

Full Length Research Paper

***In vitro* anticoccidial activity of *Thonningia sanguinea* extract on *Eimeria tenella* and *Eimeria necatrix* sporozoites cells**

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Avian coccidiosis is a parasitic disease which causes considerable economic loss in poultry. The emergence of anticoccidial drug resistance enhances the need for development of novel approach and alternative controls strategies such as plants extract. The anticoccidial activity of the aqueous extract of *Thonningia sanguinea* (AE-THOS) was evaluated on *Eimeria tenella* and *Eimeria necatrix* sporozoites cells invasions *in vitro*. The Madin-Darby-Bovine-Kidney (MDBK) cells invasions rates of *E. tenella* and *E. necatrix* sporozoites untreated and treated with AE-THOS were determined at 3, 12 and 24 h post-infection (PI). The results have shown that AE-THOS concentrations above 2.5 mg/mL inhibits significantly ($p < 0.05$) the cells invasions of sporozoites of both species of *E. tenella* and *E. necatrix*. AE-THOS could be use against avian coccidiosis.

Key words: *Thonningia sanguinea*, *Eimeria tenella*, *Eimeria necatrix*, MDBK cells, sporozoites.

INTRODUCTION

Avian coccidiosis is a parasitic disease caused by the protozoan of the genus *Eimeria*. It causes an inefficient feed utilization, mortality, reduced gain weight and eggs production (Bichet et al., 2003; Allen and Fetterer, 2002). In combination, this pathology and cost treatment cause important economic losses to the world poultry industry in excess of 3 billion dollars annually (Dalloul and Lillehoj, 2006; Li et al., 2005; Yun et al., 2000). Coccidiosis is controlled by chemotherapy using anticoccidial drugs in feed. However, the emergence of resistance and consumer demand for fewer feed additives, have stimulated a new approach and alternatives control strategies against coccidiosis (Chapman and Rathinam, 2007; Williams, 2006). These new strategies include plant extract that have been the subject of several studies

for the coccidiosis control and treatment (Chandrakesan et al., 2009; Christaki et al., 2004; Du and Hu, 2004; Kurkure et al., 2006; Youn and Noh, 2001).

Thonningia sanguinea (THOS) is well known in the African traditional medicine. It is used traditionally to treat hemorrhoids (Gyamfi et al., 2004). The studies have reported an antioxidant activity of *T. sanguinea* (N'guessan et al., 2007) and antisalmonella activity in laying hens (M'baïasbe et al., 2002; Ouattara et al., 2007).

The aim of this study is to evaluate the anticoccidial activity of aqueous extract of *T. sanguinea* on *E. tenella* and *E. necatrix* sporozoites cells invasions *in vitro*.

MATERIALS AND METHODS

Parasites

The species of *Eimeria* were obtained by *in vivo* multiplication. Twelve chicks (Isabrown) of one-day-old were divided in three

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groups of four chicks each. They were fed on unmedicated broiler diet *ad libitum*, raised in cages under coccidian-free conditions. Daily, their faeces were collected and examined for the presence of oocysts in order to monitor environmental contamination. At four weeks age, the first group of chicks was challenged with 5.10^3 oocysts of *E. tenella* and the second group with 2.10^3 oocysts of *E. necatrix*. The third group unchallenged was control. Seven days post infection, chicks were sacrificed and the contents of caecum (chicks challenged with *E. tenella* oocysts) and the intestine contents of chicks challenged with *E. necatrix* oocysts were collected. The collected faeces were filtered and centrifuged at 3000 rpm for 15 min. The pellet was stained in 5% of sodium hypochlorite and incubated at 4°C for 60 min. Then, the oocysts were washed and stored in 2.5% of potassium dichromate at 4°C (Allen, 2007).

Purification of sporozoites

Sporulated oocysts were washed three times with Phosphate Buffer Saline (PBS). The oocysts were cracked with Potter (Elvehjem, Sigma). The liberated sporozoites were excysted with solution containing 0.2% trypsin and 0.7% sodium taurodeoxycholate in a shaking water bath at 41°C for 90 min (*E. tenella*) or 15 min (*E. necatrix*). The liberated sporozoites were freed from debris by passing them through Nucleopore filter (10 µm) pads and washed twice in PBS.

Plant materials

The flowers of *Thonningia sanguinea* were collected at Daloa (central-west of Côte d'Ivoire, west of Africa). The plant was identified and authenticated by Pr AKE Assi at Department of Botany, University of Cocody. After identification, a voucher specimen (N°8355) was deposited at the herbarium of "Centre National de Floristique" of the University of Cocody-Abidjan.

Extraction procedure

The flowers of *T. sanguinea* were cut and air-dried, then reduced to powder. 20 g of the powder was extracted at a temperature of 30°C (by the process of maceration with magnetic stirrer) with 2 L of distilled water. The macerated mixture was filtered and the filtrate was evaporated at 30°C (Rotavapor Büchi) to yield 1 g of solid extract. The extract was stored at -4°C in a dry place before utilization.

Cell culture

75.10^5 cells/ml of Madin-Darby-Bovine-Kidney (MDBK) from Pan African Veterinary Vaccine Center (Origin: ECACC-PANVAC, Ref No: 121/11, Ethiopia) were stained in Eagle's minimum essential medium (EMEM, Gibco) supplemented with 2 mM glutamine, 5% New Born Calf Serum (BCS), 1000 IU/ml penicillin/streptomycin. The cells were cultured on plastic cover-slips using 12-flat bottom well sterile tissue culture plates. The cultured plates were incubated for 24 h at 37°C in an atmosphere of 5% CO₂. The cell confluency was approximately 80%.

Treatment of sporozoites

2.10^5 sporozoites/ml of *E. tenella* freshly prepared, were suspended in 1 ml of PBS containing 0 mg/ml (control); 0.625; 1.25; 2.5; 5; 10;

20 and 40 mg of AE-THOS at 40°C for 1 h. The same treatment was applied with 4.10^3 sporozoites/ml of *E. necatrix*.

In vitro cells invasions by the sporozoites

Following treatment, control and AE-THOS treatment solutions were removed from sporozoites by centrifugation and washed with PBS. The treated and washed sporozoites were added in well containing MDBK cells (approximately cell confluency 80%). Then, the plates were incubated at 37°C in an atmosphere of 5% CO₂. At 3, 12 and 24 h post-infection (PI), culture fluids which included any free, dead or live sporozoites were removed. Cells layers were washed once with sterile PBS and cover slips were fixed in formalin (10%) for 20 min. Cover slips were then colored with technical May Grünwald Giemsa. The slides were examined at 500X magnification and sporozoites within cells and outside cells were counted in 10 microscopic fields per culture (three cultures for each treatment) and the rates of sporozoites that have invaded MDBK cells were calculated from the total observed.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnett's test (Graph Pad software, USA). At 95% confidence interval $p < 0.05$ was considered statistically significant.

RESULTS

At 3 h PI, the cells invasions rates of sporozoites treated with different AE-THOS concentrations were lower compared to untreated sporozoites of control culture. The highest cell invasion rate ($54.97 \pm 5.77\%$) was observed for control culture (Figure 1). The cells invasions rates of *E. tenella* and *E. necatrix* sporozoites suspended in 0.625 and 1.25 mg/ml of AE-THOS did not significantly differ from control culture. The cells invasions rates of the two species of sporozoites suspended in concentrations varying from 2.5 to 40 mg/ml were significantly ($p < 0.05$) lower compared to the control culture.

At 12 h PI, the cells invasion rates of sporozoites suspended in 1.25 mg/ml of AE-THOS was significantly ($p < 0.05$) lower than untreated sporozoites of control culture (Figure 2). The sporozoites cells invasions rates of the both *Eimeria* species treated with AE-THOS varying from 2.5 to 40 mg/ml were lower than those of control culture. The highest cell invasion rate of these concentrations was $31.07 \pm 1.46\%$ for *E. tenella* sporozoites suspended in 2.5 mg/ml of AE-THOS. The minimum cell invasion rate ($10.73 \pm 1.80\%$) was recorded in assay culture containing *E. necatrix* sporozoites treated with 40 mg/ml of AE-THOS. The ability of the sporozoites to invade the MDBK cells has been significantly ($p < 0.05$) inhibited by the treatments with AE-THOS for concentrations varying from 2.5 to 40 mg/ml.

At 24 h PI, the results have shown that 100% of *E. tenella* sporozoites and *E. necatrix* sporozoites have invaded the MDBK cells in the control group (Figure 3).

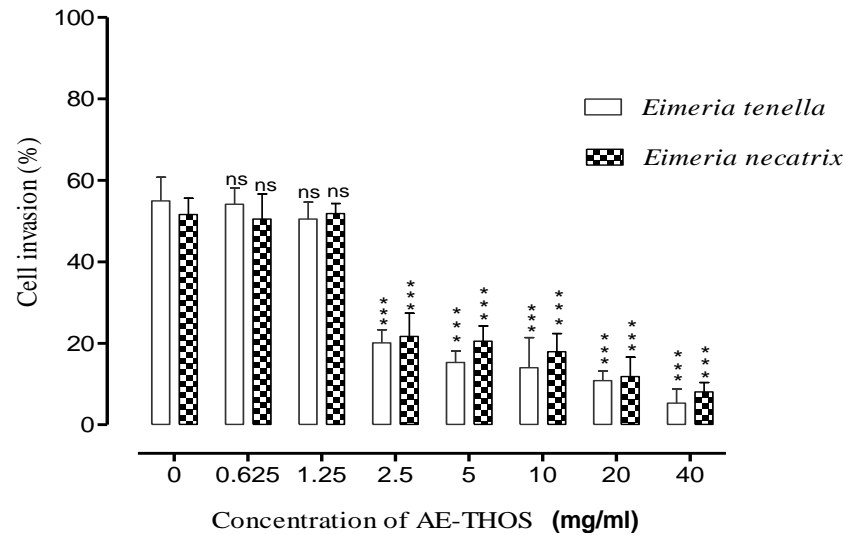


Figure 1. Sporozoites cells invasions at 3 h PI. Values are expressed as mean \pm SEM (n = 3); p < 0.05, * compared to control group; p < 0.01, ** compared to control group; p < 0.001, *** compared to control group and ns no significant compared to control group.

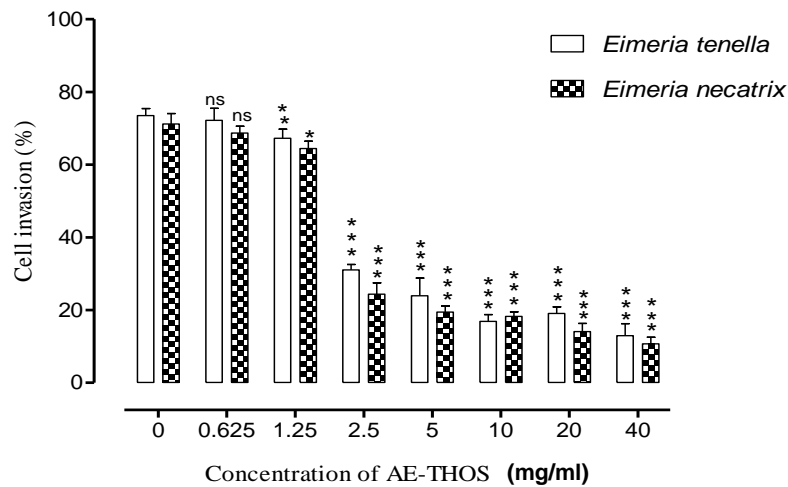


Figure 2. Sporozoites cells invasions at 12 h PI. Values are expressed as mean \pm SEM (n = 3); p < 0.05, * compared to control group; p < 0.01, ** compared to control group; p < 0.001, *** compared to control group and ns no significant compared to control group.

Conversely, the highest cell invasion rate of treated sporozoites was $90.47 \pm 2.01\%$ for *E. tenella* sporozoites suspended in 0.625 mg/ml of AE-THOS. The treatment with concentrations varying from 5 to 40 mg/ml have significantly (p < 0.05) reduced the ability of sporozoites of the both species to invade the MDBK cells. These cells invasion rates were lower more than 50%. The minimum cell invasion rate ($13.87 \pm 1.94\%$) was observed for the culture containing *E. necatrix* treated with 40 mg/ml of

AE-THOS.

DISCUSSION

In this study, the aqueous extract of *T. sanguinea* (AE-THOS) on the ability of *E. tenella* and *E. necatrix* sporozoites to invade MDBK cultured cells was evaluated. The cells invasions rates of control culture and

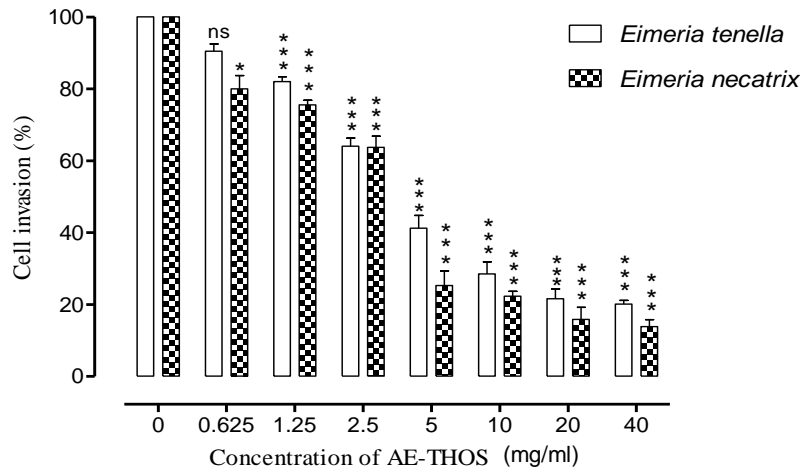


Figure 3. Sporozoites cells invasions at 24 h PI. Values are expressed as mean \pm SEM (n = 3); p < 0.05, * compared to control group; p < 0.01, ** compared to control group; p < 0.001, *** compared to control group and ns no significant compared to control group.

culture containing sporozoites treated with 0.625, 1.25 and 2.5 mg/ml of AE-THOS increased highly during the experiments. Conversely, the cells invasions rates of sporozoites suspended in 5, 10, 20 and 40 mg/ml increased lowly. The treatments with AE-THOS have significantly ($p < 0.05$) inhibited the ability of *E. tenella* and *E. necatrix* sporozoites to invade the cultured cells. Indeed, the aqueous extract of *T. sanguinea* contains phenolic components (N'guessan et al., 2007). These substances are known to possess antioxidant and cytotoxic activities (Francis et al., 2002; Gyamfi et al., 2004; Makkar, 2003; Ohtani et al., 2000), which could explain the inhibitory effect of AE-THOS on sporozoites cells invasions demonstrated in this study. Williams (1997) has shown the oocystocides activities of phenolic disinfectants against the chicken coccidium *E. tenella* in laboratory test. Several studies *in vitro* and *in vivo* have reported the inhibitory effect of plants extracts containing phenolic components. Thus, Abbas et al. (2010) and Khalafalla et al. (2011) have demonstrated that the curcumin, natural polyphenolic component derived from *Curcuma longa*, inhibited the cells invasions of *E. tenella* sporozoites *in vitro* and *in vivo*. The antimicrobial effects of phenolic compounds, known for more than a century, are targeted against the bacterial cell wall affecting the cell wall structure (Botta et al., 2005; Go et al., 2004; Iwashita et al., 2000). Allen (2007) has reported that the xanthohumol, a prenylated chalcone from the hops flowers, reduces the cells invasions rates of *E. tenella* sporozoites *in vitro*. Similarly, Augustine et al. (1997) have demonstrated that the betaine partially inhibits sporozoites cells invasions *in vitro*. Moreover, the beneficial effects of AE-THOS in the treatment of avian coccidiosis caused by *E. tenella* and *E. necatrix* justify

the inhibitory activity of AE-THOS on the cells invasions of sporozoites *in vitro*. These findings are in concordance with the results of Kouakou et al., (2010) who showed that the aqueous extract of *Thonningia sanguinea* (10 mg/mL) reduces the morbidity and the mortality in layers coccidiosis caused by *E. necatrix* and *E. tenella*. Kouakou et al. (2010) have also shown that the aqueous extract of *T. sanguinea* is not toxic for chickens.

Conclusion

This study has shown that the aqueous extract of *T. sanguinea* (AE-THOS) inhibits the ability of *E. tenella* and *E. necatrix* sporozoites to invade the MDBK cultured cells. Sporozoites of *E. necatrix* were more sensitive than *E. tenella* sporozoites. The treatments with the concentrations of AE-THOS above 2.5 mg/ml significantly ($p < 0.05$) inhibit sporozoites cells invasions compared to others treatments. The inhibitory effects of AE-THOS on both *E. tenella* and *E. necatrix* suggested that AE-THOS could be used against avian coccidiosis.

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