RESEARCH ARTICLE Indian Journal of Animal Research, Volume 59 Issue 3: 457-466 (March 2025)

Cassia alata Extract Exerts Antioxidant Power to Mitigate *Eimeria papillata*-Induced Liver Damage in Mice

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10.18805/IJAR.BF-1910

ABSTRACT

Background: This study was intended to investigate the prospective role of *Cassia alata* extract as an anticoccidial, anti-inflammatory and hepatoprotective agent against *Eimeria papillata* parasitic infection. Therefore, this study was intended to investigate the potential role of *Cassia alata* extract as an anticoccidial, anti-inflammatory and hepatoprotective agent against *Eimeria papillata* parasitic infection.

Methods: For this investigation, a total of 25 male swiss albino mice were allocated into 5 equal groups. Group I: negative control (Cont), Group II: administrated with *Cassia alata* extract (CE) at a dosage of 500 mg/kg body weight, Group III: infected group with *E. papillata* (Inf), Group IV: infected-treated with *Cassia alata* extract (Inf-CE) at a dosage of 500 mg/kg body weight and Group V: infected-treated with amprolium at a dosage of 120 mg/kg body weight (Drug). Mice were infected orally with an approximate 1×10^3 *E. papillata* sporulated oocysts excluding I and II groups. One-hour post infection, mice of groups IV and V were received the recommended doses of treatments orally once a day for 5 sequential days. Upon treatment for 5 days, all mice were sacrificed and growth performance, histopathological, histochemical and oxidative stress assessments were examined.

Result: An enhancement in body weight (-2.33±0.58%) and feed intake (130.33±4.51 grams) in relation to the body weight (-4.67±0.58%) and feed intake (98.00±4.36 grams) of the Inf group was observed. Improvements in liver structure alterations induced by *E. papillata* was recorded with a noticeable increase in its carbohydrates (6.04±0.45%) and protein (12.46±1.13%) content. Moreover, considerable reduction in hepatic nitric oxide (59.13±4.59 Umol/g) and malonaldehyde (22.07±1.20 nmol/mg) and an elevation in glutathione (18.20±1.79 ng/mg) and glutathione peroxidase (111.53±8.95 ng/mg) were documented. In summary, CE treatment could significantly ameliorate liver injury, oxidative stress and inflammatory reactions caused by *E. papillata* parasitic infection.

Key words: Anticoccidial, Antioxidant, *Cassia alata*, Hepatoprotective, Histochemistry.

INTRODUCTION

Coccidiosis is a cosmopolitan disease affecting a wide variety of vertebrates (Dalloul and Lillehoj, 2006; Alajmi *et al*., 2023). It is caused by an apicomplexan parasite from the genus *Eimeria* (family Eimeriidae). *E. papillata* was recognized in the jejunum of mouse as an eimerian parasite (Ernst *et al*., 1971; Abdel-Latif *et al*., 2016), responsible for murine jejunal coccidiosis and increased oxidative conditions (Dkhil *et al*., 2023). Decreased feed intake, reduced body weight gain, dehydration and higher vulnerability to other diseases are all consequences of *Eimeria* species infection (Abdel-Gaber *et al*., 2023). It is an intracellular obligate parasite with a complex life cycle, consisting of two developmental stages: Exogenous sporogony and endogenous schizogony and gametogony inside the host (Abdel-Gaber *et al*., 2024b). Coccidiosis primarily damages the digestive tract causing severe enteritis; heightened vulnerability to subsequent bacterial infections, as it disturbs the equilibrium of gut microbiota (Abdel-Gaber *et al*., 2023). However, it affects some auxiliary associated organs of the digestive tract indirectly as the liver (Thagfan *et al*., 2023), severely impacting the hepatic health status (Dkhil *et al*., 2012) and also raising the oxidative conditions that affects the general body growth and performance (Abdel-Latif *et al*., 2016).

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How to cite this article: Al-Malki, E.S., Elshershaby, R.E., Abdel-Gaber, R., Thagfan, F.A., Dkhil, M.A. and Kasem, S.M. (2025). *Cassia alata* Extract Exerts Antioxidant Power to Mitigate *Eimeria papillata*-Induced Liver Damage in Mice. Indian Journal of Animal Research. 59(3): 457-466. doi: 10.18805/IJAR.BF-1910.

Submitted: 25-10-2024 **Accepted:** 08-12-2024 **Online:**28-12-2024

Coccidiosis can be treated with a variety of synthetic coccidiostats of chemotherapeutic origin, such as

amprolium, which limit coccidia's normal growth, metabolism and reproduction (Abdel-Gaber *et al*., 2024a). However, the continuous use of anticoccidiants has been related to immune system interference (Yunus *et al*., 2005), resulted in multi-drug resistance (Abbas *et al*., 2011), hazardous effects on animal health (Nogueira *et al*., 2009) and remaining of parasite infections and drugs in animal tissues products (Onyiche *et al*., 2021). Consequently, in response to these problems, different coccidiosis control tactics have quickly emerged and developed. Live anticoccidial vaccinations, immunomodulators, prebiotics and natural herbs are some of these tactics. (Hamzicì *et al*., 2015). The exploitation of various plants and their component parts has been recorded in mice for the control and management of *E. papillata* infection. These plants have confirmed their ability in using as therapeutic agents against coccidiosis through reducing death rate, oocyst output records and diarrhea, as well as enhancing lesion scores and production performance (Zaman *et al*., 2012). These natural materials not only attack parasites but may also protect tissue in parasitized target hosts (Wunderlich *et al*., 2014). Several medicinal plant extracts that are environmentally friendly were proven for their anticoccidial activity as *Zingiber officinale* (Mubaraki *et al*., 2022), *Calotropis procera* (Murshed *et al*., 2023), *Rosmarinus officinalis* (Kasem *et al*., 2024), *Artemisia monosperma* (Maodaa *et al*., 2024b) (*Krameria lappacea* (Abdel-Gaber *et al*., 2024b), *Teucrium polium* (Maodaa *et al*., 2024c) and others. *Cassia alata* is a conventional herb from the Fabaceae family. Its therapeutic qualities include antifungal, anti-inflammatory, analgesic, antidiabetic and anthelmintic benefits (Keng *et al*., 2024). In addition, it exhibits anti-hepatotoxic and hepatoprotective impacts (Mohammed *et al*., 2017). It has been informed that *C. alata* have diverse bioactive compounds in its leaves with certain alkaloids, flavonoids, saponins, tannins and terpenoids (Veerachari and Bopaiah, 2012; Angelina *et al*., 2021). Furthermore, our recent study (Elshershaby *et al*., 2024), proved that *C. alata* extract had a critical anticoccidial capacity with its role in the management of goblet cells response following *E. papillata* infection.

Owing to all the formerly cited biological properties, this herein study was planned to scrutinize the anticoccidial, antioxidant activity and the ameliorative potential effects of *C. alata* extract on *Eimeria papillata*-induced liver damage in mice.

MATERIALS AND METHODS

Cassia alata extract (CE) preparation

C. alata plant leaves were collected from the botanical garden at Helwan University, Egypt. The plant was authenticated by a taxonomist at Helwan University, Egypt. The leaves were air-dried and pulverized with an electric mixer into a fine powder, extracted using 70% methanol as described by Elshershaby *et al*. (2024).

Phenolics, flavonoids content and antioxidant capacity

Folin-Ciocalteu calorimetric assay was operated to estimate the total phenolics for the CE (Sánchez-Rangel *et al*., 2013). The total phenolics were expressed as mg of the gallic acid equivalent (GAE) per gram of the dry extract. Additionally, the total flavonoids of CE were estimated as described by Chang *et al*. (2002) using the aluminum chloride ($AlCl_3$) solution. The total flavonoids were expressed as mg of the quercetin equivalent (QE) per gram of the dried extract. Antioxidant activity of CE was judged using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method (Akillioglu and Karakaya, 2010). The antioxidant capacity was represented as suppression % of DPPH radicals.

Eimeria papillata collection and sporulation

Unsporulated *E. papillata* oocysts were collected from the feces of infected mice on day 5 post infection. Oocysts were purified by flotation method using saturated saline and allowed to be sporulated in 2.5% potassium dichromate solution for 5 days at 27±2°C (Dkhil *et al*., 2013) and then preserved at 4°C for the following steps.

Experimental animals

This research was conducted between January 2024 and July 2024 at the Zoology Department, Faculty of Science, Kafrelsheikh University, Kafr Elsheikh, Egypt, while the phytochemical analysis and histological evaluation was carried out in Saudi Arabia. Twenty-five male swiss albino mice weighing about 20-25 g were included in this study. Mice were allocated into 5 groups (5 mice per group) in separate plastic well-ventilated cages that readily accessible to food and water *ad libitum* with 12 h of light and 12 h of darkness, confirming good sanitary condition. The mice were allowed to adapt to the surrounding environmental conditions for 10 days prior to *E. papillata* infection. The experimental groups were labelled as Group I: non-infected, non-supplemented negative control (Cont), Group II: administrated group with *Cassia alata* extract at 500 mg/kg body weight (CE), Group III: infected group with *E. papillata* (Inf), Group IV: infected then treated with *Cassia alata* extract at 500 mg/kg body weight (Inf-CE) and Group V: infected then treated with amprolium at 120 mg/kg body weight (Drug). Following the 10 days adaptation, mice were infected orally with *E. papillata* sporulated oocysts (approximate 1×10^3 oocysts in 100 µl normal saline) (Abdel-Tawab *et al*., 2020) using a stomach tube excluding groups I and II. One-hour post infection, mice of groups IV and V were received the recommended doses of treatments orally once a day for 5 sequential days following the earlier study investigated by Elshershaby *et al*. (2024).

Five days following treatment, all the survived mice were sacrificed by cervical dislocation. The effect of CE on murine eimeriosis was investigated by growth performance, histopathological and histochemical and oxidative stress parameters examinations.

Growth performance assessment

Body weight of the mice were measured on days 0 and 5 to calculate the body weight change according to the method of Al-Quraishy *et al*. (2020). In addition, the offered and residual diet were weighed on days 0 and 5, respectively to calculate the feed intake by mice in each group (Al-Otaibi *et al*., 2023).

Histopathological examination

The collected liver samples were washed using saline and fixed in 10% neutral buffered formalin, dehydrated, cleared and embedded in paraffin wax. Tissue cutting was performed into 5 µm thick sections as illustrated by Drury and Wallington (1980). Liver sections were stained with hematoxylin-eosin (H and E) to reveal the presence of any pathological changes. The slides were inspected and photographed using a LEICA microscope (DM650, Germany).

Histochemical analysis

Other cutting liver sections were processed and stained using periodic acid-Schiff's method (Hotchkiss, 1984) for detection of total carbohydrates. Also, histochemical total protein analysis using mercuric bromophenol blue was performed according to Mazia *et al*. (1953). The slides were photographed using a LEICA microscope (DM650, Germany).

Determination of hepatic oxidative stress

Liver samples were aseptically removed, washed using ice-cold phosphate buffer (pH 7.4), cut up into small tissue pieces and stored at -80°C for subsequent analysis. An ice-cold solution had 300 mM sucrose and 50 mM Tris-HCl was used to prepare 10% hepatic tissue homogenate to be analyzed according to Tsakiris *et al*. (2004) methodology. The hepatic tissue homogenates were centrifuged and the supernatant was used for analysis of antioxidant enzymes markers including nitric oxide (NO), malonaldehyde (MDA), glutathione (GSH) and glutathione peroxidase (GPx) by enzyme linked immunosorbent assay (ELISA) technique. The appropriate used commercial kits were obtained from MyBioSource, Inc. for NO (Catalog no. MBS723386,) and MDA (Catalog no. MBS263626), as well as from CUSABIO TECHNOLOGY LLC, USA for GSH (Catalog no. CSB-E12144r) and from BT LAB Biotech Co., Ltd. for GPx (Catalog no. E1172Ra). The absorbance was measured at 450 nm using an automated ELISA technique (Chemwell 2099; USA, Awareness Technology, Inc.).

Statistical analysis

All results were presented as means ± standard deviation (SD). Statistical changes ($p<0.05$) among groups was judged using one-way analysis of variance (ANOVA) by Tukey test using SigmaPlot® version 15.0 (Systat Software, Inc., Chicago, IL, USA).

Results and Discussion

C. alata have numerous bioactive compounds in its leaves with certain alkaloids, flavonoids, saponins, tannins and terpenoids (Angelina *et al*., 2021). A recent study by Elshershaby *et al*. (2024) indicated that *C. alata* extract diminishes oocyst output and alleviates necrotic enteritis and inflammatory alterations caused by *E. papillata*, demonstrating its anticoccidial, antioxidant and anti-inflammatory characteristics. In this study, the Folin-Ciocalteu colorimetric analysis assay demonstrated that the total phenolics of CE was 277.4±1.7 mg GAE/g dry weight extract. Besides, the aluminum chloride-based colorimetric method implied that the total flavonoids in CE was 63.6±0.2 mg QE/g dry weight extract. Furthermore, the DPPH radical scavenging activity was 89.9±2% for CE (Table 1).

Coccidiosis is one of the world's most devastating livestock illnesses, caused by parasite protozoa from the genus *Eimeria* following fecal-oral uptake of sporulated oocysts, which affects a variety of animal species and results in significant economic losses (Al-Quraishy *et al*., 2011; Blake and Tomley, 2014). It affects critical animals leading to weight loss and reduced feed efficiency (Schito *et al*., 1996). Anticoccidial synthetic chemicals are routinely used to treat coccidiosis, however misuse can consequence in to drug resistance (Flores *et al*., 2022). The medication amprolium has a wide spectrum of harmful effects on animal host tissues (Noack *et al*., 2019). Diverse animal species have been effectively treated and controlled using various strategies and alternatives globally. Among these choices, various compounds derived from natural herbal medicinal plants (Lee *et al*., 2022). Recent previous searches signposted the effective role of plant extracts as anticoccidials as *Persea americana* (Al-Otaibi *et al*., 2023a), *Krameria lappacea* (Alamari *et al*., 2024) and *Holothuria polii* (El-Sayed *et al*., 2024). For this, this study inspected the ameliorative role of CE as a hepatoprotective agent in addition to its anticoccidial, anti-inflammatory and antioxidant properties.

According to the present results, *E. papillata* infection induced loss in body weight by -4.67±0.58% and diminish in feed intake (98.00±4.36 grams) on 5th day post infection in comparison to the control group (Fig 1). The same results were proved by Abdel-Gaber *et al*. (2024a) and Al-Otaibi *et al*. (2023a), respectively. Several previous studies illuminated that this weight loss is the result of consuming the nutrients of the intestinal epithelium by the different *Eimeria* stages that leads to notable alterations in the intestinal nutrient contents (Hamid *et al*., 2019; Qaid *et al*., 2022). Also, Anwar *et al*. (2008) indicated that this considerable weight loss could be caused by various factors, including decreased daily food and drink intake.

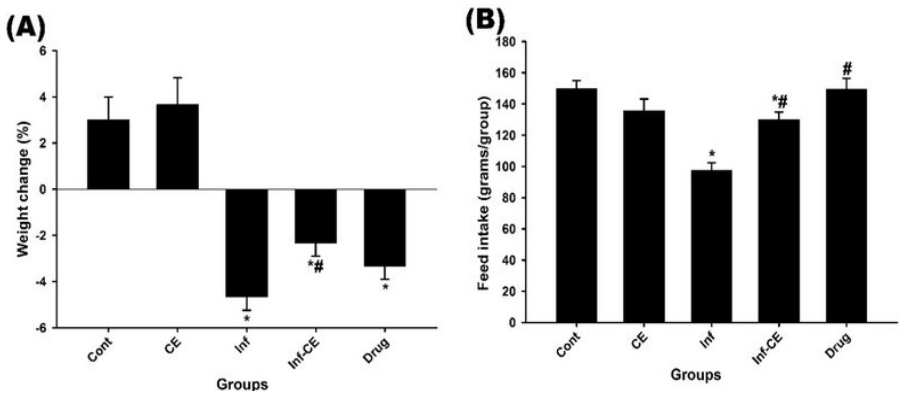
Table 1: Total content of phenolics, flavonoids and radical scavenging activity of *C. alata* extract.

Total phenolics (mg GAE/g dry extract)	Total flavonoids (mg QE/g dry extract)	Relative scavenging activity (%)
277.4±1.7	63.6±0.2	89.9±2

On the other hand, the use of CE as a therapeutic natural anticoccidial agent against *E. papillata* in this study resulted in a significant enhancement in body weight (-2.33±0.58%) and feed intake (130.33±4.51 grams), in relation to the Inf group (Fig 1). Similarly, Al-Otaibi *et al*. (2023a) enhanced the loss of body weight and feed intake that resulted from *E. papillata* infection. This significant development may result from the phytochemicals in the plant extract, which improved the nutritional status (Al-Otaibi *et al*., 2023a). *C. alata* includes innumerable types of phytochemical constituents that denote to its biological activities that possess anti-inflammatory and antioxidant activities such as flavonoids including isoflavones, kaempferol and its derivatives and rutin (Angelina *et al*., 2021) as well as anthraquinones, such as phenolic compounds, alkaloids, coumarins, saponins are also present (Muhammad *et al*., 2021).

The liver is the first point of interaction with the gut via the portal vein. *E. papillata* mainly infects the intestinal tract, predominantly the jejunum and does not usually infiltrate into the liver, however other lymphoid organs including the spleen and lymph nodes might be impacted by similar coccidian protozoa as *E. coecicola* parasites (Renaux *et al*., 2001). In the current study, light microscopic investigations of hematoxylin and eosin stained liver sections of *E. papillata* infected group showed focal hepatic necrosis associated with marked infiltration with mononuclear cells as lymphocytes and macrophages as well as marked periportal hepatic vacuolar changes consistent with marked fatty changes (Fig 2). These results were in consistent with Maodaa *et al*. (2024a), who proved that *E. papillata* infection had resulted in a moderate pathological inflammatory reaction in mice's hepatic tissue that is dominated by an increase in Kupffer cells and leukocytes, mainly lymphocytes, as a typical tissue response to hepatic destruction. Also, Dkhil and Al-Quraishy (2012) revealed that Eimeria infection caused cell membrane permeability and damage in the hepatocytes. Moreover, Dkhil *et al*. (2011) indicated that the structural evidence of mild inflammatory response in the liver correlate with oxidative tissue damage. However, upon treatment with CE in this study, a remarkable decrease in the hepatic vacuolar changes within the cytoplasm of the hepatocytes was observed showing its hepatoprotective effect (Fig 2). Similarly, the use of *Teucrium polium* leaves ethanolic extract had the ability to diminish the liver injury induced by *E. papillata* and restored the number of hepatic Kupffer cells (Maodaa *et al*., 2024a). The hepatoprotective efficacy of CE could be contributed to the anthraquinones and flavonoids contents that considered good anti-inflammatory agents (Chew *et al*., 2022).

Coccidiosis frequently affects host cell metabolism (Al-Quraishy *et al*., 2014). As well, Eimeria parasite has a noteworthy capability to benefit from the host cells by scavenging their available nutrients (Hermosilla *et al*., 2012). In the herein study, a remarkable diminution in both content of carbohydrates (0.37±0.15%) and proteins (7.06±0.67%) of *E. papillata* infected group was observed in periodic acid-Schiff and mercuric bromophenol blue stained liver sections, respectively (Fig 3, 4). One possible explanation for the reduced carbohydrate content is the excessive consumption of stored carbohydrates in the jejunal tissues by parasite stages (Metwaly *et al*., 2013). Kouwenhove (1971) stated that intestinal coccidial infections were categorized as protein-losing enteropathy. Many investigations revealed that the infected coccidial tissues had a reduced quantity of total proteins (Bangoura and Daugschies, 2007). Upon treatment with CE in the present study, the hepatic carbohydrate (6.04±0.45%) and protein (12.46±1.13%) contents was returned in comparison the infected group (Fig 3, 4). These reached results were the same that informed by Abdel-Gaber *et al*. (2024b), who indicated that the use of *Krameria lappacea* root extract had resulted in a significant improvement in the jejunal carbohydrate and protein content. Al-Otaibi *et al*. (2023a) revealed that *Persea americana* fruit extract could

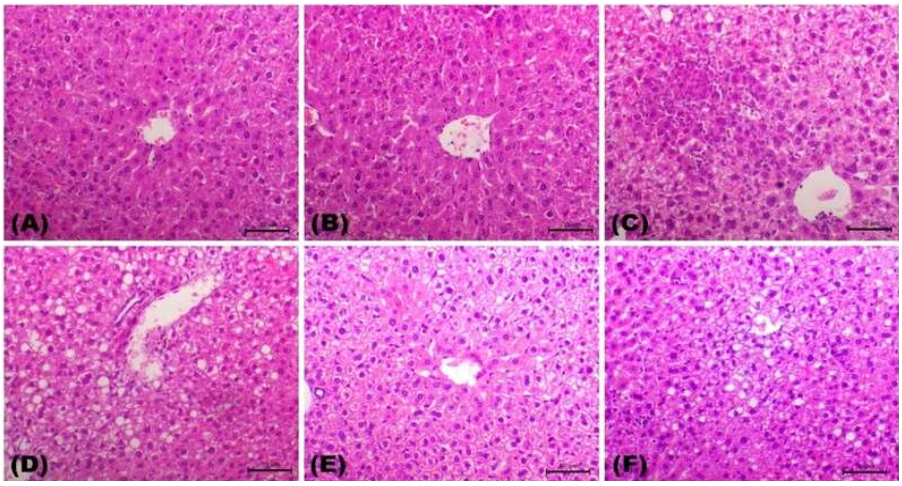


(A) Weight change (%) and (B) Feed intake (grams) of infected mice with *E. papillata* on 5th day after treatment. Results presented as averages ± SD. *Significant difference (P<0.05) related to cont group. #Significant difference (P<0.05) related to inf group.

Fig 1: Impact of *Cassia alata* extract.

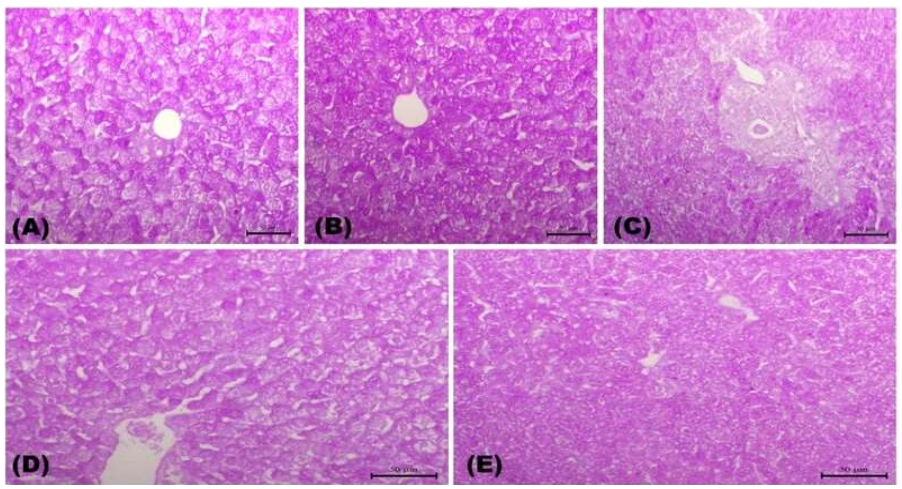
restored its jejunal content of carbohydrates through to its bioactive constituents, which reduced the activity of the glucose-6-phosphatase enzyme that most likely resulted in the reestablishment of tissue carbohydrate content. As well, Al-Otaibi *et al*. (2023a) and Abdel-Gaber *et al*. (2024b) explained that *Persea americana* fruit extract and *Krameria lappacea* root extract enhanced jejunal protein content by reducing catalytic pathways of tissue protein and nucleic acid breakdown.

Concerning Georgieva *et al*. (2006), Eimeria infection leads to an imbalance in the body's natural antioxidant defense and release of free radicals. The current findings



(A) Cont group showing normal hepatocytes with normal pink-coloured cytoplasm which arranged in cords around the central vein (CV) and separated with blood sinusoids. (B) CE administrated group showing normal hepatocytes arranged in cords around the central vein (CV) and detached with blood sinusoids. (C, D) *E. papillata* infected group displayed focal hepatic necrosis coupled with noticeable mononuclear cells infiltration consisted from lymphocytes and macrophages as well as marked periportal hepatic vacuolar changes consistent with marked fatty changes. (E) Inf-CE treated group showing marked decrease the hepatic vacuolar changes within the cytoplasm of the hepatocytes reveals mild to moderate hydropic changes. (F) Drug treated group showing decrease hepatic fatty accumulation within the hepatocytes which limited to centrolobular area. Scale bar = 50 µm.

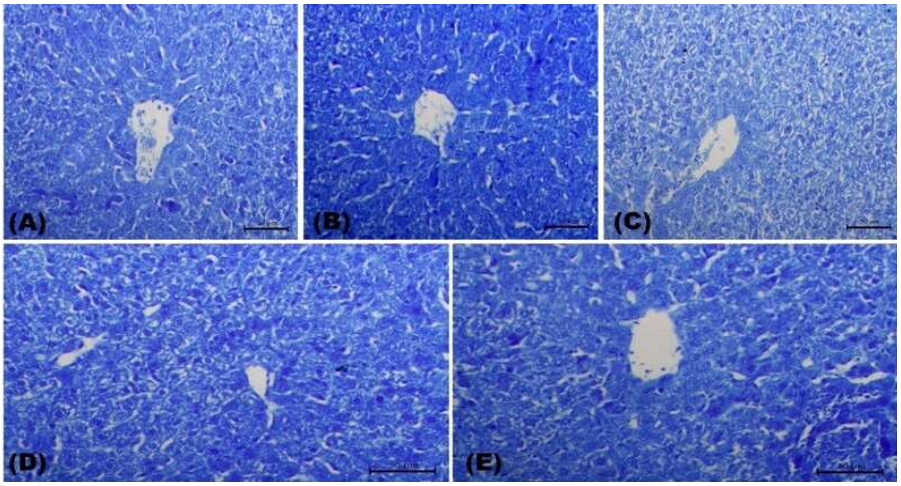
Fig 2: Microscopic photographs of H and E stained liver sections.



(A, B) Cont and CE administrated groups, respectively showing normal carbohydrate stained with deep magenta colour within the cytoplasm of the hepatocytes around the central vein (CV) (C) *E. papillata* infected group showing focal hepatic necrosis associated with depletion in carbohydrate content within the hepatocytes. (D, E) Inf-CE and drug treated groups, respectively showing marked increase of hepatic carbohydrate storage within their cytoplasms. Scale bar = 50 µm.

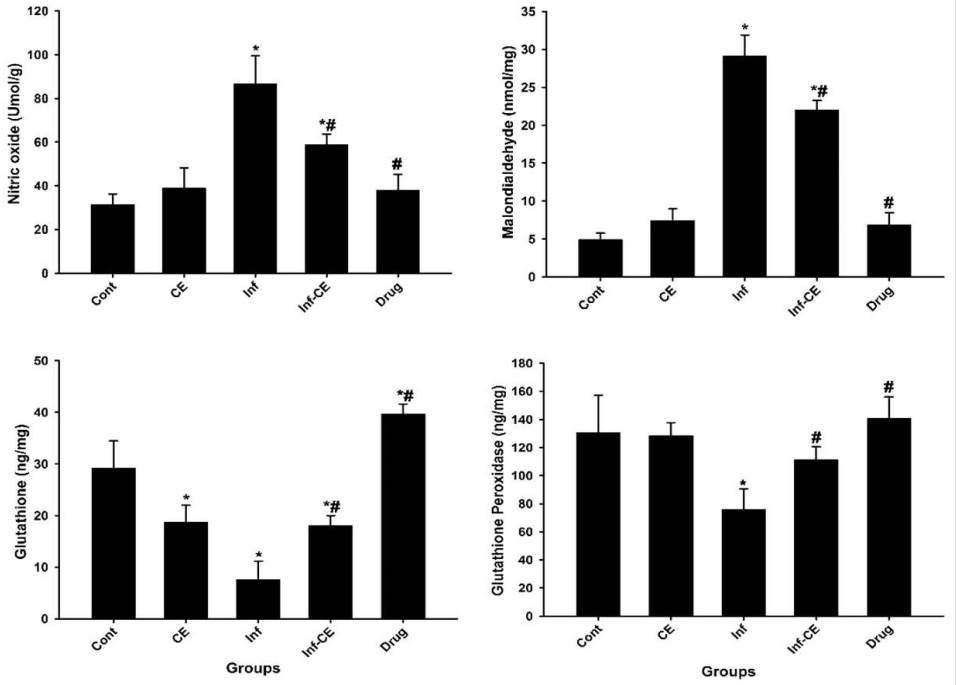
Fig 3: Microscopic photographs of periodic acid-schiff (PAS) stained liver sections.

showed that *E. papillata* infected mice exhibited hepatic injury resulting in the elevation of its antioxidant enzymes including NO and MDA (Fig 5). These results totally in agreement with Abdel-Gaber *et al*. (2024a). The higher levels of NO and MDA were due to their imbalanced status. This suggested the presence of oxidative stress resulting from the Eimeria infection and the disruption of the equilibrium between pro-oxidants and antioxidants (Abdel-Gaber *et al*., 2024a). in addition, Koinarski *et al*. (2005) and Georgieva *et al*. (2006) realized that elevated MDA and NO



(A, B) Cont and CE administrated groups, respectively showing deep bluish cytoplasmic staining. (C) *E. papillata* infected group showing hepatic vacuolation associated with decrease the cytoplasmic staining of bromophenol blue. (D, E) Inf-CE and drug treated groups, respectively showing increased hepatic cytoplasmic staining for bromophenol blue. Scale bar = 50 µm.

Fig 4: Microscopic photographs of mercuric bromophenol blue (BP) stained liver sections.



Results presented as averages ± SD. *Significant difference (P<0.05) related to cont group. #Significant difference (P<0.05) related to inf group.

Fig 5: Therapeutic effect of *Cassia alata* extract on hepatic antioxidant enzymes of infected mice with *E. papillata* on 5th day after treatment.

levels were an immune response due to the presence of infectious sporozoite stages, which infiltrate intestinal cells and induce inflammation. Whilst, the usage of CE against *E. papillata* infection had resulted in significant reduction in hepatic NO and MDA levels, compared to the infected mice (Fig 5). Al-Otaibi *et al*. (2023b) proved that the improvements of NO and MDA levels in the jejuna of *E. papillata* coccidian infected mice after medication with *Persea americana* extract as a result of the existence of phenolic compounds. This matches with Al-Otaibi *et al*. (2023a), who showed that phytochemicals in *Persea americana* extract could bind to important macromolecules on parasite membranes, disrupting them and subsequent death of the parasite.

Additionally, infected mice with *E. papillata* presented significant reduction in GSH and GPx levels in liver tissues (Fig 5). Similarly, Alajmi *et al*. (2023) and Al-Quraishy *et al*. (2024) indicated that GPx and GSH levels were decreased, respectively due to *E. papillata* infection. During Eimeria infection, GPx and GSH enzymes are crucial for safeguarding the host's body from damage caused by free radicals (Al-Sayed *et al*., 2022). Dkhil *et al*. (2015) and El-Ghareeb *et al*. (2023) clarified that Eimeria infection produces an imbalance in the antioxidant defense body system, whichdenotes to unfavorable cellular effects. Therapeutic treatment with CE enhanced hepatic levels of GSH and GPx antioxidant enzymes, relative to the infected group (Fig 5). The same results were proved by Alajmi *et al*. (2023) and Al-Quraishy *et al*. (2024). Awaad *et al*. (2016) hypothesize that the antioxidant components found in several different herbals have played a significant role in enhancing the defense against coccidiosis. *C. alata* leaves have been reported to contain a wide variety of bioactive phytochemicals (Veerachari and Bopaiah, 2012). Numerous phytochemical substances, including flavonoids, phenols, alkaloids, anthraquinones, tannins, saponins and cardiac glycosides were reported (El-Mahmood and Doughari, 2008; Eliakim-Ikechukwu, 2013). These phytochemicals have a crucial role in pharmacology through their anti-inflammatory, anti-bacterial, antifungal, antioxidant properties as its polyphenols could demonstrate strong scavenging action against free radicals and oxidative agents (Chew *et al*., 2022).

CONCLUSION

In summary, the current findings demonstrated that *C. alata* extract not only targets Eimeria stages within targeted diseased tissue, nevertheless also has anti-inflammatory properties that protect other host tissues as liver. The exclusion of phenolic and flavonoid components in CE may be responsible for its antioxidant, anti-inflammatory and hepatoprotective characteristics, which reduce the negative impacts of Eimeria infection on the affected hosts's vital biological parameters.

ACKNOWLEDGEMENT

The present study was supported by the Researchers Supporting Project (RSP2025R25), King Saud University, Riyadh, Saudi Arabia, and also supported by Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2025R96), Princess Nourah Bint Abdulrahman University, Riyadh, Saudi Arabia.

Disclaimers

The views and conclusions expressed in this article are for the authors solely and do not represent the views of the affiliated institutions.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques of the Institutional Animal Care and Use Committee of Kafrelsheikh University (KFS-IACUC) with an ethical permission number of KFS-IACUC/220/2024.

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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