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RESEARCH ARTICLE

Chlorogenic acid derived from *Moringa oleifera* leaf as a potential anti-inflammatory agent against cryptosporidiosis in mice

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ABSTRACT

Cryptosporidiosis is a serious illness in immunodeficient patients, and there is still no drug that can completely remove the parasite from the host. The present study represents the first report investigating the impact of the active molecule chlorogenic acid (CGA), naturally isolated from *Moringa oleifera* leaf extract (EMOLE), on immunosuppressed, *Cryptosporidium parvum*-infected BALB/c mice. Mice were divided into five groups: normal mice, infected immunosuppressed mice, and infected immunosuppressed mice treated with EMOLE, CGA, and nitazoxanide (NTZ) drugs. Parasitological, immunological, and histopathological investigations were recorded besides differences in the mice' body weight**.** Infected control mice showed elevated levels of oocyst shedding throughout the study. The EMOLE- and CGA-treated groups showed 84.2% and 91.0% reductions in oocyst shedding, respectively, with no significant difference compared to the drug control. The inflammatory markers IFN-γ, IL-6, IL-1β, and TNF-α were significantly higher in the infected control group. Treatment with 300 mg/kg/day of EMOLE or 30 mg/kg/day of CGA significantly downregulated pro-inflammatory cytokine levels compared to the infected group, although they did not change significantly compared to the NTZ-treated group. Histopathology of intestinal sections showed inflammatory and pathological changes in the infected control group. Low-grade tissue changes and an obvious improvement in villi structure were seen in mice treated with CGA. This study highlighted the role of CGA, isolated and purified from EMOLE, as an effective anti-inflammatory agent in eradicating *C. parvum* infection.

Keywords: *Cryptosporidium parvum*; chlorogenic acid; *Moringa oleifera*; pro-inflammatory cytokines.

INTRODUCTION

Cryptosporidiosis is caused by the protozoan parasite *Cryptosporidium parvum* and is considered a significant health problem. Saudi Arabia has the highest known prevalence of human *Cryptosporidium* infections in the Gulf region (Ahmed & Karanis, 2020). The prevalence of the disease has been studied in different areas, including Al-Taif, Dammam, Alkhobar, Jeddah, Makkah, Gizan, and Maddina (Hawash *et al*., 2014; Amer *et al*., 2016; El-Malky *et al*., 2018). *C. parvum* infects human small intestinal epithelial cells (IECs), causing diarrhea, weight loss, abdominal pain, fatigue, fever, headache, and vomiting (Hawash *et al*., 2014). In immunocompetent individuals, the cellular immune response mediated by both helper T cells, Th1 and Th2 can naturally clear the infection through the production of chemokines and antimicrobial peptides. The pro-inflammatory cytokine interferon-gamma (IFN-γ) is thought to be the main cytokine that controls *C. parvum* infection (Laurent & Lacroix-Lamandı, 2017); thus, infection in immunocompetent individuals is mostly asymptomatic, mild, and self-limiting (Ivanova *et al*., 2019). However, the infection can be life-threatening to immunocompromised individuals, as previous work has reported that extra-intestinal biliary or respiratory system involvements may occur in such patients (Khalil *et al*., 2018; Hafez & Hamed, 2021). In addition, immunosuppressed individuals may develop colorectal cancer (Osman *et al*., 2017).

At present, there are no completely effective drugs or vaccines to treat or prevent cryptosporidiosis. Nitazoxanide is used to treat immunocompetent patients, yet it is ineffective against immunodeficient individuals (Dumaine *et al*., 2019). Therefore, there is a strong need to discover alternatives to that available drug.

Moringa oleifera Lam. (Moringaceae) is a widely cultivated tree that is known for its medicinal uses. Studies on both humans and animals, including rodents, poultry, and aquatic animals, have disclosed that *M. oleifera* is safe for consumption (Mahfuz & Piao, 2019; Abidin *et al*., 2022); extracts from many parts of the tree, including flowers, leaves, and roots, have shown pharmacological properties (Stohs & Hartman, 2015; Lin *et al*., 2018; El-Sayed *et al*., 2019). *M. oleifera* has been described to possess antibacterial properties such as pyogenic bacteria (Arıvalo-Hɒjar *et al*., 2018; Fouad *et al*., 2019), antifungal properties such as *Botrytis cinerea*

(Ahmadu *et al*., 2021), and anti-inflammatory activities such as asthma, ulcerative colitis, and metabolic diseases (Alhakmani *et al*., 2013; Xiao *et al*., 2020) and anti-protozoal activities in killing *trypanosomes*, *Eimeria*, *Leishmania donovani*, and *Plasmodium malaria* (Stohs & Hartman, 2015; Somsak *et al*., 2016).

Reports have indicated that *M. oleifera* is a rich source of natural antioxidants and phytochemicals such as flavonoids, alkaloids, steroids, and tannins (Anzano *et al*., 2021; Bhalla *et al*., 2021). Moreover, it can reduce immunosuppression through the induction of cellular and humoral immune responses (Yasoob *et al*., 2021). Recent studies have reported the influence of *M. oleifera* on cryptosporidiosis in different experimental models, in *vivo* as well as in *vitro* (Laurent & Lacroix-Lamandꞌ, 2017; Aboelsoued *et al*., 2019; El-Sayed *et al*., 2019; Abidin *et al*., 2022). However, only a few reports have emphasized the effects of the active compounds isolated from these plants, and to the best of our knowledge, the active molecule in *M. oleifera* leaf extract (EMOLE) that plays the key role in the induction of immune responses against *C. parvum* infection remains unidentified. Phenolic compounds are among the major secondary metabolites that are found in EMOLE. Chlorogenic acid (CGA) is one of the well-known polyphenolic compounds that are produced by EMOLE and other plants. CGA is known for its anti-inflammatory, antidiabetic, antitumoral, and antiprotozoal activities (Wang *et al*., 2015; Majumder *et al*., 2020). In addition, CGA is well absorbed orally and may be a suitable natural compound for the prevention and treatment of gastric lesions caused by different etiologies (Wu *et al*., 2007; Shimoyama *et al*., 2013). Therefore, CGA might play an important role in protecting IECs against *C. parvum* infection.

The present work aimed to evaluate the effect of *M. oleifera* leaf extract and its isolated compound chlorogenic acid against *C. parvum* infection in mice.

MATERIALS AND METHODS

Plant material

Moringa oleifera Lam. leaves (MOL) were collected from 4-year-old *M. oleifera* trees cultivated in the King Faisal University Agriculture and Veterinary Research and Training Centre, Al-Ahsa, Saudi Arabia. *M. oleifera* Lam. was authenticated by the Department of Biological Science, College of Science, King Faisal University. The voucher specimen was maintained in the herbarium of the Biological Science Department, College of Science, King Faisal University (KFU/B.S./M-53). The fresh MOL was cleaned, sun-dried until a constant weight was reached, then ground to a fine powder and prepared for further investigation.

Preparation of *M. oleifera* leaf ethanol extract (EMOLE)

Three kilograms of dried MOL were extracted with aqueous ethanol (70%) using a Soxhlet apparatus at 80°C. This extraction process was sustained until the solution became clear. The EMOLE was filtered and evaporated under reduced pressure to achieve dryness. The dried EMOLE was kept in the fridge at 3°C for further investigation.

Phytochemical screening for EMOLE bioactive compounds

A variety of phytochemical tests have been performed on alkaloids, proteins, carbohydrates, flavonoids, saponins, tannins, and terpenes. The Dragendorff test was used to estimate the presence of alkaloids (Alam & Najum us Saqib 2015), while proteins and carbohydrates were determined by the Biuret and Molisch tests, respectively (Ezeonu *et al*., 2016). Flavonoids were determined by alcoholic sodium hydroxide and Pew's test. Saponin was determined by the foam test, tannins were determined by the ferric chloride test, and Salkowski's test was used to determine terpenes (Annapandian & Rajagopal, 2017).

Quantitation of EMOLE bioactive compounds

The total phenolic acids found in EMOLE were determined spectrophotometrically according to the Folin-Ciocalteu (FC) colorimetric method of Naczk and Shahidi (2004). Flavonoid content was determined by the colorimetric method, according to Naidoo *et al*. (2021). Terpenoid content was determined spectrophotometrically using the colorimetric method developed by £ukowski *et al*. (2021). Tannins were estimated using the spectrophotometric method according to Alwala *et al*. (2014). Saponins were estimated by the colorimetric method according to Obadoni and Ochuko (2002), and alkaloids were assayed by the gravimetric method according to Shamsa *et al*. (2008).

Investigation of phenolic acids

Isolation

The EMOLE was chromatographed on one-dimensional paper using the solvent systems B: A: W (4:1:5) and AcOH 15%. The AcOH (15%) system revealed the presence of principal flavonoids and phenolic acids. EMOLE was then exposed to multiple PPC (Whatman No. 3 mm) and TLC elution systems utilizing AcOH (15%) and ethanol: chloroform (1:9). For preparative paper chromatography (PPC), employ Whatman No. 3 mm and thin-layer chromatography (Harborne, 1989), the previous procedures of the investigation were used.

The component with the largest yield, pure phenolic acid, was purified using Sephadex LH-20 (Riedel-DE Heaen AG Sellze-Hannover). The main pure compound was analyzed using a ^{1}H-NMR spectrometer (JEOL EX-270 NMR).

Toxicity testing of CGA

To examine the toxicity of the active molecule CGA isolated from EMOLE, mice were treated in successive doses of 10, 20, 30, and 40 mg/kg/day for 14 days, according to the guidelines of the Organization for Economic Cooperation and Development (OECD, 2018). Following treatment with various doses, mice were closely observed for 10-15 minutes for any signs of acute toxicity, including shortness of breath, lethargy, or death. In addition, treated mice were observed for their overall health and daily activity and weighed daily throughout the experiments. In the present study, a daily dose of 30 mg/kg/day for 14 days was selected for oral treatment.

C. parvum oocysts isolation and mice infection

C. parvum oocysts were collected from naturally infected calves and exposed to the concentration technique with Sheather's sugar flotation method and stained with modified Ziehl–Neelsen (Henriksen & Pohlenz, 1981) to identify positive cases of *C. parvum*.

Sucrose flotation isolation procedures were performed to concentrate *C. parvum* oocysts that were preserved in a 2.5% aqueous potassium dichromate solution and stored at 4°C. For the mice's infection, *C. parvum* oocysts were washed three times with phosphate-buffered saline (PBS), and the number of oocysts was adjusted to 10^4 oocysts/ml PBS (Abdou *et al*., 2013).

Animal experiment

This study was approved by the Scientific Research Ethics Committee of King Faisal University. Internationally valid guidelines were applied to all animal experiments after acceptance by the institutional ethical committee. Fifty male BALB/c mice, 6 to 7 weeks old (weight 18 to 25 g), and clean from any parasitic infection, were maintained and bred at the animal house of the Biological Sciences Department, College of Science, King Faisal University, in accordance with the protocol approved by the Standing Research Ethics Committee. The animals were housed in well-ventilated cages with perforated covers

and supplied with standard pellet food and water. Body weight (BW) changes were measured daily. Before the experiment, the mice were given a week to adjust to their new surroundings.

Immune suppression of BALB/c mice was performed by oral injection with dexamethasone at a dose of 0.25 mg/g/day for 14 days before infection (Abdou *et al*., 2013) and also in mice after infection throughout the experiment.

Mice were divided into five groups of 10 mice each. **Group I:** normal mice, representing the negative control; the remaining mice were immunosuppressed with dexamethasone for 14 days, then divided into the following groups: **Group II:** immunosuppressed mice infected with 10^4 *C. parvum* oocysts/ml orally (infected control); **Group III:** immunosuppressed mice infected with 10^4 *C. parvum* oocysts/ml orally and treated with EMOLE at a dose of 300 mg/kg/day for 14 days (El-Sayed *et al*., 2019); **Group IV:** immunosuppressed mice infected with 10^4 *C. parvum* oocysts/ml orally and treated with CGA at a dose of 30 mg/kg/day for 14 days (CGA-treated); **Group V:** immunosuppressed mice infected with 10^4 *C. parvum* oocysts/ml orally and treated with 100 mg/kg nitazoxanide (NTZ) drug (NTZ-treated) daily starting on day 15 PI (Sadek & El-Aswad 2014). To confirm the infection, mice' feces were examined daily for oocyst recovery. Mice were sacrificed 4 weeks post-infection (Figure 1).

Parasitological examination

Feces samples of mice from each group were collected regularly post-infection until day 30 (the end of the experiment). Stained fecal smears were examined microscopically, and *C. parvum* oocysts were counted (Abdou *et al*., 2013).

Fecal smear examinations

Bright-field and fluorescence observations of fecal smears (1 slide per smear for each detection method) were performed at magnifications of 200x and 400x. The entire smear was examined to verify the presence of oocysts. If smears contained many oocysts, only a portion of the smear was examined. Oocyst numbers were demonstrated per 400x field and were classified as follows: 1+ for <2 oocysts, 2+ for 3 to 5 oocysts, 3+ for 5 to 7 oocysts, and 4+ for >8 oocysts (Rasmussen & Healey, 1992).

Efficacy of treatment

The efficacy of EMOLE and its active molecule CGA treatment against *C. parvum* oocysts was calculated according to the following formula (Egerton *et al*., 1964):

$$\text{Efficacy (\%)} = \frac{\text{Total oocysts before treatment (Group II)} - \text{Total oocysts after treatment}}{\text{Total oocysts before treatment (Group II)}} \times 100$$

Measurement of serum inflammatory marker levels

Interferon-gamma (IFN-γ), interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α) were measured in serum samples from the different studied groups using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc. and MyBioSource, USA).

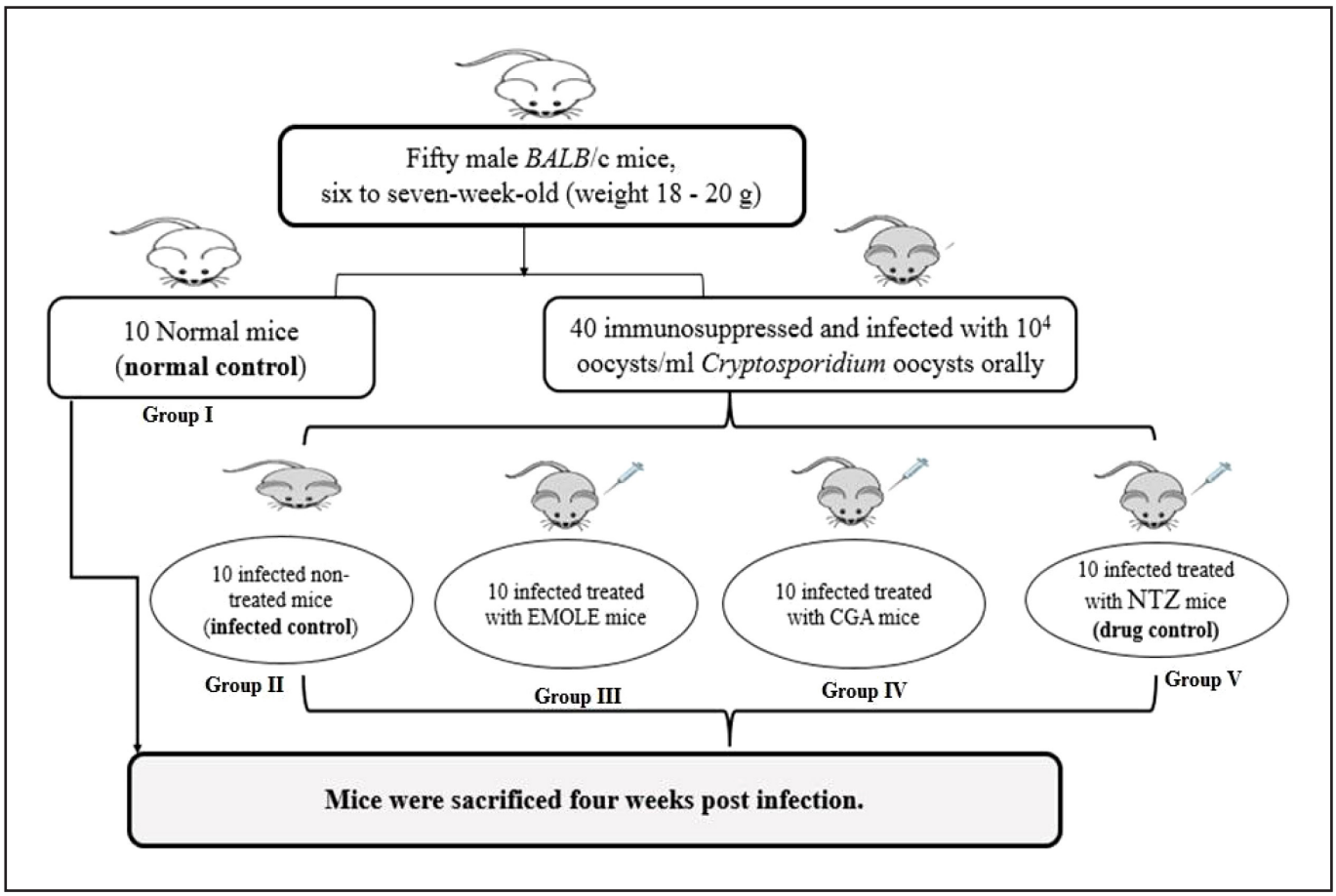

Figure 1. Experimental design flow chart.

Histopathological examinations

Small intestine sections (2 cm portions) were stained with haematoxylin and eosin in addition to Ziehl–Neelsen acid fast stain. The intestine sections were then examined with a light microscope (Gaafar, 2012). A histopathological scoring method was applied to evaluate the modifications in the tissues' structure (Hafez & Hamed, 2021), where histopathological lesions were scored in accordance with their severity.

Statistical analyses

Data were first tested for normality using the Kolmogorov-Smirnov test, and after ensuring the normality of the data, a one-way ANOVA was used, followed by a Tukey test to determine statistical significance, using SPSS version 20 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Descriptive analyses and measures of central tendency were performed to describe the sample's characteristics. Mean ± standard error of the mean (SEM) are used to express data. All statistical analyses were considered significant at $P < 0.05$.

RESULTS

Phytochemical analysis

The phytochemical tests on EMOLE showed the existence of flavonoids, alkaloids, carbohydrates, saponins, terpenoids, and tannins. According to the findings in Table 1, the highest concentration of bioactive compounds in EMOLE was phenolics (74.32 mg/g), while the lowest concentration was alkaloids (0.62 mg/g).

Table 1. Bioactive compounds of EMOLE

Bioactive compound	Concentration (mg/g) mean ± SEM
Phenolics	74.32 ± 3.10
Flavonoids	62.01 ± 2.95
Tannins	17.21 ± 0.12
Alkaloids	0.62 ± 0.02
Terpenoids	1.68 ± 0.04

Investigation of phenolic acids by ^{1}H-NMR spectra

Five compounds were isolated from EMOLE, while only one compound had an appropriate yield (40 mg) to perform the spectra analysis and the biological tests. The ^{1}H-NMR spectrum was 9.4 (s-OH), 9.1 (s-OH), 7.47 (1H, d, J = 17 Hz, α-H), 7.2 (1H, d, J = 2.5 Hz, H-2), 6.7 (1H, d, J = 7 Hz and 2.5 Hz, H-6), 6.6 (1H, d, J = 7 Hz, H-5), 6.2 (1H, d, J = 17 Hz, B-H), 5.5 (s-OH), 1.8, 2.2, 3.7, 4.5, 7. The purified molecule is recognized as chlorogenic acid (CGA) (Figure 2) based on previously revealed data and comparisons to those previously published (Mabry *et al*., 1970; Abd El-Moaty *et al*., 2020).

Toxicity test in CGA-treated uninfected mice

There was no death or any other interactive or toxic variation in all tested mice that received successive doses (10, 20, 30, or 40 mg/kg/day) of CGA. Also, there was a regular increase in body weight during the test. Consequently, these results revealed that CGA was not toxic to the treated mice throughout the experiments.

Impact of treatments with EMOLE and its active molecule CGA on *C. parvum* oocyst count in fecal samples of infected mice

The acid-fast-stained oocysts were identified as spherical, pink organisms with a diameter of about 5 µm (Figure 3). Throughout the study, immune-compromised, infected control BALB/c mice

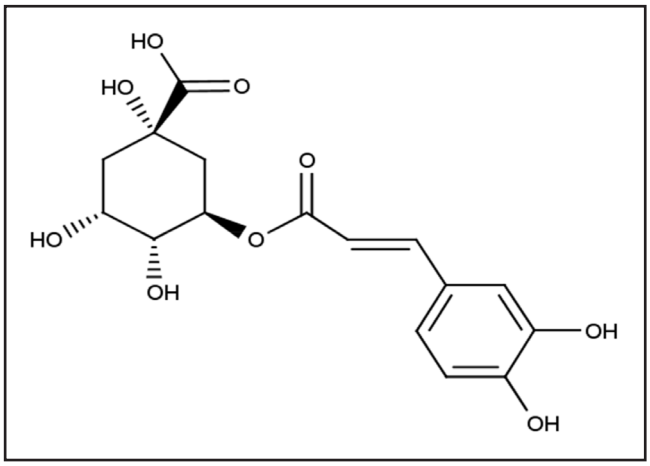

Figure 2. Chemical Structure of Chlorogenic acid.

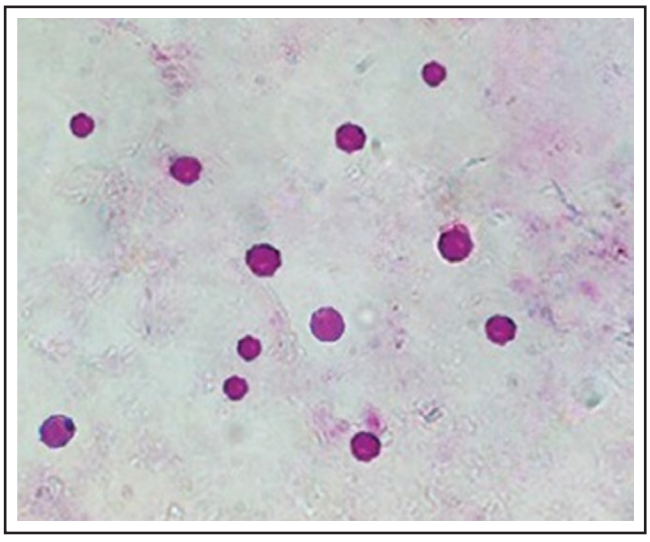

Figure 3. *C. parvum* oocysts shed in the stool of immunosuppressed infected control mice stained with modified Ziehl–Neelsen stain (400x).

Table 2. Measurement of oocyst shedding in stool samples of the studied groups on different days post-infection

Mouse groups	Day 4		Day 10		Day 15		Day 21	
	Mean±SE	% Reduction	Mean±SE	% Reduction	Mean±SE	% Reduction	Mean±SE	% Reduction
Normal	0	–	0	–	0	–	0	–
Infected	126.4±2.51	–	87±2.33	31.2%	69.2±1.14	45.3%	58.5±0.95	53.7%
EMOLE treated	74.5±2.08[a,b]	–	49.9±1.1[a,b]	33.0%	21.4±1.53[a,b]	71.3%	11.3±0.94[a,b]	84.8%
CGA treated	46.8±1.87[a]	–	20.2±1.56[a]	56.8%	7.6±0.90[a]	83.8%	4.2±0.72[a]	91.0%
NTZ treated	32.6± 1.42	–	11.1±0.67	65.9%	4.3±0.73	86.8%	2.9±0.52	91.1%

The *p*-value represents the relationship between the EMOLE- and CGA-treated groups, as well as the *C. parvum*-infected controls and NTZ-treated groups. [a] a statistically significant difference from the infected control group; and [b] a statistically significant difference from the NTZ-treated group.

shed more oocysts. As shown in Table 2, when compared to the infected control mice, the immunosuppressed infected and treated groups showed a highly significant ($P < .0001$) decrease in oocyst shedding until reaching minor numbers on day 21 post-treatment. The EMOLE-treated group showed an 84.8% reduction. However, the CGA-treated group showed a 91.0% reduction in *C. parvum* oocyst shedding with no significant difference from the NTZ-treated group, which reached a 91.1% reduction on day 21 post-treatment.

Impact of treatments with EMOLE and its active molecule CGA on bodyweight changes (Figure 4)

At the study's beginning, there was no significant variance in BW between the normal control group and all other groups. At the end of the experiment, a one-way ANOVA revealed statistically significant differences in mouse body weights between groups [$F(4,20) = 26.5$, $P < .0001$]. A Tukey post-hoc test revealed a significant decrease in BW in the infected group (22.83 ± 0.35, $P < .0001$) compared to the normal control (29.12 ± 0.77). There was no significant difference between the BWs in the CGA- and NTZ-treated groups when compared to the normal controls. Also, the CGA-treated group showed no significant difference from the NTZ-treated group.

Impact of treatments with EMOLE and its active molecule CGA on inflammatory markers (Figure 5)

Levels of IFN-γ (Figure 5A): A one-way ANOVA revealed a significant difference in IFN-γ inflammatory levels [$F(4,20) = 3474$, $P < .0001$]. A Tukey post-hoc test revealed a significant increase in the infected control group (584.10 ± 5.55, $P < .0001$) when compared to the normal control group (118.0 ± 1.64) and NTZ-treated group (164.0 ± 1.61). When compared to the infected control group, IFN-γ levels were significantly lower in the EMOLE (243.0 ± 2.59, $P < .001$), CGA (198.0 ± 2.62, $P < .0001$), and NTZ-treated groups ($P < .0001$).

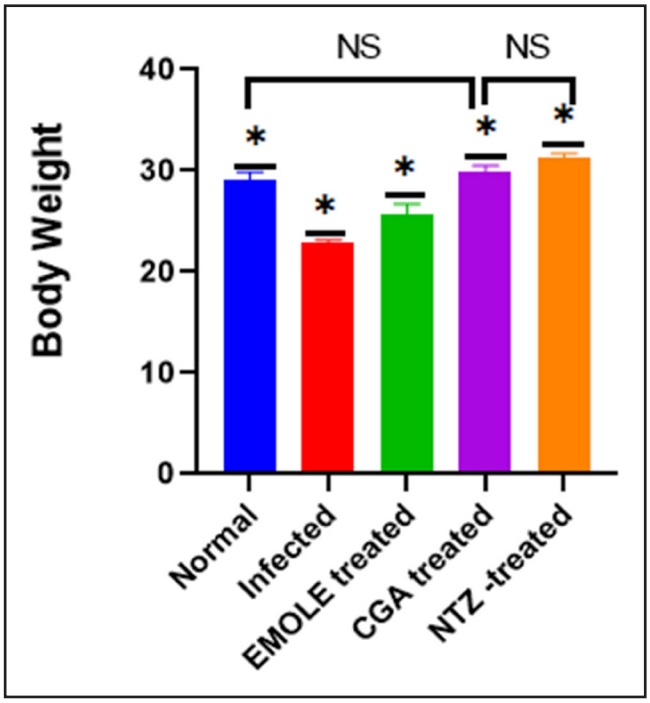

Figure 4. Impact of treatments with EMOLE and its active molecule CGA on bodyweight changes in different studied groups. All data are expressed as mean ± SE (n = 10 mice per group). *p*-value represents the relationship between EMOLE- and CGA-treated groups and normal controls, and between *C. parvum*-infected controls and drug controls. *Statistically significant in comparison to the normal and/or infected control groups. NS = non-significant in comparison to the normal control and/or NTZ groups.

Levels of IL-1β (Figure 5B): A significant difference in IL-1β levels was detected [F (4,20) = 67.80, *P* <.0001], with a significant upregulation in the infected group (44.0 ± 0.88, *P* <.0001) when compared to both the normal control (22.60 ± 0.83) and NTZ groups (25.9 ± 0.95). Levels were downregulated significantly on treatment with EMOLE (30.10 ± 1.29, *P* <.01), CGA (26.30 ± 1.054, *P* <.001) and NTZ (25.90 ± 0.95, *P* <.001). However, in the CGA group, IL-1β did not change significantly compared to the normal and NTZ-treated groups.

Levels of IL-6 (Figure 5C): A significant difference in IL-6 levels was detected [F (4,20) = 75.32, *P* <.0001], with significant upregulation in the infected group (59.0 ± 2.02, *P* <.0001) when compared to both normal controls (22.9 ± 1.08) and the NTZ group (26.7 ± 1.70). Levels were downregulated significantly on treatment with EMOLE (37.9 ± 1.92, *P* <.01), CGA (28.2 ± 1.45, *P* <.0001), and NTZ (26.7 ± 1.70, *P* <.0001). However, IL-6 levels did not change significantly in the CGA group compared to the normal and NTZ-treated groups.

Levels of TNF-α (Figure 5D): A one-way ANOVA revealed a significant difference in TFN-γ inflammatory levels [F (4,20) = 993.5, *P* <.0001]. A Tukey post-hoc test revealed a significant increase in the infected control group (298.6 ± 4.40, *P* <.0001) when compared to the normal control group (86.50 ± 1.55) and NTZ-treated group (108.7 ± 2.92). When compared to the infected control group, TNF-α levels were significantly lower in the EMOLE (147.8 ± 1.73, *P* <.001), CGA (125.7 ± 1.52, *P* <.0001), and NTZ-treated groups (*P* <.0001).

Histopathological investigations

The changes in the histopathology of intestinal sections stained with H&E stain are represented in Figure 6 and Table 3. The control group showed normal histology of villi and mucosal thickness. The *C. parvum*-infected control group showed numerous inflammatory and pathological changes consistent with the disease, including loss of epithelium with short, fused, and blunted villi; altered mucosal architecture; a compact inflammatory infiltrate; and increased oocyst numbers with a severe interstitial inflammatory infiltrate.

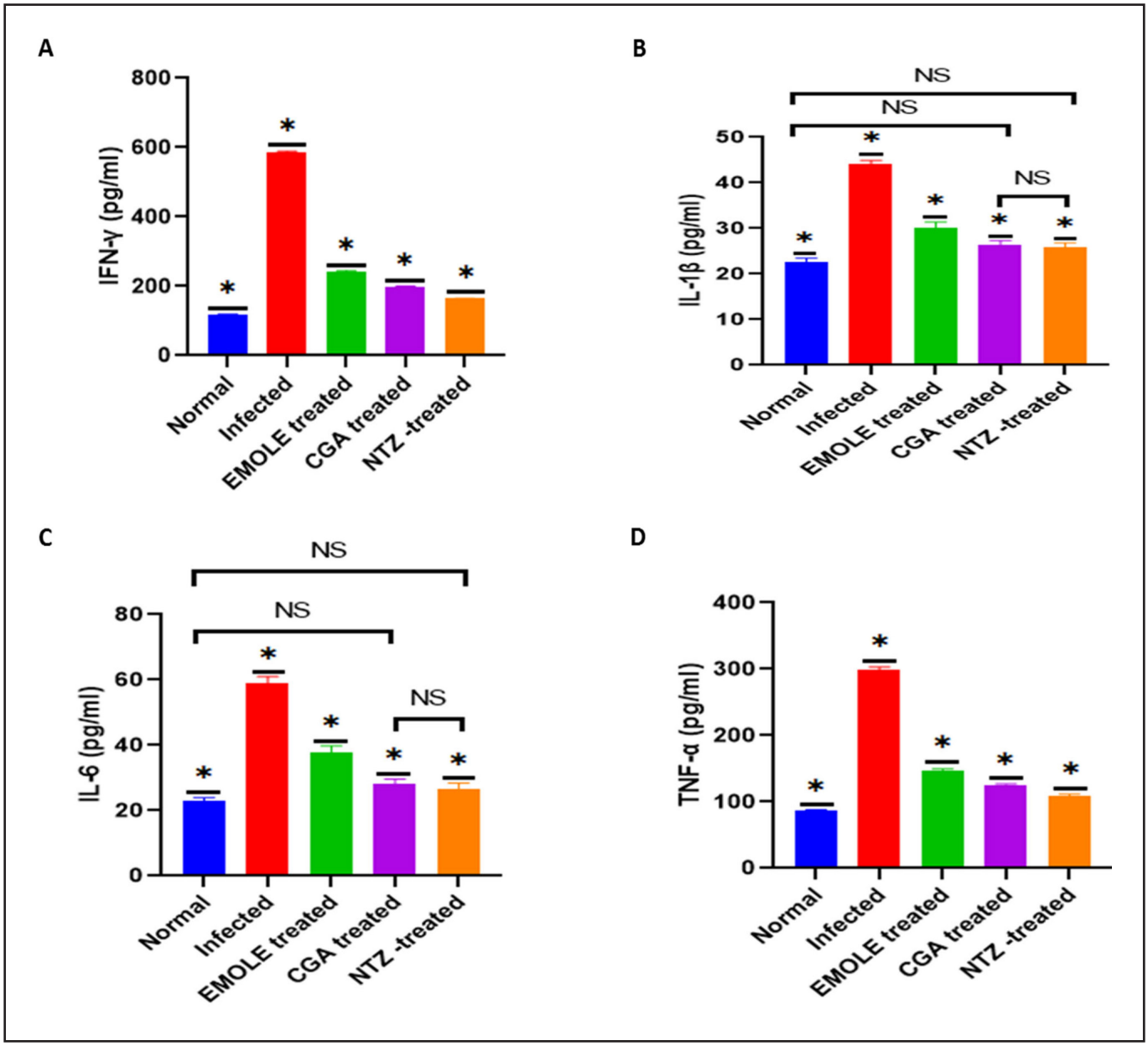

Figure 5. Impact of treatments with EMOLE and its active molecule CGA on inflammatory markers in different studied groups. **A) IFN-γ, B) IL-1β C) IL-6 and D) TNF-α.** All data are expressed as mean ± SE (n = 10 mice per group). *p*-value represents the relationship between EMOLE- and CGA-treated groups and normal controls, and between *C. parvum*-infected controls and drug controls. *Statistically significant in comparison to the normal and/or infected control groups. NS = non-significant in comparison to the normal control and/or NTZ groups.

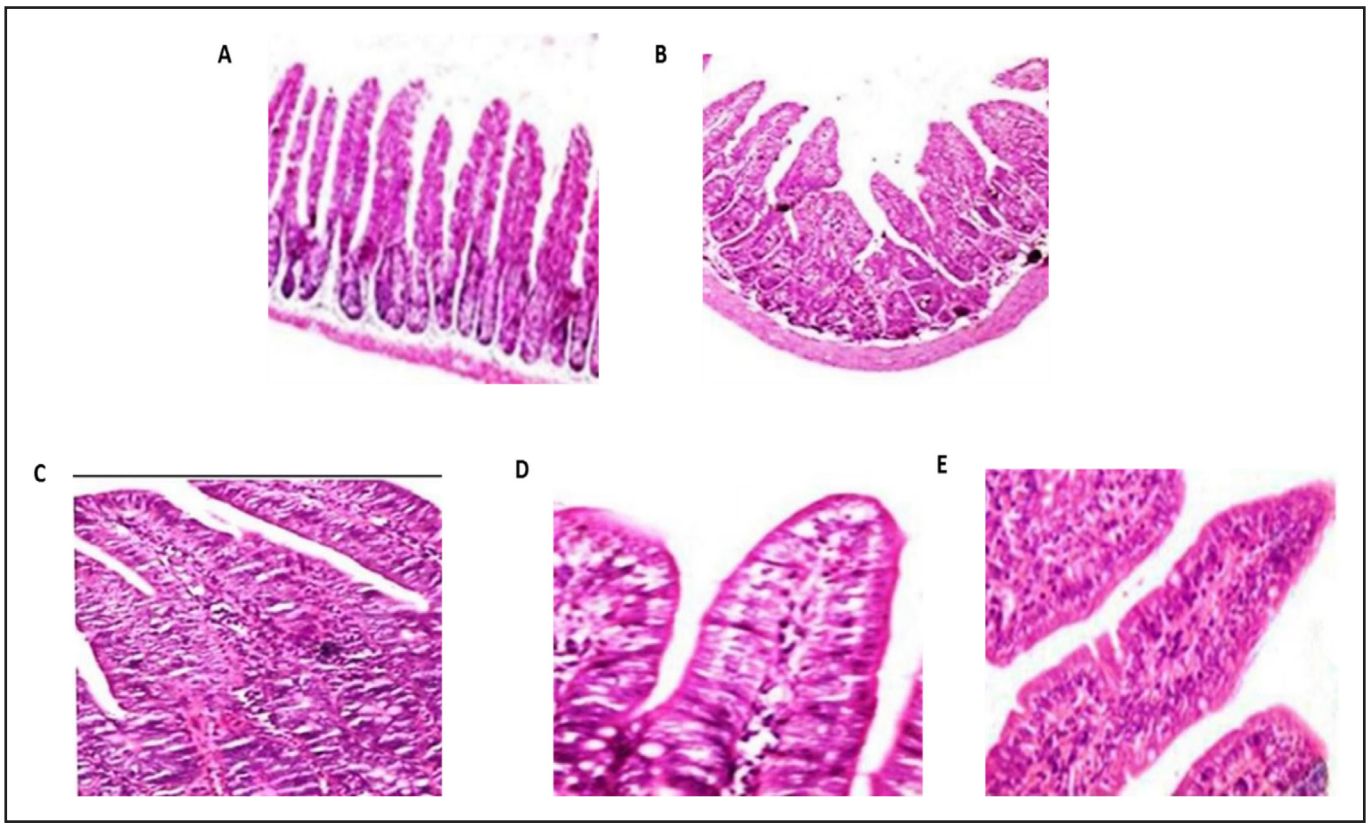

Figure 6. Light microscopy of the small intestine shows changes in intestinal sections of different studied groups stained with haematoxylin and eosin (H&E): **A)** Normal control group shows normal histology of villi. **B)** Infected control group illustrates loss of epithelium with short, fused, and blunted villi and altered mucosal architecture with a compact inflammatory infiltrate. **C)** EMOLE-treated *C. parvum*-infected mice show a significant decrease in induced mucosal damage and relatively small vacuoles when compared to the infected control mice. **D)** CGA-treated *C. parvum*-infected mice show notable improvement, where most of the villi are intact. **E)** NTZ-treated shows improved villi structure. Haematoxylin and eosin stain; scale bars, 200 µm.

Table 3. Histopathological lesions in the intestinal sections of different studied groups

Mouse groups	Mucosal thickness	Shortening of villi	Blunting of villi	Inflammatory infiltrate	Oocyst
Normal	0.00± 0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
Infected	2.80±0.20[b]	2.60±0.54[b]	2.40±0.54 [b]	2.10±0.55[b]	2.60±0.54[b]
EMOLE treated	1.40 ± 0.54[a]	0.80±0.83[a]	1.60±0.54[a]	1.40±0.55[a]	1.20±0.47[a]
CGA treated	0.40 ± 0.54[a]	0.30±0.54[a]	0.10±0.24[a]	0.30±0.45[a]	0.00±0.00[a]
NTZ treated	0.00 ± 0.00[a]	0.20±0.44[a]	0.00±0.00[a]	0.20±0.54[a]	0.00±0.00[a]

Histopathological lesions were scored in accordance with their severity. Mean±SE of the mean are used to express all data. The *p*-value represents the relationship between the EMOLE- and CGA-treated groups, as well as the *C. parvum*-infected controls and NTZ-treated groups. [a] statistically significant difference from the infected control group; and [b] a statistically significant difference from the NTZ-treated group.

Concerning the EMOLE-treated group, mild tissue changes were observed. However, on treatment with the active molecules CGA and NTZ and compared to the infected control group, significant low-grade tissue changes were seen in mice, including an obvious improvement of villi structure in both shortening [F (4,20) = 18.33, $P < .0001$] and blunting [F (4,20) = 19.82, $P < .0001$], with average mucosal thickness [F (4,20) = 44.75, $P < .0001$] and inflammatory infiltrate [F (4,20) = 22.091, $P < .0001$] as well as *C. parvum* oocysts [F (4,20) = 33.01, $P < .0001$].

DISCUSSION

Immunocompromised patients continue to suffer from the serious sickness caused by *C. parvum* infection, and there is still no medication that can eradicate the parasite completely from the host. Therefore, supportive therapy from medicinal plants is a good alternative to synthetic drugs (WHO, 2010; Jin *et al*., 2019). Although some recent studies have discussed the activity of *M. oleifera* against *C. parvum* infection (Aboelsoued *et al*., 2019; El-Sayed *et al*., 2019), to the best of our knowledge, the present study represents the first report to investigate the activity of the active molecule chlorogenic acid naturally isolated from *M. oleifera* against *C. parvum* infection.

The present study showed that EMOLE contains high levels of phenolic compounds, flavonoids, and tannins, in which is consistent with previous published works (Anzano *et al*., 2021; Bhalla *et al*., 2021; Kerdsomboon *et al*., 2021). In agreement with Amaglo *et al*. (2010), we found that CGA was one of the major phenolic compounds in *M. oleifera* leaves; this result highlights its role as a primary bioactive phytoconstituent.

Previous studies have shown a high degree of safety using *M. oleifera* leaf extract, with no side effects on humans or animals (Stohs & Hartman, 2015; El-Sayed *et al*., 2019). Regarding CGA toxicity, our results revealed that treatment with 10, 20, 30, or 40 mg/kg/day of CGA did not yield any toxic symptoms or body weight loss in mice. This result agrees with the earlier report of Alarcɳn-Herrera (2017), who indicated that *in vivo* administration of 100 mg/kg of CGA has no toxic effects.

In this study, treatment with EMOLE led to a significant decrease in *C. parvum* oocyst count in comparison to the immunosuppressed infected mice, which reached an 85.2% reduction at the end of the experiment. Similarly, a number of studies have shown that oral injection of immunosuppressed *C. parvum*-infected mice with *M. oleifera* ethanol or methanol extract reduced the oocysts' shedding (Aboelsoued *et al*., 2019; El-Sayed *et al*., 2019; Hafez & Hamed, 2021).

Many studies have disclosed that phenolic acids, among other natural compounds, have inhibitory effects against protozoan parasites (Friedman *et al*., 2018; Majumder *et al*., 2020). Consistent with this idea, our results showed that treatment with CGA led to a highly significant decrease in *C. parvum* oocyst shedding, reaching 91.0%, which was nearly equivalent to that of NTZ-treated mice (91.1%). This may suggest that the therapeutic potential of *M. oleifera* is related to its content of active molecules, particularly CGA. Many previous studies have documented the antiprotozoal activity of CGA. Majumder *et al*. (2020) showed that CGA completely cleared *Leishmania* amastigotes from infected cells *in vitro*.

Cryptosporidiosis causes long-term pan-body effects, mainly bodyweight loss. This study detected an improvement in body weight after all treatments, which may have contributed to the host immune response improvement (Gaber *et al*., 2022).

The effect of EMOLE and CGA on inflammatory markers was investigated to determine the host immune response. In this study, *C. parvum* infection resulted in a significant increase in the pro-inflammatory cytokines IFN-γ, IL-6, IL-1β, and TNF-α when compared to control mice. This suggests a potential upsurge of the Th1 response in *C. parvum* infection through the modulation of inflammatory markers to eradicate the infection. This result runs parallel with previous studies, which proved that the acute phase of *C. parvum* infection coincided with elevated levels of pro-inflammatory cytokines that control the parasite in infected IECs, and these inflammatory markers play the main role in the eradication of infection through the recruitment of specialized immune cells (Laurent & Lacroix-Lamandꞌ, 2017).

Compared to control mice, our data showed that treatment with EMOLE and CGA in *C. parvum*-infected mice led to highly significant downregulation in levels of IFN-γ, IL-6, IL-1β, and TNF-α, consistent with previous findings (Aboelsoued *et al*., 2019; El-Sayed *et al*., 2019), suggesting the positive effect of EMOLE and CGA extracts in eradicating *C. parvum*. Our data showed that CGA injection into immunosuppressed *C. parvum*-infected mice led to a highly significant downregulation in levels of pro-inflammatory cytokines, including IFN-γ, IL-6, IL-1β, and TNF-α, compared to the infected control and with no significant difference from NTZ-treated mice. Meanwhile, our data indicated the anti-inflammatory role of both EMOLE and CGA through the reduction of pro-inflammatory cytokines to downregulate the severity of the infection. CGA has been shown to reduce hepatic mRNA expression and serum levels of TNF-α, IL-6, and IL-1 in rats with carbon tetrachloride-induced inflammation (Han *et al*., 2017; Bagdas *et al*., 2020; Yu *et al*., 2021; Lee *et al*., 2022). Thus, when combined with the present data, these observations support the hypothesis that CGA directly reduces pro-inflammatory cytokines, which in turn leads to a decrease in inflammation.

The histopathological intestinal sections from the immunosuppressed *C. parvum*-infected mice showed the loss of epithelium with short, fused, and blunted villi and altered mucosal architecture because of the release of pro-inflammatory mediators due to the host immune response to the infection. Meanwhile, treatment with EMOLE led to a significant decrease in induced mucosal damage and relatively small vacuoles when compared to infected control mice. CGA-treated *C. parvum*-infected mice showed significant improvement, with most villi intact and no significant difference from NTZ-treated mice. Studies conducted *in vitro* and *in vivo* demonstrate that an excess of pro-inflammatory cytokines might have a detrimental effect on the integrity, permeability, and epithelial functions of the intestinal mucosa (Ruan *et al*., 2016; Chen *et al*., 2018; Yu *et al*., 2021). In the current investigation, CGA treatment decreased the levels of pro-inflammatory cytokines. These results suggest that CGA is effective at lowering inflammation of the intestinal mucosa.

In conclusion, this study highlights the role of the active molecule chlorogenic acid, isolated and purified from *M. Olivera* leaf extract, as an effective anti-inflammatory agent in eradicating *C. parvum* infection. Further studies are recommended to investigate CGA's mechanism of action in order to understand the inflammatory responses.

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Conflict of Interests

Authors declare that there is no Conflict of Interests.

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