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Evaluating *in vitro* Efficiency of *Calotropis procera* against *Eimeria flavescens* Oocysts, Isolated from Domestic Rabbits

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ABSTRACT

Background: Rabbit farming is important to boost wealth and increase protein intake. However, common parasitic infestations pose a threat to the survival *Eimeria* spp. The study aims to assess the effect of *Calotropis procera* leaves on *Eimeria flavescens* oocysts and determine the time and concentration to inhibit oocysts.

Methods: Twenty-four-well plates with a volume of 3 mL, each containing one thousand un-sporulated oocysts/mL, were subjected to seven different treatments: Used was 2.5% potassium dichromate solution as the non-treated control; five extract concentrations (10, 20, 40, 80 and 100 mg/mL); In addition, 25 mg/ml toltrazuril as the reference medicine.

The oocysticidal properties of the mixtures were assessed after 24, 48, 72 and 96 h.

Result: The *C. Procera* extract was analyzed and quantitative results showed phenolics, flavonoids and tannins with different rates of bioactive compounds. Moreover, LC_{50} was obtained at 44.2±0.007 g/mL for Hep-G2 cell lines. After 72 and 96 h, it was able to block the *E. flavescens* oocysts at 80% and 100% doses by approximately 81% and 89%, respectively. The relationship between sporulation and concentration was highly negative at all times. Results provide scientific validation for the application of *C. procera* extracts in the prevention of coccidiosis.

Key words: Anticoccidial, Destroyed *eimeria*, Inhibition, Oocysticidal, Sporulation.

INTRODUCTION

The commercial cultivation of rabbits (Oryctolagus cuniculus) as a source of protein has increased recently. Due to consumer preference for rabbits due to their low fat and cholesterol levels, the production of rabbits has become one most valuable animal resources (Lukefahr *et al*., 2000; Mensah *et al*., 2014; Murshed *et al*., 2024). Because of their prolificacy, low herbage intake and high feed conversion, rabbits can produce tonnes of healthy lean meat in a relatively short period (Beaumont *et al*., 2003; Birolo *et al*., 2022). Apart from their commercial worth, these animals serve as valuable models for medical study and are also kept as pets (Mapara *et al*., 2012). Rabbit production has few constraints; however, parasite infections are a problem. Infections of rabbits have the major intestinal parasite species, including nematodes, trematodes, cestodes and coccidia (Eslampanah *et al*., 2014; Raue *et al*., 2017; Marhoon *et al*., 2018). One widespread protozoal illness that affects rabbits worldwide is coccidiosis, which results in a high death rate among domestic rabbits. The protozoan *Eimeria*, which causes coccidiosis, is still one of the most important parasites that hinders rabbit production, generating significant economic losses in commercially raised rabbits (Yin *et al*., 2016). The disease affects organs such as the bile duct, the intestines and the liver and losses may occur as a result of significant morbidity and death (Maertens *et al*., 2000; Abdel-Megeed *et al*., 2005; Manjunatha *et al*., 2019). *Eimeria* species infesting rabbits are numerous, with varying degrees of pathogenicity. They dwell in a variety of habitats in the intestinal and liver (Pakandl, 2009; Pilarczyk *et al*., 2020). There are two anatomic forms: Intestinal (induced by *Eimeria intestinalis, Emagna, E irresidua, E media, E perforans, E flavescens*, or other *Eimeria* spp.) and hepatic (caused by *Eimeria stiedae*). Ingestion of the sporulated oocysts, which are typically found in tainted feed or water, is the means of transmission for both the intestinal and hepatic forms (Siotas *et al*., 2021)**.** The severity of the infection varies according to the species of *Eimeria* involved, the dose of oocysts used and the age and physiological status of the rabbit. Young rabbits often die at high rates during the early phases of weaning (Chowdhury and Fraser, 2008; El-Ghoneimy A, El-Shahawy, 2017). The widespread spread of *Eimeria* spp. necessitates continuous monitoring and the implementation of preventive measures to reduce the rate of infestation. *Calotropis Procera* can be used as an alternate anticoccidial agent to chemotherapy medications for the management of *Eimeria* species (Zaman *et al*, 2012). *C. Procera* is a

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member of the Asclepiadaceae family and widely grows in many regions of Egypt and other nations. An evergreen perennial shrub with soft wood and few stems, branches and leaves that are mostly concentrated towards the growing point (Mutiso, 2023). The primary components of *C. Procera* latex are cardenolides, triterpenoids, anthocyanins, alkaloids, resins and proteolytic enzymes (Balekar, 2016).

Calotropis procera grows well in dry habitats and is frequently observed in open environments with little competition. It has been discovered that the gigantic milkweed is useful in treating leprosy, fever, menorrhagia, malaria and snake bites (Sharma *et al*., 2012). Potential uses for the plant include anthelmintic, analgesic, anticoagulant, anticancer, antipyretic, purgative, antibacterial and inflammatory qualities (Aggarwal *et al*., 2016). In naturally infected, *C. procera* flowers significantly diminish the egg count % of gastrointestinal nematodes and temporarily paralyse red stomach worms (Iqbala *et al*., 2005).

Therefore, the purpose of the study was to ascertain how *C. Procera* affected *Eimeria flavescens* that infected rabbits.

MATERIALS AND METHODS

Plant materials and sample gathering

Fresh *C. procera* leaf samples were collected from the desert of Saudi Arabia. The plant was identified by a specialist in botany at the Faculty of Sciences of Riyadh of King Saud University. After cleaning with distilled water to get rid of any dust, the samples were left to dry for a week at room temperature in the shade. Before being analyzed, about 0.5 kg of leaves were ground into a powder in a grinder for five minutes and kept in dark bags to keep out light and moisture.

Crude extract preparation

The dried powder leaves of 200 gm in 80% methanol were maceration with under stirring at room temperature for 2 days. After filtration (Whatman sheets), the extracts were evaporated to dryness under vacuum (Yamato RE300, Tokyo, Japan) at 40°C., the extract was kept at "20°C until they were employed in an experiment. To dissolve the powder for the various experiments distilled water was used (Zhang *et al*., 2018).

Measurement total phenolic content (TPC)

The Folin-Ciocalteu test was used to calculate the extract total phenolic content (Singleton 1999). Gallic acid was used in the external calibration at several concentrations, including 0.00, 0.25, 0.50, 0.75 and 1 mM. 2.0 mL of solution A (mixing 10 mL of 2% Na_2CO_3 with 0.1 mL of CuSO4 and 0.1 mL of sodium and potassium tartrate) and 200 µL of extracts (1 mg/mL) were combined and after 4 minutes, 0.4 mL of 0.5 M sodium hydroxide was added. 0.2 mL of the Folin-Ciocalteu reagent (1:1 v/v with water) was added after 10 minutes. A UV–Vis spectrophotometer was used to measure the solution's absorbance at 765 nm after it had been left for 30 minutes. Using the gallic acid calibration curve (y = 0.005–x–0.0088), the total phenolic content was determined in mM of gallic acid equivalent (mM GAE). where (y) is absorbance and (x) is concentration.

Measurement content of total tannin (TTC)

The total tannin content was determined by utilizing this approach. An overall volume of 0.1 mL of the extract samples was put into an Eppendorf tube with a capacity of 2 mL, which already included 1.5 mL of Milli-Q water and 0.1 mL of the Folin-Ciocalteu phenol reagent. This mixture was incubated for 8 minutes. After that, to neutralize the solution, 0.3 mL of a sodium carbonate solution containing 35% was added to the combination. After that, the ingredients were thoroughly combined and then placed in a dark, ambient temperature area for twenty minutes. The measured value for the wavelength was 700 nm. The following equation, (Y=0.0013x+0.0052 with R2 = 9937) was utilized to determine the tannin content total that was calculated was given in units of mg/g dry weight (DW).

Measurement content of total flavonoid (TFC)

CPLE's total flavonoids were measured according to the method Ordonez *et al* (2006). 2% AlCl3 (1.0 mL) water solution was combined with 1.0 mL of plant extract (1 mg/mL). Incubation at room temperature for an hour yielded absorbance at 420 nm. The standard solution and standard curve (R2= 0.9996) were prepared using 50-800 g/mL quercetin solution. Flavonoids in the extracts were expressed as quercetin (mg/g DW) using the calibration curve equation, y= 0.0011x+0.0928, where y is absorbance and x is quercetin equivalent concentration (mg/g).

Oocysts preparation

The experiment was conducted at the King Saud University Parasite Laboratory, Zoology. Samples of rabbit feces were collected and examined for Eimeria infection. Using flotation, oocysts were cleaned and concentrated before experimental use. The McMaster method was used to examine stool for *Eimerea* spp. oocytes. More than one type was obtained. The oocysts were distinguished based on their morphological characteristics. 10 oocysts were separated for each species, *E. flavescens* injected was into rabbits free of Eimeria to obtain pure oocysts. Both the prasite oocysts field isolates were kept alive by undergoing periodic transmission (passage) through young rabbits.

Application of the experiment

After obtaining pure Unsporulated E. flavescens oocysts. A test was conducted on the oocyst's sporulation in the solution of 2.5% Potassium dichromate $K_2Cr_2O_7$ (10^3 oocysts/mL) with 3 mg/mL of C. procera dissolved in distilled water at 23°C for 96 h. Used was 2.5% potassium dichromate solution as the non-treated control; five different extract concentrations (10, 20, 40, 80 and 100 mg/mL); In addition, 25 mg/ml Toltrazuril as the reference medicine. The mixture's oocysticidal properties were assessed after 24, 48, 72 and 96 hours. The percentage of sporulated oocysts was assessed by microscopic observation and counting the number of sporulated oocysts in a total of 100 oocysts.

$$\text{Oocysts decrease\%} = \frac{\text{Mean oocyst number of control-Mean oocyst number of treatment}}{\text{Mean oocyst number of control}} \times 100$$

Statistical analysis

The data was analyzed using one-way ANOVA and provided as mean ± SD of three replications. The levels of significance were set at $P \leq 0.05$.

RESULTS AND DISCUSSION

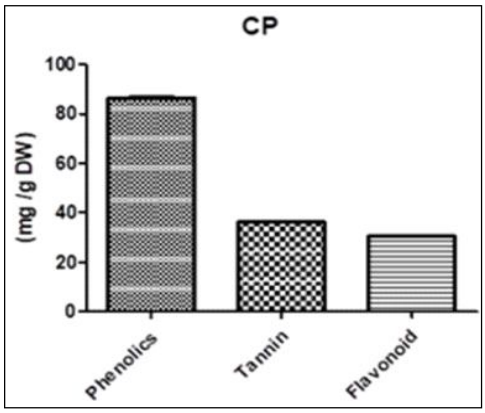


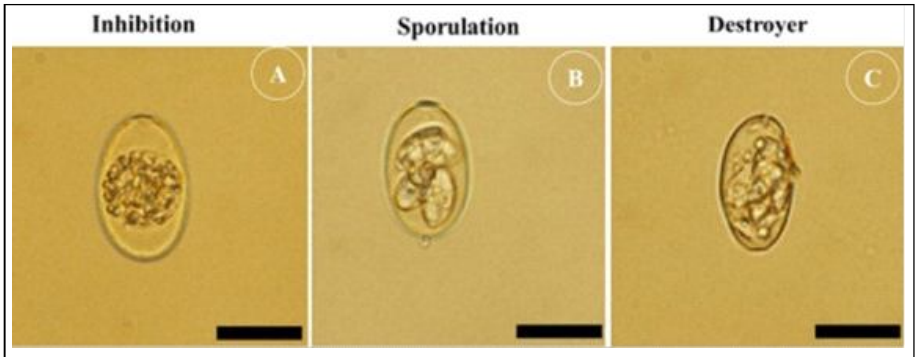
Fig 1: Total of phenols, tannin and flavonoids in methanolic extract of *C. procera* leaf.

Plant extracts have garnered interest as potential anticoccidial agents in the poultry and rabbit industry (MUSA, 2021; Murshed *et al*., 2023). This is because coccidial infections result in production losses, treatment expenses and the prevention of disease (Abebe and Gugsa 2018). Environmental circumstances that are conducive to infection sporulation, are all factors that contribute to the development of coccidiosis (Knight *et al*., 2018). To control this parasite, it is necessary to prevent the process of sporulation from occurring by interrupting the life cycle. In addition to elevating the likelihood of contracting coccidiosis, it has the potential to raise the risk of contracting other infectious diseases and parasitic infections (McDougald *et al*., 2020). CPLE and its metabolites have the potential to serve as an alternate method to anticoccidial medication for coccidiosis administration (Zaman *et al*., 2012).

Fig 1 shows the results of the phenolics TPC, flavonoids TFC and tannin TTC measurements taken of the extract. The total potency concentration of the extract was determined to be 160.22±0.652 mg GAE/g DW, 34.046±0.545 mg QE/g DW and 65.869±0.263 mg TAE/g DW, respectively (Fig 1).

Microscopic examination and enumerating of oocysts treated with *C. procera* at different concentrations demonstrated, inhibited, sporulated and lysis, which contained deformed and deteriorated oocysts with serrated walls, as shown in Fig 2.

The inhibition rates after four days were (89.12 ± 2.11) (78.42 ± 7.34) At the doses of 100 and 80% of extract, compared to the reference drug (91.66 ± 3.13) of 25 mg/ml



(A) Inhibitory oocysts. (B) Sporultory oocysts (C) Destructed oocysts after incubation. Scale-bar = 10 µm.

Fig 2: The morphology of *E. flavescens* oocysts treated with *C. procera* in different concentrations.

Table 1: Effect of *C. procera* different concentrations on rates of inhibitory, sporulation and destructed of *E. flavescens* oocysts.

Concentrations	Inhibited oocysts %	Sporulated oocysts %	Damaged and Ruined oocysts%
Control+2.5 % DPS	00.0	96±1.55^{a}	0.55±0.23^{e}
10 mg/mL CPE	17.21±5.15	82.32±2.45^{e}	5.39±1.1
20 mg/mL CPE	29.12±1.5	54.56±2.39^{d}	12.74±4.52
40 mg/mL CPE	43.25±5.61	33.71±5.35^{db}	29.33±3.35^{cd}
80 mg/mL CPE	78.42 ± 7.34	18.22±4.26^{bc}	50.66 ± 7.02^{bc}
100 mg/mL CPE	89.12±2.11	08.04±2.21^{a}	83.43±7.43^{a}
Toltrazuril 25 µg/mL	91.66 ± 3.13^{e}	05±0.99^{a}	71.33±5.87^{b}

Toltrazuril. CPLE significantly reduced sporulation rate (P≤0.05) and it stopped at a dose of 100 % AT 96 h.

The sporulation rates of CPLE relative to control were 100%, 96±1.55 and 61.85% for 5, 2.5 and 1.25 mg doses, respectively (Table 1). Also, the oocysticidal effect was confirmed by the higher ratio of damaged and ruined oocysts 83.43±7.43, 71.33±5.87 and 50.66±7.02, for concentrations 80, 100% of CPLE doses and 25 mg/ml Toltrazuril, respectively compared to the control.

The highest rates of inhibition and the lowest rates of sporulation were seen when extract concentrations of 40%, 20% and 10% were compared to the control group. Lower concentrations, on the other hand, did not exhibit any statistically significant changes. It is consistent with the findings of (Murshed *et al*., 2022), which stated that *C. procera* has the potential to diminish *E. stiedae* oocysts when present in high concentrations. In several cases, the inhibition rate was enhanced by increasing the dosages and extending the incubation period. who discovered that the level of oocyst inhibition in potassium dichromate might reach roughly 96% at higher concentrations of C. procera. According to the findings, there was a gradual and dose-dependent reduction in the amount of oocyst being produced. extract inhibited at dosages of 40%, 20% and 10%, with corresponding inhibition rates of 79%, 34.32% and 26%.

The results showed variation in sporulation and inhibition with different concentrations during an incubation period of up to 96 hours (four days), during which oocyst test results were taken every 24 hours.

The results showed the highest inhibition of oocyst at the concentration of 100 mg/mL and the reference drug, where the rate of inhibition was almost constant during the periods (24, 48, 72 and 96 hours), compared to the other concentrations where the rate of inhibition decreased at lower concentrations, while the increase in sporulation continued in the control with increasing time (Fig 3 and 4). The results showed the highest sporulation rate of oocysts at 100% in the control group, while the sporulation rate was low at high concentrations despite the increase in incubation periods up to 96 hours, which decreased to a minimum with the reference drug, concentrations 100 and 80% of the extract, while sporulation rates increased at lower concentrations as the incubation period increased (Fig 5 and 6).

The inhibition percentage of the *E. flavescens* oocysts increased with increasing concentration. The inhibition was highly negatively correlated with concentration $R^2 = 0.9274$, p ≤0.01, at 96 h. At Control, 10 mg/ml, 20 mg/ml, 40 mg/ml, 80 mg/ml, 100 mg/ml and Toltrazuril 25 mg/ml (Fig 7). The overall results of the current study suggest that the leaf extract of *C. procera* possesses potential oocysticidal properties, which could potentially be employed in coccidial administration.

The destructed *E. flavescens* oocyst was highly negatively correlated with concentrations $R^2 = 0.921$, p≤0.01(Fig 8).

inhibition were found to have a direct relationship over a considerable amount of time (Han *et al*., 2022). The efficiency of several other extracts varied according to the percentage of concentration and the amount of time spent incubating them. There were no statistically significant differences seen between the low concentrations of

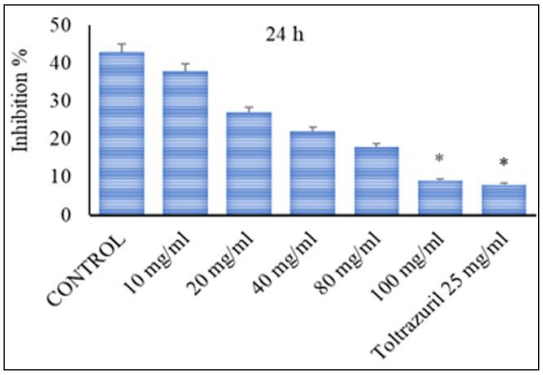


Fig 3: Mean inhibition oocyst % of *E. flavescens* oocysts at all concentrations for exposure duration 24 (hrs). (*): *p*-value < 0.05.

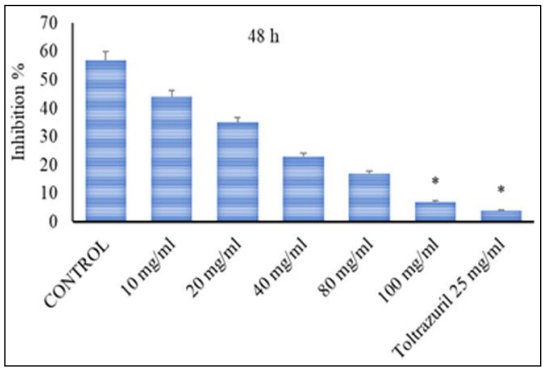


Fig 4: Mean inhibition oocyst % of *E. flavescens* oocysts at all concentrations for exposure duration 48 (hrs). (*): p-value < 0.05.

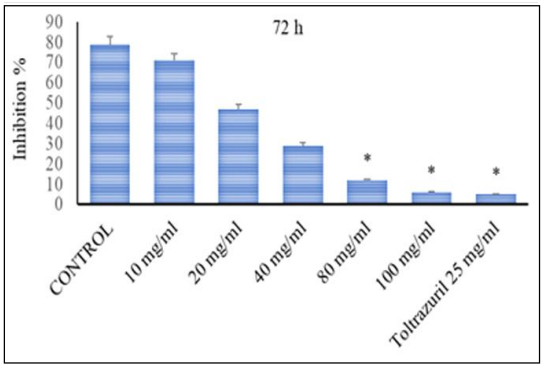


Fig 5: Mean inhibition oocyst % of *E. flavescens* oocysts at all concentrations for exposure duration 72 (hrs). (*): *p*-value < 0.05.

C. procera, which were 50%, 25% and 12.5% respectively. These findings agree with the findings of (McDougald *et al*., 2020), who found that high quantities of sheep bile could inhibit the growth of *E. stiedae* oocysts. According to (Bezerra *et al*., 2017), who discovered that larger doses of extract could inhibit oocysts by around 96% in potassium dichromate, the data presented here are consistent with those findings. As a result, a direct connection between inhibition and sporulation was found to exist throughout a considerable amount of time (Murshed *et al*., 2022). Experimentation revealed that *Punica granatum* L., *Plantago asiatica* L., *Bidens pilosa* L., *Acalypha australis* L., Pteris multifida Poir and *Portulaca oleracea* L. extracts, possessed the capacity to prevent the invasion of Eimeria tenella h *in vitro* and *in vivo* (Han *et al*., 2022). *P. macrophylla*

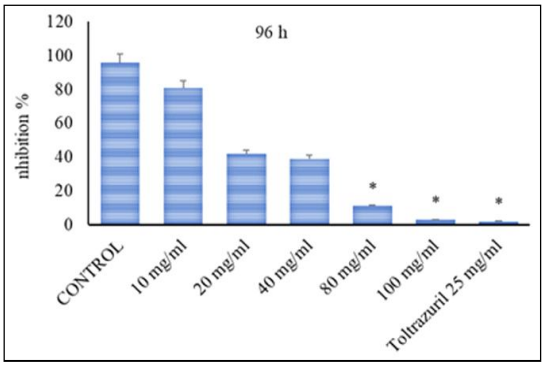


Fig 6: Mean inhibition oocyst % of *E. flavescens* oocysts at all concentrations for exposure duration 96 (hrs). (*): p-value < 0.05.

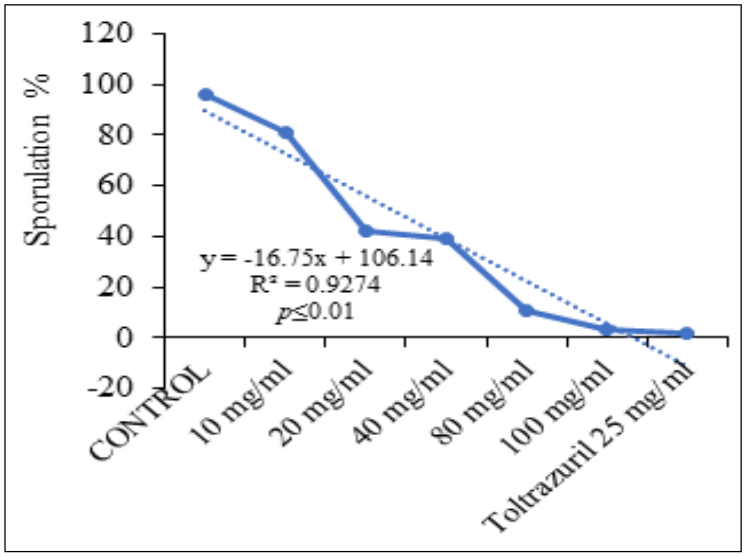


Fig 7: Mean sporulation oocyst % of *E. flavescens* oocysts at all concentrations for exposure duration 96 (hrs).

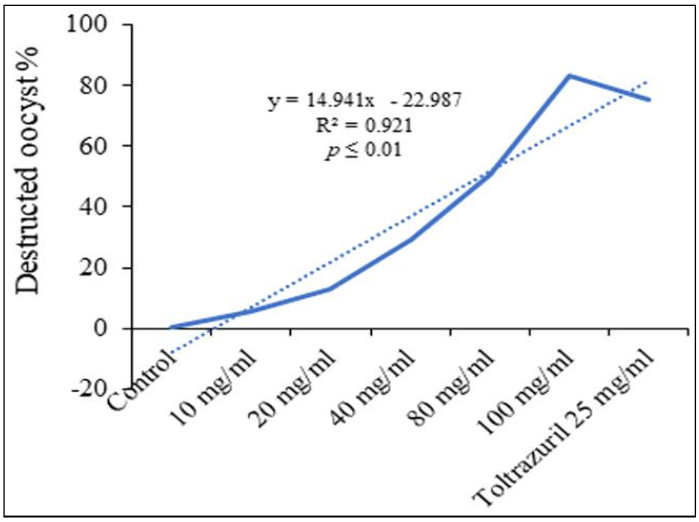


Fig 8: Mean Destructed oocyst % of *E. flavescens* oocysts at all concentrations for deference exposure times (hrs).

extracts may have been responsible for the decreased sporozoite viability, according to a study that was conducted by (Cedric *et al*., 2017). This occurred because the extracts interfered with calcium-mediated signaling in the sporozoites. The results of the study demonstrate that CPLE, which is an extract of Calotropis Procera leaf, is an efficient method for preventing the formation of oocysts.

CONCLUSION

The extract of *Calotropis Procera* leaves was found to play a vital function in suppressing sporozoites and inhibiting their viability, as well as having a damaging effect on sporozoite oocysts. More experimental and clinical investigations are needed to better understand the plant's pharmacological and therapeutic capabilities and isolate active components.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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