



***In Vitro* Oocysticidal Effect of *Vernonia amygdalina* Del, Leaf Extract and Fractions on Sporulating *Eimeria tenella* Oocysts**

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Abstract

Poultry not only provides good animal protein but also provides a source of livelihood and economic growth for individuals and society. Poultry production is threatened by coccidiosis caused by *Eimeria* species. The aim of this study was to evaluate *in vitro* oocysticidal activities of *Vernonia amygdalina* (VA) leaf extract and its fractions. Sporulation inhibition bioassay was used to evaluate the activity of the crude methanol extract and its solvent (n-hexane, ethyl acetate and methanol) fractions on sporulation of *Eimeria* oocysts. The extract and fractions were subjected to phytochemical analysis. Oral inoculation of the experimental broiler birds with oocysts was used to determine the specie of *Eimeria* through postmortem examination. VA extract and fractions were administered in concentrations of 0.1; 0.2 and 0.4 mg/ml in a McMaster culture chamber of concentrated sporulating oocysts under microscopic observation. Amprolium (0.1; 0.2 and 0.4 mg/ml), was used as control. Crude extract showed high levels of terpenoids and alkaloids, moderate concentrations of saponins, reducing sugars, total phenolics and steroids; ethyl acetate fraction had high concentration of terpenoids, moderate concentration of total phenolics, reducing sugars, saponins and alkaloids; n-hexane fraction had high concentration of steroids and terpenoids, moderate concentration of tannins, reducing sugar and alkaloids, while methanol fraction showed steroids, total phenolics, reducing sugars and saponins in moderate concentration. Postmortem examination of dead birds showed lesions consistent with *Eimeria tenella* infection. Oocysticidal activity was evidenced by lack of sporulation of oocysts when fractions were mixed with oocysts in the McMaster chamber slide. The highest oocysticidal activity (62.67 %) was recorded with ethyl acetate fraction. These results provide evidence for the use of VA as a preventive agent against coccidiosis, and as feed additive in the control of coccidiosis

Keywords: Anticoccidial, *Vernonia amygdalina*, oocysticidal amprolium, *Eimeria tenella* oocysts.

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Introduction

Avian coccidiosis is an important enteric parasitic disease of poultry associated with significant economic losses to poultry farmers (Zahraddeen *et al.*, 2010). The parasites multiply in the intestine resulting in tissue damage, lowered feed intake, poor absorption of nutrients from the feed, dehydration, blood loss, and death (Nematollahi *et al.*, 2009; Ola-Fadunsin, *et al.*, 2013). In Nigeria like in most developing nations, chickens are the most important class of the poultry species in terms of number and investment in poultry production (Zahraddeen *et al.*, 2010). Poultry coccidiosis, caused by the protozoan parasite of the genus *Eimeria tenella*, remains one of the most important parasitic diseases in poultry industry (Nematollahi *et al.*, 2009). Infection with this parasite normally occurs through ingestion of feed or water contaminated with sporulated oocysts. Fresh oocysts are not infective until they sporulate under optimal conditions [21°–32°C] with adequate moisture and oxygen in 1–2 days (Allen and Fetterer, 2002).

Current approaches to constrain avian coccidiosis include anticoccidial chemicals, vaccines, and natural products. Anticoccidial chemicals, coccidiocides, coccidiostats, and ionophores, have long been used as a mainstream strategy to control avian coccidiosis in modern poultry production (Chapman *et al.*, 2000). Indiscriminate use of anticoccidial drugs in feed and water has led to serious drug resistance problems (Usman *et al.*, 2011; Abbas *et al.*, 2011),

The presence of drug resistance and public demands for residue free meat have encouraged the development of alternative control strategies. Natural products are emerging as alternatives to combat coccidiosis. In contrast to anticoccidial chemicals and vaccines, the use of medicinal plants and phytochemicals as natural remedies for avian coccidiosis has become an alternative strategy that is easily embraced by eco- and health-conscious consumers. Plants produce a broad spectrum of phytochemicals such as phenolics, flavonoids, polyacetylenes, alkaloids, polysaccharides, terpenoids, and essential oils which are known to have antioxidant and anti-inflammatory activities (Majaro *et al.*, 2001; Bera *et al.*, 2010; Jadhav *et al.*, 2011). Plant compounds target different stages of the life cycle of *Eimeria tenella* species (Hadipour *et al.*, 2011).

Vernonia amygdalina Del, belongs to the Asteraceae family commonly called bitter leaf, is variously known as, 'Ewuro' (Yoruba), 'Etidot' (Ibibio), 'Onugbu' (Igbo), 'Itiyuna' (Tiv), 'Oriwo' (Edo) and 'Chusar-doki' (Hausa) tribes of Nigeria (Egedigwe, 2010). VA is probably the most used medicinal plant in the genus *Vernonia* (Erasto *et al.*, 2006). The

observation that an apparently sick wild chimpanzee chewed *V. amygdalina* and seemed to return to normal activity after a while (Huffman and Seifu 1989, Ohigashi *et al.*, 1991) elicited the attention of the phytomedicine community to investigate the efficacy of different extracts of the plant in managing a wide array of medical ailments. Igile *et al.*, (1994) reported that in traditional medicine, practitioners use the plant as an anti-helminth, anti-malarial, and as a laxative. Others use it as a digestive tonic, appetizer, febrifuge and for the topical treatment of wounds (Iwu, 1986). Dalziel (1937) reported that the root and twig of the plant are used for the treatment of stomach and gastro-intestinal problems by the Hausas of Northern Nigeria, while the decoction of the leaves is used in treating malaria fever in Guinea, and cough in Ghana. In some parts of Nigeria, the stems are used as chewing sticks for oral hygiene, and for the management of some dental problems. In Malawi and Uganda, VA is used by traditional birth attendants to aid the expulsion of the placenta, after birth, aid post-partum uterine contraction, induce lactation and control post-partum hemorrhage (Bullough and Leary, 1982; Kamatenesi-Mugisha, 2004). The blood sugar lowering effect of the aqueous extract of the leaves of VA was reported by Akah and Okafor (1992). VA has been reported to have a myriad of anti-infective activities such as antibacterial (Akinpelu 1999, Ijeh *et al.*, 1996 Jisaka *et al.*, 1993), antiplasmodial (Njan *et al.*, 2008, Abosi and Raseroka 2003); amoebicidal (Moundipa *et al.*, 2005; Huffman *et al.*, 1996); antifungal (Alabi *et al.*, 2005; Erasto *et al.*, 2006; Wedge *et al.*, 2000), and antileishmanial (Tadaesse *et al.*, 1993; Huffman *et al.*, 1996). This study was to evaluate *in vitro* anticoccidial (oocysticidal) activities of different fractions of *Vernonia amygdalina* leaf extracts and fractions.

Materials and Methods

Collection and preparation of plant materials

Fresh leaves of *Vernonia amygdalina* leaves were collected from a farmland at Obechera, Nsukka, Enugu State, Nigeria, in October 2017, followed by identification at the taxonomy unit of International Center for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Nigeria by Mr Osmond Okeke. The leaves were rinsed with clean water, dried at room temperature for two weeks and then pulverize into coarse particles using electric blender. The resulting coarse particles were stored in polyethylene bag pending extraction and fractionation.

Extraction and fractionation of the plant material

The pulverized leaves (400 g) were macerated in 1000 ml of methanol for 48 hours and filtered with Whatman filter No 1. The resulting filtrate was concentrated using rotary evaporator.

The concentrated crude extract was successively eluted with n-hexane, ethyl acetate and methanol using column chromatography technique to obtain n-hexane, ethyl acetate and methanol fractions respectively. The crude extract and the concentrated fractions were stored in sealed containers at -4°C in a refrigerator before commencement of the experiment.

Phytochemical analysis

The methods of Harbone (1973) and Trease and Evans (1989) were used to qualitatively and quantitatively determine the presence of the secondary metabolites in the crude extract and fractions.

Inoculation of coccidian oocysts

Twenty (20) broiler birds (2-3weeks old) were each inoculated orally with 1ml of Eimeria solution containing about 2000 oocysts. The birds were fed with standard starter feed, given adequate water with good light supply. They were observed for 7 days after inoculation to determine the species of Eimeria.

Collection and Preparation of Coccidia Oocysts.

Fresh fecal samples from chickens showing signs of clinical coccidiosis were collected and homogenized in 100 ml of water. The homogenized suspension was filtered using a coffee sieve and 10 ml of the filtrate was centrifuged at 4000 g for 10 minutes. The supernatant was carefully removed while the sediment was re-suspended in saturated salt solution and centrifuged at 1500 g for 20 minutes. The supernatant was carefully collected into a beaker. A drop of the supernatant was placed on a clean microscope slide, covered with a cover slip and viewed under x40 objective lens for the presence of oocysts. On confirmation of the presence of oocysts, the supernatant was diluted five times with water and vigorously shaken and centrifuged at 4000 g for 10 minutes following which the supernatant was discarded while the sediment containing oocysts was transferred to another beaker. After concentrating on the oocysts, they were sporulated in 2.5% potassium dichromate and kept at room temperature for use not later than 2 weeks to make sure that the oocysts maintain viability. Sporulation of the oocysts was performed according to the descriptions of Levine, (1973). VA extract and fractions were administered in

concentrations of 0.1; 0.2 and 0.4 mg/ml in a McMaster culture chamber of concentrated sporulating oocysts under microscopic observation. Amprolium (0.1; 0.2 and 0.4 mg/ml), was used as control.

Quantification of the sporulated Oocysts

The sporulated oocysts were quantified using the McMaster method (Long et al., 1976). Briefly, 1 ml of the oocyst sample was aspirated into a test tube and was diluted with 9 ml of saturated sodium chloride resulting in 1:10 dilution. The diluted sample (1 ml) was collected and used to fill both chambers of the McMaster slide. All the oocysts in both chambers were enumerated and their average number/mean calculated.

The oocyst per ml was obtained using the formular below:

$$P \times D \times K$$

Where P = the average/mean oocyst count from both chambers of the McMaster slide

D = Dilution factor (10)

K = Constant (6.67)

Statistical analysis

The data obtained were analyzed using Two-Way analysis of variance (ANOVA) and presented as mean $\pm$ standard deviation (SD). The Posthoc tests were determined with the aid of computer based GraphPad Prism version 5.0, and values were considered significant at $P < 0.05$.

Results

Phytochemical Analysis

Crude extract showed high concentrations of terpenoids and alkaloids, whereas steroids, total phenolics, reducing sugars and saponins were in moderate concentrations, flavonoids, glycosides and tannins were in low concentration. Ethyl acetate fraction had high concentration of terpenoids while reducing sugars, saponins, alkaloids and total phenolics were in moderate concentration. n- Hexane fraction had high concentrations of steroids and terpenoids whereas alkaloids, reducing sugars and tannins were moderate. Methanol fraction had moderate concentrations of reducing sugars, saponins, total phenolics and steroids whereas tannins, glycosides, alkaloids, flavonoids and terpenoids were in low concentrations (Table 1)

Table 1. Qualitative phytoconstituents

Qualitative Phytochemical	Methanol crude extract <i>V.</i> <i>amygdalina</i>	Methanol fraction <i>V. amygdalina</i>	Ethyl acetate fraction <i>V.</i> <i>amygdalina</i>	n-hexane fraction <i>V.</i> <i>amygdalina</i>
Tannins	+	+	+	++
Steroids	++	++	+	+++
Terpenoids	+++	+	+++	+++
Total phenolics	++	++	++	+
Flavonoids	+	+	+	+
Glycosides	+	+	+	+
Reducing sugar	++	++	++	++
Saponin	++	++	++	ND
Alkaloids	+++	+	++	++

Key

+	=	Present in low quantity
++	=	Present in moderate quantity
+++	=	Present in high quantity
ND	=	Not detected

Gross observation after inoculation

The birds were observed continuously for seven days after inoculation and the features displayed are presented in Table 2

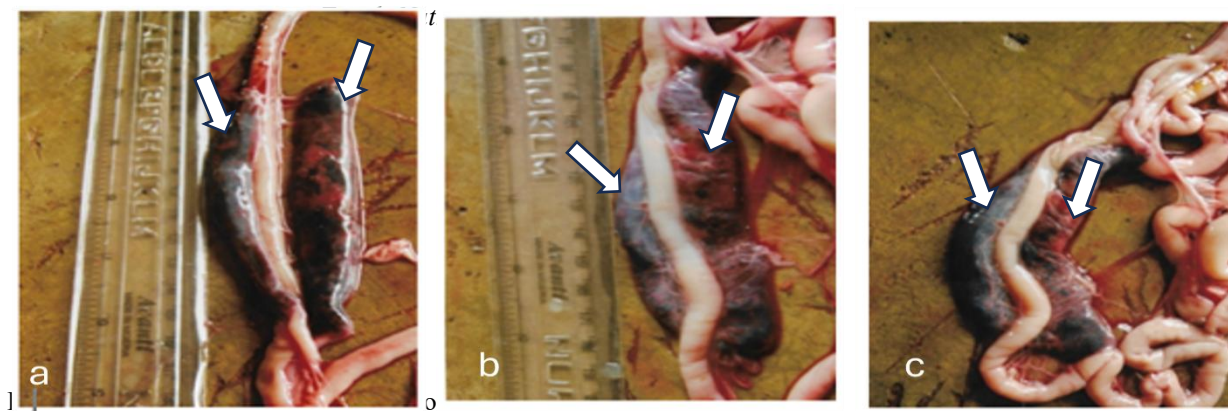
Table 2: Observations after inoculation

Days of Inoculation	Observation
1	No marked physical signs.
2	Drooping of feathers, reduction in physical activity.
3	Blood stained droppings, drooping of feathers, reduction in food intake, reduction in water intake, diarrhea.
4	2 birds died, most features of day 3 present.
5	Blood stained diarrhea droppings, drooping feathers
6	1 bird died [2 weeks] remaining birds still showed signs of drooping of feathers and reduced physical activity].
7	Bloody diarrheal dropping still present.

Postmortem Results

The postmortem findings were consistent with lesions caused by *Eimeria tenella* infection. The caecum was affected. (Figure 1). The arrows indicate the caecal lesions, while the other intestinal segments remain

free of lesions, which is characteristic of *Eimeria tenella* infection. Figures 1 a, b and c represent the same image taken from different views



Effect of the extract and fractions on oocysts

Sporulation was seen as clearly separated entities in the oocysts while unsporulation was seen as round undivided mass of cytoplasm in the oocyst. The sporulated and unsporulated oocysts were counted. Blue arrows depicted unsporulated oocysts. White arrows depicted sporulated oocysts. Freshly voided sporulating/sporulated oocysts were depicted in Figure 2. Figure 3 showed sporulating/sporulated oocysts in the presence of amprolium an anticoccidial agent. Figure 4 showed oocysts of *Emieria tenella* cultured with methanol crude fraction of *Vernonia amygdalina* with signs of inhibition of sporulation or unsporulation

as depicted by the blue arrows while the white arrows depict the sporulated oocysts. Figure 5 showed oocysts of *Emieria tenella* cultured with n-Hexane fraction with signs of sporulation and unsporulation as depicted by the blue arrows and the white arrows while figure 6 showed oocysts of *Emieria tenella* cultured with Ethyl acetate fraction with marked signs of inhibition of sporulation or unsporulation as depict by the blue arrows. The highest oocysticidal activity was observed with ethyl acetate fraction in a dose dependent manner with the highest (62.67 ± 2.5 %).oocysticidal activity at the highest concentration. This was by the methanol fraction whereas the crude extract and the n-hexane fraction showed mild oocysticidal activity

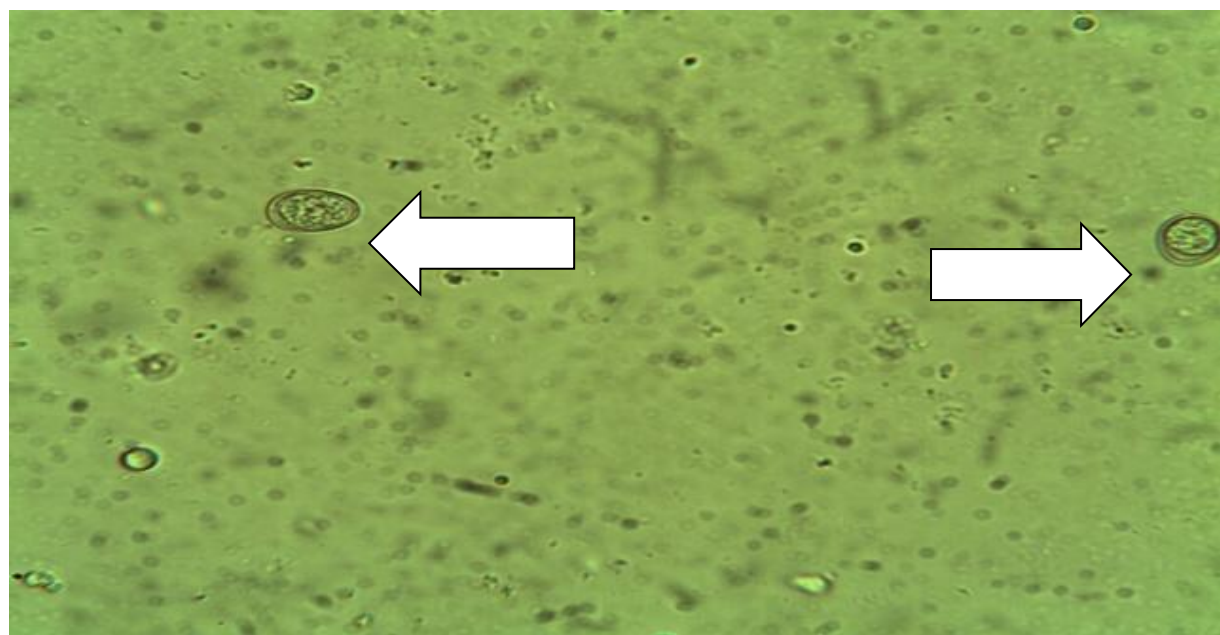


Figure 2: Freshly voided oocysts of *Eimeria tenella*

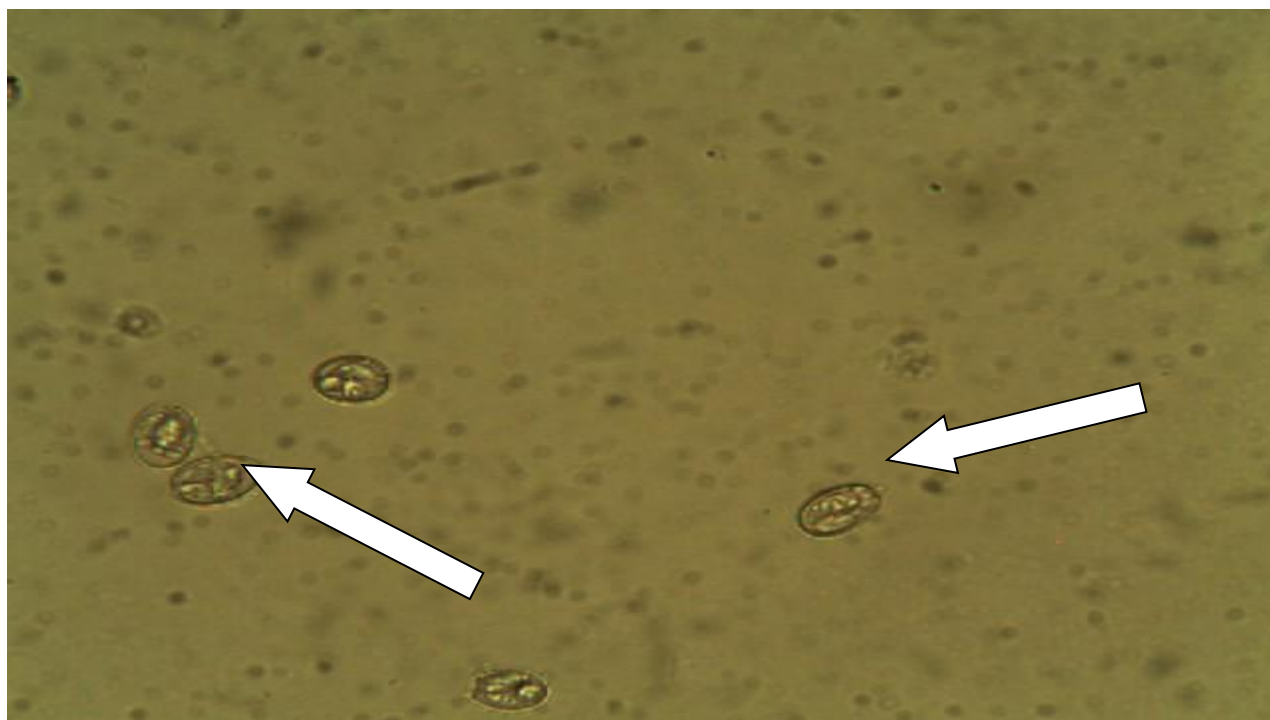


Figure 3: Slide representation of oocysts of *Eimeria tenella* cultured with Amprolium control

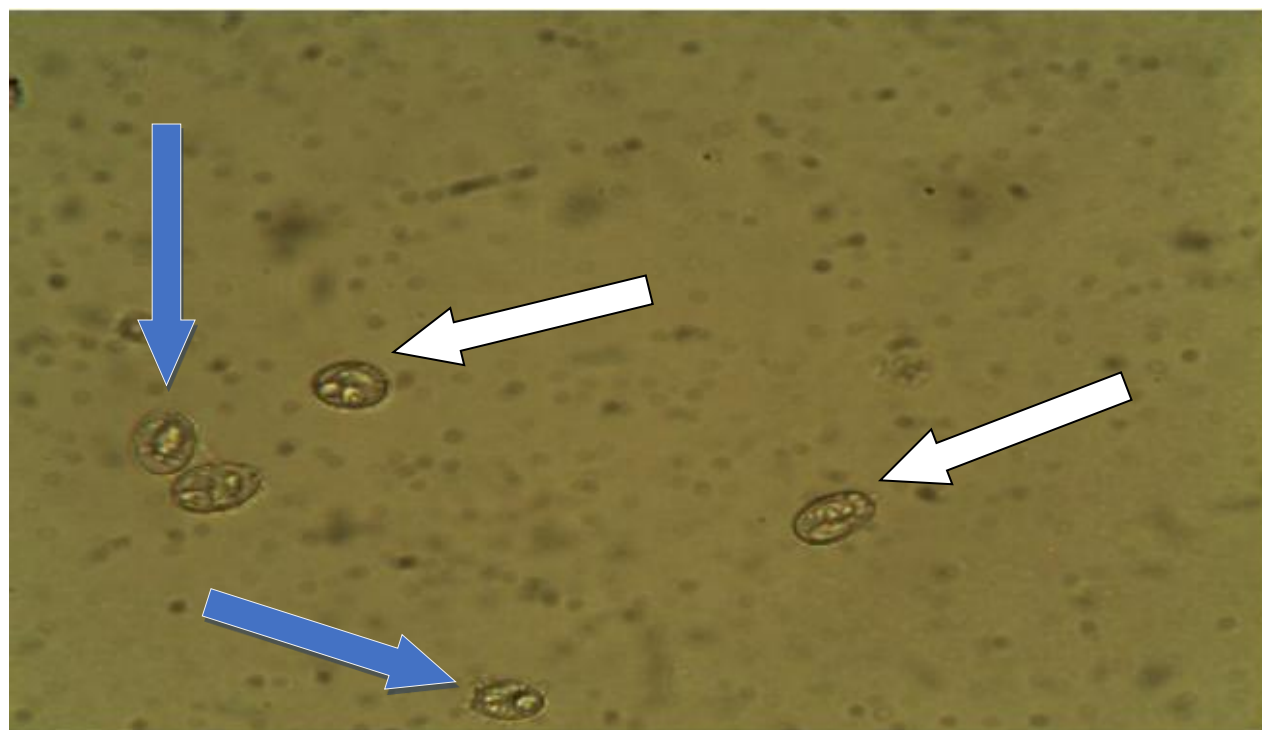


Figure 4: Slide representation of oocysts of *Eimeria tenella* cultured with methanol crude extract of *Vernonia amygdalina*

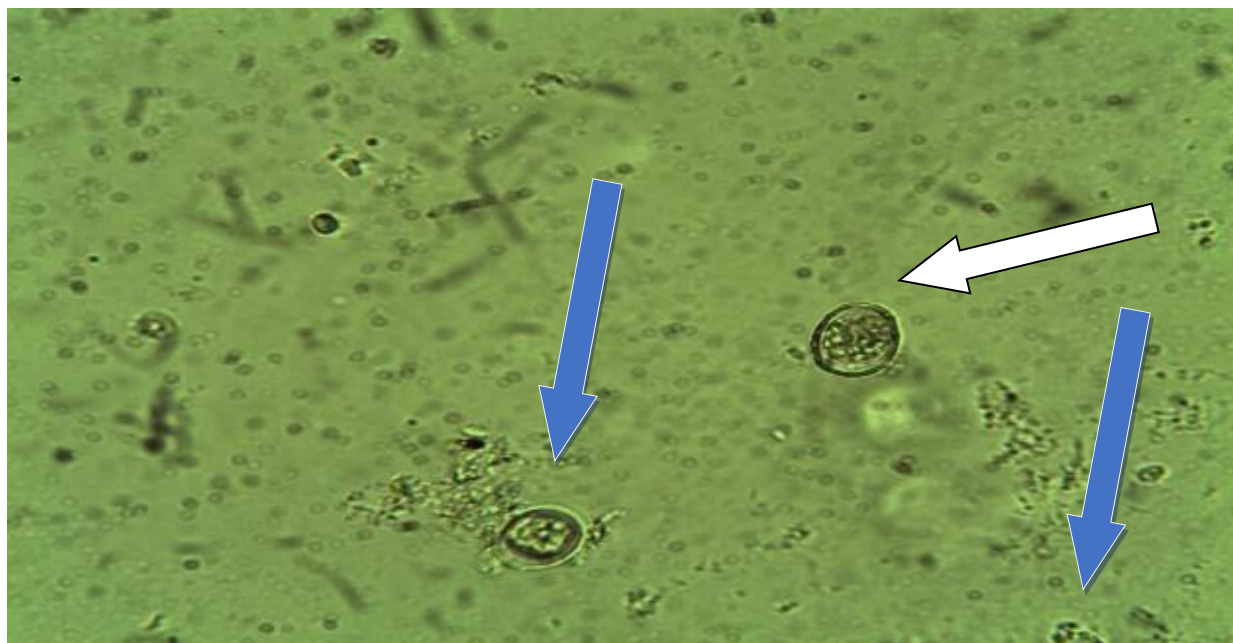


Figure 5: Slide representation of oocysts of *Eimeria tenella* cultured with n-Hexane fraction of *Vernonia amygdalina*

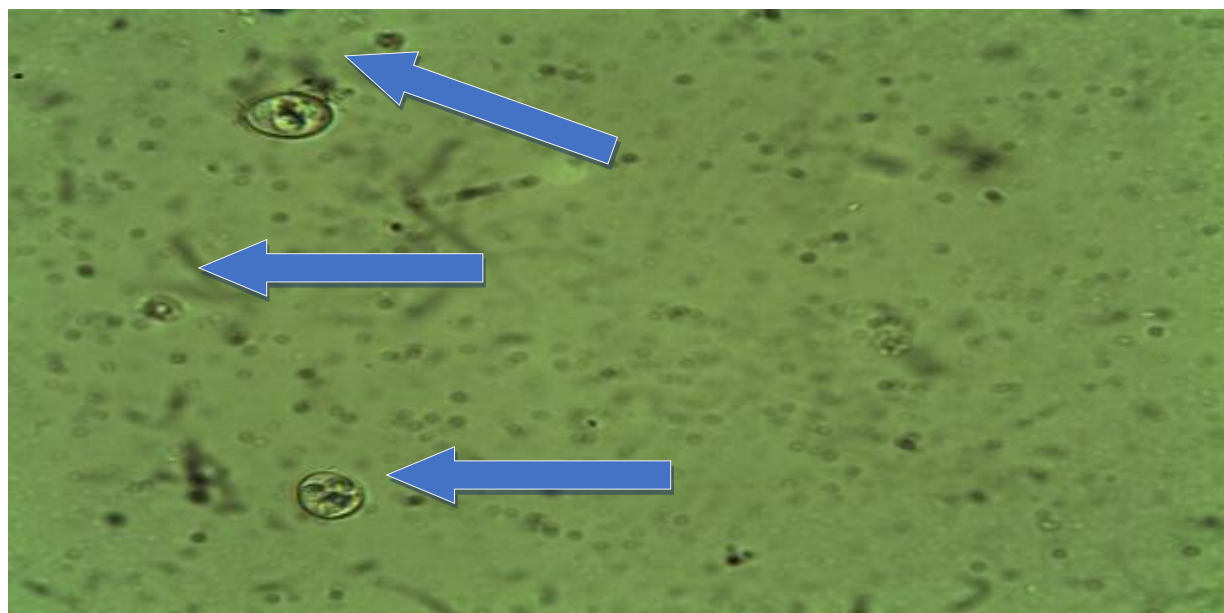


Figure 6: Slide representation of oocysts of *Eimeria tenella* cultured with Ethyl acetate fraction of *Vernonia amygdalina*

Effect of the methanol crude extract on sporulating Eimeria tenella.

There was no significant $P > 0.05$ difference between the sporulated and unsporulated *Eimeria tenella* cultured in crude methanol extract of VA when compared with the control (Figure 7).

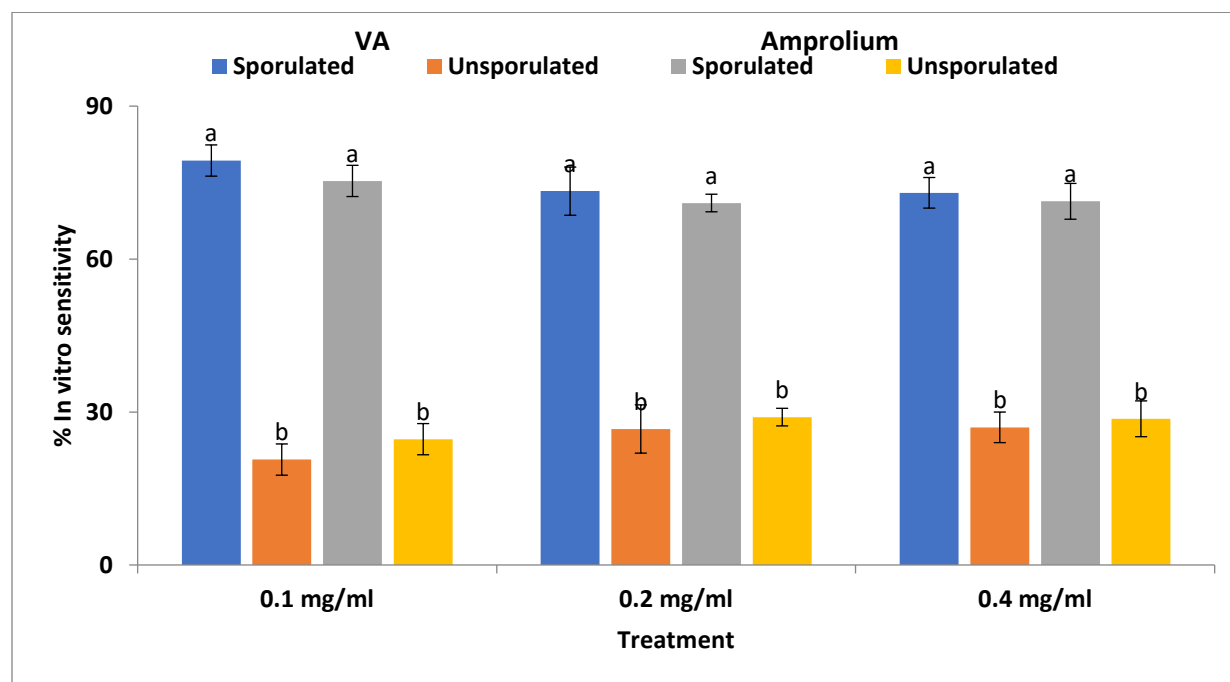


Figure 7: Sensitivity of *Eimeria tenella* cultured in *Vernonia amygdalina* methanol leaf crude extract and Amprolium. Bars bearing different alphabet letters per Extract/Amprolium dose group are statistically significant ($p < 0.05$). $n=5$

Effect of ethyl acetate fraction on sporulated Eimeria tenella

There was a significant ($P < 0.05$) increase in the unsporulated to the sporulated in a dose dependent manner for *Eimeria tenella* species cultured in the ethyl acetate fraction of VA when compared with the standard drug as shown in Fig 8

Effect of methanol fraction on sporulated Eimeria tenella specie

Figure 9 showed a significant ($P < 0.05$) increase in the unsporulated to the sporulated *Eimeria tenella* species cultured in methanol fraction of VA in a dose dependent manner when compared with the standard drug.

Effect of n-hexane fraction on sporulated Eimeriatenella specie

There was no significant $P > 0.05$ difference between the sporulated and the unsporulated of the *Eimeria tenella* species cultured in n-hexane fraction of VA for the doses when compared with the control.

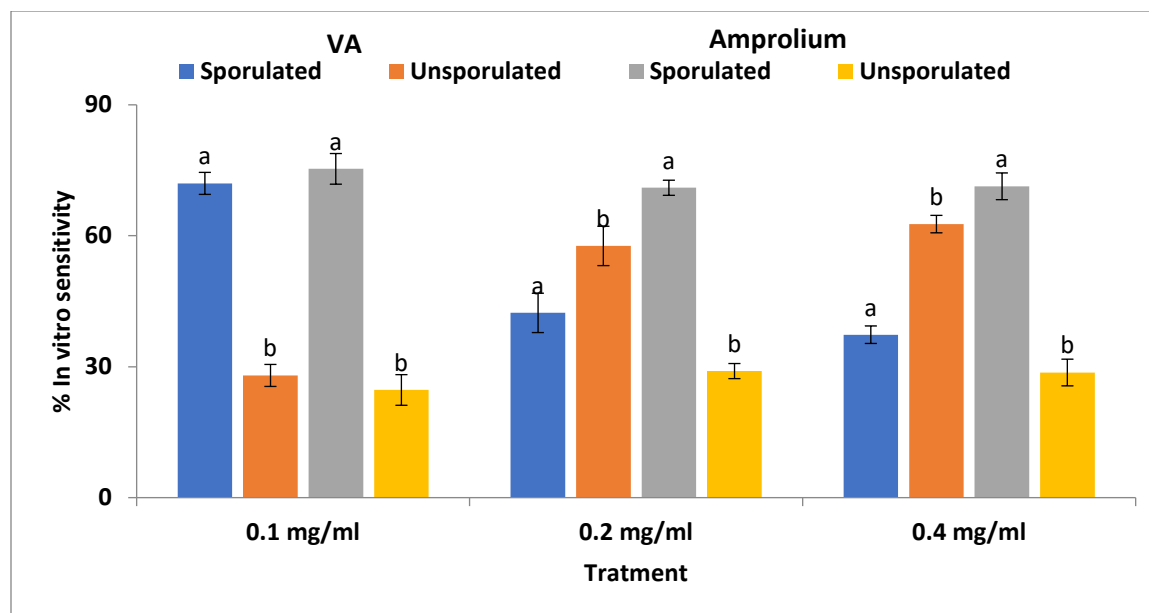


Figure 8: Sensitivity of *Eimeria tenella* cultured in *Vernonia amygdalina* ethyl acetate fraction. Bars bearing different alphabet letters per Extract/Amprolium dose group are statistically significant ($P < 0.05$). n=5

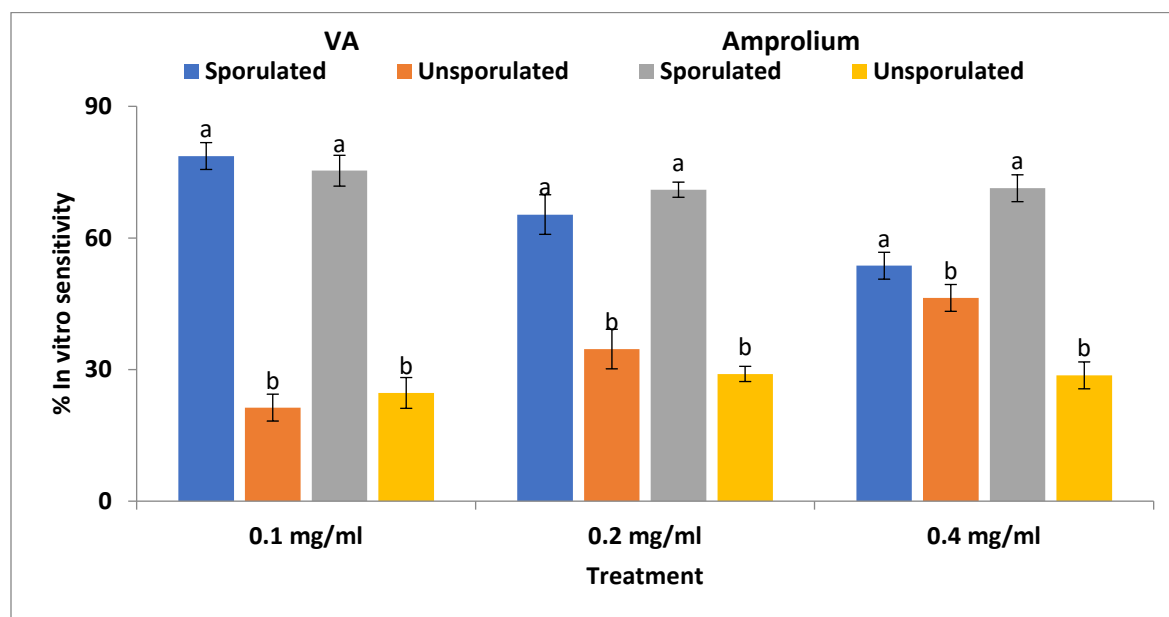


Figure 9: Sensitivity of *Eimeria tenella* cultured in *Vernonia amagdalina* methanol fraction of leaf extract and Amprolium. Bars bearing different alphabet letters per Extract/Amprolium dose group are statistically significant ($P < 0.05$). n=5

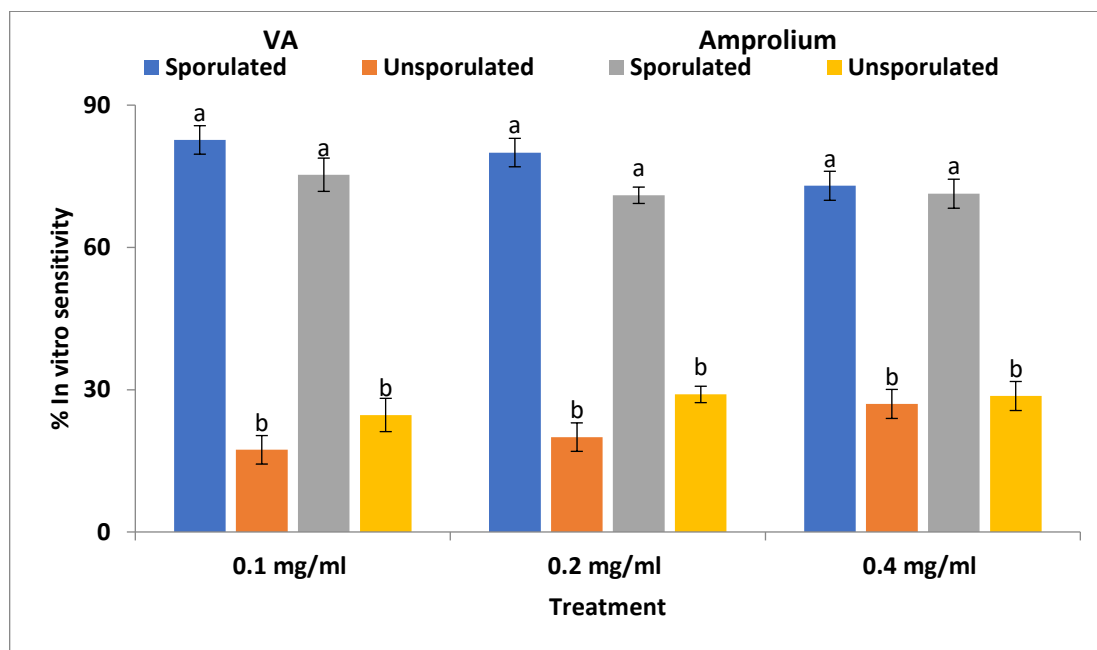


Figure 10: Sensitivity of *Eimeria tenella* cultured in *Vernonia amygdalina* n-hexane leaf extract. Bars bearing different alphabet letters per Extract/Amprolium dose group are statistically significant ($P < 0.05$) $n=5$

Discussion

Coccidiosis is a widespread disease affecting poultry with huge economic loss. The causative agents are *Eimeria* spp., protozoan parasites characterized by an intricate life cycle (Felici *et al.*, 2021). Oocysticidal activity is measured by the number of unsporulated oocysts against sporulated oocysts for various concentrations of agents. High numbers of unsporulated oocysts suggested oocysticidal activity while high numbers of sporulated oocysts signified lack of oocysticidal activity.

Vernonia amygdalina widely studied for medicinal uses (Akah and Okafor, 1992; Akah *et al.* 2009), was evaluated for oocysticidal activity on sporulating oocysts of *Eimeria tenella*. The significant oocysticidal activity of the ethyl acetate fraction could be related to the presence of highly bioactive phytoconstituents, notably terpenoids, total phenolics, saponins, alkaloids and reduced amounts of tannins and flavonoids. Terpenoids have been reported to exhibit inhibitory properties against oocysts sporulation and increase the proportion of degenerated oocysts (Hu *et al.*, 2018, 2011; Wu *et al.*, 2010; Zhang *et al.*, 2010; Yong *et al.*, 2020). Alkaloids and phenolics have also been reported to exhibit

antioxidant activity (Dakpogan *et al.*, 2019). Saponins binds to the 4-sterol molecules on *Eimeria* cell membrane, disturbing the lipids in the parasite cell membrane, therefore affecting enzymatic activity and metabolism leading to cell death, it also enhances the nonspecific immunity (Wang *et al.*, 1998; Cheeke, 2000). Tannins penetrate the coccidia's oocyst wall and destroy the cytoplasm, probably inactivating the endogenous enzymes responsible for the cycle of sporulation in poultry (Kheirabadi *et al.*, 2014; Muthamilselvan *et al.*, 2016; Kaingu *et al.*, 2017; Isah *et al.*, 2019; Desalegn and Ahmed 2020). Flavonoids have been reported to have antioxidant capacity due to their redox activities (Isah *et al.*, 2019). Steroids are also known to suppress oocyst sporulation (Ogbe *et al.*, 2010; Ahad *et al.*, 2016). With such an array of phytoconstituents oocysticidal activity is plausible. This study is in agreement with other *in vitro* assays showing that different plant extracts/fractions inhibit the sporulation of oocysts of different *Eimeria* species of poultry (Oviedo-Rondón *et al.*, 2005; Rajaei *et al.*, 2017; Gadelhaq *et al.*, 2018;).

In conclusion the use of plant products being advocated because of the harmful effects of synthetic agents as additives to feed or chemotherapeutic

agents. Natural products like *Vernonia amygdalina* which have been widely studied is strongly advocated for use as feed additive for poultry and other animals prone to coccidiosis.

Conflict of Interest

None

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