IN VITRO ANTICOCCIDIAL EFFECTS OF DAFLON® 500 mg ON *Eimeria tenella* OOCYSTS

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ABSTRACT

Avian coccidiosis is an intestinal infection caused by an intracellular parasitic protozoan of the genus Eimeria. Synthetic anticoccidial drugs are generally used to control coccidiosis. However, coccidial resistance has reduced the effectiveness of these agents. To overcome the alarming situation of resistance, integrated approaches such as the use of phytochemical substances with antioxidant activity may be the required practices for the control of coccidiosis in poultry. This study was carried out to examine the in vitro anticoccidial effect of Daflon 500® mg (DF) on Eimeria tenella oocysts. Sporulation inhibition and sporozoites viability assays were employed to evaluate the activity of DF on E. tenella oocysts. The experimental set-up was incubated at room temperature and examined after 48 hours. Daflon® 500 mg exhibited good anti-sporulation and anti-sporozoidal activities against E. tenella oocysts in a concentration dependent manner. There was a significant difference ($p < 0.001$) in the sporulation inhibition activity, with the highest (100.00 ± 0.00) activity recorded at 40 mg/mL and the lowest activity (72.30 ± 0.88) at 2.5 mg/mL concentration of DF after 48 hours of incubation. The highest ($p < 0.001$) viability inhibitory activity of 100.00 ± 0.00 at a concentration of 40 mg/mL of DF against E. tenella sporozoites was also recorded. It is concluded that DF possesses good anti-sporulation and anti-sporozoidal activity against E. tenella.

Keywords: Daflon® 500 mg, Anticoccidial, *Eimeria tenella*, In vitro

INTRODUCTION

Avian coccidiosis is a debilitating disease of poultry that is caused by a protozoan parasite of the genus *Eimeria*, which belongs to the family *Eimeriidae*, and has multiple species with complex life cycle stages (Khater *et al*., 2020). The disease mostly affects bird intestines and causes bloody diarrhoea, poor feed conversion ratios, and high mortality (Chapman, 2014; Abbas *et al*., 2017). The poultry industry spends a huge amount of money in the prevention, management, and treatment of the disease, costing more than £10.4 billion annually (Blake *et al.,* 2021). Seven intracellular protozoan species of the genus *Eimeria* are known as the causative agent of coccidiosis in broilers. Among all the *Eimeria* species, *E. tenella* and *E. necatrix* are the most pathogenic, causing high mortality in chickens. Transmission is usually through the ingestion of faecal droppings from infected broiler chickens which may contain sporulated oocysts of *E. acervulina, E. tenella, E. maxima, E. necatrix, E. brunetti, E. mitis, and E. praecox* (Shetshak *et al.,* 2021). The virulence of *Eimeria* species, high oocyst challenge, insufficient ventilation, high stocking density, immune-compromised status of the animal, bacterial enteritis, high humidity in the litter, and lack of efficacy of anticoccidial medications are some of the factors that aid the development of the disease (Habibi *et al*., 2014). The continuous use of anticoccidial drugs has led to the development of drug-resistant strains. This coupled with the effect of drug residue in meat and toxic effects of disinfectants on birds and poultry farms has led to the search for alternative methods of control of parasitic diseases using plant compounds (Ishaq *et al.*, 2022).

MATERIALS AND METHODS

Source of *Eimeria tenella* Unsporulated Oocyst

Unsporulated oocysts were harvested from the cecal tissue of two sacrificed chickens that were infected with *E. tenella* in the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria Nigeria. They were harvested according to the standard protocol of harvesting oocysts as outlined by the Royal Veterinary College, London (Blake and Tomley, 2014). First, a glass microscope slide was used to carefully scrape the cecal contents, plus the mucosal and deeper muscle layers, away from the remaining tissue. The scrapings were placed in a beaker containing phosphate buffered saline (PBS). It was then homogenised, incubated in a water bath at 41°C for 90 minutes, centrifuged at 2000 rpm, and washed the oocysts in tap water.

In vitro anti-sporulation effect of Daflon® 500 mg

In vitro sporulation inhibition effect of DF was evaluated in petri dishes. Each well contained a total volume of 2 mL of each concentration of DF (2.5, 5, 10, 20, and 40 mg/mL) incubated with equal number of unsporulated oocysts (1 x 10^3) and incubated at 28°C for 48 hrs. For comparison, $K_2Cr_2O_7$ (purchased from a reputable chemical shop in Zaria) was used as the reference. The number of sporulated and unsporulated oocysts were counted in each petri dish and sporulated percentage was estimated by counting the number of sporulated oocyst in a total of 100 oocysts. Potassium dichromate ($K_2Cr_2O_7$) and amprolium (150 g/mL) were used as negative and positive controls, respectively. The sporulation inhibitory percentage was calculated as follows (Cedric *et al*., 2018):

$$\text{Sporulation (SP) inhibition percentage (\%)} = \frac{\text{SP\% of control - SP of DF}}{\text{SP\% of control}} \times 100$$

***In vitro* anti-sporozoidal effect of Daflon® 500 mg**

Stored oocysts in $K_2Cr_2O_7$ were washed several times with Phosphate-buffered saline (PBS) solution (pH 7.4) until the $K_2Cr_2O_7$ was fully eliminated. The oocysts were then incubated in a water bath at 41°C for 60 min and being shaken during incubation. The suspension was centrifuged for 10 min at 3,000 rpm and resuspended in PBS. Liberated sporozoites were washed with PBS. The McMaster counting chamber was used to count the sporozoites. Petri dishes were used to evaluate the in vitro sporicidal activity of DF. Each well contained a total volume of 2 mL of each concentration of DF (2.5, 5, 10, 20 and 40 mg/mL) and incubated with equal number of sporozoites (100 sporozoites). While amprolium (150g/mL) was used as the positive control, $K_2Cr_2O_7$ was used as the negative control. The setup was examined after 48 hrs. The number of viable and non-viable sporozoites were counted and the percentage viability was estimated by counting the number of viable sporozoites in a total of 100 sporozoites. The viability inhibitory percentage was calculated as follows (Cedric *et al*., 2018):

$$\text{Viability (Vi) inhibition percentage (\%)} = \frac{\text{Vi\% of control – Vi\% of DF}}{\text{Vi\% of control}} \times 100$$

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's posthoc test. Values of $P < 0.05$, were considered significant. All the data were analysed using Graphpad Prism, version 8.0 for windows (Graphpad software, San Diego, California, USA).

RESULTS

Anti-Sporulation Effect of Daflon® 500 mg against *Eimeria tenella* Oocysts

Table 1 shows the percentage sporulation inhibition efficacy of Daflon 500 mg. The percentage sporulation inhibition efficacy of DF was concentration dependent. Daflon® 500 mg at the concentration of 40 and 20 mg/mL exhibited the highest sporulation inhibition efficacy of 100 ± 0.00 after 48 h of incubation.

Anti-Sporozoidal Effect of Daflon® 500 mg against *Eimeria tenella* Sporozoites

The antisporozoidal activity of DF against *E. tenella* showed highest viability inhibition of 100.00 ± 0.00 at a concentration of 40 mg/mL. The lowest efficacy was 78.00 ± 0.58 at the concentration of 2.5 mg/mL of DF (Table 2).

Table 1: Anti-sporulation effect of Daflon 500® mg against *Emeria tenella* oocysts

Concentration (mg/mL)	Sporulated oocysts (%)	Unsporulated oocysts (%)
40	0.00 ± 0.00^a	100.00 ± 0.00^a
20	0.00 ± 0.00^a	100.00 ± 0.00^a
10	5.00 ± 0.33^b	94.00 ± 0.00^b
5	13.00 ± 0.58^b	86.00 ± 0.58^b
2.5	26.00 ± 0.88^b	72.30 ± 0.88^b
K2Cr2O7 2 % (-ve control)	100.00 ± 0.00^c	0.00 ± 0.00^c
Amprolium (150 g/mL) (+ve control)	0.00 ± 0.00^a	100.00 ± 0.00^a

Values are represented as mean ± SD of triplicate tests. Means with different superscript letters within the column are statistically significant (a,b,c, $p < 0.001$). Potassium dichromate solution ($K_2Cr_2O_7$) – negative (-ve) control; Amprolium - positive (+ve) control.

Table 2: Anti-Sporozoidal Effect of Daflon® 500 mg against *Eimeria tenella* Sporozoites

Conenetration (mg/mL)	Normal oocysts (%)	Abnormal oocysts (%)
40	0.00 ± 0.00^{a}	100.00 ± 0.00a
20	0.67 ±0.33	99.33 ± 0.33^{a}
10	3.33 ± 0.33^{b}	96.67 ± 0.33^{b}
5	11.00 ± 0.58^{b}	89.00 ± 0.58^{b}
2.5	22.00 ± 0.58^{b}	78.00 ± 0.58^{b}
$K_2Cr_2O_7$ 2% (-ve control)	100.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Amprolium (150 g/mL) (+ve control)	0.00 ± 0.00^{a}	100.00 ± 0.00^{a}

Values are represented as mean ± SD of triplicate tests. Means with different superscript letters within columns are statistically significant (a,b,c, $p < 0.001$). Potassium dichromate solution ($K_2Cr_2O_7$) – negative (-ve) control; Amprolium - positive (+ve) control.

DISCUSSION

In Nigeria as in all developing countries, plants containing bioactive compounds such as flavonoids, alkaloids, tannins and phenolic compounds are regularly employed by farmers to treat recurrent coccidiosis (Mgbojikwe *et al*., 2024). In this study, DF, a flavonoid mixture containing 10% hesperidin and 90% diosmin (Rizk and Sabri, 2009) was evaluated for its anticoccidial activity. Daflon® 500 mg had a concentration dependent effect on sporulation of coccidia oocysts and sporozoites viability. This observation is in agreement with the works of Cedric *et al*. (2017) and Ishaq *et al*. (2022) who reported the anticoccidial effects of the leaf's extracts of *Psidium guajava* and *Citrus aurantium L*., respectively. Plant extracts containing flavonoids and other phytochemicals have been reported to inhibit endogenous enzyme activities (Cedric *et al*., 2017).

It is possible that DF, which is a flavonoid mixture, was able to reduce the proportion of sporulation by inhibiting or inactivating the enzymes responsible for the sporulation process as in helminth eggs (Oh *et al*., 1986). Jones *et al.* (1994) suggested that plant extract may penetrate the cell wall of oocysts and cause a loss of intracellular components. In this present study, DF which contains flavonoids like plant extracts might have penetrated the cell wall of the oocysts and damaged the cytoplasm (sporont) as evidenced by the appearance of abnormal sporocysts in the oocysts. The percentage of cells (sporozoites) viability in this study was comparable with the work of Cedric *et al*. (2017) who used the methanolic, hexane and ethyl acetate leaf extract of *Psidium guajava* as sporozoites inhibitor of different *Eimeria* species *in vitro*. Schubert *et al*. (2005) demonstrated that extracellular calcium and Ca^{2+} signalling is essential for the invasion of *E. tenella* sporozoites into host cells. Extract containing flavonoids have been shown to activate and desensitise receptors in calcium channels (Sarkozi *et al*., 2007). It is possible that DF contributed to the observed inhibition of sporozoite viability by disrupting calcium-mediated signalling in the sporozoites.

CONCLUSION

The result of this *in vitro* study has shown that Daflon® 500 mg possesses anticoccidial activity evidenced by the concentration dependent anti-sporulation and anti-sporozoidal activity observed against *E*. *tenella* oocysts.

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