



PHYTOCHEMISTRY AND *IN VITRO* ANTI COCCIDIAL EFFECTS OF *ARTEMISIA ANNUA* METHANOL LEAF EXTRACTS ON *EIMERIA TENELLA* OF CHICKEN

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ABSTRACT

Coccidiosis is a grave predicament in the poultry industry and it is one of the top financial burdens. The escalating threat of drug-resistant *Eimeria* species coupled with rising global needs for organic food, has spurred the quest for alternative treatment. The study was aimed at evaluating the *in vitro* anti coccidial effects of methanol leaf extract of *Artemisia annua* (MLEA) on *Eimeria tenella* (*E. tenella*) in chicken. Ninety (90 g) of ground *Artemisia annua* (*A. annua*) leaves was extracted with methanol using cold maceration method. Phytochemical screening showed the presence of carbohydrates (324 ug/ml), phenols (129.9 ug/ml), flavonoids (84 ug/ml), tannins (55 ug/ml), triterpenes (23 ug/ml), alkaloids (22 ug/ml), cardiac glycosides (16 ug/ml), steroids (13 ug/ml) and anthraquinones (0.0012 ug/ml). Four concentrations of MLEA were used (5 mg/ml, 10 mg/ml, 20 mg/ml and 40 mg/ml) to study its effects on sporulation of *E. tenella* oocysts. Amprolium 15 mg/ml was used as the positive control, while 2% potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) was used as the negative control. The extract showed a dose-dependent anti coccidial effect. The highest sporulation inhibition of 100% was obtained at the highest concentration of MLEA used in the study (40 mg/ml). There was no significant (P ≤ 0.05) difference between sporulation inhibition produced by MLEA at 20 mg/ml and 40 mg/ml compared to the standard drug Amprolium. This study showed that methanol leaf extract of *Artemisia annua* possesses anti coccidial effects against *Eimeria tenella* and thus supports the traditional use of *Artemisia annua* as an anticoccidial.

**Keywords:** Anticoccidial, *Artemisia annua*, *Eimeria tenella*, Inhibition, Sporulation

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INTRODUCTION

Coccidiosis is of profound importance in the poultry industry as it poses a substantial threat to poultry health [1]. It is caused by a unicellular protozoan parasite belonging to the genus *Eimeria* [2]. *Eimeria tenella* is one of the coccidial parasites with the highest economic importance in poultry production [3]. Globally, coccidiosis control has relied predominantly on chemical prophylaxis and treatment [4]. In recent times, there has been an alarming upward rise in antimicrobial resistance, coupled with growing concerns regarding the safety of antimicrobials and their negative impact on human, animal and environmental health [5]. Consequently, this has led to an increasing interest in herbal extracts as alternative treatments [6].

MATERIALS AND METHODS

Plant material

*Artemisia annua* leaves were obtained from a local market in Zaria city, Kaduna state and submitted to the Herbarium at the Department of Botany, Faculty

of Life Science, Ahmadu Bello University, Zaria, Nigeria, for identification. The specimen identification number ABU0485 was assigned. The leaves were air dried in the laboratory and then ground into powder form. This was kept in air tight polythene bags until further required.

Preparation of extract

Ninety (90) g of the powdered stem-bark was extracted by cold maceration using 98% methanol with agitation of the mixture at intervals over a period of 72 hrs. A solvent to dry weight ratio of three (3): one (1) was used for the first 48 hrs of extraction, this was followed by rinsing for 24 hrs using a solvent to dry weight ratio of two (2): one (1). The extract was filtered into a beaker and then concentrated in an evaporating dish placed in an oven at 40°C. The extract was then left to stand at room temperature for two days to ensure complete evaporation of any remaining solvent. The extract which yielded 17.8% (W/W) was then stored in a refrigerator till future use.

### **Qualitative and quantitative phytochemical screening**

Qualitative phytochemical screening of AME was conducted to determine the presence of secondary metabolites using standard procedure as described by Evans [7]. All quantitative tests were carried out in triplicates.

### **Total cardiac glycosides**

This was determined according to the method described by [8]. One ml solution of the extract was mixed with 10 ml freshly prepared Baljet's reagent (95 ml of 1% picric acid + 5 ml of 10% NaOH). After an hour, the mixture was diluted with 20 ml distilled water and the absorbance was measured at 495 nm UV/VIS spectrophotometer. For preparation of the standard curve, 1 ml of different concentrations (20-100 mg/L) of securidaside were prepared. Securidaside was isolated from *Securigera securidaca* extract. Total glycosides from triple replicates were expressed as milligram of securidaside per gram of dried extracts.

### **Total steroid concentration**

This was done using the Liebermann-Burchard reaction method [9]. This produced a dark pink to green colour, due to the hydroxyl group reacting with acetic anhydride and H<sub>2</sub>SO<sub>4</sub>. Varying concentrations of cholesterol (10-100 µg/ml) was used for standard calibration curve, which was read at 640 nm using a spectrophotometer. The concentration of steroids was expressed in mg/g of the crude extract.

### **Total triterpenoid concentration**

One ml of the solution of the extract was mixed with vanillin-glacial acetic acid solution 1.5 mls, 5% w/v) and perchloric acid solution (5 ml). The sample solution was heated for 45 min at 60°C and then cooled in an ice-water bath to the ambient temperature. After the addition of glacial acetic acid (2.25 ml), the absorbance was measured at 548 nm, using a UV-visible-light spectrophotometer. Ursolic acid/Cholesterol (20–100 µg/mls in methanol) was used as a standard. Results were expressed as milligram ursolic/cholesterol/linalool acid equivalents (mg ursolic acid/cholesterol/g extract) [10].

### **Total anthraquinone concentration**

This was done using Bornträger-reaction. To 1.0 ml of the extract solution, 1.0 ml solution of 10% H<sub>2</sub>SO<sub>4</sub>, was added and then stirred in a hot water bath for 5 min. The insoluble parts were filtered out and the filtrate solution was left to cool to room temperature. To the filtrate, 0.5 ml of 10% NH<sub>3</sub> was added and then agitated. 5 ml of 0.5N KOH was added. A pinkish red color indicates anthraquinone glycoside. The concentration was determined by checking the dilution factor of each sample compared to the

standard anthraquinone solution (Rhein/Anthracene) at 515 nm [11].

### **Total flavonoid concentration**

This was determined using aluminium chloride colorimetric assay method [12]. To 0.5 ml of the *Artemisia annua* methanol leaf extract (MLEA) and standard solution (0.01-1.0 mg/ml) of quercetin, 2 ml of distilled water was added and subsequently 0.15 ml of sodium nitrite (5% NaNO<sub>2</sub>, w/v) solution was also added. After 6 minutes, 0.15 ml of (10% AlCl<sub>3</sub>, w/v) solution was added. The solutions were allowed to stand for 6 min and after that 2 ml of sodium hydroxide (4% NaOH, w/v) solution was added to the mixture. The final volume was adjusted to 5 ml with immediate addition of distilled water, mixed thoroughly and allowed to stand for another 15 min. The absorbance of each mixture was determined at 510 nm against the same mixture. The total flavonoid was determined as mg quercetin equivalent per gram of sample with the help of calibration curve of quercetin.

### **Total phenolic content**

Total phenol content was quantified using Folin–Ciocalteu colorimetric method [13], using Gallic acid as the standard and expressing results as Gallic acid equivalent per gram of sample. Different concentrations (0.01-0.1 mg/ml) of Gallic acid was prepared in methanol. 0.5 ml of MLEA and each sample of the standard solution was measured then mixed with 2 ml of Folin–Ciocalteu reagent (1:10 in deionised water) and 4 ml of saturated solution of sodium carbonate (7.5% w/v). The tubes were covered and incubated at room temperature for 30 minutes with intermittent shaking. The absorbance was taken at 765 nm using methanol as blank. The total phenol content was determined with the aid of standard curve prepared from pure phenolic standard (Gallic acid).

### **Total saponin content**

Total saponin content was measured according to method described by [14]. A known quantity of freeze-dried extract was dissolved in aqueous 50% methanol and a suitable aliquot (5 mg/ml) was taken. Vanillin reagent (0.25 ml; 8%) were added followed by sulphuric acid (2.5 ml; 72% v/v). The reaction mixtures were mixed well and incubated at 60°C in a water bath for 10 min. After incubation, the reaction mixtures were cooled on ice and absorbance at 544 nm (UV visible spectrophotometer) was read against a blank that does not contain extract. The standard calibration curve was obtained from suitable aliquots of diosgenin (0.5 mg/ml in 50% aqueous methanol). The total saponins concentration was expressed as mg diosgenin equivalents per g dry weight.

### **Total alkaloid content**

This was quantified using the method described by Sharief *et al.* [15]. 1 mg/ml of MLEA was dissolved in 2 N HCl and then filtered. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. 1 ml of this solution was then transferred to a separating funnel together with 5 ml of bromocresol green (BCG) solution and 5 ml phosphate buffer. The mixture was agitated and the complex formed was extracted with chloroform. The extract was collected in a 10 mls volumetric flask and diluted with chloroform. The absorbance was measured at 470 nm. Total alkaloid content was determined as mg atropine equivalent per gram of sample with the aid of calibration curve of atropine.

#### Total tannin content

This was determined by Folin-Ciocalteu method. 0.1 ml of MLEA was added to a 10 ml volumetric flask containing 7.5 ml of distilled water, 0.5 ml of Folin-Ciocalteu phenol reagent, 1 ml of 35% Na<sub>2</sub>CO<sub>3</sub> solution and then made up to 10 ml with distilled water. The mixture was agitated well and kept at room temperature for 30 min. A set of reference standard solutions of Gallic acid (20, 40, 60, 80 and 100 µg/ml) was prepared in the same manner as described earlier. Absorbance was measured at 725 nm with a UV/Visible spectrophotometer. The tannin content was expressed in terms of mg of GAE/g of extract [16].

#### Eimeria tenella oocyst collection.

*Eimeria tenella* oocysts used for this study were isolated from infected chicken caeca. Infected chicken caeca were collected from a slaughter slab in Samaru, Zaria in air tight packs and transported to the Helminthology laboratory, Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine Ahmadu Bello University Zaria. The caeca were sliced open longitudinally to obtain the contents and scrapings, which were then processed using simple flotation method and the oocysts were then prepared in phosphate buffered solution. The oocysts were enumerated with the aid of a microscope and adjusted to a concentration of 200 oocysts/ml in 2% w/v potassium dichromate in distilled water.

#### In vitro sporulation inhibition test

The methanol leaf extract of *A. annua* was tested at 4 concentrations (5, 10, 20 and 40 mg/ml), amprolium 15 mg/ml was used as the positive control, while 2% potassium dichromate was the negative control and amprolium 15 mg/ml was used as the positive control. The experiment was carried out in three replicates. Two hundred (200) unsporulated oocysts were transferred to the wells of a microtitre plate with the aid of a micropipette. The extract at the different concentrations and both positive and negative controls were added to the labelled microtitre plates which were then kept in an incubator at a temperature of 27° C with a relative humidity of 65%. After 48 hrs, the oocysts were washed with physiological buffered saline (PBS) and then checked for sporulation under the microscope. Percentage sporulation inhibition of oocysts was calculated using the following formula:

$$\text{Sporulation(\%)} = \frac{\text{number of sporulated oocyst}}{\text{total number of oocysts}} \times 100$$

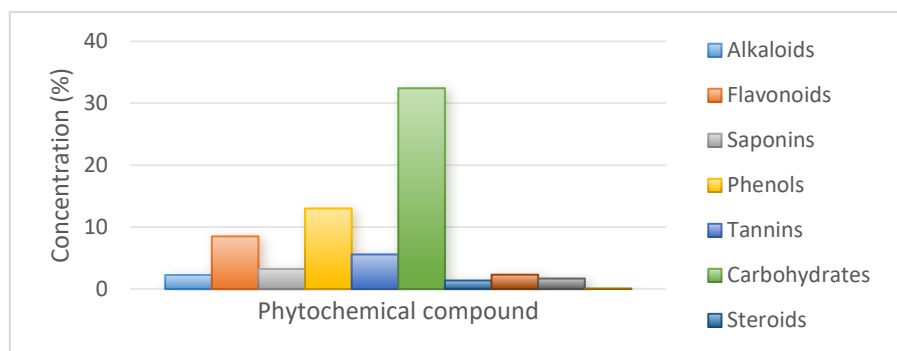
#### Statistical analysis

Results were subjected to analysis of variance (ANOVA). Tukey's post hoc test (Graph Pad prism version 5.0) was used to test for differences in sporulation inhibition between the treatment groups. Values of P ≤ 0.05 were considered significant.

## RESULTS

#### Phytochemical screening

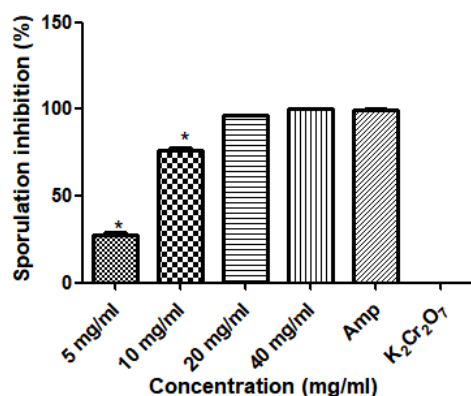
Phytochemical screening of methanol leaf extract of *A. annua* showed the presence of alkaloids, anthraquinones, carbohydrates, cardiac glycosides, flavonoids, saponins, tannins, phenols, steroids and triterpenes. The compound with the highest concentration detected was carbohydrates (324 µg/ml), followed by phenols (129.9 µg/ml), flavonoids (84.9 µg/ml) tanins (55 µg/ml), saponins (32.2 µg/ml), triterpenes (23.0 µg/ml), alkaloids (22.5 µg/ml), cardiac glycosides (16.8 µg/ml), steroids (13.6 µg/ml) and anthraquinones (0.3 µg/ml). Figure 1 shows the different concentrations of the compounds.



**Figure 1:** Percentage concentration of phytoconstituents in methanol leaf extract of *Artemisia annua*

### Sporulation inhibition test

*Artemisia annua* methanol leaf extract produced a concentration dependent sporulation inhibition against *Eimeria tenella* oocysts as shown in figure 2. There was no significant difference between the sporulation inhibition produced by AME at 20 mg/ml and 40 mg/ml when compared to amprolium, but there was a significant ( $p < 0.05$ ) increase in sporulation inhibition produced by amprolium and the extract at 20 mg/ml and 40 mg/ml concentration respectively when compared to extract at 5mg/ml and 10 mg/ml.



**Figure 2:** Effects of methanol leaf extract of *Artemisia annua* on sporulation of *Eimeria tenella* oocysts

\*means with different superscripts are significantly ( $p < 0.05$ ) different

Amp: Amprolium K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>: potassium dichromate

### DISCUSSION

Qualitative phytochemical screening of methanol leaf extract of *A. annua* revealed the presence of alkaloids, anthraquinones, carbohydrates, cardiac glycosides, flavonoids, saponins, tannins, phenols, steroids and triterpenes. Ashok and Upadhyaya [17] detected carbohydrates, saponins, phytosterol, proteins, tannin, phenolic compounds and flavonoids on phytochemical analysis. Ogbonna *et al.* [18], demonstrated the presence of alkaloids, cardiac glycosides, flavonoids, phenols saponins, steroids, tannins and terpenes. The difference in the phytochemical compounds detected is likely due to factors such as variations in the solvent and extraction methods used [19], genetic composition as well as the environment from which the plant was harvested [20]. In this study the phytochemical compound with the highest concentration was carbohydrates, which is similar to the result obtained by [21]. The *in vitro* anticoccidial effect of MLEA against *Eimeria tenella* at 20 mg/ml (96.5%) and 40 mg/ml (100%) concentration was similar to the anti coccidial effect produced by the standard drug amprolium (99.8%). Maodoa *et al.* [22], also obtained similar results using ethanol extract of *Artemisia sieberi* where they recorded a 100%

sporulation inhibition using 300 mg/ml methanol extract of *Artemisia sibirica* leaves. Fatemi *et al.* [23], recorded 31% inhibition at 5 mg/ml using *Artemisia annua* aqueous extract. The anticoccidial effect exhibited by MLEA could be attributed to the phytochemical constituents like polyphenols, flavonoids and terpenoids present in the plant [24]. *Artemisia annua* has been reported to exert its anticoccidial effects due to the presence of artemisinin, a sesquiterpene lactone that is one of the most important compounds isolated from *Artemisia annua* [25, 26]. Artemisinin is thought to act by inhibiting sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) expression by macrogametes [27]. SERCA has an important function in maintaining calcium equilibrium and it also influences the secretion of cell wall-forming structures [28]. It has also been proposed that artemisinin helps to trigger programmed cell death of infected host cells and also suppresses the hosts inflammatory reaction to infection with *E. tenella* [29].

### CONCLUSION AND RECOMMENDATIONS

Methanol leaf extract of *Artemisia annua* (MLEA) showed very good anticoccidial effect by inhibiting the sporulation of *Eimeria tenella* oocysts of chicken *in vitro*. Its activity is comparable to that of Amprolium and could serve as a potential alternative to chemotherapy. We however recommend further studies to evaluate the toxicity profile of the plant and its efficacy *in vivo*.

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