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In vitro anticoccidial activity of ethanolic leaf extract of *Citrus aurantium* L. against *Eimeria tenella* oocysts

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Abstract

This study was carried out to examine the anticoccidial effect of *Citrus aurantium L* ethanol leaf extract against the oocysts of *Eimeria tenella* isolated from broiler chickens. The fresh leaves of *C. aurantium* were collected from Emirate Garden, Katsina, authenticated, air-dried at room temperature, pulverised by milling and subjected to extraction. Sporulation inhibition bioassay was employed to examine the activity of *C. aurantium* ethanol extract on the sporulation of *E. tenella* oocysts. In this assay, deep well petri dishes containing 100 unsporulated oocysts were subjected to 2 ml of five different concentrations of the extract (2.5, 5, 10, 20 and 30 mg/ml) in triplicates while oocysts sporulated in 2.5% potassium dichromate solution ($K_2Cr_2O_7$) and phenol served as control groups. The content of the Petri dishes was stirred to ensure adequate oxygenation. The experimental set-up was incubated at room temperature and examined after 24 and 48 hours for sporulation inhibition. The sporulated and unsporulated oocysts were determined by counting using the Mcmaster apparatus. Phytochemical screening of *C. aurantium* revealed the presence of alkaloids, saponins, carbohydrates, steroids and tannins. The result showed that ethanolic leaf extract of *C. aurantium* to possess anticoccidial activity against unsporulated oocysts of *E. tenella* in a concentration-dependent manner. There was significant difference ($p < 0.05$) in the sporulation inhibition activity, with the highest (97 ± 0.8%) at 30 mg/ml and the lowest activity (8 ± 1.0%) at 2.5 mg/ml concentration of the extract after 48 hours of incubation. There was a general trend of sporulation inhibition with an increase in the concentration of the plant extract. The findings from this study showed ethanol leaf extract of *C. aurantium* possesses a remarkable *In vitro* anticoccidial effect that may be further scientifically explicated.

Keywords: Anticoccidial, *Citrus aurantium*, *Eimeria tenella, In vitro,* Inhibition

Introduction

The poultry industry plays a critical role for mankind through the supply of food in the form of high-quality protein, income generation, provision of employment and as a source of raw materials to some industries (El Banna *et al.,* 2016). Globally, the poultry industry spends a significant sum of money on the prevention, control and treatment of several diseases. One of the diseases is avian coccidiosis, which is caused by several species of a protozoan parasite that belongs to the phylum Apicomplexa, family Eimeriidae and genus *Eimeria* (Quiroz-Castañeda & Dantán-González, 2015). *Eimeria* parasite undergoes a direct life cycle with transmission between hosts by way of a resistant oocyst (Quiroz-Castañeda & Dantán-González, 2015). Otu *et al.* (2020) reported that the infection can occur in both local and exotic chickens with the former serving mainly as the reservoir hosts. Protozoan *Eimeria* spp. are the most economically significant parasites of chickens, incurring global costs of more than £10.4 billion per annum (Blake *et al*., 2021). There are seven major *Eimeria* species that are responsible for coccidiosis, including *E. tenella, E. necatrix, E. acervulina*, *E. brunetti, E. maxima, E. mitis and E. Praecox* (Blake & Tomley, 2014). Of all these species, *E. tenella* that causes caecal coccidiosis and *E. necatrix* that is responsible for chronic intestinal coccidiosis are considered to be the most pathogenic (Blake & Tomley, 2014). Infection of the *E. tenella* in poultry injures the intestinal mucosa, which leads to a decrease in nutrient absorption, feed efficiency, and increases secondary bacterial infection (Cooper & Songer, 2009). Coccidia oocysts are highly resistant to both physical and chemical treatment due to the two proteinous layers derived from the amalgam of wall-forming bodies; wall forming bodies type 1 (WFB1) and type 2 (WFB2) found in the macrogamete stage of the parasite (Belli *et al*., 2006). The oocyst wall is impermeable to many common disinfectants, allowing the oocysts to survive and remain infectious in a humid environment for long periods (Ryley, 1973). Phytocompounds can enhance the damaging of a microbial colony (Kasem *et al.,* 2019). On account of constraints due to drug resistance, effects of chemical residue in meat and toxic effects of disinfectants on birds and poultry farm workers, parasitic problems are now being addressed with an alternative approach (Hamad *et al*., 2014). Plant compounds and their derivatives have shown better therapeutic effects against various parasitic, viral, and bacterial poultry diseases (Kiran *et al*., 2018). In this regard, quite a good number of herbal products have been discovered and experimentally validated to have a chemotherapeutic effect against coccidiosis in poultry and are and now being commercialised as a cheaper alternative in the control of poultry coccidiosis (Zaman *et al*., 2012). Botanicals such as *Garcinia kola* (Shetshak *et al*., 2021), *Vitis vinifera* (Abbas *et al*., 2020), *Pentaclethra macrophylla* (Cedric *et al*., 2018) and *Boesenbergia pandurata* (Jitviriyanon *et al*., 2016) have been documented to have good *In vitro* effect against *Eimeria* oocyst. *Citrus aurantium*, commonly known as bitter orange, belongs to the Rutaceae family and possesses multiple therapeutic potentials. It is known to contain several pharmacological important biomolecules whose efficacy is well established by several biochemical and pharmacological studies (Karthikeyan & Karthikeyan, 2014). Its biological credentials include anticancer, antianxiety, anti-inflammatory, antiobesity, antibacterial, antioxidant, pesticidal, and antidiabetic activities (Shen *et al*., 2017; Suntar *et al*., 2018). Based on its reported therapeutic potentials, the current study was conducted to examine the phytochemical constituents and *In vitro* anticoccidial activity of *C. aurantium* against the oocyst of *E. tenella* isolated from broiler chickens.

Materials and Methods

Ethical clearance

The approval for this study was obtained from Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC).

Plant material collection and identification

Fresh leaves **of** *C. aurantium* were collected from Emirate Garden, Katsina town, Katsina State, North-Western Nigeria, in the month of January, 2021. Identification and authentication were done by the curator of the herbarium unit of Botany department, Ahmadu Bello University, Zaria, Nigeria, and a voucher specimen numbered ABU01432 was deposited in the herbarium.

Preparation of plant extract

The leaves of the plant were washed in salt solution in order to get rid of possible microbial contaminants, rinsed in clean water and air-dried at room temperature (25-27^{o}C) under shade (Ahmed *et al*., 2019). The dried leaves were pulverised by milling and subjected to extraction using absolute ethanol for 72 hours at room temperature (Tekwu *et al*., 2017). The solution was sieved and filtered using a filter paper of pore size 2.5µm (Arlette *et al*., 2019). The filtrate was then evaporated using rotary evaporator and the crude extract was stored under freezing temperature until used.

Source of Eimeria tenella unsporulated oocysts

Unsporulated oocysts were harvested from the caecal tissue of three sacrificed chickens that were infected with *E. tenella* in the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria Nigeria. They were harvested according to the standard protocol of harvesting oocysts as outlined by the Royal Veterinary College, London (Blake & Tomley, 2014). First, a glass microscope slide was used to carefully scrape the caecal contents, plus the mucosal and deeper muscle layers, away from the remaining tissue. The scrapings were placed in a beaker containing phosphate-buffered saline (PBS). It was then homogenised, incubated in a waterbath at 41^{o}C for 90 minutes, centrifuged at 2000 rpm, and washed the oocysts in tap water.

Qualitative phytochemical screening of C. aurantium

The phytochemical analysis of C*itrus aurantium* was conducted in the Department of Pharmacognosy and Drug Development of Ahmadu Bello University, Zaria, Nigeria. The analysis was done to test for the presence of anthraquinones, tannins, saponins, flavonoids, cardiac glycosides, terpenoids, steroids, carbohydrates, phenols, alkaloids and other metabolites according to the standard assay procedure as described by Sofowora (1993).

In vitro anticoccidial assay of ethanol extract of C. aurantium

The *In vitro* anticoccidial activity was conducted by observing the effect of the crude ethanol extract of *C. aurantium* on the sporulation time of the *E. tenella* oocysts. The oocysts number was determined using the McMaster counting apparatus. Using the method described by Cedric *et al.* (2018) and with little modification, well petri dishes were used to evaluate the *In vitro* disinfectant activities of the extract. A total volume of 2 ml of each concentration of the extract (2.5, 5, 10, 20 and 30 mg/ml) was placed in each well containing an equal number of unsporulated oocysts (100) and incubated at room temperature. 30 mg/ml concentration was prepared separately, while the remaining concentrations were prepared in two-fold serial dilution. Phenol and potassium dichromate ($K_2Cr_2O_7$) were used as control, and the set-up was examined after 24 and 48 hours. The total number of unsporulated and sporulated oocysts was counted, and the percentage of sporulation and unporulation (sporulation inhibition) was calculated by counting the number of sporulated and unsporulated oocysts in a total of 100 oocysts.

$$\text{Percentage of sporulated oocysts} = \frac{number\ of\ sporulated\ oocysts}{100} \times 100$$

$$\text{Percentage of unsporulated oocysts} = \frac{number\ of\ unsporulated\ oocysts}{100} \times 100$$

Data analysis

The phytochemical analysis data were presented in a Table and the data obtained from the *In vitro* assay were subjected to one-way analysis of variance (ANOVA) using GraphPad Instat, version 3.10 and presented as mean ± standard deviation (SD) of 3 replications. Values of $p < 0.05$ were considered statistically significant.

Results

The phytochemical screening showed the presence of major classes of phytochemicals such as glycosides, saponins, alkaloids, phenolic compounds, tannins, steroids, carbohydrates, flavonoid and terpenoids (Table 1). The photomicrograph of the sporulated and unsporulated oocyst is shown in Figure 1. The crude extract at the concentration of 30 mg/ml shows the highest sporulation inhibition efficacy of 98 ± 0.8% though not significant ($p > 0.05$) when compared to the positive control (5% Phenol).

Table 1: Qualitative phytochemical screening of ethanol leaf extract of *Citrus aurantium* L.

S/No	Phytoconstituents	Test	Inference
1	Alkaloids	Dragendorff test	+
2	Glycosides	Keller-Kiliani test	+
3	Saponins	Frothing test	+
4	Phenolic compounds	Lead acetate test	+
5	Tannins	Ferric chloride test	+
6	Steroids	Salkowiski test	+
7	Carbohydrates	Molisch test	+
8	Flavonoids	Shinoda test	+
9	Terpenoids	Liebermann Bucchard test	+
10	Anthraquinones	Bontragers test	–

Keys: (+) = present; (–) = absent

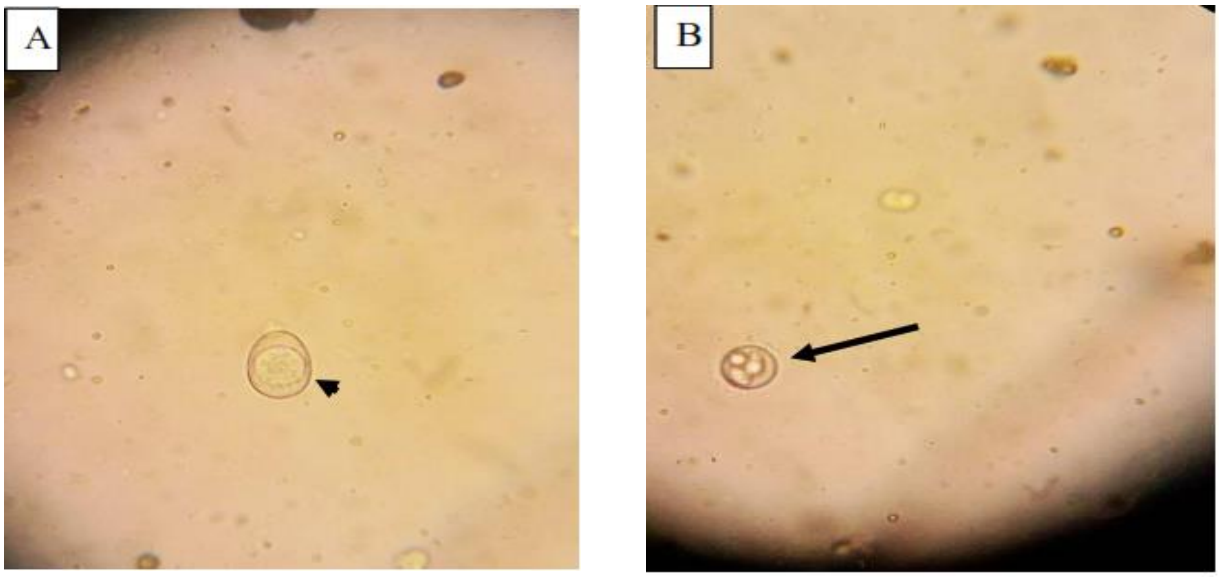

Plate I: (A); Photomicrograph of unsporulated *E. tenella* oocyst (arrow head); (B) sporulated *E. tenella* oocyst (arrow)

Table 2: Percentage sporulation inhibition of *E. tenella* oocysts after 24 hours of incubation

Concentration (mg/ml)	Sporulated oocysts (%)	Unsporulated oocysts (sporulation inhibition) (%)
2.5	91 ± 1.3^{a}	9 ± 1.3^{a}
5	77 ± 0.8^{b}	23 ± 0.8^{b}
10	54 ± 4.6^{c}	46 ± 4.6^{c}
20	16 ± 1.2^{d}	84 ± 1.2^{d}
30	2 ± 0.8^{e}	98 ± 0.8^{e}
$K_2Cr_2O_7$ (-ve control)	98 ± 0.8^{f}	2 ± 0.8^{f}
Phenol 5% (+ve control)	0 ± 0.3^{e}	100 ± 0.3^{e}

values are represented as mean ± SD of triplicate tests. Mean with different superscript letters within column are statistically significant ($^{a,b,c,d,e,f}p < 0.001$). $K_2Cr_2O_7$; potassium dichromate solution (+ve); positive (-ve); negative

However, the Inhibition was noticed to be concentration-dependent (Table 2).

The crude extract at the concentration of 30 mg/ml shows the highest sporulation inhibition efficacy of 97 ± 0.8% though not significant ($p > 0.05$) when compared to the positive control, while the sporulation inhibition of 8 ± 1.0% was observed in oocysts treated with 2.5 mg/ml of the plant extract after 48 hrs of incubation (Table 3). A significant ($p < 0.05$) sporulation inhibition of 8.0 ± 1.0% was observed in the group treated with 2.5% potassium dichromate solution ($K_2Cr_2O_7$) when compared with 100 ± 0.3% that was recorded after 48 hours of incubation in the phenol group (Table 3).

Discussion

The most essential plant bioactive compounds are flavonoids, alkaloids, tannins and phenolic compounds (Mehmood *et al*., 2015). This phytochemical screening carried out in this study is supported by Rauf *et al.* (2014), who reported the presence of phenols, flavonoids, steroids and tannins in *Citrus* species; *C. limonum*, *C. aurantifolia*, and *C. sinensis*. The result is however contrary to the findings of Gunwantrao *et al.* (2016), who established the absence of flavonoids and terpenoids in ethanol extract of *C. aurantium* peel. The difference in the presence or absence of secondary metabolites may depend on the type of solvent, type of extraction or climatic conditions of the region from which the plant samples were taken (Gunwantrao *et al.,* 2016). Several studies have documented the inhibitory potential of plant extracts containing phenolic compounds. Natural polyphenolic components obtained from medicinal plants have been reported to inhibit cell invasion of *E. tenella* sporozoites *In vitro* (Arlette *et al*., 2019). The result is further supported by the study that ascribed

Table 3: Percentage (%) sporulation inhibition of *E. tenella* oocysts after 48 hours of incubation

Concentration (mg/ml)	Sporulated oocysts (%)	Unsporulated oocysts (Sporulation inhibition) (%)
2.5	92 ± 1.0^{a}	8 ± 1.0^{a}
5	79 ± 1.0^{b}	21 ± 1.0^{b}
10	56 ± 2.8^{c}	44 ± 2.8^{c}
20	17 ± 1.0^{d}	83 ± 1.0^{d}
30	3 ± 0.8^{e}	97 ± 0.8^{e}
$K_2Cr_2O_7$ (-ve Control)	99 ± 1.0^{f}	1 ± 1.0^{f}
Phenol 5% (+ve Control)	0 ± 0.3^{e}	100 ± 0.3^{e}

Values are represented as mean ± SD of triplicate tests. Mean with different superscript letters within column are statistically significant ($^{a,b,c,d,e,f}p < 0.001$). $K_2Cr_2O_7$; potassium dichromate solution (+ve); positive (-ve); negative

anticoccidial activity of *Moringa oleifera* to its biological constituents which include flavonoids and phenolic compounds that have an anti-inflammatory properties and antioxidant activities (Gadelhaq *et al*., 2018). The result obtained in this experiment showed that the ethanol leaf extract of *Citrus aurantium* possess *In vitro* anticoccidial effect against the unsporulated oocysts of *E. tenella* in a concentration-dependent manner. This is in accordance with the findings of Cedric *et al.* (2018), who reported the anticoccidial, antioxidant and cytotoxicity activity of *Psidium guajava* extracts in a concentration-dependent manner against four different species of *Eimeria*. The inability of $K_2Cr_2O_7$ to inhibit sporulation could be attributed to the fact that $K_2Cr_2O_7$ is a bactericidal agent (Arlette *et al*., 2019). The interplay between these compounds and the organic system aids in the loss of membrane integrity and loss of cellular materials such as ions, Adenosine Triphosphate (ATP) and genetic material (de Oliveira *et al*., 2019). *C. auratium* is rich in saponins, and studies on saponins have shown that these compounds interact with cholesterol on the sporozoite membrane and thus interfere with the life cycle of *Eimeria* (Felici *et al*., 2020). Remmal (2011) noted the presence of distorted oocyst walls and debris among oocysts treated with essential oil extract. Therefore, in this study, the extract of *C. aurantium* was postulated to have perforated the wall of the oocyst and destroyed the cytoplasmic contents thereby inhibiting sporulation. This is consistent with the findings of Cedric *et al.* (2018), who noted the appearance of abnormal sporocysts in oocysts exposed to higher concentrations of *Pentaclethra macrophylla* and suggested that the extract might have penetrated the wall of the oocysts and damaged its cytoplasm (sporont). It also agrees with the findings of Mikail *et al*. (2016), who showed that the methanolic leaves extract of *Lannea schimperi* were able to dose-dependently weakened the *E. tenella* oocysts wall with subsequent lysis of the oocyst at different concentrations. Phenol was used as a reference disinfectant in this study, and it exhibited a 100% sporulation inhibition efficacy after 48 hours of incubation at room temperature. This is supported by the results of Arlette *et al*. (2019) and Toah *et al*. (2021), who reported a perfect oocysticidal sporulation inhibition by phenol against *E. tenella* after 48 hours of incubation. Disinfection plays a vital role in coccidiosis control, and the purpose of disinfection is to damage the exogenous forms of the parasite (Chroustova & Pinka, 1986). The oocysts of coccidia are resistant to all water-soluble disinfectants, even to the most antagonistic ones like 10% sodium-hydroxide or potassium-hydroxide (Hilbrich, 1975).

In conclusion, the present study revealed the presence of bioactive compounds in the ethanol leaf extract of *C. aurantium,* and the result obtained from the *In vitro* assay revealed a promising anticoccidial activity of ethanol extract of *Citrus aurantium* L. at various concentrations. Further investigation on the isolation, purification, toxicity and mode of action of active compounds in the *C. aurantium* is suggested.

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Conflict of interest

The authors declare that there is no conflict of interest.

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