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In vivo Anticoccidial Efficacy of *Calotropis procera* Leaf Extract as an Alternative Treatment against *Eimeria papillate* Infection in Mice

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ABSTRACT

Background: Coccidiosis is still a serious parasite illness caused by Eimeria pps in many areas throughout the globe. As a consequence of eimeria medication resistance. Coccidiosis needs new drugs that contain natural antiparasitic chemical compounds. This study was conducted to investigate the *in vivo* anti-coccidia activity of *Calotropis Pocera* leaf extract (CPLE) against infection of mice with *Eimeria papillate*.

Methods: The first and second mouse groups were not infected, while the third, fourth, fifth and sixth were given 1×10^3 *E. papillate* sporulated oocysts. The fourth and fifth groups received 100 and 200 mg/kg CPLE orally and the sixth received toltrazuril 25 mg/kg in physiological saline. On day 5 p.i., animals were slaughtered and jejunum samples were prepared.

Result: CPLE phytochemistry revealed 13 chemical compounds. 200 mg/kg was the most efficacious dose, reducing oocyst discharge in mouse faces by 68% and parasite stages in jejunal sections. Infection by *E. papillata* in mice causes inflammation, epithelial vacuolation, villi loss and a decrease in goblet cell density. When infected mice were given CPLE, histological damage was reduced by 69% and goblet cell number was restored to near-control levels. The infected group lost weight owing to emesis. However, the treated group CPLE gained weight.

Key words: *Calotropis procera,* Eimeriosis, Goblet cells, Histology, Jejunum, Mice.

INTRODUCTION

Eimeria is the most prevalent protozoan parasite in the apicomplexan phylum. These protozoan parasites pose a risk to poultry and animals since they are the primary agents in the transmission of avian coccidiosis (Blake *et al*., 2015). The life cycle of Eimeria takes place in two phases: one takes place outside of animals and the other takes place inside the intestines of animals (Lal *et al*., 2009; Mesa-Pineda *et al*., 2021). Eimeria causes damage to the host's digestive tracts and proliferates rapidly in their cells (Metwaly *et al*., 2014; Adhikari *et al*., 2020). In addition to nutrient malabsorption, bloody diarrhea, sometimes weight loss due to inefficient food conversion, increased susceptibility to bacterial infections and mortality in extreme cases (Alnassan *et al*., 2014). Infection with *E. papillate*, one of the species of Eimeria, occurs in the jejunum of mice. This infection results in substantial damage to the intestinal mucosa as well as inflammation (Abdel-Latif *et al*., 2016; Qasem *et al*., 2020). Consequently, *E. papillate* is an excellent model for the investigation of avian coccidiosis (Dkhil, 2013).

There are several medications to treat *Emeria*, such as amprolium, sulfonamides, diclazuril, halofuginone, nicarbazine, robenidine and toltrazuril. However, there are many problems, including parasite resistance to drugs and a lack of effective vaccines, which make the treatment of Emeria with chemical drugs somewhat ineffective and has many side problems (Shirley *et al*., 2007). The pathogenicity and mortality burden of Emeria could be reduced by improving *Emeria* diagnosis, strengthening prevention, using correct therapies and adopting strategies aimed at preventing drug resistance (Hafez, 2008). Medicinal plants have been used to treat Eimeria since ancient times and these plants are promising sources for identifying new types of highly effective compounds for coccidiosis control (Stephen *et al*., 1997). The use of natural plant sources has been used in recent studies for the safe treatment of different parasitic infections. One of the most important of these plants is Calotropis Procera leaf a traditional medicinal plant used to treat pain because of its analgesic and anti-inflammatory properties in mice infected with *E. papillate* (Mai *et al*., 2009).

Calotropis Procera leaf extract, often known as Madar, is a medicinal herb used in a range of herbal compositions

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to treat a variety of diseases and body ailments (Kumari and Chaudhary 2021). The potential therapeutic benefits of CPLE, including its antiviral, anti-stress, anti-aging, anti-radiation, anti-microbial and anti-cancer capabilities, have been described in numerous research papers. The aqueous flower extract has been shown to possess analgesic, antipyretic and anti-inflammatory activities (Mascolo *et al*., 1988; Sriranjini *et al*., 2015). It is a well-known tribal shrub used in numerous folk remedies to cure skin diseases, elephantiasis, toothaches, asthma, leprosy and rheumatism (Sriranjini *et al*., 2015). Calotropis Procera is also important in Ayurvedic medicine. Many polyherbal preparations contain it to cure ailments. Ayurveda is one of the plants that has been evaluated for anticonvulsant action (Balkrishna and Misra, 2017). The whole dried herb can be used as a tonic and it also acts as an expectorant and an anthelminthic agent (Agharkar, 1991). Calotropis Procera has phytochemical elements such as saponins, alkaloids, tannins, flavonoids, glucosides and terpenoids that have medicinal characteristics such as anti-microbial, anti-oxidant, anti-inflammatory, analgesic, spasmolytic, anti-fertility and anti-ulcer. Results indicated that Astragalus membranaceus showed significant *in vivo* anticoccidial effects against *Eimeria papillate* infection (Abdel-Tawab *et al*., 2020). According to the findings, CPLE has a beneficial, inhibiting potential and protective effect on the sporulation of coccidian oocysts and sporozoites *in vitro* (Mutee *et al*., 2022).

The goal of this study is to find out what effect CPLE has on mice that have been infected with *Eimeria papillate*. More experimental investigations and clinical research are needed to determine the plant's additional pharmacological and therapeutic capabilities, which will aid in the creation of key therapeutic medications based on the active phytochemical constituents of *Calotropis procera*.

MATERIALS AND METHODS

Five days before the start of the experiment mice were screened to confirm that they were free of *Eimeria* infection via fecal examination and no eimerian oocysts were found.

Ethical approval

The research was carried out in accordance with the ethical guidelines for the use of animals that were established by the Kingdom of Saudi Arabia (Ethic Committee King Saud University, ethical approval number: KSU-SE-21-86).

Calotropis Procera leaf extract

The CPLE was prepared using a wild plant obtained from the desert of Riyadh, Saudi Arabia and the plant's identification was validated by a taxonomist at the University of King Saud's Department of Botany. The leaves (500 g) were air dried at 42°C, ground into a powder and then extracted for 24 hours at +4°C with 70% methanol (Dkhil, 2013). The resulting extract was concentrated and dried in a rotating vacuum evaporator (Yamato RE300, Japan). The powder was dissolved in distilled water for various investigations or experimental studies.

Phytochemical analysis

The phytochemical analysis of CPLE was performed to identify chemically active compounds according to the recommended protocol of Harris *et al*., (Harris and Galhena 2011). The Direct Analysis in Real Time - Time of Flight - Mass Spectrometry (DART-ToF-MS) analysis was performed using AccuTOF LC-Plus (JEOL, Japan). The extract's volatile components were evaporated in a stream of helium heated to 350°C, then ionized by excited metastable helium atoms before entering the time of flight mass spectrometer's ion source. Most of the molecules are protonated in the positive ionization mode and they don't break apart. Each peak in the spectrum is an [M+H]+ ion.

Mice

In this study, adult male C57BL/6 mice aged 10 to 12 weeks, with an average weight of 21 g/mice, were subjected to the experiment. The animals were kept in pathogen-free environments according to the protocols specified, at a controlled temperature (21°C) with 12 h of light and 12 h of darkness, by using a standard diet and water ad libitum.

Oocysts sporulation

Fresh *E. papillate* un-sporulated oocysts were originally obtained from Prof. Mehlhorn at Duesseldorf University (Duesseldorf, Germany) and maintained by periodic passage through coccidian-free mice. Un-sporulated oocysts were obtained from the feces of mice four days after infection and allowed to sporulate in readiness for use in the experiment. For oocyst flotation, the fecal pellets from each mouse were suspended and diluted using 2.5% (w/v) potassium dichromate in saturated sodium chloride (NaCl). Then it was washed to remove the solution. The number of these newly acquired oocysts was adjusted such that each mouse was given 1×10^3 sporulated oocysts in 100 µl of saline by oral gavage. A McMaster chamber was used to count the sporulated oocysts and the following equation was used to figure out the percentage of sporulation:

$$\% \text{ Efficacy} = \frac{\text{OPG prior to treatment - OPG post-treatment}}{\text{OPG prior to treatment}} \times 100$$

Treatment design

The mice were divided into six groups, with five mice in each group. In Group 1, the control received 100 µl of sodium chloride (0.9% NaCl) daily for 5 days, while in Group 2, the infected received 1×103 with *E. papillate* oocyst without treatment. Group 3 was treated daily with 200 mg/kg of CPLE via gavage, without challenge to determine toxicity. Groups 4 and 5 were orally infected with 1×10^3 *E. papillate* oocysts. Of these, one hour after infection. The last three groups were treated with 100 and 200 mg/kg of CPLE, while the sixth group received 25 mg/kg of Toltrazuril daily for five days, respectively.

The change in weight

Mice were weighed at the beginning of the experiment and on the fifth day before slaughter for all groups to calculate the change in weight.

Sample collection

Each mouse was separated on day 5 after infection in a small cage. Fresh feces samples were collected from the mice and weighed for each mouse once every 24 h and the bedding was changed to eliminate reinfection and oocysts per gram (OPG) of feces were estimated using the McMaster modified technique (Schito *et al*., 1996). Then, all the mice were euthanized and parts of the jejunum were collected and fixed in formalin (10%) for histological and histochemical examination.

Number of oocysts in the jejunum

Jejunum tissue was fixed in 10% neutral formalin buffer, dehydrated in ethanol, embedded in paraffin wax and cut into 5 µm thick sections. Hematoxylin and eosin (H&E) staining was used on the sections (Drury and Wallington, 1980). This fixed tissue was then processed to assess the parasitic developmental stages in count Oocysts were counted in 10 well-oriented villous-crypt units (VCU) for each animal using Olympus BX61 light microscope (Tokyo, Japan).

Histochemistry-counting goblet cells

The prepared paraffin sections were deparaffinized with xylene and then rehydrated gradually in descending ethanol and finally with water. Sections were then stained with Alcian blue (Sigma) to estimate the goblet cells (Allen *et al*., 1986). The number of goblet cells in the jejunum was counted for each animal in at least ten well-orientated villous crypt units (VCUs). Results were given as the mean number of goblet cells per ten villi.

Statistical evaluation

Analysis of ANOVA was carried out in one way and statistical comparisons between groups were made using the Duncan method. Values have been expressed as mean±SD, at a significance level of ≤0.05. GraphPad Prism 5 was used for statistical analysis.

RESULTS AND DISCUSSION

The active chemical compounds produced by plants were obtained (Fig 1) as follows: 2-acetamido butanoate, 1-(3,4-dimethoxy phenyl)-N methanidylmethanamine, benzoxazole, 3-ethyl-2-methyl-, iodide, 1-(3,4-dimethoxy phenyl)-N methanidylmethanamine1,3-diphenyl propane, hydroxycadalene, hexanedioic acid, mono cyclohexyl ester, 2,3-dihydroxy cyclopentane-1-carboxylate, N-1-adamantyl-2-ethylbutanamide, benzyl beta-D-glucopyranoside, dicyclohexyl succinate, cannabioxepane and 2,4-dinaphthyl pentane (Table 1). Many scientific types of research have shown that CPLE has promising efficacy against a wide variety of diseases and body disorders (Auyeung *et al*., 2016). According to subchronic toxicity studies by Thagfan *et al*., (2020), on Mulberry extract, the dose of 200 mg/kg is safe without any distinct toxicity and side effects (Thagfan *et al*., 2020). It was also discovered that a dose of 50 mg/kg of Astragalus root extract can improve the immunity of mice given cyclophosphamide and has significant activity against cholinesterase and oxidative stress in a retrograde amnesia mouse model (Qiu and Cheng 2019; Abdelaziz *et al*., 2019). On day 5 post-infection, the infected group's fecal oocyst output reached its highest level, which was approximately 5966±210.14 oocysts/g feces. PCE was able to suppress the oocysts by about 69% when treating the *E. papillate*-infected mice with 200 mg/kg. Also, the output of oocysts was reduced by about 39% when treating the *E. papillate*-infected mice with 100 mg/kg. While in the group treated with the reference drug Toltrazuril, the oocyst output was about 73% and reached 39.25±5.66 × 10^5/g feces (Fig 2). It was therefore very clear that the dose of 200 ml/kg had the greatest potential to inhibit the production of fecal oocysts. As a result, for the subsequent investigations, we only used the dose of 200 ml/kg. Three doses of CPLE (100, 150 and

Table 1: Identification of chemical compounds by real-time, time of flight and mass spectrometry (DART-ToF-MS) in C. procera leaf extracts (CPLE).

RT*	Experimental mass	Identification	Molecular formula	Unsaturation degree
81	144.06676	2-Acetamidobutanoate	C6H10NO3	2.5
62	150.09531	1-Hydroxy-1-phenyl-2-propanaminium	C9H12NO	4.5
71	162.09011	Benzoxazolium, 3-ethyl-2-methyl-, iodide	C10H12NO	5.5
100	18.10332	1-(3,4-dimethoxy phenyl)-Nmethanidylmethanamine	C10H14NO2	4.5
47	196.12685	1,3-Diphenylpropane	C15H16	8
55	211.13927	Hydroxycadalene	C15H18O	8
42	230.12366	Hexanedioic acid, mono cyclohexyl ester	C12H19O4	3.5
40	241.15942	2,3-dihydroxy cyclopentane-1-carboxylate	C17H21O	7.5
60	253.17408	N-1-adamantyl-2-ethylbutanamide	C16H27NO	7.5
76	270.11055	Benzyl beta-d-glucopyranoside	C13H18O6	5
46	283.17597	Dicyclohexyl succinate	C16H26O4	4
51	306.16096	Cannabioxepane	C21H22O2	11
58	324.18714	2,4-Dinaphthyl pentane	C25H24	14

*RT: Retention time.

200 mg/kg) were tested as a target natural product against coccidia in this study. In terms of anticoccidial efficacy, this study clearly demonstrated that 200 mg/kg was the most effective of all tested doses. Also, we showed that CPLE interfered with the life cycle of *E. papillate* at all stages and also with oocyst sporulation. This was demonstrated by a significant reduction in both of the developmental stages in mice jejunum and the fecal oocyst excretion. Furthermore, significant suppression in the rates of oocyst sporulation was observed in a dose-dependent manner. In addition, the weight of the affected mice decreased compared with the control, while the treatment mice improved by CPLE significantly. *E. papillate* infection significantly (P≤0.05) reduced the weight of mice, where the weight loss rate in the infected group was significantly reduced compared to the control group, in which the weight of mice increased. While in the 200 mg/kg dose treated group with CPLE, the weight increase has been preserved compared to the infected group (Fig 3). Epithelial cells of the jejunum of mice that had been experimentally infected with *E. papillate* oocysts developed different stages of the parasite (Fig 4). When compared to the infected group, the treatment with 200 mg/kg of CPLE resulted in a significant reduction of 68.5% in the number of parasitic stages that were counted per ten villous-crypt units (P≤0.001). The effect of CPLE on the goblet cells in the jejunum. *E. papillate* infection was found to result in a statistically significant reduction (P≤0.001) in the number of goblet cells in the jejunum when compared to the non-infected group through the use of microscopic analysis of Alcian blue-stained jejuna sections. On the other hand, in comparison to the infected group, the jejunum of mice given CPLE had a significant increase in the number of goblet cells (Fig 5, 6). The findings of this study demonstrated the efficacy of CPLE in the treatment of coccidiosis. CPLE anticoccidial effect may be attributed to its saponin content, which acts on protozoan development by interacting with cholesterol present on the parasitic cell membrane, resulting in parasitic death (Zaman *et al*., 2011).

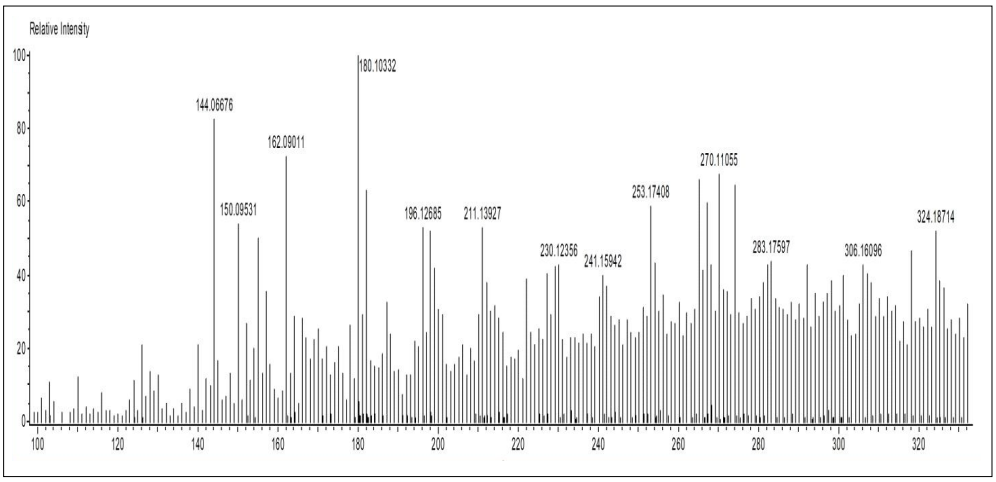


Fig 1: Infrared spectroscopy of calotropis procera leaf extract.

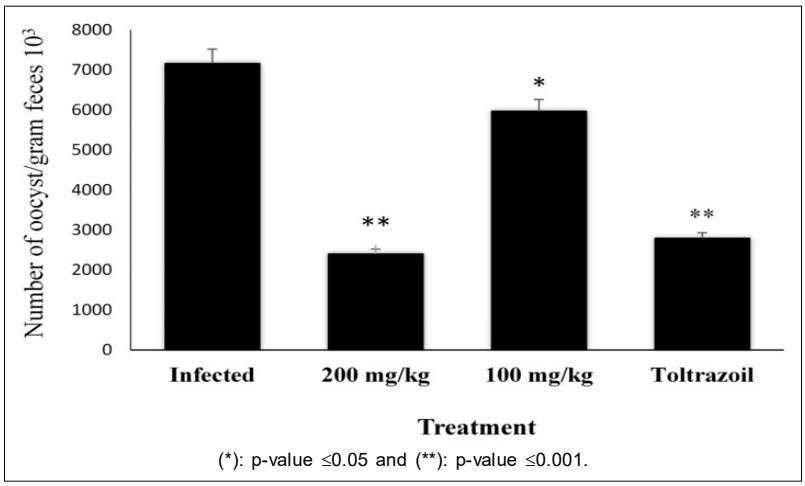
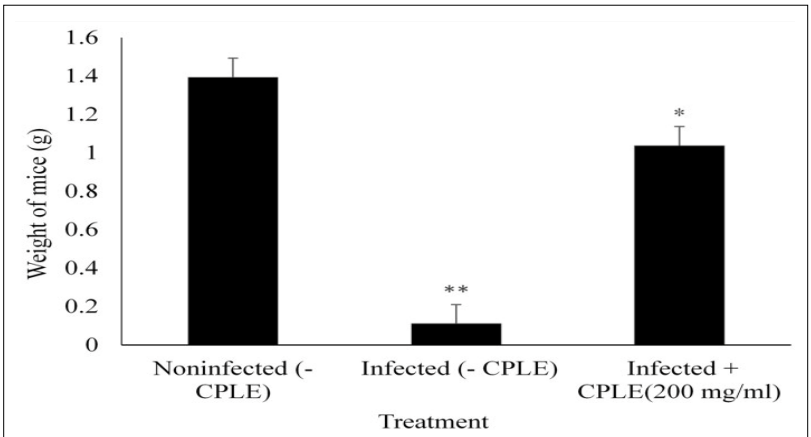


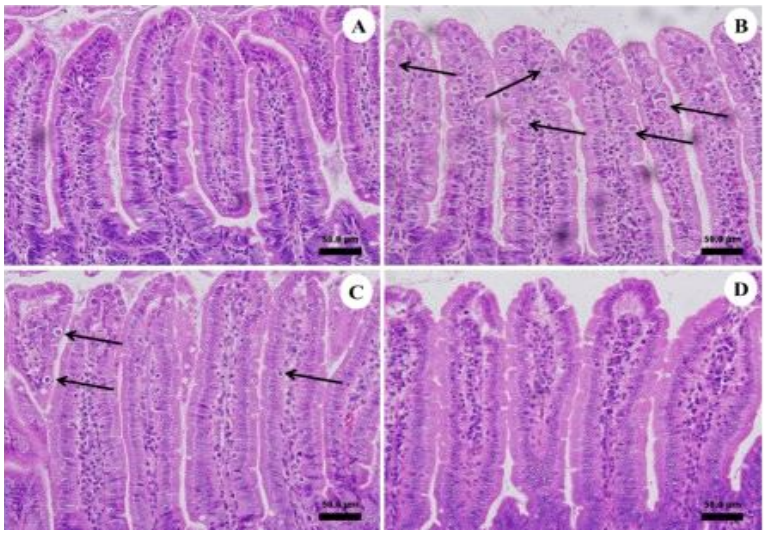
Fig 2: Signs of oocyst output of mice infected with *E. papillate* and treated with various concentrations of CPLE on day 5 p.i. to obtain the best concentration.

Inhibiting oocyst sporulation and sporozoite invasion into cells is a similar mechanism used by Bidens pilosa to treat coccidiosis in hens (Yang *et al*., 2011). Additionally, CPLE suspension seems to prevent oocyst sporulation, which will finally curtail the spread of infection (Fatemi *et al*., 2015; Yang *et al*., 2011). It is recognized that goblet cells can serve as a dynamic protective mechanism against pathogenic bacteria, viruses and parasites by altering the contents of mucus and by increasing their number and size (Khan, 2008). Cheng (1974) Proved that demonstrated stem cells that form goblet cells are confined to the intestinal crypts (Cheng, 1974). Similar to the findings of Thagfan *et al*. (2017), the study of histological sections of the jejunum revealed that the parasitic stages of *E. papillate* were most commonly identified in the crypt region (Thagfan *et al*., 2017). Through the course of infection, the considerable decrease in goblet cell numbers in the infected group may be explained (Fig 7), A result of exposure stem cells being parasitized and losing their ability to create theirs (Dkhil 2013; Lin and Liang, 2019). In this study, while using plant extracts as anticoccidial agents against *E. papillate* infection, we found that treatment with CPLE interferes with the development of the parasite and, as a result, increases the number of goblet cells resulting from injury, as previously reported by Dkhil (2013) and Thagfan *et al*., (2017). Demonstrated that CPLE treatment has an effective effect and activity against *Eimeria* (Forder *et al*., 2012; Thagfan *et al*., 2017). It is inferred that CPLE has strong anticoccidial



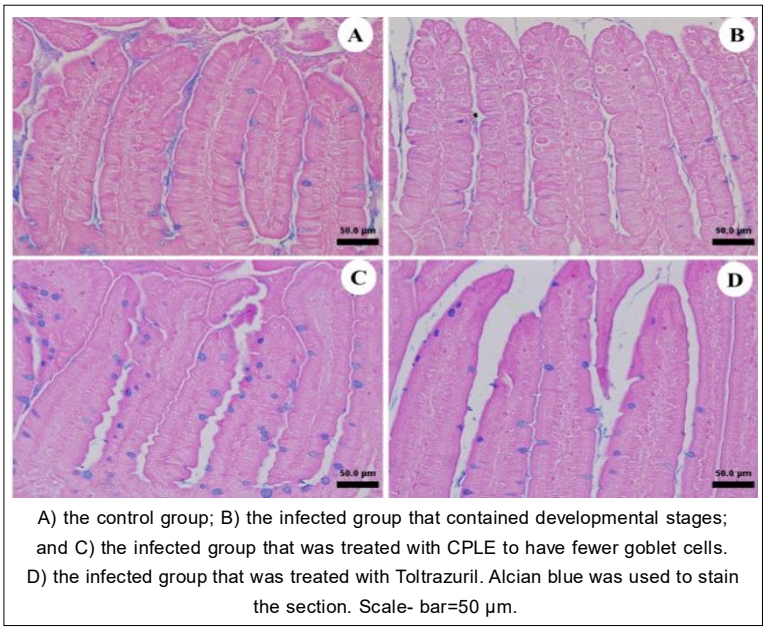
(*): *p*-value ≤0.05 and (**): *p*-value ≤0.001.

Fig 3: Weight changes of mice due to infection with *E. papillate* on day 5 p.i.



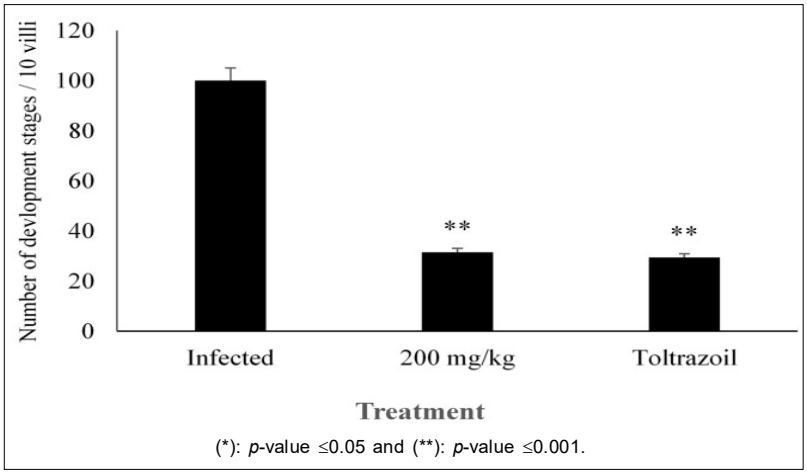
A) the uninfected control; B) an infected sample with development stages indicated by arrows; C) an infected sample that has been treated to have fewer developmental stages; and D) the infected sample that was treated with Toltrazuril with reduced numbers of developmental stages. Scale-bar = 50 µm.

Fig 4: The effect of CPLE on the development stages of *E. papillata* in jejunum histological sections that have been stained with hematoxylin and eosin.



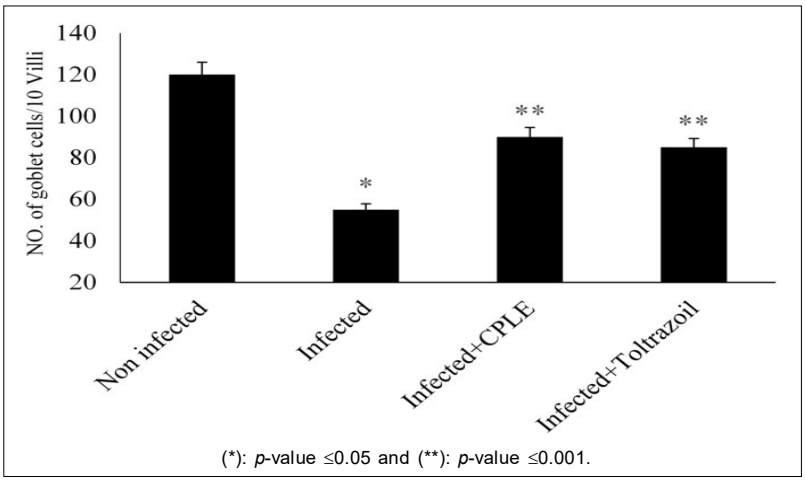
A) the control group; B) the infected group that contained developmental stages; and C) the infected group that was treated with CPLE to have fewer goblet cells. D) the infected group that was treated with Toltrazuril. Alcian blue was used to stain the section. Scale- bar=50 µm.

Fig 5: The effect of CPLE on the number of goblet cells present in the jejunum of mice that had *E. papillate* infection.



(*): *p*-value ≤0.05 and (**): *p*-value ≤0.001.

Fig 6: Effect of CPLE-induced decrease in the number of goblet cells present in the jejunum of mice that had *E. papillata* infection.



(*): *p*-value ≤0.05 and (**): *p*-value ≤0.001.

Fig 7: The effect of CPLE on the reduction in the number of goblet cells in the mice jejuna that had *E. papillate* infection.

CONCLUSION

Calotropis Procera leaf extract has been proven to have anti-inflammatory properties. It is inferred that CPLE has strong anticoccidial activity in light of the aforementioned results. This is corroborated by decreased oocyst release and sporulation, fewer parasite developmental stages in the jejunum and a return to normal goblet cell counts. The results also suggest that CPLE can prevent the development of oocysts.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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