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An in vitro evaluation of the inhibitory effects of an aqueous extract of Acacia nilotica on Eimeria tenella

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Abstract

Eimeria tenella is one of the most important species of *Eimeria* that infect domestic fowl, causing coccidiosis in the poultry industry associated with drastic economic loss. Alternative treatment options are often necessary since anticoccidial drugs are prohibitively expensive, have serious side effects, or develop resistance. The role that herbal therapy plays in basic healthcare has been rediscovered worldwide. Consequently, our research assessed the in vitro inhibitory effect of escalated concentrations (6.25 mg, 12.5 mg, 25 mg, 50 mg, and 100 mg/ml) of Acacia nilotica aqueous extract (ANAE) on *Eimeria tenella* sporulation. Statistical analysis revealed that ANAE decreased the percentage of oocyst sporulation in a dose-dependent manner. Furthermore, ANAE showed abnormal sporulation and morphological deterioration of *E. tenella* oocytes. Area Under the Curve (AUC) calculation was used to determine the efficacy of ANAE and revealed that ANAE concentrations significantly reduced the coccidial score index. At 100 mg/ml, ANAE completely suppressed the sporulation of *E. tenella* oocysts, with obvious changes to their morphology and size. The phytochemical analysis of ANAE has shown that ANAE contains several active principles that possess anthelmintic activities. These compounds include tannins, saponins, flavonoids, terpenoids, and alkaloids, which can be attributed to the anticoccidial activity of ANAE. Considering our findings, we recommend that ANAE be used to prevent and control *Eimeria*.

Keywords: *Acacaia nilotica*, anthelmintic, *Eimeria tenella*, phytochemical analysis, Sporulation.

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Competing interest: The authors have declared that no competing interest exists

Introduction

In the industry of poultry, Coccidiosis is a main economic disease (Williams, 2005). Coccidiosis is the most prevalent disease among growing chickens all over the world. Its spread causes a restriction on the development of the poultry industry. Coccidia has nine species, but seven *Eimeria* are known among chickens, which are *E. maxima, E. acervulina, E. tenella, E. brunetti, E. praecox, E. necatrix, and E. mitis* (Conway and McKenzie, 2007). Avian coccidiosis is categorized as intestinal and cecal forms. *E. tenella* is highly pathogenic, causing bloody cecal coccidiosis (Kaufmann, 1996). Most anticoccidial drugs, such as Amprolium, inhibit the normal growth, metabolism moreover reproduction of coccidia. When anticoccidial drugs are overused, there is a risk of resistance and residues in the poultry (Chapman et al., 1997; Tajick and Shohreh, 2006). Thus, finding new drugs with fewer drug residues and low drug resistance is urgent. Herbal therapy and their byproducts have many advantages, such as few drug residues, side effects, less drug resistance, and low prices. Moreover, they have bioactive components, for instance, tannins, alkaloids, phenolic acids, flavonoids, and terpenes (Abbas et al., 2017a). These components have antioxidant and anticoccidial activity (Abbas et al., 2017b) more than synthetic drugs (Mohiti-Asli and Ghanaatparast-Rashti, 2015). *Acacia nilotica* has various phytoconstituents, including alkaloids, volatile essential oils, phenols, phenolic glycosides, and terpenes. These phytoconstituents have a high therapeutic potential and can be used to prevent and treat various infectious diseases and harmful conditions (Sadiq et al., 2015). Therefore, the study aims to evaluate in vitro inhibitory effect of *Acacia nilotica* pods aqueous extract (ANAE) on *Eimeria tenella* sporulation.

Materials and methods

Collection and processing of plant samples

Taxonomy of *Acacia nilotica* was conducted by the Botany Department, Faculty of Science, South Valley University, Egypt. *Acacia nilotica*'s pods were collected from Qena Province. After a thorough cleaning, they were shaded and dried for 5–7 days at 32–35°C and 50–60% relative humidity. The dried pods were mechanically ground in a commercial stainless-steel blender.

Aqueous extract preparation

The aqueous extract of the *Acacia nilotica* pods sample was prepared via adding distilled water (2 L) to 200 g of powdered pods sample and boiling it for 15 minutes, then filtered by a muslin cloth and Whatman® filter papers. The extracts were concentrated by a laboratory rotary vacuum evaporator at 40°C. The dried extract (chocolate-colored crystals) was weighed, labeled, stored in a clean glass bottle, and kept in the refrigerator at 4°C until needed for usage. The yield was estimated concerning the powdered pod's sample.

Preliminary qualitative phytochemical screening of ANAE

To identify secondary metabolites, present in ANAE, a preliminary qualitative phytochemical analysis was conducted (Harborne, 1998; Trease and Evans,1983).

In vitro inhibitory study:

Eimeria tenella oocysts were obtained from the infected chicken

cecum. Feces obtained from the cecum of infected broilers were dissolved in a saturated salt solution for flotation. Oocysts were then collected and washed with saline before being purified, indicating that they were sedimented by centrifugation at 1300 x G for eight minutes, discarding the supernatant and making a total amount of 6 ml. Using Mc-Master Slides, 13000 oocysts were counted in every 1 ml. we added 1 ml of different concentrations of ANAE (12.5, 25, 50, 100, and 200 mg) and 1 ml of the oocyst solution in potassium dichromate 2.5% to each well. For 5 days, oocyst sporulation was examined and counted daily in each well. A 26-well plate incubated in a humidified chamber was utilized in a modified protocol for *Eimeria* sporulation. A Beaker was filled with water as a humidity source. The oocysts were counted in each well (Williams, 2006). The counting of oocysts in each well was performed as follows: (Williams, 2006). The suspension in a well was properly mixed, and then 1 mL of this sample was diluted with 9 mL of distilled and deionized water before being centrifuged at 400 g for 10 minutes. Using a pipette, 9 mL of the supernatant was withdrawn, and 1 mL of the remaining supernatant was resuspended with the sediment. A 100 μL subsample was immediately extracted, deposited on a clean, grease-free-standard glass microscope slide, and covered with a 64 × 22 mm no. 1 glass coverslip. The slide was inspected with low and high magnifications by an inverted microscope. Oocysts with/without sporulation were counted. Every oocyst observed beneath the coverslips corresponded to one oocyst mL-1 of the suspension in the well. This process was continued until no suspension remained in the well. The total number of oocysts (unsporulated and sporulated) in the well suspension was measured by the average count of two subsamples.

Statistical analysis

GraphPad Prism software was used to perform the One-Way ANOVA and to generate the primary graphs. The Percentage-Maximum-Possible Effect (% MPE) was calculated as the percentage difference between the measured (treated sample) and baseline response (control sample) and divided by the difference between the maximum response and the baseline response. All data were presented as Mean ± SD.

Results

The phytochemical profile of ANAE confirms its presence of tannins, saponins, flavonoids, terpenoids, steroids, proteins, amino acids, carbohydrates, and the absence of alkaloids and anthraquinones (Table 1).

Inhibitory effect of different concentrations of ANAE on sporulation rate:

The sporulation rate of different concentrations of ANAE is shown in (Fig. 1). The control group's sporulation rate was 98%, while oocyst sporulation rates were 96, 89, 62, 44 and 3% for 6.25, 12.5, 25, and 50 mg/ml of ANAE, respectively. While ANAE at 100 mg/ml completely inhibited *E. tenella* oocyst sporulation. Sporulation index recorded a highly significant decline ($P < 0.0001$) with ANAE 25, 50 and 100 mg/m. In contrast, the index was found to be insignificant with ANAE 6.25 and 12.5 mg/ml compared to the control (Fig 1A). Data analysis revealed that ANAE inhibited oocyst sporulation with the inhibition

concentration 50% (IC_{50}) was 40.27 mg/ml (Fig 1B).

The impact of ANAE on sporulated oocysts count:

The number of sporulated oocysts in ANAE 50 and 100 mg/ml decreased on day 3 ($P < 0.01$, $P < 0.001$) and significantly on day 5 ($P < 0.0001$) compared control (Fig 2A). Coccidial score of all concentration of ANAE 6.25, 12.5, 25, 50, and 100 mg/ml recorded a highly significant ($P < 0.001$) decrease compared to control group (Fig 2B). The Percentage Maximum Possible Effect (%MPE) was calculated as the percentage difference between the measured and baseline response and divided by the difference between the maximum response and the baseline response. The percentages 10, 20, 53, 73, 98% for 6.25, 12.5, 25, 50, and 100 mg/ml concentrations of ANAE respectively, as represented in (Fig. 3).

Table 1. Phytochemical constituents of the ANAE (+ve present; -ve absent):

Phytochemicals	Test	Result
Alkaloids	Mayer	-ve
	Dragendorff	-ve
Carbohydrate	Molisch	+ve
	Fehling's	+ve
Flavonoids	Lead acetate	+ve
Glycosides	General test	+ve
Saponins	Frothing	+ve
Tannins	Ferric chloride	+ve
Terpenes and steroids	Lieberman- Salkowski's	+ve
Anthraquinones	Free anthraquinones	-ve
	Combined anthraquinones	-ve

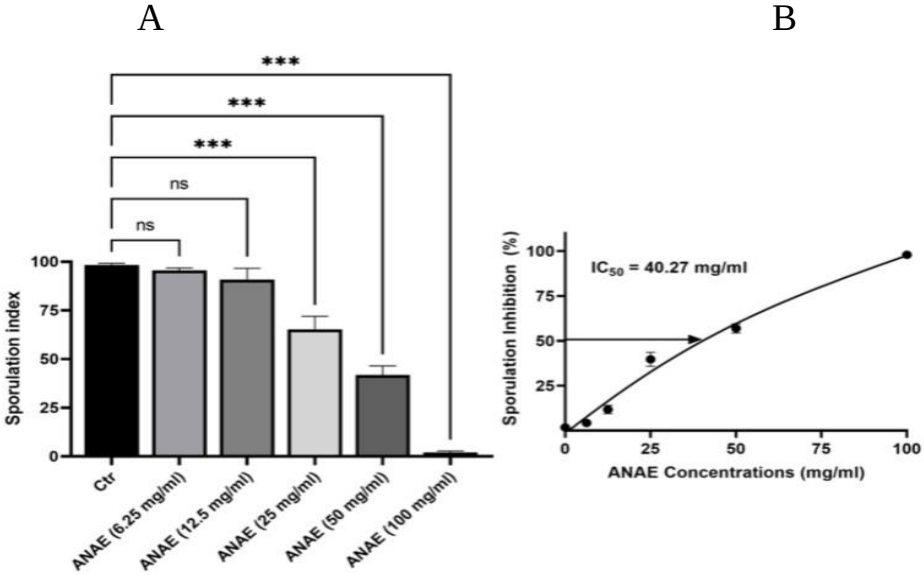


Fig. 1. Sporulation index and IC50 of different doses of ANAE (6.25, 12.5, 25, 50, and 100 mg/ml) for 5 days. The values are presented as the mean ± S.D. of 3 for each group. ***p < 0.001 compared with control group. Sporulation index of ANAE 25, 50 and 100 mg/ml recorded a highly significant ($P < 0.001$) decline compared to control. ANAE exhibits in vitro anti-sporulation activity against *Eimeria tenella* with IC_{50} = 40.27 mg/ml.

A B

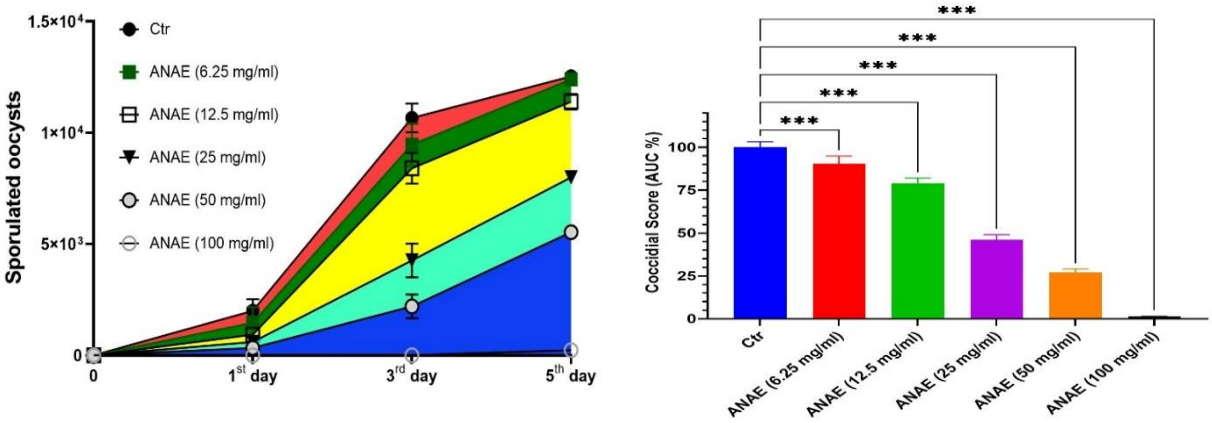


Fig. 2. Sporulated oocysts count and Coccidial score of different doses of ANAE (6.25, 12.5, 25, 50 and 100 mg/ml) on the number of sporulated oocysts for 5 days. The values are presented as the mean ± S.D. of 3 for each group. ***p < 0.001 compared with control group. The number of sporulated oocysts in ANAE 50 and 100 mg/ml decreased on day 3 (P < 0.01, P < 0.001) and significantly on day 5 (P < 0.0001) compared control. Coccidial score of all concentration of ANAE (6.25, 12.5, 25, 50, and 100 mg/ml) recorded a highly significant (P < 0.001) decline compared to control group.

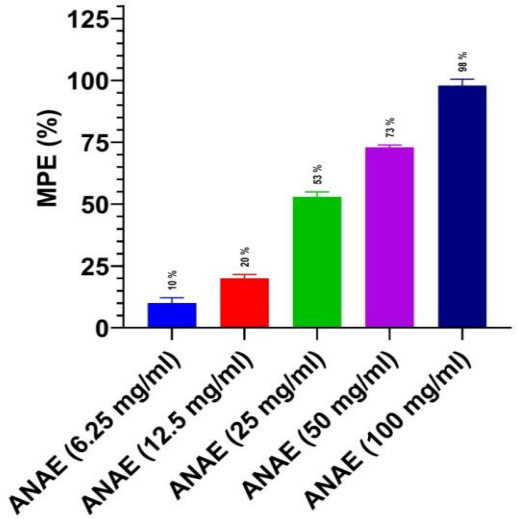


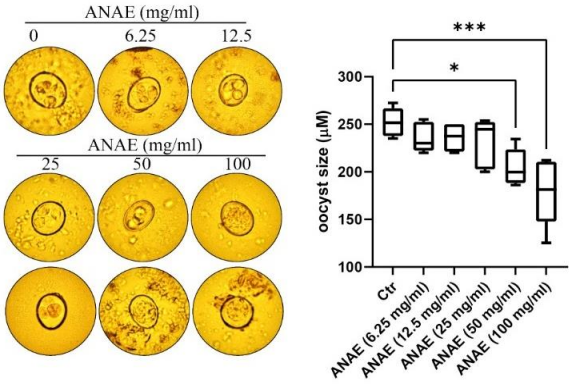
Fig. 3. The Percentage Maximum Possible Effect (% MPE) was calculated as the percentage difference between the measured and baseline response and divided by the difference between the maximum response and the baseline response. The percentages 10, 20, 53, 73, 98% for 6.25, 12.5, 25, 50, and 100 mg/ml concentrations of ANAE, respectively.

Effect of different concentrations of ANAE on morphology and size of *Eimeria tenella* oocyst:

ANAE at 6.25, 12.5, and 25 mg/ml showed morphological alterations in sporocysts and sporozoites, with aberrant sporulation, while the oocyst sporulation process is completely inhibited by damaging of oocyst wall in ANAE 50 and 100 mg/ml as shown in (Fig 4A). ANAE

50 mg/ml induced a significant decrease in oocyst size ($P < 0.05$), with a highly significant decrease in the size ($P < 0.0001$) in ANAE 100 mg/ml (Fig 4).

Fig. 4. Morphological alterations of *E. tenella* oocysts after exposure to



different ANAE concentrations at different time intervals. At varied doses of ANAE, *E. tenella* Oocysts showed obvious morphological changes (alteration of Oocyst, Sporocyst, and Sporozoite morphology) as well as aberrant sporulation. Untreated oocyst shows normal oocyst shape and size, normal sporocysts, and normal sporozoites (untreated control); (ANAE 6.25, 12.5, 25 mg/ml) exhibited significant morphological alterations in sporocysts and sporozoites, with aberrant sporulation. (ANAE 50 mg/ml) Showed significant changes in oocysts morphometry, and morphological alteration in Sporocysts and Sporozoites, the sporulation process came to a halt and did not proceed. (ANAE 100 mg/ml) The oocyst sporulation process is completely inhibited, with visible morphological alterations.

Discussion

The present study investigated the in vitro inhibitory effect of ANAE on the sporulation of *E. tenella* oocyte, which is one of the most crucial factors affecting the epidemiology of coccidiosis since poultry infected by ingesting sporulated oocyst are susceptible to infection. (Conway and McKenzie 2007; Molan et al 2009). Unfortunately, neither physical nor chemical medications have much effect on the *Eimeria* oocyst wall (Mai et al., 2009). Recently, Numerous *in vitro* studies have confirmed that herbal extracts are effective against coccidiosis in birds (Abdel-Tawab et al., 2020; Balta et al., 2021; Yang et al., 2019; Yong et al., 2020). *Acacia* is native to Egypt and widely distributed worldwide in tropical and subtropical countries like Asia, Australia, Africa, and America (Spicer et al., 2007). The plants have many secondary metabolites (Alli et al., 2011; Brenan, 1983) and these secondary metabolites represented in plant extracts have many pharmacological activities in managing and treating numerous diseases. Thus, medicinal plants have an effective role in producing and developing modern studies on the biological activities of natural substances. Phytochemical analysis of ANAE showed the presence of bioactive metabolites such as tannins, saponins, flavonoids, terpenoids, steroids, proteins, amino acids, and carbohydrates. Research showed tannins, flavonoids, and saponins are known to have anticoccidial activity by decreasing sporulation (Anteneh et al., 2021). Tannins and saponins penetrate/s the coccidia's oocyst wall and destroy the cytoplasm, as they inactivate the endogenous enzymes responsible for the cycle of sporulation in chickens (Molan et al., 2004; Mwale et al., 2006; Yim et al., 2011) and (Sanchez-Hernaandez et al., 2019) reported sporozoite damage and growth inhibition of *Eimeria* by the effect of saponins. Saponin also binds to the 4-sterol molecules on the *Eimeria* cell membrane and causes lipids disturbance leading to cell death (Wang et al., 1998; Cheeke, 2000; Alfaro et al., 2007). Du and Hu,

(2004) also demonstrated the inhibiting effect of saponins and Triterpenoid glycosides on the maturing of unsporulated and killing effect on the sporulated oocysts. Polyphenols and flavonoids alter the oocyst wall formation process and inhibit sporulation by destroying the sporozoites (Pop et al., 2019). In this work, we illustrated the ability of ANAE to inhibit *Eimeria* oocyst sporulation by different concentrations of ANAE at 6.25, 12.5, 25, 50, and 100 mg/ml for 5 days. Sporulation inhibition study showed an increase in sporulation inhibition with increasing concentration of the extract, and sporulation inhibition is commonly used to evaluate anticoccidial activity (Molan et al., 2009). It is possible that the phytochemicals had an anti-sporulation effect by interfering with sporulation-related physiological processes such as oxygen availability and suppressing the sporulation enzyme (Zaman et al., 2012). Various concentrations of ANAE not only inhibit the sporulation of oocyst but also produce some morphological changes (shape and size). Similar observations were obtained by **(**Fatemi et al., 2015**)**, who found that petroleum ether (PE) and ethanol Artemisia extracts (E) inhibited sporulation of *Eimeria* oocysts in 2 and 5 ppt concentrations, many oocysts in PE and E groups were wrinkled and contained abnormal sporocysts at the same concentration. The mechanism is unclear, but PE and E extracts of the plant may penetrate the oocyst wall and damage sporozoite in 2 and 5 ppt. Considering that the deformed oocysts are not infective, the impact of reducing the infective oocysts is, thus, doubled (McDougald, 2013; Conway and McKenzie, 2007). Also Wondimu et al., (2021) showed that the exposure to a higher concentration of crude extract (100 mg/ml) produced a greater proportion of oocyst wall deformation, which was 6,000, 9,600, and 5,400 for *Vernonia amygdalina*, *Croton macrostachyus*, and *Azadirachta indica*, respectively. Swelling and corrugation of curcumin-exposed sporozoites at concentrations of 25, 50, 100, 200, and 400 μM may reflect a drastic osmotic disturbance due to affected cell membranes of treated sporozoites (Khalafalla et al., 2011). In conclusion the present study provides insight into the therapeutic potential of ANAE as an effective inhibitor of *Eimeria tenella* sporulation and a promising agent against chicken coccidiosis.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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