (45.0 ± 9.3), 2,5-Dimethoxybenzaldehyde (40.0 ± 3.5)	2-Methoxybenzaldehyde (39.9 ± 5.9), 3-Methoxybenzaldehyde (36.3 ± 9.5), 2,3-Dimethoxybenzaldehyde (32.9 ± 5.9)	
Group 3 (15% < n ≤ 30%)	4-Methoxybenzaldehyde, 2,3-Dimethoxybenzaldehyde, 2-Hydroxy-4-methoxybenzaldehyde, 2-Hydroxy-4-methylbenzaldehyde	3,4-Dimethoxybenzaldehyde, 3-Hydroxy-4-methoxybenzaldehyde, 2-Hydroxy-5-methoxybenzaldehyde, 2-Methylbenzaldehyde, 2,4,5-Trimethoxybenzaldehyde, 3,5-Dimethoxy-4-hydroxybenzaldehyde, 4-Methoxy-2-methylbenzaldehyde, 2-Hydroxy-5-methylbenzaldehyde, 2-Hydroxy-4-methoxybenzaldehyde	3,4-Dimethoxybenzaldehyde, 2-Hydroxy-3-methoxybenzaldehyde, 3-Methylbenzaldehyde, 2,5-Dimethoxybenzaldehyde
Group 4 (n ≤ 15%)	3,4-Dimethoxybenzaldehyde, 3-Hydroxy-4-methoxybenzaldehyde, 2-Hydroxy-3-methoxybenzaldehyde, 2-Hydroxy-5-methoxybenzaldehyde, 3-Methylbenzaldehyde, 4-Methylbenzaldehyde, 2,4-Dimethoxybenzaldehyde, 3,5-Dimethoxybenzaldehyde, 2,4,5-Trimethoxybenzaldehyde, 3,5-Dimethoxy-4-hydroxybenzaldehyde, 2-Hydroxy-5-methylbenzaldehyde, 2,4-Dimethylbenzaldehyde, 2,4-Dihydroxybenzaldehyde	Cinnamaldehyde, 2-Hydroxy-3-methoxybenzaldehyde, 3-Methylbenzaldehyde, 4-Methylbenzaldehyde, 4-Methoxybenzaldehyde, 2,4-Dimethoxybenzaldehyde, 2,5-Dimethoxybenzaldehyde, 3,5-Dimethoxybenzaldehyde, 2,4-Dimethylbenzaldehyde, 2-Hydroxy-4-methylbenzaldehyde, 2,4-Dihydroxybenzaldehyde	3-Hydroxy-4-methoxybenzaldehyde, Cinnamaldehyde, 2-Hydroxy-5-methoxybenzaldehyde, 2-Methylbenzaldehyde, 4-Methylbenzaldehyde, 2-Methoxybenzaldehyde, 3-Methoxybenzaldehyde, 4-Methoxybenzaldehyde, 2,3-Dimethoxybenzaldehyde, 2,4-Dimethoxybenzaldehyde, 3,5-Dimethoxybenzaldehyde, 2,4,5-Trimethoxybenzaldehyde, 3,5-Dimethoxy-4-hydroxybenzaldehyde, 4-Methoxy-2-methylbenzaldehyde, 2-Hydroxy-5-methylbenzaldehyde, 2,4-Dimethylbenzaldehyde, 2-Hydroxy-4-methoxybenzaldehyde, 2-Hydroxy-4-methylbenzaldehyde, 2,4-Dihydroxybenzaldehyde

Results and discussion (Continued)

T. vaginalis G3 (Most susceptible)

In G3, 4-methoxy-2-methylbenzaldehyde (Group 1) exhibited the highest anti-protozoal activity, achieving 51.8 % growth inhibition at 100 μM (**Table 2**). Five benzaldehydes were further categorized as Group 2 compounds, which inhibited G3 growth 31.3% to 45.0% [Of note, the three compounds in Group 2 (cinnamaldehyde, 2-methoxybenzaldehyde and 2,5-dimethoxybenzaldehyde) previously showed potent antifungal activity against filamentous fungal pathogens (Kim et al., 2011)]. The remaining seventeen benzaldehydes belong to either Group 3 (Four benzaldehydes) or Group 4 (Thirteen benzaldehydes) compounds, thus achieved up to 30% or 15%, respectively, of growth inhibition in G3 (**Table 2**).

It is intriguing to note that the level of anti-protozoal activity of 4-methoxy-2-methylbenzaldehyde (51.8%; Group 1) was roughly equivalent to the sum of the % inhibition of both 2-methylbenzaldehyde (31.3%; Group2) and 4-methoxybenzaldehyde (20.1%; Group 3; **Table S1**), indicating methoxyl and methyl moiety at 4- and 2- position on the benzaldehyde ring, respectively, provided an additive effect for anti-protozoal efficacy.

Table S1. Percent (%) growth inhibition of G3, C1 and D1 by the treatments of benzaldehyde analogs at 100 µM.

G3, Benzaldehydes	Average Inhibition (%)	SD (%)
3,4-dimethoxybenzaldehyde (Veratraldehyde)	4.3	3.8
3-hydroxy-4-methoxybenzaldehyde	8.9	9.3
cinnamaldehyde	39.0	9.1
2-hydroxy-3-methoxybenzaldehyde (o-vanillin)	5.0	4.5
2-hydroxy-5-methoxybenzaldehyde	7.1	6.5
2-methylbenzaldehyde (o-tolualdehyde)	31.3	9.8
3-methylbenzaldehyde (m-tolualdehyde)	1.0	3.0
4-methylbenzaldehyde (p-tolualdehyde)	6.7	7.2
2-methoxybenzaldehyde (o-anisaldehyde)	36.8	4.2
3-methoxybenzaldehyde (m-anisaldehyde)	45.0	9.3
4-methoxybenzaldehyde (p-anisaldehyde)	20.1	7.7
2,3-dimethoxybenzaldehyde	16.7	9.2
2,4-dimethoxybenzaldehyde	4.8	7.0
2,5-dimethoxybenzaldehyde	40.0	3.5
3,5-dimethoxybenzaldehyde	5.7	9.9
2,4,5-trimethoxybenzaldehyde	0.5	0.8
3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde)	2.4	4.1
4-methoxy-2-methylbenzaldehyde	51.8	8.2
2-hydroxy-5-methylbenzaldehyde	3.3	5.8
2,4-dimethylbenzaldehyde	4.0	8.3
2-hydroxy-4-methoxybenzaldehyde	20.6	7.9
2-hydroxy-4-methylbenzaldehyde	27.2	6.9
2,4-dihydroxybenzaldehyde	8.2	8.8

Table S1. Percent (%) growth inhibition of G3, C1 and D1 by the treatments of benzaldehyde analogs at 100 μM. (Continue)

C1, Benzaldehydes	Average Inhibition (%)	SD (%)
3,4-dimethoxybenzaldehyde (Veratraldehyde)	18.3	7.7
3-hydroxy-4-methoxybenzaldehyde	24.4	6.5
cinnamaldehyde	2.0	5.3
2-hydroxy-3-methoxybenzaldehyde (o-vanillin)	0.5	4.5
2-hydroxy-5-methoxybenzaldehyde	25.2	6.9
2-methylbenzaldehyde (o-tolualdehyde)	28.0	9.4
3-methylbenzaldehyde (m-tolualdehyde)	9.8	9.9
4-methylbenzaldehyde (p-tolualdehyde)	2.0	0.3
2-methoxybenzaldehyde (o-anisaldehyde)	39.9	5.9
3-methoxybenzaldehyde (m-anisaldehyde)	36.3	9.5
4-methoxybenzaldehyde (p-anisaldehyde)	12.9	6.7
2,3-dimethoxybenzaldehyde	32.9	5.9
2,4-dimethoxybenzaldehyde	-13.6	7.1
2,5-dimethoxybenzaldehyde	-0.9	7.3
3,5-dimethoxybenzaldehyde	10.5	9.8
2,4,5-trimethoxybenzaldehyde	23.6	8.0
3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde)	29.4	2.9
4-methoxy-2-methylbenzaldehyde	24.8	6.7
2-hydroxy-5-methylbenzaldehyde	24.5	9.9
2,4-dimethylbenzaldehyde	7.1	6.5
2-hydroxy-4-methoxybenzaldehyde	29.1	3.7
2-hydroxy-4-methylbenzaldehyde	9.2	9.8
2,4-dihydroxybenzaldehyde	0.2	0.4

Table S1. Percent (%) growth inhibition of G3, C1 and D1 by the treatments of benzaldehyde analogs at 100 µM. (Continue)

D1, Benzaldehydes	Average Inhibition (%)	SD (%)
3,4-dimethoxybenzaldehyde (Veratraldehyde)	17.1	8.5
3-hydroxy-4-methoxybenzaldehyde	11.8	6.0
cinnamaldehyde	5.0	5.3
2-hydroxy-3-methoxybenzaldehyde (o-vanillin)	18.0	1.5
2-hydroxy-5-methoxybenzaldehyde	6.2	5.5
2-methylbenzaldehyde (o-tolualdehyde)	3.9	6.8
3-methylbenzaldehyde (m-tolualdehyde)	24.6	9.1
4-methylbenzaldehyde (p-tolualdehyde)	5.8	7.6
2-methoxybenzaldehyde (o-anisaldehyde)	0.1	3.2
3-methoxybenzaldehyde (m-anisaldehyde)	2.4	9.7
4-methoxybenzaldehyde (p-anisaldehyde)	14.2	9.1
2,3-dimethoxybenzaldehyde	8.0	9.7
2,4-dimethoxybenzaldehyde	10.6	9.3
2,5-dimethoxybenzaldehyde	15.9	9.4
3,5-dimethoxybenzaldehyde	4.9	8.5
2,4,5-trimethoxybenzaldehyde	4.7	9.5
3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde)	1.5	2.5
4-methoxy-2-methylbenzaldehyde	8.0	6.8
2-hydroxy-5-methylbenzaldehyde	2.9	3.9
2,4-dimethylbenzaldehyde	3.7	6.4
2-hydroxy-4-methoxybenzaldehyde	11.3	9.9
2-hydroxy-4-methylbenzaldehyde	10.0	9.9
2,4-dihydroxybenzaldehyde	4.0	3.8

Results and discussion (Continued)

T. vaginalis C1 (Moderately susceptible)

In C1, the highest anti-protozoal activity (32.9% to 39.9%) was achieved by three benzaldehydes, which belong to Group 2 (viz., 2- & 3-methoxybenzaldehyde, 2,3-dimethoxybenzaldehyde) compounds (**Table 2**) [2- and 3-methoxybenzaldehydes also showed similar level of anti-protozoal activity in G3 (See above & **Table 2**)].
However, other benzaldehydes belonging to Group 2 in G3 (viz., cinnamaldehyde, 2-methylbenzaldehyde, 2,5-dimethoxybenzaldehyde) did not achieve similar level of anti-protozoal activity in C1, while 2,3-dimethoxybenzaldehyde inhibited the growth of C1 around 32.9% (**Table S1**) [2,3-Dimethoxybenzaldehyde previously also showed potent antifungal activity against filamentous fungal pathogens (Kim et al., 2011)].
The remaining twenty benzaldehydes belong to either Group 3 (Nine benzaldehydes) or Group 4 (Eleven benzaldehydes) compounds.

Results and discussion (Continued)

T. foetus D1 (Least susceptible)

In D1, the highest anti-protozoal activity (15.9% to 24.6%) was achieved only by four benzaldehydes, which belong to Group 3 (**Table 2**). The remaining nineteen benzadehydes were categorized as Group 4 compounds, which achieved less than 15% of growth inhibition. In particular, 4-methoxy-2-methylbenzaldehyde or cinnamaldehyde (which were also potent anti-protozoal compounds in G3) did not achieve similar level of anti-protozoal activity to that observed in C1.

As determined in alkyl gallates (See above), there was also differential susceptibility of test strains to benzaldehyde analogs, where G3 was the most susceptible to benzaldehydes, followed by C1 and D1. Collectively, it's concluded that certain benzo derivatives (benzoic acid or benzaldehyde analogs) could be developed as potent anti-protozoal agents or leads with structural-activity relationship, where the level of activity is dependent upon types of protozoa pathogens.

Conclusions

In this study, anti-protozoal effects of selected benzo derivatives, namely ten GA alkyl esters (viz., benzoic acid analogs) and twenty-three benzaldehyde analogs, were investigated against *T. vaginalis* trophozoites (G3), *T. foetus*-like C1, and *T. foetus* bovine strain (D1). Results indicated that benzo derivatives could be developed as potent anti-protozoal drug candidates/leads, where GA having longer alkyl chains exhibited much higher anti-protozoal activity compared to that with shorter alkyl chains or all benzaldehyde analogs tested.

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