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Original Research Article

Effect of *Calotropis procera* extract against *Eimeria piriformis* oocyst- and sporozoite-infected rabbits

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Abstract

Purpose: *To investigate the effect of Calotropis procera (Asclepiadaceae) extract on Eimeria piriformis (E. piriformis) oocysts and sporozoites (coccidiosis) isolated from commercial rabbits.*
Methods: *Calotropis procera extract was obtained from dried, pulverized leaves, and macerated in 80 % methanol for 30 h. The extract was analyzed using infrared spectroscopy (IR). Twelve-well plates of 3 mL containing 1 x 104 non-sporulated oocyst were tested in 6 groups: Negative (untreated) control received 2.5 % potassium dichromate solution. Other groups received four concentrations of C. procera leaf extract (12.5, 25, 50, and 100 mg/mL) for oocysts vitality test. Toltrazuril (25 µl/mL) was used as positive control. Moreover, 250, 500, 750, and 1000 µg/mL of C. procera extract were tested for sporozoites vitality test. The mixture was examined daily for 4 days for oocysts vitality and after 12 and 24 h for sporozoite vitality.*
Results: *Findings of IR showed that extracts contained 5 biologically active chemical components, indicating the presence of alkaloids, flavonoids, saponins, and phenols. The extract showed a significant inhibitory effect on E. piriformis oocysts at 100 mg/mL with suppression rates of approximately 89 % after 96 h, nearly similar to toltrazuril ($p \leq 0.01$). Furthermore, C. procera extract showed inhibition at the highest repression ($p \leq 0.01$) of 89 % of E. piriformis sporozoites viability at 1000 µg/mL after 24 h. The inhibitory rate increased proportionately with an extended incubation duration and high concentration.*
Conclusion: *C. procera extract is as effective as the positive control in treating coccidiosis in rabbits. However, further studies are necessary to determine the active constituents of C. procera and their mode(s) of action.*

Keywords: *Calotropis procera, E. piriformis, Oocysts, Oryctolagus cuniculus, Sporozoiticidal*

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INTRODUCTION

Eimeria genus is a predominant cause of coccidiosis, which is a widespread parasitic infection affecting animals. Particularly in local rabbit populations, it leads to substantial mortality rates, contributing to annual losses reaching up to 13 billion dollars in poultry farming and 127 million dollars in rabbits [1]. *Eimeria piriformis*, identified as a slightly pathogenic species developing in the duodenum, adds to the complexity of this issue. The life cycle of *Eimeria* encompasses both exterior and internal stages and it is classified as an obligate parasite in the

Apicomplexa phylum. Entering the host through the oral route, these parasites infect and proliferate within mucosal epithelia and various digestive tract sections, causing gastrointestinal damage, inflammation, bloody or watery feces, and other symptomatic manifestations, morbidity and death [2].

This pathogen has the potential to disrupt the microbiota of the intestinal tract and make the environment of the intestinal tract more conducive to the growth of pathogenic bacteria like *Clostridium perfringens*, which ultimately lead to necrotic enteritis [3]. *Eimeria* infection destroys mucosal cells in the host, which leads to an increase in the permeability of cells, leakage of nutrients and plasma proteins, reduced digestion, and decreased protein absorption. These effects contribute to coccidiosis's clinical and subclinical manifestation. Dehydration, anemia, hypoproteinemia, and malabsorption of nutrients are among the potential outcomes of induced villi atrophy, which leads to electrolyte imbalance. The consequence of this problem is that commercial rabbits suffer economic loss. Managing these disorders necessitates costly therapeutic interventions, including chemoprophylaxis and live-attenuated vaccines, both with notable downsides such as potential parasite resurgence and medication resistance [3]. Given the existing challenges, searching for novel agents with unique modes of action is imperative. Natural products, such as *Calotropis procera*, have emerged as a potential supplementary and alternative means of controlling coccidiosis. *Calotropis procera's* anticoccidial action suggests that it may be a valuable substitute for chemotherapeutic drugs for *Eimeria* species [4].

Calotropis procera (Madar), (giant milkweed) belonging to the Asclepiadaceae family, is an evergreen perennial shrub with unique medicinal properties. Its latex contains triterpenoids, alkaloids, cardenolides, anthocyanins, resins, and proteolytic enzymes [5]. *C. procera* thrives in arid environments and open spaces with little competition. Treatments of leprosy, menorrhagia, malaria and snake bites have all been linked to the giant milkweed [6]. With known benefits in various ailments, including analgesic, anticancer, anticoagulant, anti-inflammatory, and antimicrobial properties, *C. procera* has demonstrated its efficacy in preventing coccidian and inhibiting oocyst sporulation in *Eimeria papillata*. Recent studies have also highlighted its oocysticidal activity against *Eimeria stiedae* [4]. Considering this background, the present study aimed to evaluate the efficacy of *C. procera* against oocysts and sporozoites *Eimeria piriformis* through *in vitro* trials, providing valuable insights into potential alternative therapies for coccidiosis.

EXPERIMENTAL

Sample collection and extraction

C. procera leaves were obtained from the Herbaceous leaves in the desert of Riyadh, Saudi Arabia, and classification scientists (Ahmed Alfarhan) from the Department of Botany, King Saud University, verified the identification of the plant. The leaves were desiccated for 3 days in an incubator at 43 °C. Subsequently, they were pulverized into fine powder and immersed in 80 % methanol for 30 h at 4 °C, with periodic agitation. The leaf extract was filtered using Whatman filter paper. Afterwards, the extracts were subjected to a process of concentration and drying using a rotating vacuum evaporator (Yamato RE300, Tokyo, Japan) at 40 °C and reduced pressure. The crude extract was kept at −20 °C before analysis. Distilled water was used as the diluent for the extract in all investigations [7].

Ethical approval

Approval for animal use was obtained from King Saud University Ethics Committee (no. ID: KSU-SE-21-86). The study y followed international guidelines on the protection of animals used for experimental and other scientific purposes [8].

Infrared spectroscopy

After preparing the sample for analysis, 1 part of extract was mixed with 99 parts of potassium bromide powder (Sigma-Aldrich, Berlin, Germany). Subsequently, the mixture underwent a coarse pulverization procedure before being inserted into a die for pellet formation. Infra-red spectra were obtained using NICOLET 6700, Fourier-transform infrared spectrophotometer (FT-IR), Thermo Scientific, USA. The wave numbers (cm^{-1}) were used to represent the number of waves with significant peaks and troughs. At 25 ^{o}C, spectra were recorded with a resolution of 4 cm, and the spectral range extended from 4000 to 400 cm^{-1} [9].

Parasites

The study was carried out in the Veterinary and Sanitary Expertise Department in Saudi Arabia, specifically at the Parasitology Laboratory of the Department of Zoology at King Saud University. When it was found that the rabbits were naturally infected with Eimeria, samples of their feces

were collected for examination. The feces were examined for oocysts of *Eimerea* spp. The type obtained was from *Eimeria piriformis* oocysts. The oocysts were distinguished based on their morphological characteristics [10].

In vitro sporulation assay for _Eimeria piriformis_ oocysts using _C. procera_

To determine the impact of different concentrations of *C. procera* on the sporulation of *E. piriformis* oocysts, non-sporulated oocysts (1 x 10^4) were exposed to six treatments: 2.5 % potassium dichromate solution as control, four concentrations of *C. procera* extract (12.5, 25, 50, and 100 mg/mL), and toltrazuril 25 mg/mL as positive control for anti-oocyst activities. The mixture was examined daily for 4 days (after 24, 48, 72, and 96 h) for oocysticidal activities. Each treatment was repeated three times to ensure accuracy. After incubating each petri dish at 28 °C for four days, the percentage sporulation was calculated for each treatment by counting the number of sporulated and non-sporulated oocysts using McMaster method. Furthermore, morphological changes, abnormalities, and the percentage of destruction were assessed for each treatment method.

E. piriformis oocyst sporulation

Unsporulated *E. piriformis* oocysts from infected rabbits' duodenums were utilized to study oocyst sporulation. *E. piriformis* oocyst sporulation was undertaken in an aqueous solution of 2.5 % potassium dichromate $K_2Cr_2O_7$ (10^4 oocysts/mL) with or without 100 µg/mL of extract at 25 °C for 70 h. The proportion of sporulated oocysts was determined by inspecting 100 oocysts under a microscope and counting sporulatory and inhibitory oocysts [12].

Preparation of sporulated oocysts

After obtaining *E. piriformis* oocysts from the duodenum, they were cleaned and concentrated using the method of flotation. The matured oocyst was preserved in a potassium dichromate ($K_2Cr_2O_7$) solution with a concentration of 2.5 % at 4 °C until they were used to determine the level of infection. The *E. piriformis* field isolates were maintained by periodically transmitting them via young rabbits at the Parasitology Laboratory Animal house [12].

Anti-sporozoite activity of _C. procera_ under _in vitro_ conditions

The oocyst samples, kept in a potassium dichromate solution, were cleaned using phosphate-buffered saline (PBS) with a pH of 7.5. The centrifuge used Falcon tubes containing 10 mL of liquid, which were subjected to a centrifugal force of 1008 g for approximately 15 min. This process was repeated 3 - 6 times until the $K_2Cr_2O_7$ solution was eliminated. The oocysts were placed in a water bath and kept at a temperature of 42 °C for 1 h with regular shaking of the tubes. The parasite suspension, consisting of 1,000 sporozoites, was divided into two halves. Each portion included extract concentrations at 125, 250, 500, and 1,000 µg/mL. Subsequently, these components were placed in a 3 mL petri plates containing 24 compartments. A standard treatment of toltrazuril at 30 µg/mL was used to enable comparison. Furthermore, potassium dichromate solution was used as a negative control.

The exact conditions were maintained for each treatment, which was carried out in triplicates. Viable sporozoites were determined by recording the findings at 12 and 24 h. McMaster chamber was used for sporozoite quantification. Viability (Vi) of sporozoites was estimated by applying a mathematical rule to a total of 100 sporozoites, which were classified as either viable (v_i) or non-viable (Eq 1).

Inhibition of Vi (%) = $\{(V_{i\%\ control} - V_{i\%\ bile})/V_{i\%\ control}\}100$ ……………………….. (1)

Statistical analysis

Statistical software SPSS 22 (Chicago, IL, USA) was used to perform one-way ANOVA for each group. Furthermore, Duncan's post-hoc test was conducted to make additional multiple comparisons. Significance was established at $p < 0.05$. A linear correlation analysis used Pearson's technique to assess the relationships between variables.

RESULTS

FTIR characteristics

The finding of FTIR analysis revealed that the principal bands were located at 3347.92, 2115.89, 1508.78, 1050.52, 1050.52, and 926.18 cm^{-1}, respectively, as illustrated in (Figure 1).

The processes of N-H stretching generated the active phytochemical components, N=C=S stretching, N-O stretching, CO-O-CO stretching, and C=C bending. These processes produced absorbance values ranging from 400 to 4000 cm^{-1}.

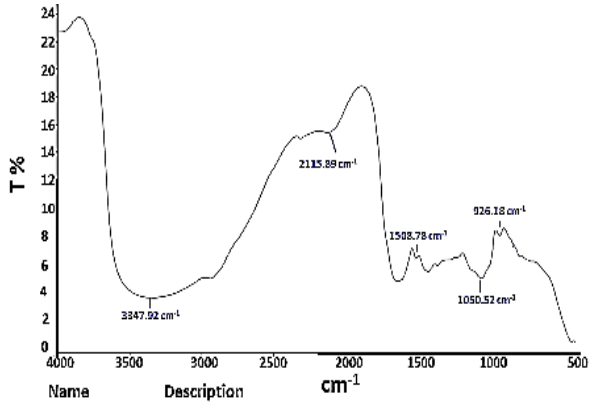

Figure 1: Infrared spectra of the leaf extract of *Calotropis procera* using a Nicolet 6700 FT-IR spectrometer, a Fourier-transform infrared spectrometer

Effect of *C. procera* against *E. piriformis* sporulation

The methanolic extract showed that the longer the incubation period, the greater the inhibition rate and the reverse for the sporulation rate. As the incubation time increased, the rate of sporulation inhibition continued to vary depending on extract concentration ($p \leq 0.05$) in treatment groups. In general, the best concentration for inhibiting the parasite was 100 mg/mL (Figure 2 A), compared with control group (2.5 % potassium dichromate) and the reference treatment (Figure 2 B).

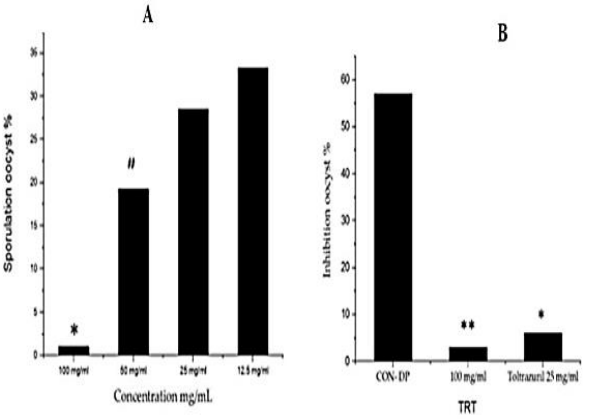

Figure 2: Effect of *Calotropis procera* on the formation of *E. piriformis* oocyst spores in a laboratory setting. *$P < 0.01$ and #$p \leq 0.05$. (B) Optimal concentration and reference treatment vs. control **$P < 0.01$ and *$p < 0.05$

The experimental groups showed highly significant ($p < 0.01$) inhibition rates with increasing doses. The difference between the 100 mg/mL concentration compared to control group was significant ($p < 0.01$), as well as the difference between reference drug toltrazuril (25 µg/mL) and control element ($p < 0.05$). Nevertheless, the remaining concentrations did not exhibit any significant disparities in comparison to control (Figure 3).

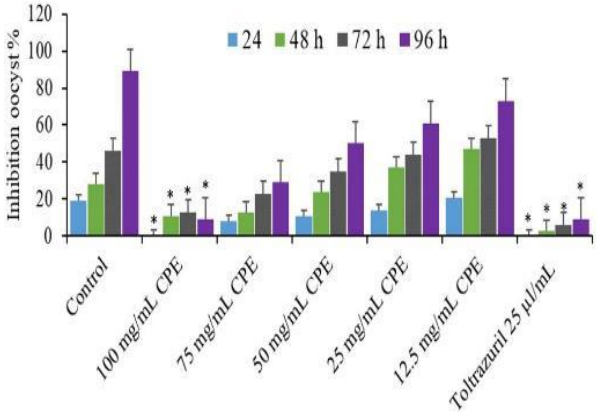

Figure 3: The impact of *Calotropis procera* on the number of *E. piriformis* oocyst *in vitro.* Extracts doses were 12.5, 25, 50, and 100 mg/mL, with a control and 25 mg/mL Toltrazuril. **$P < 0.01$ vs. control, *$p \leq 0.05$ vs. control. ***Note:*** CPE refers to *Calotropis procera* extract

Microscopic analysis and quantification

It was shown that oocysts treated with *C. procera* at different doses exhibited deformities, including fractured walls and lysis. The control had a sporulation rate of 898 ± 1.41 after three days. At a 100 mg/mL dosage, *C. procera* exhibited an inhibitory solid impact on the sporulation rate ($p < 0.05$), ultimately leading to its complete cessation. A similar result was seen with toltrazuril at 25 µg/mL. Compared to control, the sporulation inhibition rates of *C. procera* at various concentrations were 100, 99.1, 71, 50, 40, 27, and 98 %, respectively. The oocysticidal action was verified by seeing a more significant proportion of degenerated oocysts, namely 88.33, 73.33, 61.33, 50.66, 29.33, and 94.66 % for extract concentrations of 100, 50, 25, and 12.5 mg/mL, as well as 25 µg/mL of Toltrazuril, respectively, compared to control group (Table 1).

Effect of sporulation time on percentage of *E. piriformis* oocysts

Figure 4 shows the effect of sporulation time on the percentage of *E. piriformis* oocysts that had sporulation and those that did not sporulate *in vitro*. Data shows that the proportion of sporulation grew as the incubation period increased, whereas the rate of inhibited oocysts was stabilized with higher concentrations. The inhibition ratio of sporulation increased substantially with the incubation duration, reaching its highest level at the 96th h ($p < 0.05$).

This indicates significant differences in the rate of sporulation inhibition across exposures of 24, 48, and 72 h (Figure 4).

Table 1: Effect of different doses of *Calotropis procera* on sporulation and destructive rates of *E. piriformis* oocysts, *in vitro*

Concentration (mg/mL)	Sporulated oocysts (%)	Destructive oocysts (%)
Control + 2.5 % DPS	98±1.41	0.67±0.57
100 CPE	09±2.44^{a}	88.33±6.42^{a}
50 CPE	50±4.89^{ab}	61.33±6.66^{ab}
25 CPE	60±5.35^{b}	50.66±7.02^{b}
12.5 CPE	73±9.27^{c}	29.33±3.05^{c}
Toltrazuril (25 µg/mL)	09±0.99^{a}	94.66±5.13^{a}

Note: Means with distinct superscripts within a column are significantly different, (a) Significant differences at 100 mg/mL and toltrazuril $p < 0.01$ vs. control, (ab) Significant differences were minor with the highest concentration and the reference treatment, (c) There are no significant differences with the control. CPE, *Calotropis procera* Extract. DPS, a solution containing potassium dichromate

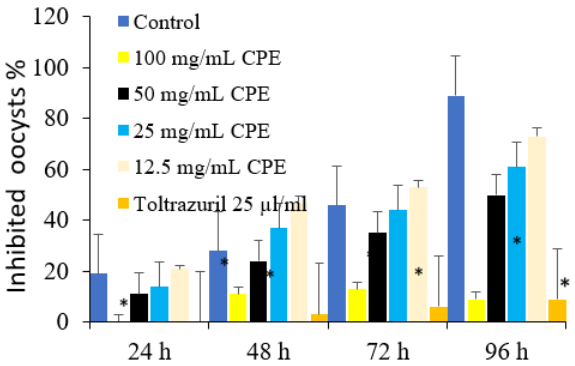

Figure 4: Effect of *Calotropis procera* on the number of *E. piriformis* oocysts inhibited *in vitro* at different Periods (24, 48, 72, and 96 h). ***Note:*** *$P < 0.01$ vs. control. CPE refers to the extract of *Calotropis procera*

Correlation between sporulation (%) and concentration

There was a negative correlation between sporulation and concentration percentage. The liner equation showed the highest coefficient of determination (R^2) p = 000244. The results are significant at $p < 0.05$ for sporulation and destruction (Figure 5 and Figure 6).

Anti-sporozoite activity of *C. procera*: *In vitro*

A test was conducted to determine the sporozoite vitality rate of *C. procera* according to the length of the incubation period and the extract concentration used to measure vitality. It was observed after 12 and 24 h of incubation that 1,000 µg/mL of extract and 25 µg/mL toltrazuril showed significant changes ($p < 0.05$) in viability rate of *E. piriformis* sporozoites when compared to control treatment ($K_2Cr_2O_7$). However, sporozoites at lower extract concentrations (500, 250, and 125 µg/mL) displayed varying degrees of viability (Figure 7).

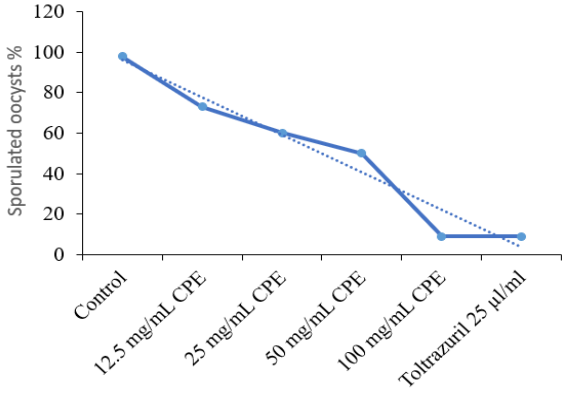

Figure 5: Liner correlation between sporulated oocytes and *Calotropis procera.* ***Note:*** CPE: *Calotropis procera* extract

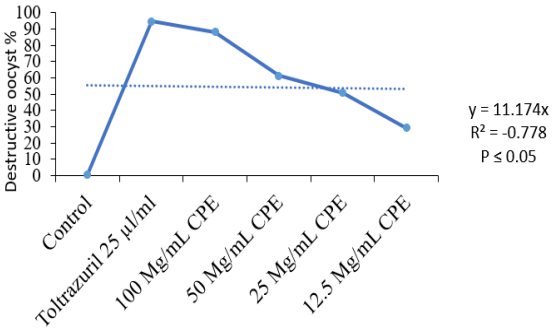

Figure 6: Linear correlation between destructed oocyst and *Calotropis procera.* ***Note:*** P = 0.039412 (significant difference *vs* control)

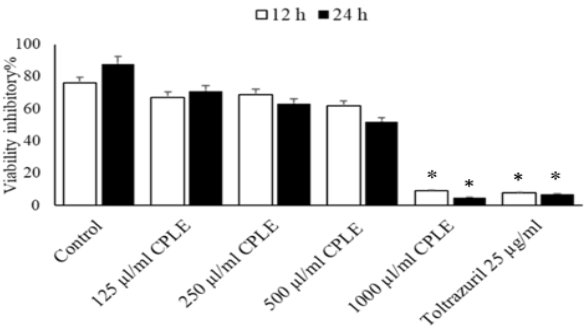

Figure 7: The effect of *C. procera* leaf extract *in vitro* on *E. piriformis* sporozoites viability after 12 and 24 h. ***Note:*** *$P < 0.05$ vs. control; n = 3; CPLE: *C. procera* leaf extract potassium dichromate

Sporozoite viability rate

Figure 8 demonstrates a significant ($p < 0.05$) impact of the different incubation periods on the rate of sporozoite viability. This rate increased as

the incubation period increased, whereas the rate of non-viability decreased. Therefore, sporozoites' inhibition rate significantly increased when the incubation duration reached 12 h ($p < 0.05$). There was no significant difference in the rate of inhibition of sporozoites between the 12th and 24th h of exposure.

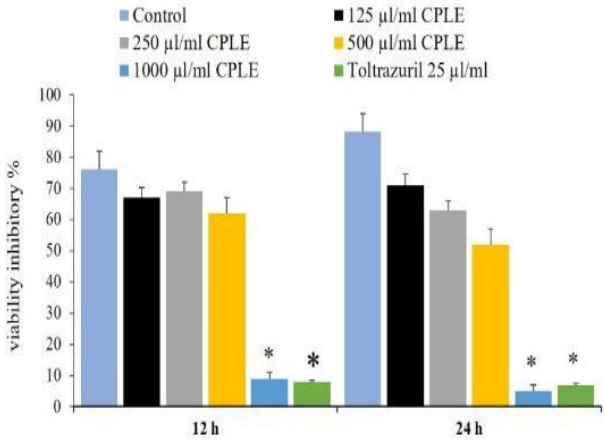

Figure 8: Effect of *C. procera* leaf extract, at varying doses, on the *in vitro* inhibition of the viability of *E. piriformis* sporozoites between 12 and 24 h. ***Note:*** **P* ≤ 0.01 vs. control. CPLE*: C. procera* leaf extract

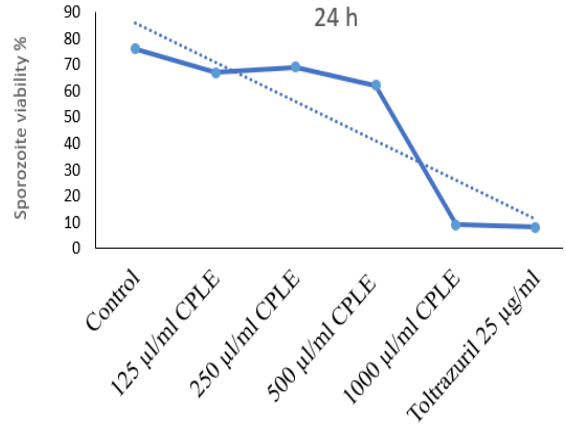

Figure 9: Liner correlation between the viability of *E. piriformis* sporozoites and *C. procera.* ***Note:*** *P* = 0.060901

Relationship between sporozoites' viability and concentration

The viability percentage of sporozoites exhibited a negative correlation with increasing concentration ($y = -14.886x + 100.6$; $R^2 = 0.7911$; $p < 0.05$), indicating a significant relationship. Nevertheless, no notable disparity was seen between the extract concentration at 1000 µg/mL and the reference medication, toltrazuril, at a concentration of 25 µg/mL. This suggests that the efficacy of *C. procera* extract at this concentration is comparable to the established reference drug. This finding raises interesting questions about the potential of CPLE as a promising alternative in the context of *E. piriformis* control, especially considering its equivalence to toltrazuril (Figure 9).

DISCUSSION

Coccidiosis, a globally prevalent disease, significantly hampers rabbit productivity, leading to substantial financial losses. The associated morbidity and mortality from these parasitic infections not only impair livestock productivity but also pose a severe health risk [13]. Understanding the current species and prevalence of intestinal parasites becomes crucial for minimizing financial losses in the rabbit sector, assessing infection risks, and implementing effective control measures [14]. Several authors have explored the impact of diverse plant extracts on oocyst viability and the duration of sporulation [15]. These extracts have proven effective in inhibiting sporulation and reducing the viability of *Eimeria* oocysts [16]. The consistent application of natural extracts in animal farms, including rabbits and poultry, could emerge as a therapeutic and preventive strategy to diminish the survival rate of oocysts belonging to the *Eimeria* and prevent them from maturing [17]. The effect of natural product extracts on the oocysts of these parasitic protozoa has been the subject of extensive research. In previous studies, *Nerium oleander* leaf extract against *E. magna* and *E. exigua* oocysts and *Calotropis procera* leaf extracts against *E. stiedae* oocysts for 96 h demonstrated a significant inhibition, ranging from 90 to 98 % [18,19].

This study investigated the impact of *C. procera* extract over varying durations (24, 48, 72, and 96 h) and concentrations (12.5, 25, 50, and 100 mg/mL) on *E. piriformis* oocyst sporulation *in vitro*. The highest effectiveness of the tested concentrations resulted in approximately 91 % inhibition of sporulation after 96 h of exposure to a 100 % *C. procera* concentration. In contrast, the control group ($K_2Cr_2O_7$) exhibited a high level of oocyst sporulation. The efficacy of different extract concentrations varied depending on the concentration and incubation period of the oocysts. Comparing extract concentrations of 50, 25, and 12.5 mg/mL to control group, the highest rates of inhibition and lowest rates of sporulation were observed. Lower concentrations showed no significant differences. These findings align with previous reports that *C. procera* may reduce *E. stiedae* oocysts at high concentrations [4].

Extended incubation times and higher doses often improved the inhibition rate. Moreover, at 1,000 µg/mL, *C. procera* exhibited the maximum

inhibition rate of *E. steal* sporozoite viability (92 %) and the lowest inhibition rate (8 %) at 125 µg/mL. These results are consistent with earlier reports that oocyst inhibition in potassium dichromate could reach approximately 96 % at higher concentrations of *C. procera*. The outcomes indicated a gradual and concentration-dependent decrease in oocyst sporulation. Extract at concentrations of 50, 25, and 10 mg/mL exhibited inhibition rates of 71.7, 33.11 and 19.88 % respectively. Sporulation and inhibition were directly related over an extended period [18,20].

Generally, the extract concentration of 100 mg/mL exhibited the highest rate of inhibition and the lowest rate of sporulation compared to control group. Lower concentrations (50, 25, and 12.5 mg/mL) of *C. procera* did not show significant differences. These findings agree with previous reports that sheep bile could suppress *E. stiedae* oocysts at high concentrations [19]. Increased incubation time and larger doses generally enhanced inhibition rate. Additionally, *C. procera* showed the highest inhibition rate of *E. stiedae* sporozoite viability (92 %) at 1000 µg/mL and the lowest inhibition rate (8 %) at 125 µg/mL. The current findings align with reports that higher concentrations of extract could inhibit oocysts by approximately 96 % in potassium dichromate [21]. Moreover, results demonstrated the ability to suppress oocyst sporulation in a concentration-dependent manner over time.

The results align with a study, where the effects of eight plant extracts, essential oils, and their combination were investigated as potential treatments for Coccidial infection, particularly against *Eimeria tenella* [20]. Remmal *et al* independently assessed the primary components of essential oils, including carvacrol, isoeugenol, thymol, eugenol, and carvone, and reported their effectiveness against coccidian [20]. *Vitis vinifera* leaf extracts also affected *E. papillae* oocysts by interfering with calcium-mediated signaling in the sporozoites [21]. These findings are consistent with the results of this study which affirm that CPLE (*Calotropis procera* leaf extract) effectively prevents the formation of oocysts.

CONCLUSION

Extract of *C. procera* leaves plays an essential role in inhibiting oocysts and preventing their sporulation. It also has a destructive effect on oocysts. Further studies are needed to understand the plant's pharmacological and therapeutic properties and isolate its active compounds.

DECLARATIONS

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Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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