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THE *IN VITRO* ANTICOCCIDIAL ACTIVITY OF AQUEOUS AND ETHANOLIC EXTRACTS OF *Ageratum conyzoides* AND *Vernonia amygdalina* (ASTERACEAE)

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ABSTRACT

The development of drug resistance to *Eimeria* species is a reality farms in poultry. Thus, use of plants extracts as an alternative to anticoccidial drugs has been an appealing approach for controlling avian coccidiosis. This study was conducted to evaluate the effect of *Ageratum conyzoides* and *Vernonia amygdalina* aqueous and ethanolic extracts on the inhibition of the sporulation of *Eimeria tenella* oocysts. In this assay, 1(ml) of the suspension containing 1000 unsporulated oocysts of *E. tenella* was introduced into Petri dishes and different concentrations (100, 50, 25 and 12.5 mg/ml) of *A. conyzoides* and *V. amygdalina* extracts were added. Tween 80 3, 5 % and Potassium Dichromate (2, 5%) were used as negative controls while phenol 5% was used as positive control. The Petri dishes were incubated for 48 h. At the end of incubation the anticoccidial activity of *A. conyzoides* and *V. amygdalina* against unsporulated oocysts of *E. tenella* was found to be concentration dependent and increased significantly at the higher concentration ($P < 0.05$) with mean inhibition rates of 78, 67 %, 54, 33% and 47, 67% respectively for ethanolic, infusion and macerated extract of *A. conyzoides* and 66,66 %, 40,66% and 20,00% respectively for ethanolic, infusion and macerated extract of *V. amygdalina*. The inhibition rate of ethanolic

extracst of both plants were significantly higher (P<0. 05) at all concentrations compared to those of aqueous extracts.

KEYWORDS: *Ageratum conyzoides*, *Vernonia amygdalina*, anticoccidial activity, *Eimeria tenella*, sporulation, oocysts.

INTRODUCTION

Avian coccidiosis is a parasitic disease caused by *Eimeria* protozoan species. This disease is transmitted through hard, thick-walled sporulated oocysts which are able to survive for lengthy periods in poultry litter and soil particles.[1] Seven species are known to infect poultry and each species have its own characteristics depending on the site of infection and pathogenicity.[2] Among these species, *Eimeria tenella*, which causes caecal coccidiosis is a major threat to poultry production.[3] *E. tenella* oocysts can remain infective for up to nine months in litter and, therefore, become a source of disease for other farms through air current dispersion.[4] Hence, substances capable of inhibiting the sporulation may be used in preventive measures.

Indeed, in the external environment, farmers usually fight against coccidiosis by applying disinfectant compounds such as Ammonia, Methyl Bromide, Carbon Disulfide and some phenolic products on soil and litter.[5] However, the toxicity of these products makes their use difficult for disinfecting the broiler house in presence of animals. On the other hand, their effects are hazardous to the farmers using them. Moreover, anticoccidial drugs have played a major role in the effective control of avian coccidiosis, but, their extensive use has resulted in the emergence of the drug resistant coccidians strains.[6, 7] So, due to the drug resistance, residual effect of drugs in meat and toxic effects of disinfectants, scientists all over the world have turned to alternative approaches for the control of coccidiosis. Herbal anticoccidials have opened new perspectives and proven suitable particularly in countries with limited economic potentials.[8]

Ageratum conyzoides L. (Asteraceae) is an annual herbaceous plant with a large spectrum of traditional medicinal and agricultural uses.[9] It has been reported to possess anthelminthic[10] antiplasmodial[11], antioxidant[12], antiradical[13], anticoccidal[14] and anti-inflammatory activities.[15] *Vernonia amygdalina* (Asteraceae), commonly known as Bitter leaf is a shrub indigenous to a variety of ecological zones in Africa especially sub-Saharan Africa.[16] Also, *V. amygdalina* is reported to have many medicinal uses which include antidiabetic[17],

antioxidant[18], and antimalarial[19], anthelminthic.[20] etc. The leaves of these plants contain many bioactive compounds which are responsible for their diverse biological activities.

Based on this background, the current study was undertaken to evaluate the *in vitro* antisporulation effects of aqueous and ethanolic extracts of *A. conyzoides* and *V. amygdalina* against oocysts of *E. tenella*, for potential use as environmental disinfectants.

MATERIALS AND METHODS

1.1. Plant collection and storage

The leaves of *A. conyzoides* and *V. amygdalina* were collected in Bamboutos Division, Western Region of Cameroon and identified by in Cameroon National Herbarium (Yaoundé) using a voucher specimen registered under the Reference No 6575 /SRF and N°9535/SRF for *A. conyzoides* and *V. amygdalina* respectively. The collected plant material was dried in shade, at ambient temperature for about two weeks after which it was blended into fine powder and stored in airtight plastic bag in the laboratory at 4°C.

1.2. Plant extracts preparation

The aqueous (hot and cold water) and ethanolic extracts were prepared to compare their activities on *E. tenella* oocysts. Extraction was done according to the procedure described by.[21]

For ethanolic extract, 100 g of stored powder were macerated in 1.5 l of ethanol 95%. The mixture was daily stirred to permit better extraction of the active ingredients. Seventy-two (72) hours later, this solution was sieved and filtered through a cotton layer and a filter paper of pore size 2.5 µm. The filtrate was evaporated in a rota vapor at 82ºC for 8 hours. The extract obtained was poured in a large Petri dish and allowed to dry at room temperature for two days.

For aqueous extracts, a similar procedure was carried out except that, hot (at 100^{o}C) and cold distilled water were used as solvent for the hot and cold water extracts respectively. Infusion took 3 hours and maceration 48 hours to avoid fungal growth. Dried aqueous extracts were obtained after 72 h in a ventilated oven heated at 50^{o}C.The ethanolic and aqueous extracts obtained were kept in a refrigerator at 4ºC for further processing.

1.3. Anticoccidial activities of extracts

1.3.1. Preparation of culture media

Dichromate ($K_2Cr_2O_7$) Potassium: 2.5% Potassium dichromate was prepared by dissolving 2.5 g of potassium dichromate in 100 ml of distilled water. This culture medium was stored and used to prepare our plant extract concentrations.

1.3.2. Preparation of stock solutions

For the aqueous extracts, 4g of each plant extract were weighed using an electronic scale balance and then 20 ml of distilled water introduced into the mortar. After homogenization, the mixture was transferred into a beaker. For the organic extract, a stock solution was equally prepared and the same amount of dry extract was first mixed with 0.7 ml tween 80 % to facilitate dissolution of the organic extract with water. Stock solutions with a concentration of 200 mg/ml were then obtained. Through successive dilutions, we obtained solutions of concentration 200, 100, 50 and 25 mg/ml.

1.3.3. Preparation of inoculum

Coccidial oocysts of *E. tenella* were obtained from the caeca of naturally infected chicks from a local market of Dschang. Following evisceration at post mortem, the caeca were separated, sliced open longitudinally and their contents washed into a beaker using tap water. The washings were put in tube for centrifugation. Oocyst mensuration was done to determine the purity of the oocysts suspension obtained. Harvested oocysts were multiplied in three healthy chicks by oral infection.[22] The chicks were routinely monitored for the development of clinical signs of coccidiosis and the presence of *E. tenella* oocysts in their faeces. Ten days (10) days post infection, fecal materials were collected and the intensity of pure species were assessed using a McMaster technique counting chamber.[23]

1.3.4. Experimental design and sporulation inhibition assay

An *in vitro* sporulation inhibition assay was used to examine the effect of *A. conyzoides* and *V. amygdalina* extracts on oocysts of *E. tenella* species. In this assay, fresh fecal samples were collected from chickens experimentally infested with oocysts of *E. tenella*, mixed with Potassium Dichromate and filtered. The prepared oocysts-potassium dichromate mixture was centrifuged so as to increase oocysts counts in 1 ml. The supernatant was discarded and oocysts suspension was obtained. Potassium dichromate was added to inhibit bacterial growth that may hinder the sporulation of oocysts.[24] 1 (ml) of the suspension containing 1000 unsporulated oocysts was introduced into each petri dish. One ml of various dilutions of the

respective extract of *A. conyzoides* and *V. amygdalina* (200, 100, 50 and 25 mg/ml) were added in these Petri dishes to have a final concentration of 100, 50, 25 and 12,5 mg/ml. 3, 5 %Tween 80 and Potassium Dichromate were used as negative control for ethanolic and aqueous extract respectively while phenol 5% was used as reference disinfectant.[25] The Petri dishes were partially covered to allow oxygen circulation and incubated for 48 h at 28ºC. The contents of the Petri dishes were stirred off and on to ensure the oxygenation. At the end of incubation, the effect of tested products on the oocysts sporulation was examined under microscope at 40X. The numbers of sporulated and non sporulated oocysts were counted and the percentage of inhibition was estimated by counting the number of unsporulated oocysts in a total of 100 oocysts. Three replications were made for each concentration.

1.4. Phytochemical screening

In the quest to study the distribution of secondary metabolites in the differents plant extracts, a phytochemical analysis was carried out to test for the presence of phenolic compounds, alkaloids, flavonoids, polyphenols, tannins, saponin, triterpenes and steroids using standard procedures described by.[26]

1.5. Statistical analysis

The data obtained was analyzed using Two-way analysis of variance (ANOVA) and presented as mean ± standard deviation (SD) of three replications. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 12.0. The post hoc statistical significance test employed was Duncan, differences between the means were considered significant at P< 0,05.

2. RESULTS

2.1. *In vitro* anticoccidial effect

The *in vitro* oocysticidal activity of the different plant extracts against *E. tenella* is summarized in Table I. This table shows that various concentrations of the different extract produced varying degrees of inhibition of sporulation. The inhibition rate was concentration dependent and increased significantly at the higher concentration (P<0. 05) with mean rates of 78, 66 %, 54, 33% and 47,66% respectively for ethanolic, infusion and maceration extracts of *A. conyzoides* and 66,66%, 40,66% and 20,00% respectively for ethanolic, infusion and macerated extract of *V. amygdalina*. At the concentrations lower or equal to 50 mg/ml the mean inhibition rates of infused extract of both plant as well as macerated aqueous extract of *A. conyzoides* were not significantly different (P> 0, 05). However macerated extracts of *V.*

amygdalina had mean inhibition rates lower or equal to 21% at all the concentrations. In the control Petri dishes containing oocysts and 3, 5 % Tween 80 or $K_2Cr_2O_7$ about 85% of oocysts of *E. tenella* managed to sporulate. The inhibition of oocyst sporulation of these plants was less than that produced by Phenol 5 % (100%). The percentage inhibition of ethanolic extract of both plants was significantly higher (P<0. 05) at all concentrations compared to that of aqueous extracts. At concentrations equal to 100 mg/ml or higher, the percentage inhibition of *A. conyzoides* was significantly higher (P<0. 05) than that of *V. amygdalina*. However, at concentrations lower than 100 mg/ml the reverse effect was observed (P<0.05).

2.2. Phytochemical screening

Phytochemical screening revealed the presence of various phytochemicals in the different extracts (Table II). This Table shows that ethanol leaf extracts of *A. conyzoides* were found to contain almost all the phytochemicals tested except steroids and terpenoids. On the contrary, saponins, steroids and terpenoids were absent in aqueous extract. However, tannins, polyphenol, Flavonoids, glycosides and alkaloids were found in all the extracts.

Table I: Sporulation inhibition percentage of *A. conyzoides and V. amygdalina* extracts on *E. tenella* oocysts after 48 h of incubation, n=3

	Ageratum conyzoides			*Vernonia amygdalina*		
Conc mg/ml	**Et**	**AI**	**AM**	**Et**	**AI**	**AM**
100	$78,66\pm3,21^{Aa}$	$54,33\pm2,51^{Ca}$	$47,66\pm2,51^{Da}$	$66,66\pm1,52^{Ba}$	$40,66\pm5,03^{Ea}$	$20,00\pm5,00^{Fa}$
50	$47,66\pm2,51^{Ab}$	$24,00\pm2,64^{Cb}$	$22,00\pm2,64^{Cc}$	$51,66\pm2,88^{Ab}$	$32,66\pm3,05^{Bb}$	$21,00\pm2,00^{Ca}$
25	$30,00\pm2,00^{Bc}$	$12,66\pm2,51^{Dc}$	$15,66\pm4,04^{Dc}$	$47,66\pm2,51^{Ab}$	$31,66\pm1,52^{Bb}$	$20,66\pm2,08^{Ca}$
12,5	$19,33\pm2,08^{BCd}$	$11,33\pm2,30^{Cc}$	$15,00\pm5,00^{BCc}$	$37,00\pm6,24^{Ac}$	$22,33\pm2,51^{Bc}$	$16,66\pm2,88^{Ca}$
$K_2Cr_2O_7$	NA	10,00±2,00	11,00±2,00	NA	11,66±1,52	12,66±3,05
Tween80 3,5 %	10,66± 1,15	NA	NA	10,66±2,08	NA	NA
Phenol 5 %	100±00	100±00	100±00	100±00	100±00	100±00

Small letters compare means in a column and capital letters means in a row. Different letters indicate significant difference ($P < 0.05$). Legend: EEt: Ethanolic extract; EI: infusion extract; ME: Maceration extract; NA: Not applicable. The results are the mean ± SD of triplicate tests evaluated after 48 h of incubation at room temperature.

Table II: Secondary metabolites found in aqueous and éthanolic extract of *Ageratum conyzoides and Vernonia amygdalina*

	Ageratum conyzoides			*Vernonia amygdalina*		
Chemical groups	**Indication**					
	EET	**EI**	**EM**	**EET**	**EI**	**EM**
Alkaloids	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Polyphenols	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Saponins	+	-	-	+	-	-
Stéroids	-	-	-	+	-	-
Terpenoids	-	-	-	-	-	-
Glycosides	+	+	+	+	+	+

EET= Ethanolic extract, EI= Infusion extract, EM= Maceration extract

3. DISCUSSION

Inhibition of sporulation is a common criterion to assess anticoccidial properties.[27] Oocysts of coccidians are very resistant to physical and chemical treatment because of the two proteinous layers of their wall.[28] Thus, drugs capable of crossing this wall and inhibit the sporulation process are the best choice for preventive measures against coccidiosis.[3, 4] The major finding of this study was that both plants (*A. conyzoides* and *V. amygdalna*) exhibited anti-sporulation activity against *E. tenella* oocysts at the highest concentration. This anti-sporulation activity could be attributed to a variety of secondary metabolites present. Indeed, phytochemical screening of these plants extract revealed the presence of Polyphenolic compounds. Several studies *in vitro* and *in vivo* have reported the inhibitory effect of plant extracts containing phenolic compounds.[29]and[30] have demonstrated that the natural polyphenolic component derived from *Curcuma longa*, inhibited cell invasion of *E. tenella* sporozoites *in vitro* and *in vivo*.[23] showed that extracts containing amounts of polyphenolic compounds may have the ability to inhibit the enzymes responsible for the sporulation process of the coccidian oocysts. This ability of polyphenolic compounds to inhibit the activities of various endogenous enzymes is well documented. Moreover,[31] noticed that, mannitol (energy source of oocyst) is necessary during sporulation process of oocysts of *E. tenella* in the presence of mannitol dehydrogenase enzyme. It can therefore be speculated that, extract of *A. conyzoides* and *V. amygdalina* exhibited anti-sporulation effect by interfering in the physiological processes necessary for sporulation like either preventing access to oxygen, or inhibiting and inactivating the enzymes responsible for the sporulation process. The observation that $K_2Cr_2O_7$ could not inhibit sporulation could be explained by the facts that since it is a bactericidal drug as well, it might have killed the bacteria present thereby enhancing the sporulation of oocysts. Therefore it could be that bacteria if present could have interfered with the sporulation of oocysts, possibly by competing for nutrients and/or feeding on the oocysts.

The efficacy of ethanolic extracts of both plants was significantly highest compared to aqueous extracts. Similarly,[32] demonstrated that ethanolic extracts of *Carica papaya* had a higher anticoccidial activity than aqueous extracts on *E. tenella.* According to this author, this could be due to the fact that ethanolic extracts have the ability to penetrate more rapidly the oocysts wall in a relatively short time than aqueous extract. Likewise,[19] showed that ethanol solvent allows the extraction of more natural constituents than aqueous extracts. Then the efficacy of ethanolic extract might be due to the amount of polar products coming from their

extraction or to the fact that these extracts have weakened oocyst walls more rapidly, inhibiting more readily the sporulation process. We also noticed that inhibition rate was concentration dependent and increased significantly at the higher concentrations (P<0, 05).[3], [4]and[33] observed the same trend in coccidial oocysts of *E. tenella and E. intestinalis.* Indeed,[10] proposed that the concentration dependent activity of extracts could result from the increased amount bioactive compounds in higher extracts concentrations.

The two plants species had different inhibition rate of sporulation (P<0. 05) since they differ in the amounts of chemicals compounds they contain. Given the inhibition rates obtained, the tested plants may not be highly effective on the sporulation of *E. tenella*.

4. CONCLUSION

The present study revealed the higher efficacy of ethanolic extracts of both plants tested in inhibiting the sporulation of *E. tenella* oocysts compared to aqueous extracts. The *in vivo* activity of these plants on the others stages of *E. tenella* life cycle are recommended.

Competing interests

Authors have declared that no competing interests exist.

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