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 h 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. brunetti, 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.

1. 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, 2005). 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.

2. 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/H₂O/CHCl₃ (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 Micro-centrifuge, Thermo Scientific, MA, USA) at 15700 × g for 15 min. The supernatant was transferred to a new Eppendorf tube, and 500 μL of MeOH/H₂O/CHCl₃ 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 h 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 h. 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 h, the sporozoite mixtures were diluted ten times, and a volume of 30 μL were filled in a Fuchs-Rosenthal chamber. The number of alive sporozoites were counted based on the method described by Jaskiewicz et al. (2018) and Yang et al. (2019). In brief, the viability of sporozoites was assessed through the motility of sporozoites under microscope with a ×40 objective lens (Nikon Eclipse Gi-l, Tokyo, Japan). Five fields of the chamber were counted and averaged for each sample, and four samples were measured as replicates. The means of the treatment were used for statistical analysis. All data were analysed in a completely randomized design by ANOVA using JMP 14.0 (SAS Institute, USA). Mean values were compared among the treatments with Tukey’s test and probability values < 0.05 were considered to be statistically significant.

Gas chromatography-mass spectrometry (GC-MS) untargeted profiling revealed a total of 102 metabolites in the Polygain® extract (Fig. 1); of these, 68 were identified unambiguously and included 14...
3. Results and discussion

Results showed that no-treatment and ethanol treated controls respectively had the highest and the lowest live counts (P < 0.001) of all Eimeria sporozoites among treatments (Table 2). Salinomycin significantly reduced the counts compared to no-treatment control but was higher than ethanol control (P < 0.001). Polygain™ decreased (P < 0.001) the counts of sporozoites compared 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; Molan et al., 2009; Molan and Faraj, 2015).

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 anticciodial 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.

### Table 1 (continued)

| Compound                  | Response area (%) | EI-MS unique fragment ion (m/z) | Retention time (min) |
|---------------------------|-------------------|---------------------------------|----------------------|
| 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.

a 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.

amino acids, 34 organic acids, 11 sugars, 5 sugar alcohols, one sugar phosphate and three other compounds (Table 1). The most abundant metabolites detected, in splitless mode, were trans-4-hydroxycinnamic acid, pyrogulmatate and vanillic acid. However, the least abundant metabolites were gluconate, butanoic acid and glycine. Similarly, in split mode, sugars such as fructose, sorbose and glucose were highly abundant in the Polygain® extract.
4. 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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**Declaration of competing interest**

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

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