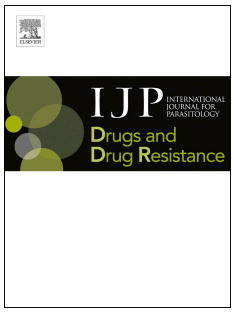

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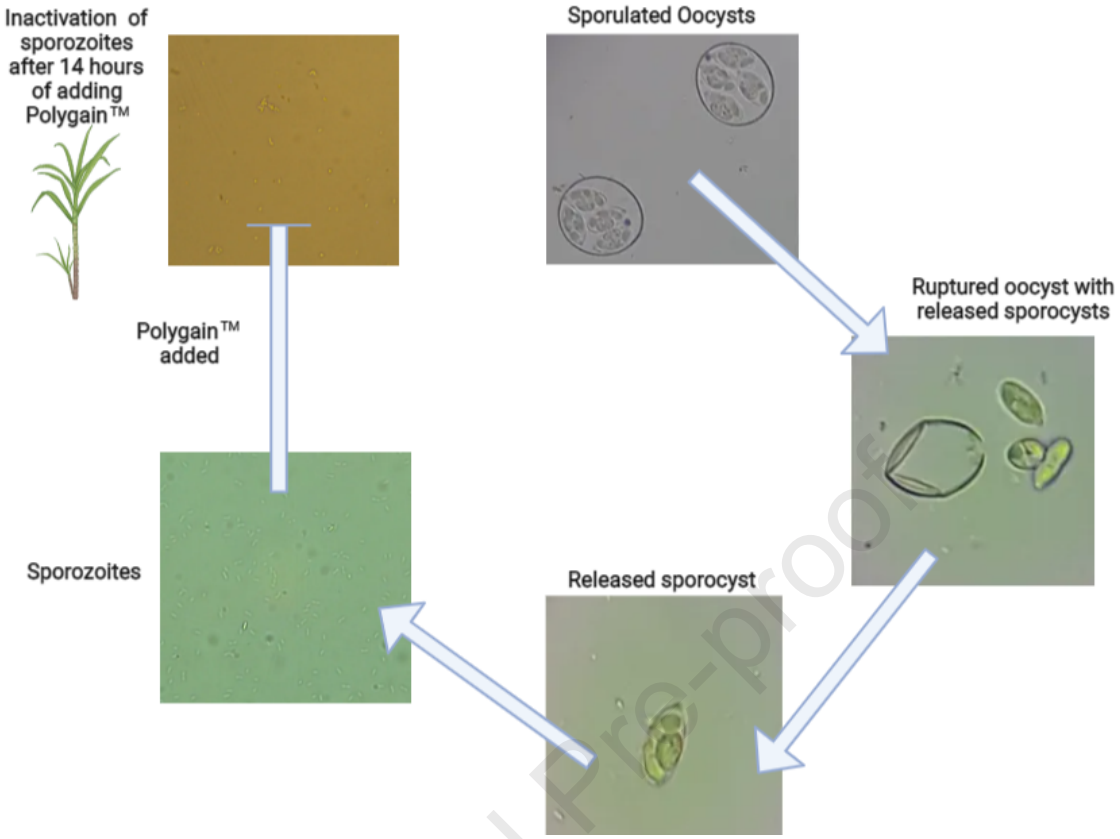

In vitro inhibitory activities of sugarcane extract on avian *Eimeria* sporozoites

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Abstract

The current *in vitro* study aimed to investigate the effects of a processed sugarcane extract on the viability of avian *Eimeria* sporozoites. Treatments were applied to hatched sporozoites: 1) without additives (no-treatment control); 2) with ethanol; 3) with salinomycin; 4) with Polygain™. All treatments were incubated in RPMI media containing live sporozoites at 37°C for 14 hrs and then the number of viable sporozoites were counted. Compared to the no-treatment control, Polygain™ decreased ($P < 0.001$) the counts of *E. maxima*, *E. acervulina*, *E. bruneti*, and *E. mitis* sporozoites to a level similar to salinomycin ($P > 0.05$). In conclusion, Polygain™ could be a potential candidate as an anticoccidial agent.

Keywords: Sugarcane extract, avian coccidiosis, sporozoites, *in vitro* excystation, anticoccidial agents, *Eimeria* species.

Introduction

Coccidiosis is an enteric poultry disease induced by protozoan parasites of the apicomplexan genus *Eimeria* (Chapman, 2014). The disease imposes more than $3 billion in annual losses on the global poultry industry (Dalloul and Lillehoj, 2006). At the beginning of the *Eimeria* life cycle in chickens, sporulated oocysts are ingested from litter, feed, drinker, and then undergo mechanical (gizzard) and biochemical (enzymes) changes passing through the gastrointestinal tract to release sporozoites (Conway et al., 1993). The motile sporozoites can invade the epithelial cells as part of sexual and asexual replication and destroy the mucosal layer and underlying tissues resulting in hemorrhagic lesions and bloody diarrhea (Blake and Tomley, 2014; Chapman, 2014). The lesions can directly decrease nutrient absorption, weight gain, and subsequently feed efficiency (Chapman, 2014), and indirectly perturb the intestinal microbiome and predispose the intestinal environment for the proliferation of pathogenic bacteria such as *Clostridium perfringens* leading to necrotic enteritis (Arakawa et al., 1981). The common methods in preventing and controlling coccidiosis comprise anticoccidial ionophorous antibiotics and vaccination (De Gussem, 2007). The emergence of antibiotic-resistant *Eimeria* (Abbas et al., 2011) and public concerns about antibiotic residues in poultry products led to the poultry producers to use live vaccination against coccidiosis. However, alternative methods are also sought to combat coccidiosis in combination with vaccination. Therefore, various additives such as prebiotics, probiotics, essential oils, and plant extracts have been introduced to the poultry industry in attempts to minimize the negative effects of coccidiosis. Several studies demonstrated that plant extracts have biologically active compounds (natural products) such as flavonoids which can play a prophylactic role as anticoccidial agents and activate the host-immune system to protect the intestinal layers from pathogenic invasion (Abbas et al., 2012; Wunderlich et al., 2014).

Sugarcane (*Saccharum officinarum* L.) is a perennial tropical plant used to produce sugar, wax, and other valuable products (Singh et al., 2015). The ability of a sugarcane extract to inhibit *Eimeria*

species and thus prevent the chickens from coccidiosis has been demonstrated in previous research (El-Abasy et al., 2003; Akhtar et al., 2008; Awais et al., 2011). It has been demonstrated that sugarcane extracts can activate the immune response against *Eimeria* spp., possibly through increasing the antibody production by polysaccharides components of the extract in broilers challenged with coccidiosis (El-Abasy et al., 2003; Akhtar et al., 2008). While previous studies showed the beneficial effects of sugarcane extracts in controlling coccidiosis in broilers, to the best of our knowledge, no reports have examined the inhibitory effects of the extract on *Eimeria* sporozoites of a wide range of species *in vitro*. Therefore, the current study aimed to evaluate the inhibitory effects of processed sugarcane extracts on avian *Eimeria* sporozoites under *in vitro* conditions. The hypothesis was that processed sugarcane extracts could reduce the number of viable sporozoites in the growth medium.

Materials and methods

To determine the bioactive compounds of sugarcane extract, metabolites were extracted from a 30 mg Polygain™ sample in a tube containing 500 µL of MeOH/H_2O/$CHCl_3$ (3:1:1, *v:v:v*). The mixture was homogenized using a MP homogeniser (FastPrep®) (1 min, 4.5 m/s) and vortexed and incubated (70 °C for 15 min) in a thermomixer at 850 rpm. Then, the mixture was centrifuged (Heraeus™ Pico™ 21 Microcentrifuge, Thermo Scientific, MA, USA) at 15700 ×g for 15 min. The supernatant was transferred to a new Eppendorf tube, and 500 µL of MeOH/H_2O/$CHCl_3$ was added into the first lysing tube containing the previously freeze-dried sample. The samples were again vortexed and centrifuged at 15700 ×g for 15 min. The resulting supernatant was then transferred into the tube containing the original supernatant from the previous centrifugation. Pooled samples were then vortexed for 30 s and 20 µL aliquots of supernatant were transferred into separate glass inserts and dried in vacuo for subsequent trimethylsilyl (TMS) polar metabolite derivatisation using GC-MS analysis as previously described by Afshari et al. (2020). One microliter of each derivatized sample

was injected into a GC-MS (Agilent, Santa Clara, USA) using either split (1:20 split ratio) or splitless mode.

Oocysts of *E. acervulina*, *E. maxima*, *E. brunetti*, *E. tenella*, *E. necatrix*, and *E. mitis* used in the current study were provided by Eimeria Pty Ltd (Ringwood, VIC, Australia). The excystation was performed as described by Tomley (1997) with some modifications. A volume of 500 µL of each *Eimeria* sporulated oocysts was pipetted into 2 ml Eppendorf tubes, an equal weight of 0.5 mm glass beads were added to the same tube, and the mixture was vortexed for about 1 min to mechanically discharge sporocysts from oocysts. The released sporocysts were centrifuged (Eppendorf centrifuge 5819R, Hamburg, Germany) twice at 1800 ×g for 10 min in phosphate buffered saline plus 1% glucose at pH 8 (mPBS) to wash the sporocysts. The washed sporocysts were incubated in hatching solution (Hank's Buffered Salt Solution, 1% taurocholic acid, 0.25% trypsin, 1M magnesium chloride solution, and adjusted to pH 8.0) at 41°C for 2 hrs with 100 rpm (Shaker-Incubator, Paton Scientific Pty. Ltd., SA, Australia). Following hatching, the sporozoites were purified using Amicon stirred cell (Merck, Germany) with 5 µm filter membrane (Durapore®, Merck, Ireland). The excysted sporozoites were suspended in mPBS and centrifuged twice at 1800 ×g for 10 min to remove any debris of excystation and also to bring the pH back to around 8 as the media becomes quite acidic during hatching. The cleaned pellet of *Eimeria* sporozoites was suspended in 12 mL RPMI medium (Gibco®, Thermo Fisher Scientific, USA). A total amount of 1980 µL medium containing sporozoites was aliquoted into 2 ml Eppendorf tubes, and then, 20 µL mPBS or respective experimental additives were added to the media and incubated at 37°C for 14 hrs. In the current study, the processed form of the sugarcane extract under the commercial name of Polygain™ was tested. Polygain™ is a commercially available sugarcane extract that is prepared via a patented filtration procedure (Patent number: WO2019213703A1). Treatments were as follows: **1)** No-treatment control; **2)** Ethanol control containing absolute ethanol to kill *Eimeria*; **3)** Salinomycin (60 ppm) as a coccidiostat treatment; **4)** Polygain™ (1%). After incubation for 14 hrs, the sporozoite mixtures were diluted ten

113 times, and a volume of 30 µL were filled in a Fuchs-Rosenthal chamber. The number of alive
114 sporozoites were counted based on the method described by Jaskiewicz et al. (2018) and Yang et al.
115 (2019). In brief, the viability of sporozoites was assessed through the motility of sporozoites under
116 microscope with a ×40 objective lens (Nikon Eclipse Ci-l, Tokyo, Japan). The microscope was
117 equipped with a camera connected to a computer operated by the software NIS-Elements
118 Documentation (Nikon, Tokyo, Japan). Five fields of the chamber were counted and averaged for
119 each sample, and four samples were measured as replicates. The means of the treatment were used
120 for statistical analysis. All data were analysed in a completely randomized design by ANOVA using
121 JMP 14.0 (SAS Institute, USA). Mean values were compared among the treatments with Tukey's test
122 and probability values < 0.05 were considered to be statistically significant.

123 Gas chromatography-mass spectrometry (GC-MS) untargeted profiling revealed a total of 102
124 metabolites in the Polygain® extract (Figure 1); of these, 68 were identified unambiguously and
125 included 14 amino acids, 34 organic acids, 11 sugars, 5 sugar alcohols, one sugar phosphate and three
126 other compounds (Table 1). The most abundant metabolites detected, in splitless mode, were *trans*-
127 4-hydroxycinnamic acid, pyroglutamate and vanillic acid. However, the least abundant metabolites
128 were gluconate, butanoic acid and glycine. Similarly, in split mode, sugars such as fructose, sorbose
129 and glucose were highly abundant in the Polygain® extract.

130 **Results and Discussion**

131 Results showed that no-treatment and ethanol treated controls respectively had the highest and the
132 lowest live counts ($P < 0.001$) of all *Eimeria* sporozoites among treatments (Table 2). Salinomycin
133 significantly reduced the counts compared to no-treatment control but was higher than ethanol control
134 ($P < 0.001$). Polygain™ decreased ($P < 0.001$) the sporozoites of *E. maxima*, *E. acervulina*, *E. brunetti*,
135 and *E. mitis* compared to no-treatment control and to the level no different ($P > 0.05$) to salinomycin.

Adding Polygain™ to the medium of *E. tenella* and *E. necatrix* decreased ($P < 0.001$) the counts of sporozoites compared to no-treatment control but were higher ($P < 0.001$) than salinomycin.

The results of the current study demonstrated that Polygain™ had similar inhibitory effects to salinomycin on the most *Eimeria* sporozoites. In agreement with the current results, Abbas et al. (2015) reported that sugarcane extract destroyed the morphology and shape of oocysts in the medium resulting in lower oocysts sporulation and consequently inactivated the *Eimeria* species. Several studies evaluated the effects of sugarcane extracts against coccidiosis in broilers and related the beneficial effects of this extract to its biologically and immunologically active ingredients like polysaccharides, polyphenols, flavonoids, and phenolic acids (El-Abasy et al., 2003; Akhtar et al., 2008; Awais et al., 2011). *Eimeria* oocysts are protected from environmental conditions by the thick wall layers, while these layers rupture through the process of excystation and the released sporozoites are susceptible to the surrounding biochemical agents (Belli et al., 2006; Mai et al., 2009). The anticoccidial effects of plant extracts have been proven in *Eimeria* species previously. It was demonstrated that polysaccharides, polyphenols, flavonoids and other biologically active natural products present from plants could impair the balance of oxidants and antioxidants on both sides of oocyst membranes, induce oxidative stress, penetrate the oocyst cytoplasm, and interfere with the cell cycle, hindering *Eimeria* replication (El-Abasy et al., 2003; Molan et al., 2009; Molan and Faraj, 2015). Therefore, it could be postulated that Polygain™, having a complex cocktail of bioactive compounds (such as polysaccharides and phenolic compounds) with synergistic biological action, might exert an antioxidant imbalance on the sporozoite membrane, disturb internal hemostasis, and subsequently sporozoites collapsed. The exact mechanism is yet to be elucidated.

Conclusion

Based on the results of the current in vitro study, it can be concluded that the sugarcane extract enriched with various bioactives (Polygain™) inhibited avian *Eimeria* spp. at the stage of sporozoites

and consequently Polygain™ can be a potential alternative for anticoccidial antibiotics. As salinomycin is able to inhibit the *Eimeria* cycle at different stages such as sporozoites, merozoites, and trophozoites, it will be interesting to examine whether Polygain has similar inhibitory capacity to salinomycin at other stages of the *Eimeria* life cycle.

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Declarations of interest

Matthew Flavel and Barry Kitchen are employees of The Product Makers (TPM) Pty. Ltd., Keysborough, VIC, Australia, the manufacture of Polygain™.

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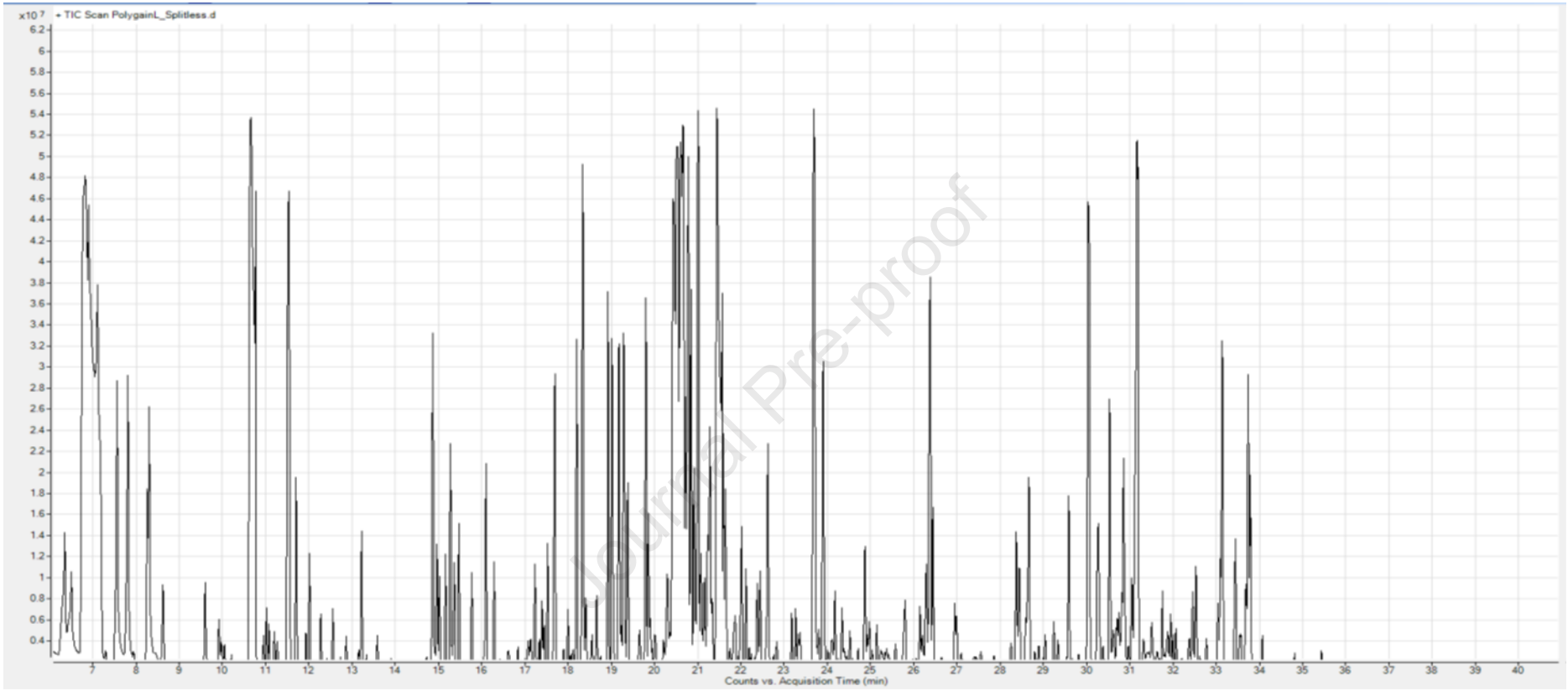

Figure 1. Gas Chromatography-Mass Spectrometry (GC-MS) untargeted profile (Total Ion Chromatogram) of metabolites identified in Polygain™.

Table 1. GC-MS untargeted profile of the Polygain™ extract.

Compound	Response area (%)	EI-MS unique fragment ion (*m/z*)	Retention time (min)
Amino acids			
Valine	0.613	144	9.60
Isoleucine	0.396	158	11.06
Proline	0.196	142	11.19
Serine	0.169	204	12.27
Threonine	0.043	218	12.73
Aspartate	0.542	232	15.15
Pyroglutamate	4.806	156	15.28
Phenylalanine	0.593	192	11.53
Asparagine	0.082	231	17.68
Tyrosine	0.376	218	21.78
Alanine	0.019	190	7.59
Beta alanine	0.008	218	9.29
Glycine	0.006	204	7.94
Homoserine	0.029	218	13.89
Organic acids			
Glycolic acid	0.085	205	7.65
Glyceric acid	0.238	189	11.7
Fumarate	0.442	245	12.17
Pipecolate	0.077	230	12.43
Malate	0.631	245	1.31
Erythronate	0.065	292	15.47
Threonate	0.035	292	15.78
Benzoic acid 4-hydroxy	2.298	282	17.06
Trihydroxypentanoic acid	0.063	245	17.23
Keto-L gluconic acid	0.114	292	19.01
4-hydroxyphenyl propionic acid	0.053	310	19.17
Ribonic acid	0.013	292	19.18
Vanillic acid	3.914	297	19.22
Shikimic acid	1.086	255	19.8
Glucaric acid	0.039	333	19.86
trans- 4-Hydroxycinnamic acid	7.579	293	21.88
Galactonic acid	0.092	319	22.38
Hexadecanoic acid	0.502	328	23.26
Lactic acid*	0.809	191	6.6
3-Hydroxypropanoic acid*	0.275	219	8.17
Succinic acid	0.045	247	11.45
cis-Aconitic acid	0.703	285	18.92
Quinic acid*	1.590	345	20.43
Nicotinic acid	0.054	232	11.26
Malonic acid	0.009	233	9.43
Benzoate	0.754	135	10.37
Itaconic acid	0.046	215	12.01

Salicylate	0.778	267	15.04
2,3-Dihydroxybutanedioic acid	0.024	219	16.43
trans-3-caffeoyl-Quinic acid	0.973	345	34.81
Butanoic acid	0.006	219	13.21
3-hydroxy-3-Methylglutaric acid	0.179	342	16.54
Citric acid	0.042	257	19.91
trans-Ferulic acid	1.338	338	23.92
Sugars			
Trehalose	0.475	191	31.17
Raffinose	0.043	204	36.99
Benzyl glucopyranoside	1.428	217	27.45
Fructose*	21.664	307	20.63
Mannose*	11.062	160	21.02
Maltose*	12.484	204	31.08
Sorbose*	17.191	20.67	20.67
Glucose*	15.555	160	20.96
Gluconate	0.006	292	22.44
Sucrose*	1.80	361	30.02
Cellobiose	0.009	480	30.77
Sugar alcohols			
Ribitol	0.194	319	18.35
Mannitol *	2.567	319	14.06
Arabitol	0.270	307	18.33
Inositol*	0.804	305	23.68
Threitol	0.024	205	14.73
Sugar phosphate			
Glycerol-3P	0.138	205	10.71
Others			
Urea	0.034	189	10.20
Uracil	0.020	241	11.93
Thymine	0.043	270	13.07

UN SUG= unknown sugar; UN= an unknown compound with a specific ion qualifier and a retention time.
*The response area (%) of these metabolites were determined from the Polygain's GC-MS (split) injection due to the high concentration of these metabolites present in the product.

250

Table 2. Effects of processed sugarcane extracts on avian *Eimeria* sporozoites: counts after 14 hours incubation at 37°C in RPMI medium

	Live counts											
	E .maxima		*E. acervulina*		*E. brunetti*		*E. tenella*		*E. necatrix*		*E. mitis*	
Treatments	Ave. No.[1]	IR(%)	Ave. No.	IR(%)	Ave. No.	IR(%)	Ave. No.	IR(%)	Ave. No.	IR(%)	Ave. No.	IR(%)
No-treatment control[2]	68.90[a]	-	52.75[a]	-	64.50[a]	-	69.45[a]	-	60.50[a]	-	86.55[a]	-
Ethanol control[3]	4.40[c]	93.6	2.05[c]	96.1	2.20[c]	96.6	3.40[d]	95.1	1.80[d]	97.0	4.80[c]	94.5
Salinomycin[4]	37.65[b]	45.4	8.65[b]	83.6	11.35[b]	82.4	32.45[c]	53.3	6.45[c]	89.3	17.70[b]	79.6
Polygain[5]	38.15[b]	44.6	9.30[b]	82.4	12.10[b]	81.3	34.75[b]	50.0	7.75[b]	87.2	19.90[b]	77.0
SEM[6]	0.929	-	0.489	-	0.572	-	0.485	-	0.281	-	1.082	-
P-value[7]	<0.0001	-	<0.0001	-	<0.0001	-	<0.0001	-	<0.0001	-	<0.0001	-

1. Ave. No: to count sporozoites (5 fields/sample; 4 samples/treatment), the sample was diluted 10 fold, and then the average number of live sporozoites was calculated. IR%: inhibition rate.
2. containing 1980µl RPMI medium+sporozoites+20µl phosphate buffer saline (PBS)
3. containing 1000µl RPMI medium+sporozoites+1000µl absolute Ethanol
4. containing 1980µl RPMI medium+sporozoites+20µl Salinomycin (60 ppm)
5. containing 1980µl RPMI medium+sporozoites+20µl Polygain™
6. standard error of means
7. [a-d] values within a column with different letters differ significantly ($P < 0.05$).

Conflict of Interest

Matthew Flavel and Barry Kitchen are employees of The Product Makers (TPM) Pty. Ltd., Keysborough, VIC, Australia, the manufacture of Polygain™.