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Phytochemical Profile of the Methanolic Leaf Extract of *Vernonia amygdalina* Del. and *In Vitro* Anticoccidial Effect of Its Fractions against *Eimeria tenella*

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Abstract:
Phytochemical screening of the crude methanolic leaf extract of Vernonia amygdalina was carried out. The extract and its fractions were tested for in vitro anticoccidial efficacy against the unsporulated oocysts of Eimeria tenella by the oocyst sporulation inhibition model in a 96-well microtitre plate. Phytochemical analysis of the extract revealed the presence of alkaloids, tannins, saponins, glycosides, and triterpenoids. Different concentrations of the extracts, including 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.625 mg/ml, and 7.8 mg/ml, were tested against freshly prepared unsporulated oocysts of E. tenella. Triplicate assays were conducted for each concentration of the extract to screen for anticoccidial activity. The methanol extract and fractions showed activity against the oocysts of E. tenella in a dose-dependent manner. The sporulation inhibition rate was determined as effective when the result showed more than 50% inhibition at each concentration level of treatment. Only the methanol extract and its butanol fraction at the lowest treatment concentration of 7.8 mg/ml showed an inhibition rate of 55% and 68%, respectively. All treatments: methanol extract (100%), hexane fraction (100%), butanol fraction (100%), and aqueous residue fraction (58%) were active at the highest concentration of 250 mg/ ml. It is concluded that the methanol leaf extract of Vernonia amygdalina and its butanol fraction had better oocyst sporulation inhibition activity against Eimeria tenella and may further be explored for their anticoccidial properties.

Keywords: *Phytochemical screening, Vernonia amygdalina, Eimeria tenella, in vitro sporulation inhibition, chicken*

1. Introduction

Avian coccidiosis, caused by multiple species of parasitic apicomplexan protozoa of genus *Eimeria*, is one of the most economically important diseases of the poultry industry (Chapman *et al*., 2013). Recent calculations of the financial impact of prophylaxis and estimates of production losses due to the disease put the costs of coccidiosis to the poultry industry worldwide in excess of 14.4 billion US dollars annually (Blake *et al*., 2020).

Generally, seven species of *Eimeria*: *E. tenella, E. necatrix*, *E. acervulina*, *E. maxima*, *E. brunetti*, *E. praecox*, and *E. mitis* are responsible for coccidiosis. In broiler chickens, the most prevalent species are *Eimeria tenella*, *E. acervulina*, and *E. maxima* (Gyorke *et al*., 2013; Chapman *et al*., 2016). *E. tenella* is the most pathogenic species that parasitizes the caeca recognized by the accumulation of blood in the caecal lumen and by bloody droppings (McDougald & Fitz-Coy, 2013).

Anticoccidial drugs are routinely and continuously applied in the feed and, to a limited extent, in the drinking water of birds raised in the poultry industry to control coccidiosis. With the passage of time, a large number of anticoccidial drugs have been introduced, but resistance to them has been described in different parts of the world (Abbas *et al*., 2011; Djemai *et al*., 2016; Tan *et al*., 2017).

Chemicals have been applied to control coccidiosis on the farm, but most of them are toxic to the live birds and the farm personnel (Fetterer *et al*., 2010; Samaha *et al*., 2013; You, 2014).

Alternative strategies are being sought for safer and low-cost control of avian coccidiosis (Muthamilselvan *et al*., 2016). Using botanical anticoccidial and natural products may provide a novel approach to controlling coccidiosis (Abbas *et al*., 2012; Quiroz-Castenada and Dantan-Gonzalez, 2015).

Several plants have been listed as having anticoccidial properties, and some have been validated using modern scientific methodologies (Zaman *et al*., 2012; Ahad *et al*., 2017). A good example is the use of *Vernonia amygdalina* (*V. amygdalina*) to control gastrointestinal parasites in both animals and man, as reported by different researchers (Toyang & Verpoorte, 2013; Abay *et al*., 2015).

Recently, some other studies on the *in vitro* efficacy of plant extracts using oocyst sporulation inhibition assays were documented (Jitviriyanon *et al*., 2016; Abbas *et al*., 2020; Sharma *et al*., 2021) with a promising result of their use in the control of coccidiosis.

The present study was carried out for qualitative phytochemical analysis of the methanolic leaf extract of *V. amygdalina* and the *in vitro* anticoccidial efficacy of its fractions against *E. tenella.*

2. Materials and Methods

2.1. Plant Collection and Identification

Fresh disease-free leaves of *V. amygdalina* were collected at the flowering stage as one batch from a private garden in Jos town. The sample was identified and authenticated at the Department of Botany, Ahmadu Bello University, Zaria, and voucher sample numbered 7183 was deposited at the herbarium of the department for future reference. The leaves were washed with tap water and air dried on galvanized-wire screens under the shade with occasional shifting until a constant weight was obtained. A sample of ten kilograms (10 kg) of leaves was then powdered with a mechanical grinder and stored in an airtight plastic container until further use.

2.2. Extraction and Fractionation of V. amygdalina Leaves

A 2 kg portion of the powder of the leaves of *V. amygdalina* obtained was subjected to extraction with 10 L of absolute methanol in Soxlet apparatus at 70^{o}C to obtain a methanolic extract. The extract was dried into powder using a rotary evaporator at a temperature of 40^{o}C and stored in an airtight amber-coloured glass bottle under refrigeration until used. For solvent fractionation, the methanol extract was suspended in water and then partitioned sequentially with hexane, ethyl acetate, and butanol, leaving a residual aqueous fraction (Fall e*t al*., 2016).

2.3. Phytochemical Analysis

Chemical tests for the screening and identification of bioactive chemical constituents of the methanolic leaf extract of the plant were carried out using standard procedures as described (Ayoola *et al*., 2008).

2.4. In Vitro Studies of Methanolic Extract of V. amygdalina Leaf and Fractions against E. tenella

2.4.1. Parasite Propagation and Purification

A local strain of *E. tenella* previously subjected to molecular characterization and maintained at the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria (Jatau *et al*., 2016) was used for the study.

To propagate the oocysts, a seed stock of 1x 10^4 oocysts suspended in 1 mL of distilled water was administered orally to 5 broiler chickens at two weeks of age, as recommended by Holdsworth *et al*. (2004).

2.4.2. Oocysts Sporulation Inhibition Assay

The sporulation inhibition assay was carried out by observing the effects of the plant extracts on the sporulation of *E. tenella* oocysts. The unsporulated oocysts suspension was prepared by serial dilution with distilled water to obtain the concentration of 1x 10^5 oocysts/ mL. A volume of 0.2 mL of this suspension was dispensed in each well of 96 flat-bottomed microtitres and mixed with 1.8 mL of six graded concentrations (7.8 mg/mL, 15.625 mg/mL, 31.25 mg/mL, 62.5 mg/mL, 125 mg/mL, 250 mg/mL) of each herbal extract. The control wells contained 1 mL of potassium dichromate and the oocysts suspension. The plates were incubated for 7 days at room temperature with continuous aeration. From incubated suspension, a 100 µL subsample was immediately removed, deposited onto a clean, grease-free microscope slide, and covered with a coverslip. Both sporulated and unsporulated oocysts were counted, and percentage sporulation was determined by counting the number of sporulated oocysts in a total of 100 oocysts as described (Saratsis *et al*., 2; Mikail *et al*., 2016). The oocyst sporulation inhibition was calculated by the formula of Gadelhaq *et al*. (2018):

[(Sporulation % of control – Sporulation % of treated) /sporulation % of control] x 100

2.4.3. Ethical Approval

Ethical approval for using live animals was obtained from the Ethical Committee of Ahmadu Bello University, Zaria.

3. Data Analysis

Data were expressed as the mean. The mean values were compared by one-way analysis of variance (ANOVA) followed by Tukey's test. The statistical analysis was performed with SPSS version 20. The differences between groups were considered significant if $p < 0.05$.

4. Result

4.1. Qualitative Screening of Phytochemical Content of the Methanolic Extract of V. amygdalina Leaf

The phytochemical constituent of the methanolic extract of the leaf of *V. amygdalina* is presented in table 1. The major phytoconstituents included: alkaloids, glycosides, saponins, tannins, and flavonoids. Also present were steroids and triterpenes, but anthraquinones were not detected.

4.2. In Vitro Sporulation Inhibition Rate

The effect of methanolic extract of the leaf of *V. amygdalina* and its fractions on the percentage sporulation inhibition of the oocysts of *E. tenella* is presented in table 2. The oocysts sporulation inhibition rate was significantly ($p < 0.05$) higher in the butanol fraction group than in other treatments. As the concentration of the extracts increased, the oocysts sporulation inhibition rates drastically increased, and the highest oocysts' sporulation inhibition rate was recorded at the concentration of 62.5 mg/mL. Butanol fraction completely inhibited oocyst sporulation (as depicted in figure 1), while some managed to sporulate in other treated groups (Figure 2). The other fractions ranged remarkably in their capacities to inhibit oocysts sporulation in a concentration and time-dependent manner.

Test	Constituents	Inference
Molisch/Wagner's Test	Alkaloids	+
Bontrager's Test	Anthraquinones	-
Fehling's Test	Glycosides	+
Keller-Killiani Test	Cardiac glycosides	+
Frothing Test	Saponins	+
Ferric chloride	Tannins	+
Shinoda Test	Flavonoids	+
Lieberman Buchard	Steroids	+
Lieberman Buchard	Triterpenes	+

Table 1: Phytochemical Constituents of Methanolic Extract of the Leaf of V. Amygdalina Collected from Jos (+)-present (-) -absent

	Concentration of Extract					
Treatment	**7.8**	**15.25**	**31.625**	**62.5**	**125**	**250**
ME	55	57	66	78	85	100
HF	28	37	26	46	57	100
BF	68	72	84	100	100	100
AF	5	15	26	33	45	58
Control	4	3	3	2	2	2

Table 2: Effect of Methanolic Extract of Leaf of V. amygdalina Leaf on Oocysts Sporulation Inhibition Rate of E. tenella ME=Methanol Extract, HF= Hexane Fraction, BF= Butanol Fraction, AF= Aqueous Residue Fraction

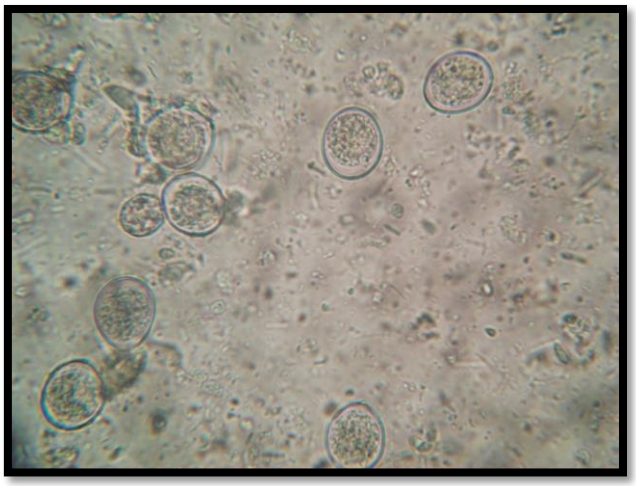

Figure 1: Unsporulted Oocyst of E. tenella from the Butanol Fraction (BF)-Treated Group

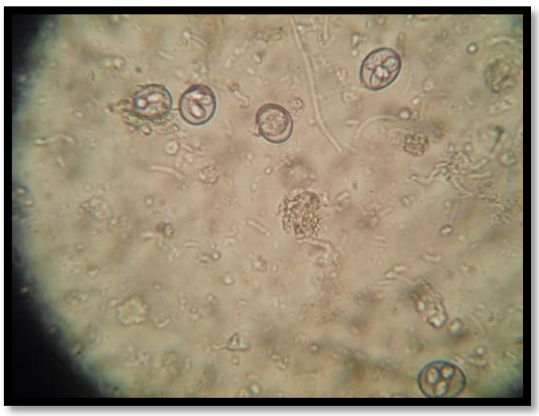

Figure 2: Sporulating Oocysts of E. tenella from Aqueous Fraction (AF) - Treated Group

5. Discussion

The present study revealed the presence of alkaloids, saponins, tannins, and flavonoids in the methanolic extract of *V. amygdalina* leaf and agreed with the findings of Ch'ng *et al*. (2017). The quantitative abundance of the phytochemicals, particularly alkaloids, saponins, and tannins, means they could exert anticoccidial activities against infection with *Eimeria* spp. Imaga and Banigbetam (2013) reported the presence of alkaloids, tannins, saponins, flavonoids, and cardiac glycosides as the most abundant phytochemicals in their study on aqueous bitter leaf extract. Alcohol is generally a better solvent for extracting tannins, flavonoids, alkaloids, and saponins (Alara *et al*., 2020). This concurs with the findings of Adebayo *et al*. (2014), who compared the antimicrobial activity against some beta-lactamase-producing bacteria between the aqueous and alcoholic extracts of the leaf of *V. amygdalina*. They observed that the alcoholic extracts yielded higher values of phytochemicals than the aqueous extracts.

The degree and rate of sporulation of oocysts in the environment usually determine the infection pressure of coccidial parasites in a poultry flock. Any substance that can interrupt the sporulation process could potentially be used to control coccidiosis.

Unsporulated oocysts of *Eimeria tenella* contain a large cargo of carbohydrates: amylopectin, mannitol, and glucose which act as energy stores required for oocyst sporulation (Schmatz *et al*., 1988; Michalski *et al*., 1992). The mechanism of inhibition of oocyst sporulation may be attributed to the ability of phytochemicals to interfere with the enzymes responsible for the utilization of the energy stored in the unsporulated oocyst (Molan *et al*., 2009; Fatemi *et al*., 2015)

Many plant-derived drugs are alkaloids; halofuginone and berberine are well-known among them with anticoccidial effects (Ramadan *et al*., 1997; Zhang *et al*., 2012, Malik *et al*., 2014, 2016).

Results of the study revealed that the leaf of *V. amygdalina* had a high concentration of saponins. The presence of saponins in the leaf of *V. amygdalina* agrees with the report of Adebayo *et al*. (2014). Saponins are present in a number of herbal preparations, which appear to have anti-inflammatory effects.

Tannins were also detected in abundance in the present study. Tannins exhibit their activity through their anti-oxidant property as assessed by their free-radical scavenging activity (Alara *et al*., 2020). Condensed tannins from *Acacia nilotica*, *Eugenia jambolana*, *Ficus reliogiosa*, *Leucaenia leucocephala*, and *Psidium guajava* were reported to exhibit anticoccidial activity by *in vitro* inhibition of sporulation of the oocyst of *Eimeria* spp. (Zargar *et al*., 2017).

Tannins are reported to exert their prospective anticoccidial activity through immunostimulatory properties. Kaleem *et al*. (2014) reported that tannins from *Emblica officinalis* protected industrial broiler chickens from the harmful effects of coccidiosis.

The presence of phenolics in the leaf of *V. amygdalina* was detected in the present study. Plant extracts with an abundance of phenolics have been reported to exhibit anticoccidial properties. The effect of *Plantago asiatica* in ameliorating coccidiosis in chickens has been reported (Hong *et al*., 2016). *Various researchers have reported that Curcuma longa*, which contains a high concentration of phenolics, possesses anticoccidial activity (Kim *et al*., 2013).

Flavonoids were abundantly detected in the methanolic extract of the leaf of *V. amygdalina* in this study. *Camellia sinensis* is a plant with abundant flavonoids whose anticoccidial activity has been reported (Jang *et al*., 2007; Abbas *et al*., 2017). Yang *et al*. (2015) reported that *Bidens pilosa*, which is rich in flavonoids, has anticoccidial activity in chickens infected with *E. tenella*. Cedric *et al*. (2017) also reported the inhibitory effect of flavonoids against oocyst sporulation of the oocyst of *Eimeria* spp. in rabbits *in vitro*. Another *in vitro* study (Allen, 2007) reported the ability of xanthohumol, which is abundant in flavonoids, to inhibit the invasion of MDBK cells by sporozoites of *Eimeria tenella*.

Cardiac glycosides were recorded in the leaf of *V. amygdalina* in this study. This finding agrees with the report of Mbatchou *et al*. (2017), who reported the presence of common secondary plant metabolites, including cardiac glycosides, in the methanolic leaf extract of *V. amygdalina.*

It is concluded that the efficacy displayed by the methanolic extract of *V. amygdalina* leaf and its fractions against oocyst sporulation of *E. tenella* can potentially be utilized as a herbal-based remedy in the control of caecal coccidiosis in broiler chickens. Future research should, therefore, focus on the *in vivo* efficacy testing of the plant extract.

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