

Invitro Anticoccidial Effect of Fractions of Chromolaena Odorata Extract on Sporulating Eimeria Tenella Oocysts

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Abstracts: Coccidiosis is an important cause of digestive system disorders in birds, especially the poultry. The aim of this study was to evaluate the in-vitro anticoccidial effects of the different fractions of Chromolaena odorata extracts on sporulated Eimeria tenella oocysts. Sporulation inhibition bioassay was used to evaluate the activity of crude methanol fraction of Chromolaena odorata extracts; n-hexane, ethyl acetate, and methanol fractions of Chromolaena odorata on sporulation of Eimeria tenella, which was used to cause coccidiosis in the birds following oral inoculation of experimental broiler birds with oocysts. Post-mortem examination of dead birds showed lesions consistent with Eimeria tenella. The fractions of the Chromolaena odorata were examined for the oocysticidal activities. Amprolium an anticoccidial drug was used as control. The fractions were used in 4 doses: 0 mg/ml; 0.1 mg/ml; 0.2 mg/ml and 0.4 mg/ml. The control drug was used in corresponding doses. The highest oocysticidal activity of tested plant extracts and fractions was recorded. The n-hexane fraction of Chromolaena odorata gave the highest oocysticidal activity. This oocysticidal activity was noticed at lower concentrations but activity was found to be dose-dependent. The highest oocysticidal activity for the n-hexane fraction of Chromolaena odorata was at 65.67±2.08%. Amprolium showed no oocysticidal activity at all concentrations. However, other fractions of the Chromolaena odorata showed different levels of oocysticidal activities. The methanol fractions were secondary to the n-hexane while the ethyl acetate was the least. In conclusion, this study provides the basis for using Chromolaena odorata, especially the n-hexane fraction as preventive agents against coccidiosis and suggests their use as a feed additive in the control of coccidiosis.

Keywords: Chromolaena Odorata, Anticoccidial, Oocysticidal.

1. INTRODUCTION

Poultry production has a significant impact on the socioeconomic development of most countries of the world [1]. It is one of the major livestock businesses in Nigeria and has developed to the level of commercial enterprises. It provides employment opportunities and a source of income to the populace and remains one of the major sources of animal protein for urban and rural dwellers as well as manure for cultivation [2].

Avian coccidiosis is the most common disease of poultry in the globe [3]. It is present where chickens are reared, both in small and large-scale farms. Poultry coccidiosis, caused by *Eimeria tenella*, remains one of the most common parasitic diseases in the poultry industry [4,5]. Coccidiosis due to *Eimeria tenella* species often presents with symptoms such as enteritis, diarrhea, weight loss, indigestion, poor sexual maturation, drooping wings, stunted growth, and low production [6,7] with ultimate high mortality and morbidity rates [8]. The mortality rates are often higher in young chicks because the *Eimeria* species affect birds between the ages of 3 and 18 weeks [4]. The combination of different species of *Eimeria tenella* and the severity of the symptoms vary considerably [9,10]. A high incidence of coccidiosis is often predominant in poultries under intensive management systems such as deep litter because of the increased chances of high oocysts accumulation in the litter [11,12]. Again, increased stocking densities have been associated with increased incidence of coccidiosis because of the high rate of infection and transmission of the coccidian oocysts in dense flocks from one poultry house to another [13].

Indiscriminate use of anticoccidial agents has accounted for most of the drug resistance [14]. It has been shown that dietary direct feed microbial (DFM) supplementation has helped in the restoration of broiler performance during the starter and early grower periods during coccidiosis [15]. This is by maintaining gut integrity through improving intestinal morphology and reducing disease severity.

Chromolaena odorata [family: Asteraceae] is a flowering shrub in the sunflower family found in both tropical and subtropical areas. Its local name is iruraazhi. It is said to be a very useful plant and an excellent fallow crop in

arable farming [16]. Smith and Alli, noted that using the plant as a mulch is effective against weed invasion [17]. The essential oils that make the plant repugnant to livestock have also been found to have insect-repelling properties [18]. The therapeutic properties of the plant were attested to by some natives interviewed at Idoani in Ondo State of Nigeria. They also stated that the plant is very potent in the management of enteritis and malaria [18]. Apart from its beneficial effects, it is reported to be toxic to cattle contains carcinogenic pyrrolizidine alkaloids [19], and also causes allergic reactions. It has been reported to have larvicidal effects against all major mosquito vectors [20]. This study was aimed at evaluating the invitro-anticoccidial effect of fractions of *Chromolaena odorata* extract on sporulated *Eimeria tenella* oocysts

2. MATERIALS AND METHODS

2.1. Collection and Preparation of Plant Materials

Fresh leaves of *Chromolaena odorata* were collected from farmland at Obechera, Nsukka, Enugu State of Nigeria. The plant was identified at the taxonomy unit of the International Center for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Nigeria. The leaves were rinsed with clean water, dried at room temperature for two weeks, and then pulverized into coarse particles using an electric blender. The resulting coarse particles were stored in a polyethylene bag pending extraction and fractionation.

The pulverized leaves of *Chromolaena odorata* (400 g) were macerated in 1000 ml of methanol for 48 hrs, and filtered with Whatman filter paper. The resulting filtrate was concentrated using a rotary evaporator.

The concentrated crude extract of *Chromolaena odorata* was successively eluted with n-hexane, ethyl acetate, and methanol to obtain n-hexane, ethyl acetate, and methanol fractions respectively using column chromatography technique. Some portion of the crude extract and the concentrated fractions were stored in sealed containers at – 40C in a refrigerator before the commencement of the experiment.

2.2. Collection and Preparation of Coccidia Oocysts.

Fresh faecal samples of chickens showing signs of clinical coccidiosis were collected into faecal sample containers and submitted to the Department of Veterinary Parasitology Laboratory, the University of Nigeria Nsukka. A representative sample of the faecal samples was poured into a beaker 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 thereafter 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 the x40 objective lens of the microscope for the presence of oocysts. On confirmation of the presence of oocysts, the supernatant was diluted five times with water. The diluted supernatant was 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 and was properly labeled. After concentrating 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 [21].

2.3. Inoculation of Coccidia Oocysts

10 broiler birds at 2 weeks and 10 broiler birds at 3 weeks were each inoculated orally with 1ml of *Eimeria* solution containing about 2000 oocysts. The birds were fed with standard starter feed and given adequate water with a good light supply.

2.3.1. Observations After Inoculation

Day 1: no marked physical signs

Day 2: decrease in physical activities and drooping of feathers,

Day 3: blood-stained droppings, drooping of feathers, reduction in food intake, reduction in water intake, diarrhea

Day 4: 2 birds died, and most features of day 3 are present. The dead birds were taken to the Department of Veterinary Pathology, University of Nigeria, Nsukka

Day 5: blood-stained diarrhea droppings, drooping of feathers with about 4 birds [2/2] becoming slightly more active

Day 6: 1 bird dead [2 weeks], The remaining birds still showing signs [drooping of feathers, reduced physical activity]. The dead bird was taken to the Department of Veterinary Pathology University of Nigeria.

Day 7: bloody diarrheal dropping still present

2.3.2. Post Mortem Results

Post-mortem of the dead birds was done at the pathology laboratory of the Department of Veterinary Pathology University of Nigeria, Nsukka. The result of the post-mortem was consistent with lesions of *Eimeria tenella*. The caecum was affected leaving the other gut free of Lesions as is found in *Eimeria tenella* infection

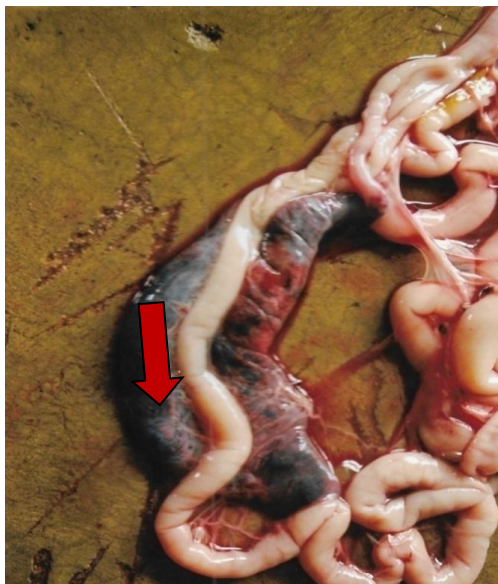


Figure 1: Caecal lesion (Red arrow) caused by *Eimeria tenella*

2.4. Quantification of the Sporulated Oocysts

The sporulated oocysts were quantified using the McMaster method [22]. 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 a 1:10 dilution. 1 ml of the diluted sample was collected and used to fill the chambers of the McMaster slide. All the oocysts in both chambers were counted and their mean number was calculated.

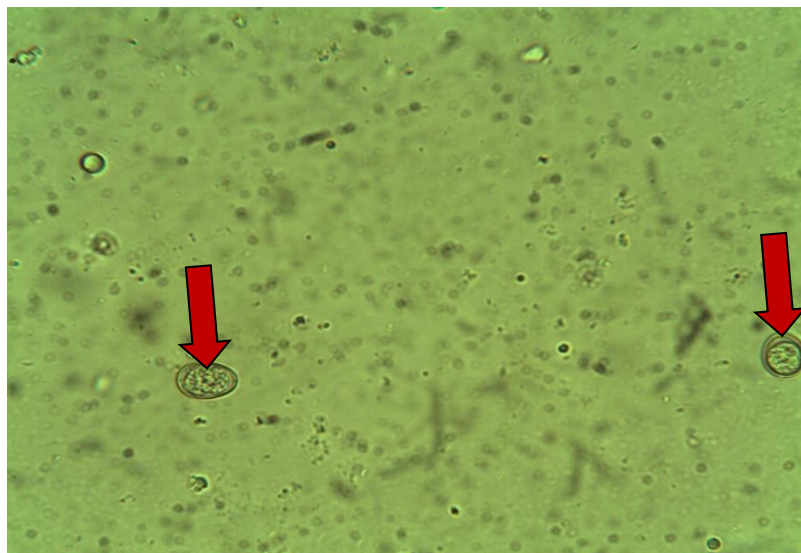
The oocyst per ml was then obtained using the formula below:

$$P \times D \times K$$

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

D = Dilution factor (10)

K = Constant (6.67)



Slide 1. Freshly voided Oocysts of *Eimeria tenella*

3. RESULTS

3.1. Qualitative and Quantitative Phytochemical Analysis of the Extract Fractions.

As presented in Tables 1 and 2, most of the phytochemicals were present in low quantities (tannins, steroids, terpenoids, flavonoids, glycosides, and alkaloids) in both the methanol and methanol crude fractions of the *C. odorata*. The phenolics and reducing sugar were in moderate quantities. In the ethyl acetate fraction, most of the phytochemicals were either in high or moderate quantities, except for saponins and alkaloids that were not detected. In the n-hexane fraction, only the steroids and terpenoids were in moderate quantities, the alkaloid was in low quantity while the others were not detected.

Table 1. Qualitative phytochemical analysis of the fractions of *C. odorata* extract

Phytochemicals	Methanol crude extract <i>C. odorata</i>	Methanol fraction <i>C. odorata</i>	Ethyl acetate fraction <i>C. odorata</i>	n-hexane fraction <i>C. odorata</i>
Tannins	+	+	++	ND
Steroids	+	+	+++	++
Terpenoids	+	+	+++	++
Total phenolics	++	++	+++	ND
Flavonoids	+	+	++	ND
Glycosides	+	+	+	ND
Reducing sugar	++	++	++	ND
Saponin	ND	++	ND	ND
Alkaloids	+	+	ND	+

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

Table 2. Quantitative phytochemical analysis of the fractions of *C. odorata* extract

Phytochemicals	Methanol crude extract <i>C. odorata</i>	Methanol fraction <i>C. odorata</i>	Ethyl acetate fraction <i>C. odorata</i>	n-hexane fraction <i>C. odorata</i>
	21.31	20.53	29.55	
Tannins (mg/100g)	22.46	21.07	32.09	ND
	22.38	21.31	34.18	
	0.306	0.386	13.18	4.05
Steroids	0.281	0.421	14.03	5.12
	0.27	0.562	14.03	4.52
	8.24	12	640.18	143.13
Terpenoids	6.59	13.65	686.44	134.18
	8.47	14.12	686.44	157.25
	5282.26	4137.1	12491.94	
Total phenolics	5951.61	4169.35	12572.58	ND
	5612.9	4193.55	12572.58	
	8.02	8.12	74.97	
Flavonoids	8.8	10.41	72.99	ND
	9.01	9.74	74.66	
	3.47	3.19	7.81	
Glycosides	3.47	1.19	7.78	ND
	3.49	3.18	7.81	
	36334	557.83	754.35	
Reducing sugar	4.35.91	590.43	765.65	ND
	371.74	572.61	766.52	
			0.243	
Saponin	ND	ND	0.234	ND
			0.239	
	1029.17	804.17		72.92
Alkaloids	891.67	716.67	ND	68.75
	1029.17	785.42		58.33

3. 2. Monolayer cell cultures of the oocysts treated with the various fractions of *C. odorata* leaf extracts.

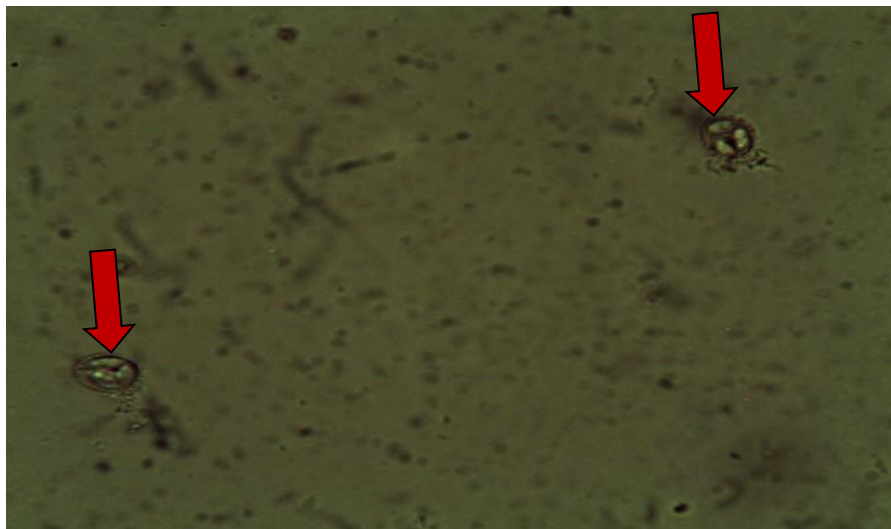
In slide 2, the cultured oocysts of *E. tenella* were treated with a known drug, "Amprolium" control. Amprolium does not affect the sporulation of the *E. tenella* oocysts. Slide 3 presents cultured oocysts of *E. tenella* treated with an Ethyl acetate fraction of *C. odorata* extract. This gave the least anticoccidial effect on the sporulated oocysts. Slide 4 shows the cultured oocysts of the *E. tenella* treated with methanol fraction of *C. odorata* extract respectively. Oocysts of the *E. tenella* treated with N-hexane fraction had the best anticoccidial activity and were shown in slide 5.



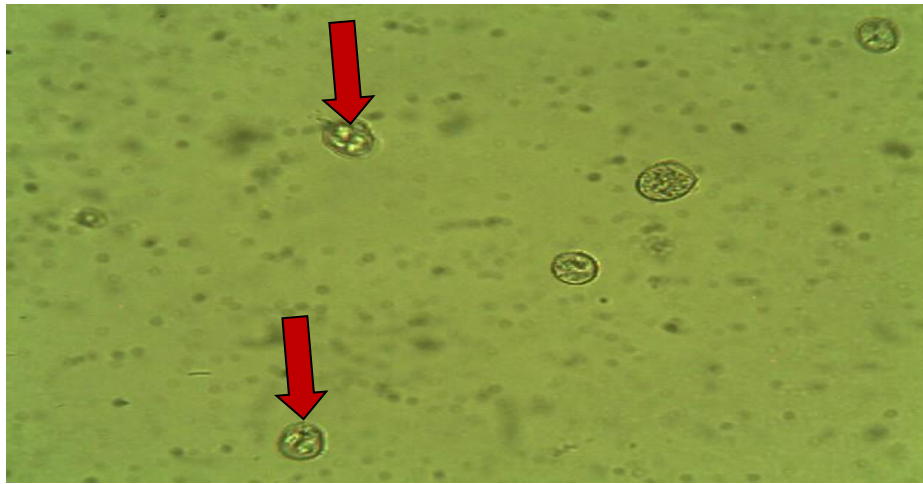
Slide 2. Oocysts of *Eimeria tenella* culture treated with Amprolium.



Slide 3. Oocysts of *Eimeria tenella* culture treated with Ethyl Acetate Fraction of *C. odorata*



Slide 4. Oocysts of *Eimeria tenella* culture treated with Methanol Extract of *C. odorata*



Slide 5. Oocysts of *Eimeria tenella* culture treated with N-Hexane fraction *C. odorata*

3.3. Percentage sensitivity of sporulated *Eimeria tenella* species cultured in *Chromolaena odorata*-hexane fraction of leaf extract and Amprolium.

The result of the normal control dose (0.0 mg/ml) showed that there was a significant ($P < 0.05$) decrease in percentage sensitivity of unsporulated ($3.33 \pm 1.53\%$) *Eimeria tenella* species compared to the sporulated form ($96.67 \pm 1.53\%$). Also, there existed a significant ($P < 0.05$) decrease in percentage sensitivity of unsporulated *Eimeria tenella* species cultured in *Chromolaena odorata* n-hexane fraction of leaf extract at 0. mg/ml and amprolium at 0.0-0.4mg/ml compared to their respective doses of sporulated form. However, *Chromolaena odorata* n-hexane fraction of leaf extract had a significant ($P < 0.05$) increase in percentage sensitivity of unsporulated *Eimeria tenella* species cultured in it compared to the sporulated cultured in it. There were significant ($P < 0.05$) differences among sporulated and unsporulated *Eimeria tenella* species cultured in *Chromolaena odorata* n-hexane fraction of leaf extract and amprolium when compared among themselves as shown in Figure 2.

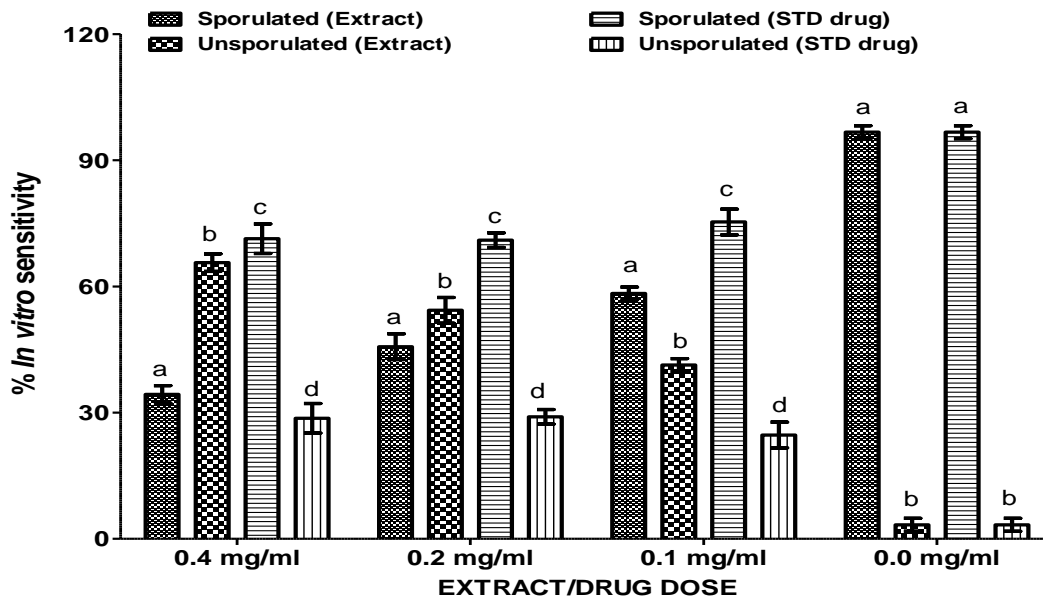


Figure 2: Percentage *in vitro* sensitivity of *Eimeria tenella* species cultured in *Chromolaena odorata*-hexane fraction of leaf extract and Amprolium.

Bars are mean \pm standard deviation of triplicate determinations. Bars bearing different alphabet letters per Extract/Drug dose group are statistically significant ($p < 0.05$).

3. 4. Percentage sensitivity of sporulated *Eimeria tenella* specie cultured in *Chromolaena odorata* methanol fraction of leaf extract and Amprolium.

The result of the normal control dose (0.0 mg/ml) indicated that there was a significant ($P < 0.05$) decrease in percentage sensitivity of unsporulated ($3.33 \pm 1.53\%$) *Eimeria tenella* species compared to the sporulated ($96.67 \pm 1.53\%$). The same trend was observed in 0.1 mg/ml of *Chromolaena odorata* methanol fraction of leaf extract and amprolium ($27.33 \pm 2.52\%$ against $72.67 \pm 2.52\%$) and $24.67 \pm 3.06\%$ against $75.33 \pm 3.06\%$ respectively. Meanwhile, there was a significant ($P < 0.05$) decrease in percentage sensitivity of unsporulated *Eimeria tenella* species cultured in *Chromolaena odorata* methanol fraction of leaf extract and amprolium at 0.2 and 0.4mg/ml when compared to their respective sporulated form. However, their mean separations showed significant ($P < 0.05$) differences among their various percentage sensitivity mean values as shown in Figure 3.

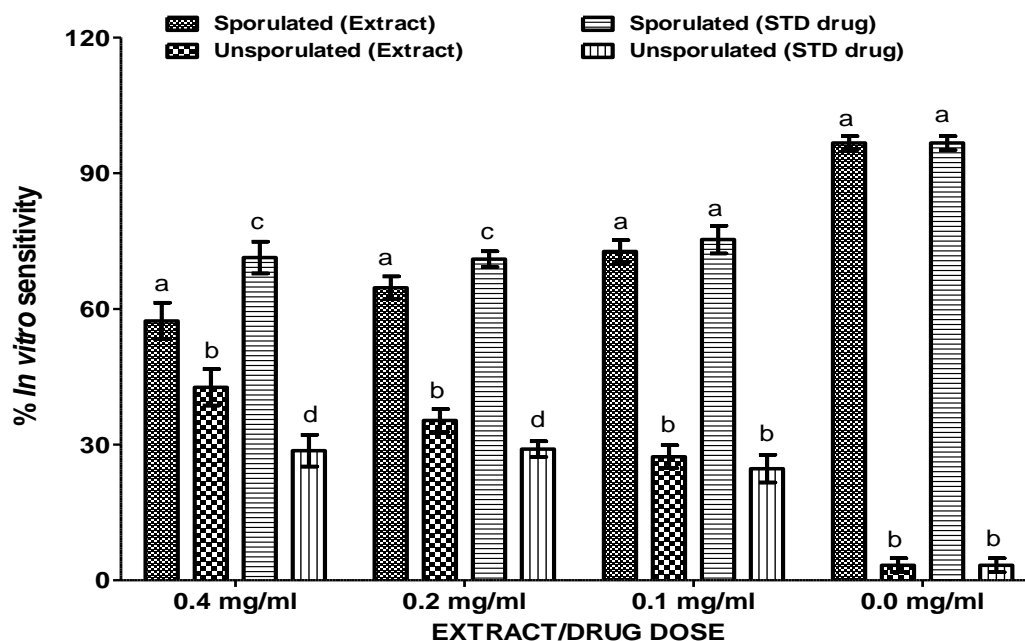


Figure 3: Percentage *in vitro* sensitivity of *Eimeria tenella* species cultured in *Chromolaena odorata* methanol fraction of leaf extract and Amprolium.

Bars are mean \pm standard deviation of triplicate determinations. Bars bearing different alphabet letters per Extract/Drug dose group are statistically significant ($p < 0.05$).

3. 5. Percentage sensitivity of sporulated *Eimeria tenella* species cultured in *Chromolaena odorata* crude methanol fraction of leaf extract and Amprolium.

The result of the normal control dose (0.0mg/ml) decrease in percentage sensitivity of unsporulated ($3.33 \pm 1.53\%$) *Eimeria tenella* species compared to the sporulated ($96.67 \pm 1.53\%$). On the other hand, there was significant ($P < 0.05$) decrease in percentage sensitivity of unsporulated *Eimeria tenella* species cultured in *Chromolaena odorata* crude methanol fraction of leaf extract and amprolium at 0.1-0.4mg/ml when compared to their respective sporulated form with the exemption of 0.4mg/ml of *Chromolaena odorata* crude methanol fraction of leaf extract that had no significant ($P > 0.05$) decrease between unsporulated and sporulated *Eimeria tenella* species. Their mean separations indicated significant ($P < 0.05$) differences among their various percentage sensitivity mean values as shown in Figure 4.

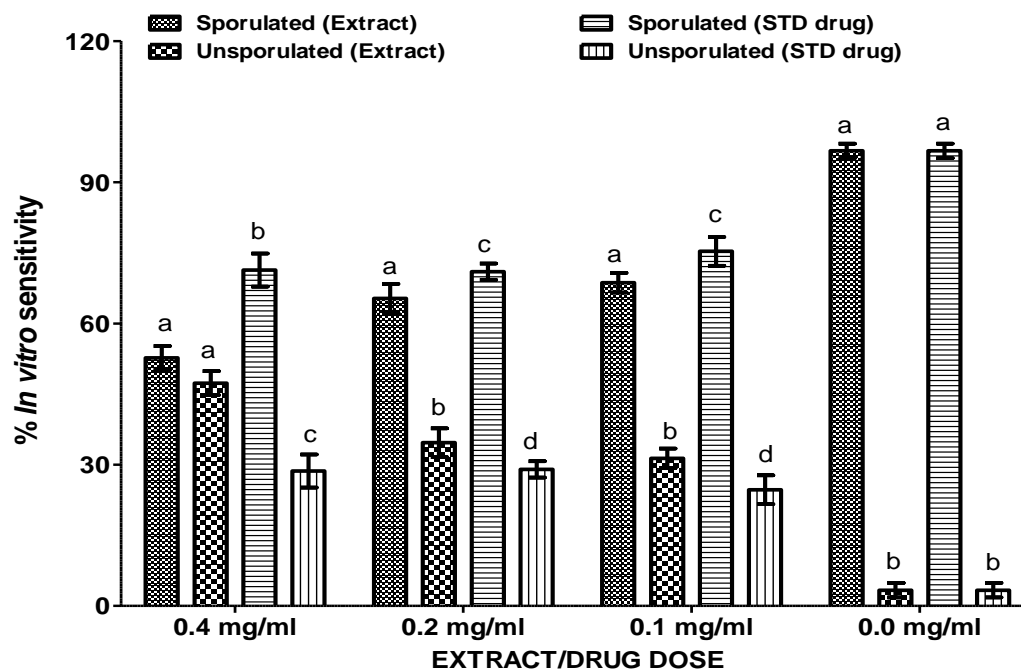


Figure 4: Percentage *in vitro* sensitivity of *Eimeria tenella* species cultured in *Chromolena odorata* crude methanol fraction of leaf extract and Amprolium.

Bars are mean \pm standard deviation of triplicate determinations. Bars bearing different alphabet letters per Extract/Drug dose group are statistically significant ($p < 0.05$).

3. 6. Percentage sensitivity of sporulated *Eimeria tenella* species cultured in *Chromolaena odorata* ethyl acetate fraction of leaf extract and Amprolium.

The result in Figure 5 showed that the normal dose (0.0mg/ml) had a significant ($P < 0.05$) decrease in percentage sensitivity of unsporulated *Eimeria tenella* species ($3.33 \pm 1.53\%$) compared to the sporulated species ($96.67 \pm 1.53\%$). On the other hand, there was a significant ($P < 0.05$) decrease in percentage sensitivity of unsporulated *Eimeria tenella* species cultured in *Chromolaena odorata* ethyl acetate fraction of leaf extract and Amprolium at 0.1-0.4mg/ml when compared to their respective sporulated form except 0.4mg/ml of sporulated *Eimeria tenella* species cultured in *Chromolaena odorata* ethyl acetate fraction leaf extract compared to the Amprolium that showed non-significant ($P > 0.05$) increase in percentage sensitivity when the former is compared with the latter. Their mean separations indicated that a significant ($P < 0.05$) difference existed among their various percentage sensitivity mean values.

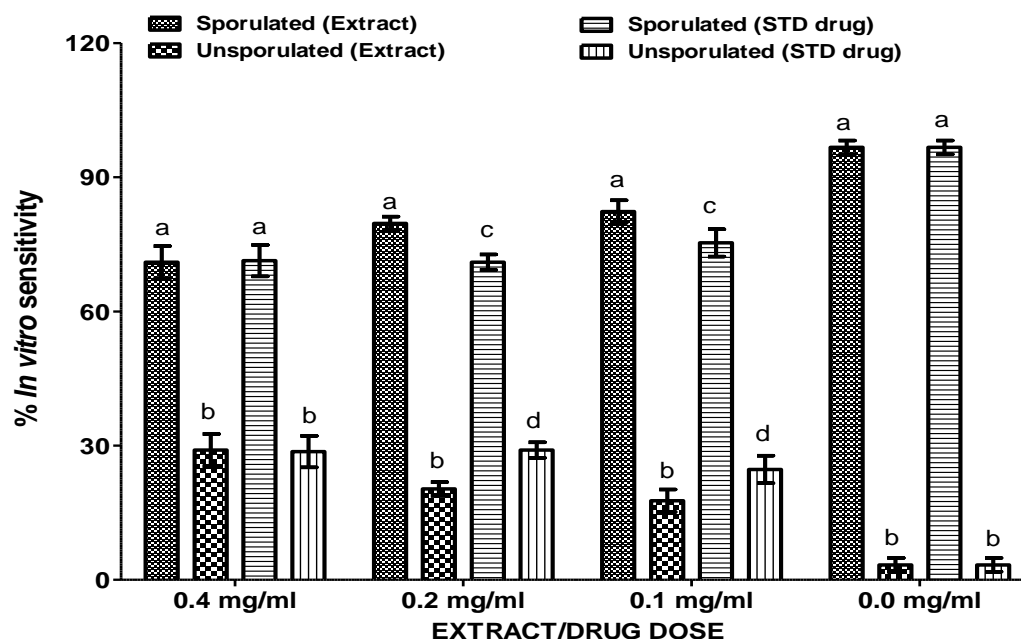


Figure 5: Percentage *in vitro* sensitivity of *Eimeria tenella* species cultured in *Chromolaena odorata* ethyl acetate fraction of leaf extract and Amprolium.

Bars are mean \pm standard deviation of triplicate determinations. Bars bearing different alphabet letters per Extract/Drug dose group are statistically significant ($p < 0.05$).

4. DISCUSSION

Many anticoccidial drugs are being banned from use in food animals. Although prophylactic vaccines emerge as the most suitable approach, successful vaccines against coccidial parasites are scarce and limited to the veterinary field [23,24]. This study was aimed at investigating the *in-vitro* anticoccidial effect of *C. odorata* leaf extracts on sporulating *E. tenella* oocyst and determining the fraction with the best anticoccidial activity.

The therapeutic potentials of medicinal herbs are usually due to the constituents of their phytochemicals [25]. The phytochemical contents of *C. odorata* leaf extract in this study were found to differ from the different fractions of the *C. odorata* extract. Only the steroids and terpenoids are present in all four fractions of *C. odorata*, though at different concentrations. Among the four fractions of the extract, the n-hexane fraction had the highest anticoccidial activity as it inhibited the sporulation of *Eimeria tenella*. This fraction was found to contain only steroids and terpenoids with low quantities of alkaloids. There is a tendency for the steroids and terpenoids to be the major potential anticoccidial constituents of the extracts. The presence of many other phytochemicals may probably have an antagonistic effect on the anticoccidial activity of the extracts. This is because the ethyl acetate fraction has higher quantities of both the steroids and terpenoids than the n-hexane fraction, yet with the least ability to inhibit the sporulation of *E. tenella*.

Crude methanol fraction was next in the order of anticoccidial activity. It has the highest quantities of some of the phytochemicals but there was the absence of alkaloids and saponins.

Next in line was the methanol fraction. This has almost similar activity as crude methanol fraction with almost similar phytochemical constituents as crude except for a reasonable quantity of saponin in the methanol fraction. The ethyl acetate fraction had the least anticoccidial activity, phytochemically it had steroids, terpenoids, and total phenolics in very high concentration, with tannins, flavonoids, and reducing sugars in reasonable concentration while glycosides were small in concentration, alkaloids, and saponin were not present. The phytochemical picture

clearly shows that the alkaloid content of the fractions influenced their anticoccidial activity. Terpenoids and steroids also have anticoccidial influence.

CONCLUSION

This study showed that the phytochemicals; steroids, terpenoids, and alkaloids appear to be the most active substances in the *Chromolaena odorata* which made it a potent anticoccidial agent. Thus the study provides the basis for the use of *Chromolaena odorata*, especially the n-hexane fraction as preventive agents against coccidiosis.

Recommendation

We recommend that the n-hexane fraction of the *Chromolaena odorata* be used as a feed additive in the control of coccidiosis.

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