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In vitro Therapeutic Evaluation of *Calotropis procera* Extract against *Eimeria perforans* Oocysts Infecting Rabbits

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ABSTRACT

Background: Plant products are reliable sources of traditional and contemporary medications for animals. This study aimed to investigate the effect of Calotropis procera on Eimeria perforans oocysts that infection rabbits.

Methods: Twelve well-plates of 3 ml containing 1×10^3 unsporulated oocysts were exposed to six treatments: 2.5% potassium dichromate solution as the non-treated control, four concentrations of C. procera extract (12.5, 25, 50 and 100 mg/mL) and toltrazuril 25 mg/mL as traditional medicine.

Result: The mixture was examined (after 24, 48, 72 and 96 hours) for oocysticidal activity. The extract exhibited notable inhibition of *E. perforans* oocysts at 100 mg/mL doses, with suppression rates of approximately 89% after 96 h, nearly similar to the utilized reference drug, toltrazuril. The inhibitory rate increased with extended incubation duration and a high concentration ratio. The current study suggests that C. procera extract possesses inhibitory properties against oocyst sporulation.

Key words: *C. procera*, Destructive, *E. perforans*, Oocysticidal, *Oryctolagus cuniculus*.

INTRODUCTION

The Eimeria genus is the predominant cause of coccidiosis, a widespread and impactful parasitic infection affecting animals. Particularly in local rabbit populations, it leads to substantial mortality rates (Madlala, Okpeku and Adeleke 2021). Adult rabbits that are carriers of a coccidial illness and are generally asymptomatic have the potential to produce infections in young, with morbidity and mortality rates reaching up to 90% and 60%, respectively with young (Fang *et al.,* 2019).

Eimeria perforans, identified as a slightly pathogenic species developing in the duodenum, adds to the complexity of this issue (Katsui *et al.,* 2022). The life cycle of *Eimeria* involves both external and endogenous phases and it is classified as an obligatory parasite in the phylum Apicomplexa (López-Osorio *et al.,* 2020). Entering the host through the oral route, these parasites infect and proliferate within the mucosal epithelia and various digestive tract sections, causing gastrointestinal damage, inflammation, bloody or watery feces, other symptomatic manifestations, morbidity and death (Ananthakrishnan and Xavier, 2020). According to scientific studies, this pathogen has the potential to disrupt the microbiota of the intestinal tract and make its environment of the intestinal tract more conducive to the growth of pathogenic bacteria such as Clostridium perfringens, which can ultimately lead to necrotic enteritis (Madlala *et al.,* 2021).

Eimeria infection destroys the mucosal cells in the host, leading to increased cell permeability, leakage of nutrients and plasma proteins, reduced digestion and decreased protein absorption (Madlala *et al.,* 2021). These side effects contribute to the clinical and subclinical manifestation of coccidiosis (Yang *et al.,* 2020). Dehydration, anemia, hypoproteinemia and nutrient malabsorption are among the potential outcomes of induced villus atrophy, which can also lead to electrolyte imbalance.Consequently, commercial rabbits were economic costs (Montoro-Huguet *et al.,* 2021). Managing these disorders requires costly therapeutic interventions, including chemoprophylaxis and live-attenuated vaccines, both of which have notable disadvantages such as potential parasite resurgence and medication resistance (Madlala *et al.,* 2021). Given existing challenges, the search for novel agents with unique modes of action is imperative (Waters *et al.,* 2023). Natural products, such as *Calotropis Procera*, have emerged as potential supplementary and alternative means of controlling coccidiosis. *Calotropis procera's* anti-coccidial action *C. procera* suggests that it may be a useful substitute for chemotherapy drugs for *Eimeria* species (Murshed *et al.,* 2023).

Calotropis procera, which belongs to the Asclepiadaceae family, is an evergreen, perennial shrub with unique medicinal properties (MANDILA, 2021). Its latex contains triterpenoids,

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alkaloids, cardenolides, anthocyanins, resins and proteolytic enzymes (Mali *et al.,* 2019). *C. procera* thrives in arid environments and open spaces with little competition (Siraj *et al.,* 2023). Treatments for leprosy, fever, menorrhagia, malaria and snake bites have been linked to giant milkweed (McGaw *et al.,* 2022). With known benefits in various ailments, including analgesic, anticancer, anticoagulant, anti-inflammatory (Khan *et al.,* 2020a) and antimicrobial properties, *C. procera* has demonstrated its efficacy in preventing coccidian and inhibiting oocyst sporulation in *Eimeria papillata* (Mpofu, 2022; Murshed *et al.,* 2024). Recent studies have highlighted the oocysticidal activity against Eimeria stiedae (Murshed *et al.,* 2023). Considering this background, the current investigation aims to assess the effectiveness of *C. procera* in handling rabbit *Eimeria perforans* oocysts and sporozoites through in-vitro trials, providing valuable insights into potential alternative therapies for coccidiosis.

MATERIALS AND METHODS

Experiment Implementation

The experiment was carried out in the Parasitology Laboratory, Department of Zoology, College of Science (Kingdom of Saudi Arabia). The experiment was carried out 3-4 months in 2024.

Collected plant leaf

Calotropis procera leaf extracts were obtained from an herbal plant in the desert of Riyadh, Saudi Arabia and the identification of the plant was confirmed by a classification scientist in the Botany Department of King Saud University. Distilled water was used to dissolve the powder in various experiments distilled water was used (Begashaw *et al.,* 2017).

Parasite

This study was conducted by Veterinary and Sanitary Expertise (Saudi Arabia) at the King Saud University Parasitology Laboratory of the Department of Zoology. Samples of rabbit feces were collected for analysis when it was discovered that they were naturally infected with *Eimeria*. Feces were examined for oocysts of *Eimerea* spp. This strain was obtained from *E. perforans*. Oocysts are distinguished based on their morphological characteristics (Ogedengbe, 2011).

In vitro: *E. perforans* oocysts sporulation test by *C. procera*

To determine the effects of different concentrations of *C. Procera* on the sporulation of *E. perforans* oocysts, an experiment was conducted *In vitro*. In this particular test, *C. procera* was introduced into a mixture of 2.5% potassium dichromate, which included 1×10^3 oocysts. The final concentration of the mixtures ranged from 5 to 1.25%. Oocytes in the control group were not treated. Each treatment was performed thrice to ensure accuracy. After each Petri dish had been incubated at a temperature of 28°C for four days, the sporulation percentage was determined for each treatment by counting the number of oocysts that had sporulated and those that had not sporulated using the McMaster technique. Morphological alterations, malformations and destruction percentages were evaluated for each therapeutic intervention.

Preparation of sporulated oocysts

Oocysts of *E. perforans* were obtained from the duodenum. The method according to (Schito *et al.,* 1996).

Statistical analysis

One-way analysis of variance (ANOVA) was performed for every experimental group using the statistical program SPSS 22 (SPSS, Chicago, IL, USA), along with a Duncan post-hoc test for multiple comparisons. The significance level was set at $p \leq 0.05$. To evaluate the correlations between variables, a straightforward linear correlation study was conducted using Pearson's approach.

RESULTS AND DISCUSSION

The effects of different doses of *C. procera* over different periods on the prevention of *E. perforans* sporulation were tested. The methanolic extract showed that the longer the incubation period, the greater the rate of inhibition; the opposite was true for sporulation rate. As the incubation time increased, the rate of sporulation inhibition continued to vary depending on the extract concentration ($p \leq 0.05$) in the treatment groups. In general, the best dose for inhibiting the parasite was 200 mg/ml, compared with the control group (2.5% potassium dichromate) and the reference treatment (Fig 1). The consistent application of natural extracts on animal farms, including rabbits and poultry, could emerge as a therapeutic and preventive strategy to diminish the survival rate of oocysts belonging to the genus Eimeria and prevent them from maturing (Kuralkar and Kuralkar, 2021). The effects of natural product extracts on the oocysts of these parasitic protozoa have been the subject of extensive research. In our previously published study, we investigated the effects of Nerium oleander leaf extract on *E. magna* and *E. exigua* oocysts, bile on *E. columbae* and *E. papillate* oocysts and *Calotropis procera* leaf extracts on *E. stiedae* oocysts (Murshed *et al.,* 2025). In a methanolic extract exposed to oocysts for 96 h, the results demonstrated a significant inhibition ranging from 90% to 98%.

Coccidiosis, a globally prevalent disease, significantly hampers rabbit productivity and leads to substantial financial loss. The associated morbidity and mortality from these parasitic infections not only impair livestock productivity but also pose a severe health risk (Sander *et al.,* 2020). Understanding the current species and prevalence of intestinal parasites is crucial for minimizing financial losses in the rabbit sector, assessing infection risks and implementing effective control measures (Pinto *et al.,* 2024). Numerous authors have explored the effects of diverse plant extracts on oocyst viability and sporulation duration (Murshed *et al.,* 2024; Abd-ELrahman *et al.,* 2022). These extracts have proven effective in inhibiting

sporulation and reducing the vitality of Eimeria oocysts (Khan *et al.* 2020b).

The experimental groups showed significantly higher rates of inhibition with increasing doses and the statistical analysis showed that there were statistically significant differences between the 100 mg/mL dose compared to the control group ($p<0.01$) and between the reference drug toltrazuril (30 µg/mL) compared to the control group ($p<0.05$). The other doses did not show any significant differences (Fig 2). This study investigated the effect of *C. procera* extract over varying durations (24, 48, 72 and 96 h) and concentrations (12.5%, 25%, 50% and 100%) on *E. perforans* oocyst sporulation *in vitro*. The highest effectiveness of the tested concentrations resulted in approximately 91% inhibition of sporulation after 96 h of exposure to the 100% *C. procera* extract. In contrast, the control group ($K_2Cr_2O_7$) exhibited higher levels of oocyst sporulation. The efficacy of different extract concentrations varied depending on the concentration percentage and incubation period of the oocysts. Comparing extract concentrations of 50%, 25% and 12.5% to the control group, the highest rates of inhibition and lowest rates of sporulation were observed, whereas lower concentrations showed no statistically significant differences. These findings align with those of (Murshed *et al.,* 2023b), who reported that *C. procera* may reduce *E. stiedae* oocysts at high concentrations. Extended incubation times and doses often improve inhibition rates. Extract inhibited at doses of 50%, 25% and 10%, with corresponding inhibition rates of 71.7%, 33.11% and 19.88%. Sporulation and inhibition are directly related over an extended period (Murshed *et al.,* 2024).

Fig 3 apparition the main effects of sporulation time and experimental groups on sporulation and non-sporulation (%) of *E. perforans* oocysts sporulation *in vitro.*

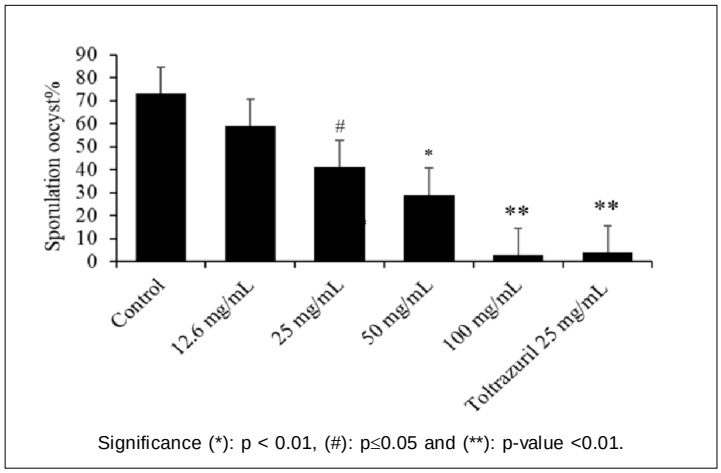


Fig 1: Influences of *C. procera* on the sporulation of *E. perforans* oocysts *in vitro* throughout different periods determine the best concentration of the extract compared to the reference treatment with the control.

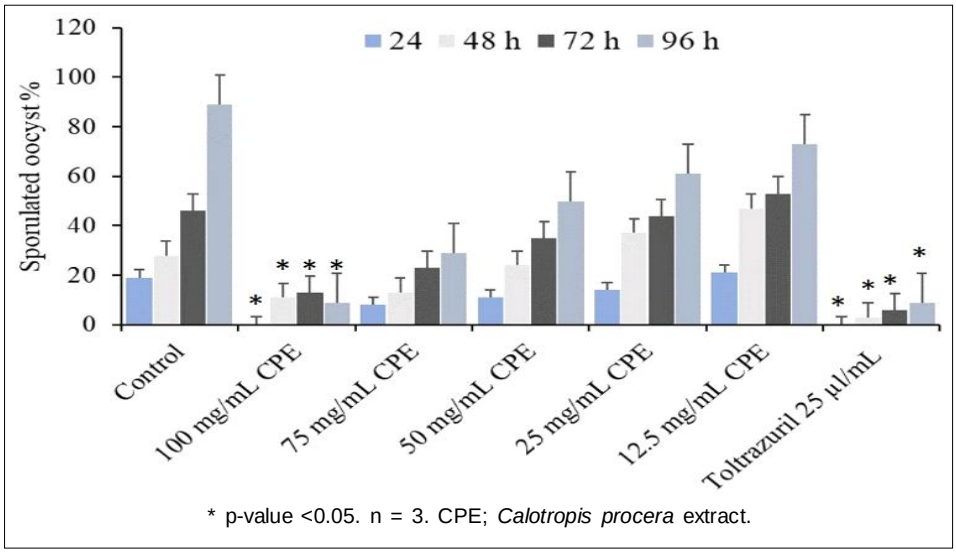


Fig 2: The results show that *C. procera* impacts the number of *E. perforans* oocysts inhibited *in vitro* at different concentrations (Control, 12.5, 25, 50, 100 and 25 Toltrazuril mg/mL).

It demonstrates that the sporulation percentage increased with incubation time and, conversely, the non-sporulation percentage decreased. The rate of sporulation inhibition rose considerably with incubation time up to 96 h ($p<0.05$), showing that the rate of sporulation inhibition differed significantly between 24, 48 and 72 h exposures (Fig 3).

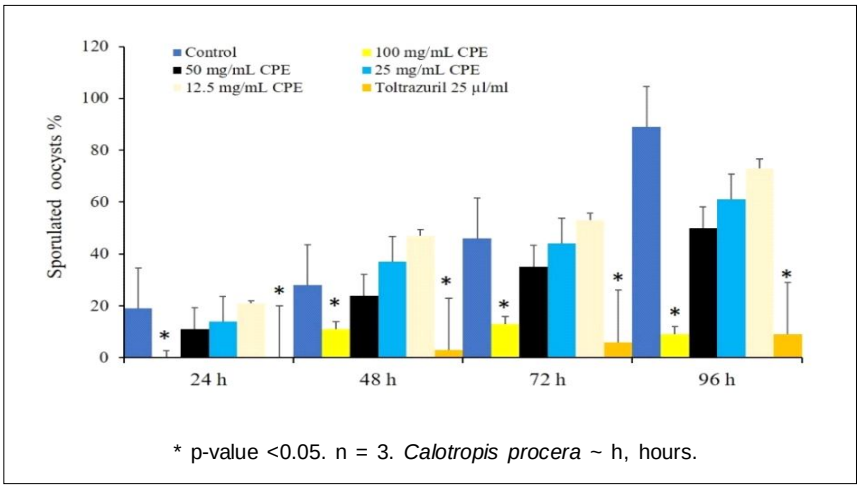
Microscopic examination and counting of the oocysts treated with *C. procera* at various concentrations of *C. Procera* revealed deformed oocysts with cracked walls and lysis. The sporulation rate in the control after three days was 898±1.41. At the doses of 100 mg/mL and the reference drug 30 µg/mL of toltrazuril ($p\leq0.05$), *C. Procera* significantly reduced the sporulation rate ($p\leq0.05$) and it stopped at a dose of 100 mg/mL the sporulation inhibition rates of *C. procera* relative to control were 100%, 99.1%, 71%, 50%, 40%, 27% and 98%, respectively (Table 1).

In vitro experiments show the major impacts of sporulation times and tested groups on the number of *E. perforans*

Table 1: Effect of different doses of *C. procera* on sporulation and destructive rates of E. *perforans* oocysts, *In vitro.*

Concentration Mg/mL	Sporulated oocysts%	Destructive oocysts%
Control	92±1.11^{a}	0.57±0.27^{e}
100 mg/mL extract	10±2.1^{e}	79.21±5.22^{a}
50 mg/mL extract	53±3.9^{d}	61.13±7.23^{b}
25 mg/mL extract	64±4.13^{db}	45.33±7.12^{bc}
12.5 mg/mL extract	76±9.7^{bc}	28.31±4.04^{cd}
Toltrazuril 25 µg/mL	11±0.9^{a}	89.43±5.13^{e}

At a significance level of $p<0.05$, means with distinct superscripts within a column were found to be significantly different.



* p-value <0.05. n = 3. *Calotropis procera* ~ h, hours.

Fig 3: The results show that *C. procera* impacts the number of *E. perforans* oocysts inhibited *in vitro* at different periods (24, 48, 72 and 96 hours).

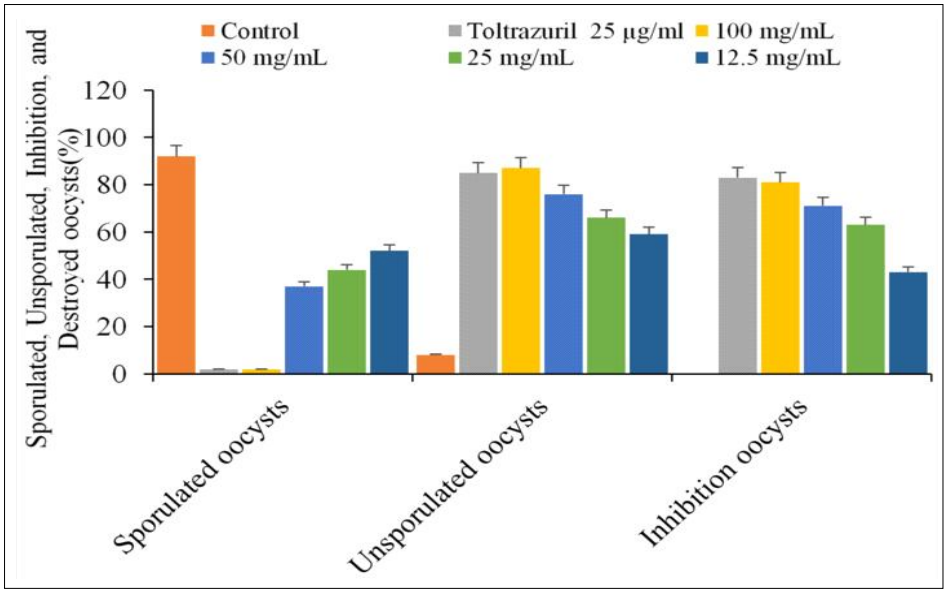


Fig 4: The major *in vitro* impacts of *C. procera* extracts on the percentage of sporulation, non-sporulation and sporulation inhibition oocysts of *Eimeria perforans* oocysts at varying concentrations with the reference drug and control.

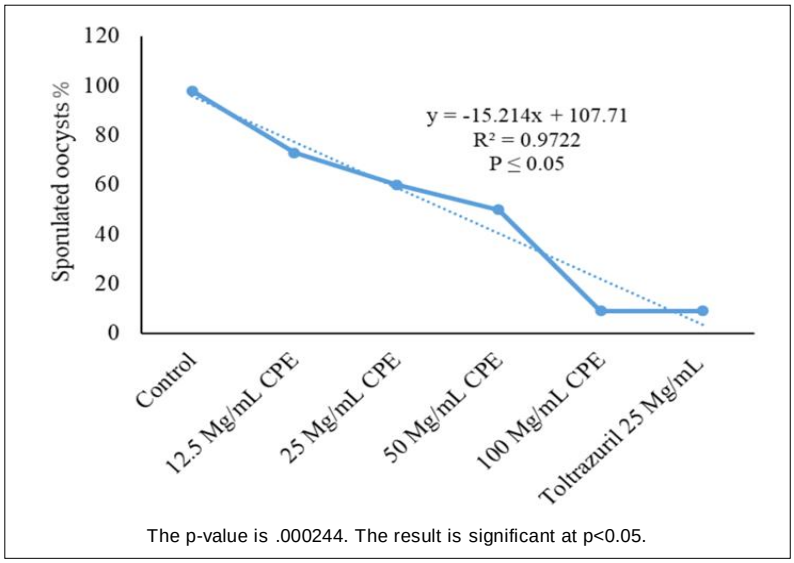


Fig 5: Liner correlation between sporulated and doses of *C. procera*.

oocysts that sporulate (%), don't sporulate (%) and inhibitory (%). The results revealed that as the extract's concentration ratio grew, so did the proportion of inhibition. In the extract groups, on the other hand, when the concentration went down, the number of non-sporulating oocysts and destroyed oocysts also went down (Fig 4).

A negative correlation was observed between the sporulation and concentration. The linear equation had the highest coefficient of determination (R^2) p-value. 000244, The result is significant at p<0.05. for sporulation. The p-value is .039412. The result is significant at p<0.05, for destruction (Fig 5).

The results align with Remmal's study, where the effects of eight plant extracts, essential oils and their combination were investigated as potential treatments for coccidial infections, particularly against Eimeria tenella (Arczewska-Włosek *et al.,* 2023). Remmal independently assessed the primary components of essential oils, including carvacrol, isoeugenol, thymol, eugenol and carvone, were found to be effective against coccidians. In another study, the anticoccidial effects of essential oils from *Artemisia argyi* and *Camellia sinensis,* along with extracts from *Punica granatum* L., *Plantago asiatica* L., *Bidens pilosa* L., *Acalypha australis* L., *Pteris multifida* Poir and *Portulaca oleracea* L., were explored for their potential as anticoccidial agents. These substances demonstrated the ability to inhibit *Eimeria tenella* invasion both *in vitro* and *in vivo* (Han *et al.,* 2022). A study by (Murshed *et al.,* 2023) suggested that *P. macrophylla* extracts might have influenced the decreased sporozoite viability by interfering with calcium-mediated signalling in the sporozoites. Vitis vinifera leaf extracts also affected *E papillae* oocysts (Murshed *et al.,* 2025), which is consistent with our results. The findings affirm that CPLE (Calotropis procera leaf extract) effectively prevents the formation of oocysts.

CONCLUSION

The elimination of parasites is critical to the health of organisms, which in turn can contribute to the development of sustainable production methods that maintain the health of consumers. It was concluded that the extract of *C. procera* leaves plays an important role in inhibiting oocysts and preventing sporulation and it also has a destructive effect against oocysts. Further experimental and clinical studies are needed to understand the pharmacological and therapeutic properties of plants to isolate the active compounds.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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