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RESEARCH ARTICLE

Elettaria cardamomum essential oil; immunomodulatory, antioxidant, and anti-inflammatory effects for controlling acute *Toxoplasma gondii* infection

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ABSTRACT

The present study was conducted to investigate the immunomodulatory and anti-inflammatory effects of *Elettaria cardamomum* essential oil (ECEO) for the control of acute *Toxoplasma gondii* infection. The effect of ECEO on *T. gondii* tachyzoites was measured by the tetrazolium bromide method. Mice received ECEO orally at doses of 1-4 mg/kg/day for 14 days. Once acute toxoplasmosis was induced in mice, their mortality rate and parasite load were recorded. The level of liver antioxidant/oxidant enzymes and the level of mRNA expression of interleukin-1 beta and interferon-gamma were also investigated. ECEO particularly at a concentration of 150 µg/ml has promising *in vitro* anti-*Toxoplasma* effects (p<0.001). After treatment with ECEO, the mortality rate (9th day) and parasite load decreased (p<0.001) in the infected mice. ECEO markedly (p < 0.05) restored hepatic oxidant and antioxidant enzyme levels, as well as increased cytokines. These results report a significant inhibitory effect of ECEO mainly at a dose of 4 mg/mL, against the *T. gondii* Rh strain through strengthening the immune system and reducing inflammation and oxidative stress; however, further research is needed to verify these results.

Keywords: *Toxoplasma*; herbs; tachyzoites; *in vitro*; *in vivo*.

INTRODUCTION

Toxoplasmosis (TOP), which is derived from the *Toxoplasma gondii* parasite, is recognized as one of the most common parasitic diseases in the world (Saadatnia & Golkar, 2012; Molan *et al*., 2019). Humans acquire this infection by ingesting *T. gondi*i cysts in food and water, by ingesting tissue cysts in raw and undercooked meat (Martinez *et al*., 2018), and via spreading to the placenta during pregnancy (Al-Agroudi, *et al*., 2017). In people with well-functioning immune systems, TOP is usually asymptomatic and rarely causes severe symptoms; however, in people with weakened immune systems, serious signs such as encephalitis, pneumonia, and seizures can be seen (Dubey *et al*., 2012). The main chemotherapeutic agents for the treatment of TOP are pyrimethamine, sulfadiazine, and atovaquone (Gajurel *et al*., 2015). Many studies have shown that these substances have various unpleasant side effects, such as hematuria, gastrointestinal disturbances, hypersensitivity reactions, and teratogenicity (Gajurel *et al*., 2015). Therefore, finding new drugs with the fewest side effects is a top priority for scientists in this field. Due to the lack of highly effective *Toxoplasma* vaccines, clinicians have strongly advocated prevention strategies, mainly for individuals such as patients with weakened immune systems and pregnant women (Wang *et al*., 2017).

Medicinal herbs are commonly used to treat and prevent various infections due to their high efficacy and accessibility, low cost, and minimal side effects (McFarland *et al*., 2016). Many studies have been reported on the preventive capacity of many plants in TOP, for example, *Zataria* spp*, Allium* spp. and *Curcuma* spp. (Montazeri *et al*., 2017; Cheraghipour *et al*., 2021); however, their commercial use to control TOP has not been verified due to their different findings as well as their safety.

Green cardamom, or *Elettaria cardamomum* L., of the family Zingiberaceae, is recognized as a flavorful spice commonly used in food and conventional remedies (Ashokkumar *et al*., 2020) to cure diseases such as asthma, infections, and gastrointestinal ones (Sharma *et al*., 2011). In modern medicine, this plant shows promising anti-diabetic, antinociceptive, anti-inflammatory, anticancer, insecticidal, and antimicrobial activities (Sharma *et al*., 2011; Saeed *et al*., 2014; Garg *et al*., 2016; Ashokkumar *et al*., 2021). Reviews reported that *E. cardamomum* essential oil (ECEO) contains certain components, such as 1,8-cineole, alpha-terpinol, alpha-terpineol, linalyl, and camphor (Kaskoos *et al*., 2006; Goudarzvand Chegini & Abbasipour, 2017). The present study was conducted to investigate the immunomodulatory and anti-inflammatory effects of ECEO in the prevention of acute *T. gondii* infection.

MATERIALS AND METHODS

Plant collection

E. cardamomum seeds were obtained from a shop in the city of Riyadh, Saudi Arabia. Purchased seeds were labeled by a botanist, and a voucher sample (No. 1805) was registered at the herbarium of the Shaqra University, Saudi Arabia.

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Isolating essential oil
The ECEO was obtained through the hydro-distillation approach. Briefly, 200 g of powdered seeds were mixed with distilled water in a Clevenger glass apparatus for four hours, then dehydrated with sodium sulfate and stored in a refrigerator until testing (Mahmoudvand *et al*., 2016; Shaapan *et al*., 2021).

Gas chromatography-mass spectrometry (GC-MS)
Hewlett-Packard 6890 (USA), equipped with an HP-5MS column (30 m × 0.25 mm, film thickness 0.25 mm), was used to determine chemical combinations of ECEO based on the previous study (Almohammed *et al*., 2022). The analysis was done based on the difference between their mass spectra with the NIST mass spectral library (NIST, 2014) and literature values (Adams, 2007; Mahmoudvand *et al*., 2017; Alyousif *et al*., 2021)

Parasites and cell culture
Tachyzoites from the RH strain (*T. gondii*) were used and adjusted to 1×10^6 and 1×10^4 parasites/mL for *in vitro* and *in vivo* experiments, respectively. Vero cells were cultured in RPMI-1640 with 10% FBS and penicillin/streptomycin (100 µg/mL) at 37°C with 5% CO_2.

Effects of ECEO on tachyzoites
First, 0.2 mL of tachyzoites were exposed to ECEO at the concentrations of 32.5–150 µg/mL for 0.5–3 h at 37°C. Then, 0.05 mL of MTT liquid was transferred to the test wells and incubated four hours at 37°C with 5% CO_2. Next, DMSO stop solution was added to the well, and the OD of the samples was reported at 570 nm by an ELISA reader (LX800 USA) (Teimouri *et al*., 2018). The negative and positive controls were normal saline + 0.1% tween-20 and atovaquone 100 µg/mL, respectively, whereas all tests were done in triplicate.

Flow cytometry analysis
Annexin-V (AV) analysis was used to determine the early and late apoptosis after tachyzoite treatment with ECEO at concentrations of 32.5, 75, and 150 µg/ml utilizing an APOAlert® kit (CA, USA) based on the manufacturer's instructions. Briefly, followed by treatment for 180 min, the cells were washed and removed with AV-FITC and propidium iodide. Finally, the level of apoptotic and necrotic cells was determined using a flow cytometer (Biosciences, USA) (Zaki *et al*., 2020).

Inhibitory effects on infectivity and parasites in the cells
To assess the effects of ECEO on infection rates and intracellular parasites, firstly, Vero cells (1×10^5) were seeded on a coverslip and incubated at 37°C for 24 h. Then, cells from each well were infected with 0.1 mL of *T. gondii* tachyzoites (1×10^6 cells/mL) for 24 h. In the next step, after removing the supernatant, cells were infected with tachyzoites and treated with different concentrations of ECEO (32.5–150 µg/mL), normal saline plus 0.1% tween-20 (negative control), and atovaquone 100 µg/mL (positive control) for 48 h. After providing slides and staining with Giemsa, the percentage of infected cells and the number of parasites were examined in 100 cells under a light microscope (Teimouri *et al*., 2018). We estimated the means of cells treated with normal saline plus 0.1% tween-20, which is related to 100% of *T. gondii* infection and proliferation rates.

Cytotoxicity on Vero cells
Vero cells were exposed to different concentrations of ECEO in a 96-well plate for 48 h at 37°C with 5% CO_2. The MTT colorimetric assay was applied to study the cytotoxic effect of ECEO on the viability of Vero cells. Then a cytotoxic concentration of 50% (CC_{50}) was determined for ECEO-treated Vero cells (Albalawi *et al*., 2021a, 2021b).

Animals
A total of 84 male BALB/c mice (42–56 days old) weighing 20–25 g was used. The animals were kept in optimal conditions with an unlimited supply. The required agreement was obtained from the Ethics Committee of Almaarefa University, Saudi Arabia (IRB07-18052022-46).

Effects of ECEO on acute *T. gondii* infection
Sixty mice were divided into 5 groups (12 mice per each), including those given orally for 14 days with normal saline plus 0.1% Tween 20 (as solvent) (E1), atovaquone 100 mg/kg/day (E2), and ECEO 1-4 mg/kg/day (E3-E5) (Keyhani *et al*., 2020). On the day 15 (one day after the treatment), mice in all groups were infected intraperitoneally with 200 µL of tachyzoites.

Mortality rate and parasite load
Tested mice were examined regularly, and their mortality rate was recorded. In addition, on day 3, the peritoneal fluid of the tested mice was obtained, and the amount of tachyzoites was recorded under the microscope (Shakibaie *et al*., 2020).

Preparation of liver homogenates
Three days after infection, in each group, six mice were euthanized (by ketamine-xylazine). After the collection of the liver organs, 0.1 g of liver tissue was ground and homogenized in cold PBS buffer using a steel homogenizer. After centrifuging the homogenate at 5,000 rpm and 4°C for 15 min, the upper phase was used to assess inflammatory, antioxidant, and proinflammatory markers (Zakaria *et al*., 2018).

Evaluation of inflammatory markers
The levels of lipid peroxidation (LPO) and nitric oxide (NO) were checked using the Lipid Hydroperoxide (LPO) Assay Kit (ab133085, abcam, USA) and the Nitric Oxide Assay Kit (ab272517, abcam, USA), based on the manufacturer's instructions, respectively (Albalawi *et al*., 2022).

Evaluation of antioxidant enzymes
The assessment of hepatic levels of glutathione peroxidase (GPx) and superoxide dismutase (SOD) enzymes was performed using the GPx Assay Kit (ab102530, abcam, USA) and SOD Activity Assay Kit (ab65354, abcam, USA) consistent with the method explained by the manufacturer, respectively.

Evaluation of mRNA expression level by real-time PCR
First, RNA was extracted through the Qiagen RNeasy kit (Germany) as ordered by the company. Using random primers, the cDNA was produced by the Qiagen's cDNA synthesis kit (Germany) based on the company orders and used for the real-time PCR via the oligonucleotide primers showed in Table 1 (Albalawi *et al*., 2021c). The reaction conditions were 95°C for 6 min, 40 cycles at 95°C for 10 s, and 56°C for 35 s, respectively. The $2^{-\Delta\Delta CT}$ method was used by the Bio-Rad system software (Hercules, USA) to study the expression level.

Table 1. The primers were applied for real-time PCR

Amplicon	Primers	Sequence (5'–3')	Size (bp)
IL-1β	F	AACCTGCTGGTGTGTGACGTTC	78
	R	CAGCACGAGGCTTTTTTGTTGT	
IFN-γ	F	ATGAACGCTACACACTGCATC	182
	R	CCATCCTTTTGCCAGTTCCTC	
β-actin	F	GTGACGTTGACATCCGTAAAGA	245
	R	GCCGGACTCATCGTACTCC	

Toxicity effects on healthy mice

Twenty-four healthy mice were divided into four groups, a control group receiving normal saline and three experimental groups receiving oral ECEO at doses of 1-4 mg/kg/day for 14 consecutive days. After this time, mice blood samples were obtained through cardiac puncture to obtain serum (Mahmoudvand *et al.*, 2019). Commercial kits from Roche (Germany) were used to assess the amounts of liver enzymes (e.g., AST, ALT, and ALP) and renal function factors (e.g. Cr, and BUN).

Statistical analysis

All *in vitro* experiments were performed in triplicate. The collected data were analyzed using SPSS software (version 22.0). P<0.05 is reported as a significant difference.

RESULTS

GC/MS analysis

The yield of white essential oil was 3.7% (v/w). As shown in Table 2, GC/MS contained 19 compounds in ECEO, accounting for 98.2% of the total essential oil. The highest components were 34.3, 23.3, and 17.7% for 1,8-cineole, α-terpinyl acetate, and α-pinene, respectively.

Effects of ECEO on tachyzoites

As shown in Figure 1, ECEO showed a strong *in vitro* antiparasitic effect (p<0.001) on the viability of *T. gondii* tachyzoites in a dose- and time-dependent manner compared with the control group. So, ECEO completely inhibited the growth rate of tachyzoites at 75 and 150 µg/mL followed by 3 and 2 h of exposure, respectively.

Flow cytometry analysis

The results showed that ECEO raised (p < 0.01) the level of necrotic cells from 0.12% to 3.02%. After treating tachyzoites with ECEO, the percentage of early and late apoptotic cells considerably increased from 0.29 and 0.47% to 53 and 19%, respectively (Figure 2).

Effect on infectivity rate and cytotoxicity activity

As shown in Figure 3B, the microscopic examination revealed the infection rates for 32.5, 75, and 150 µg/mL were 62.1, 51.3%, and 20.2%, respectively (p<0.001) (Figure 3A). *In vitro* results also showed that the intracellular reproduction of tachyzoites in infected Vero cells was markedly reduced (p<0.001) after treatment with different doses of ECEO (Figure 3B). The CC_{50}

Table 2. GC/MS analysis of chemical compositions of *Elettaria cardamomum* essential oil

No	Compound	RI[a]	RI[b]	Composition (%)
1.	α-thujene	927	921	1.6
2.	α-pinene	937	930	17.7
3.	Camphene	952	951	1.4
4.	Sabinene	968	975	3.2
5.	Myrcene	986	990	1.3
6.	1,8-cineole	1028	1031	34.3
7.	β-cymene	1041	1030	1.1
8.	γ-terpinen	1062	1059	1.2
9.	Trans-sabinene hydrate	1069	1060	1.6
10.	Linalool	1103	1101	3.1
11.	Trans-pinocarveol	1131	1140	0.6
12.	Pinocarvone	1160	1164	0.7
13.	Terpinen-4-ol	1169	1173	1.8
14.	Cis-4-decenal	1188	1193	0.9
15.	Linalyl acetate	1258	1253	1.9
16.	Bornyl acetate	1288	1289	0.8
17.	α-terpinyl acetate	1319	1300	23.3
18.	Geranyl acetate	1377	1381	0.8
19.	(E)-nerolidol	1567	1565	0.9
	Total			**98.2**

a: calculated retention index; b: retention index in literature.

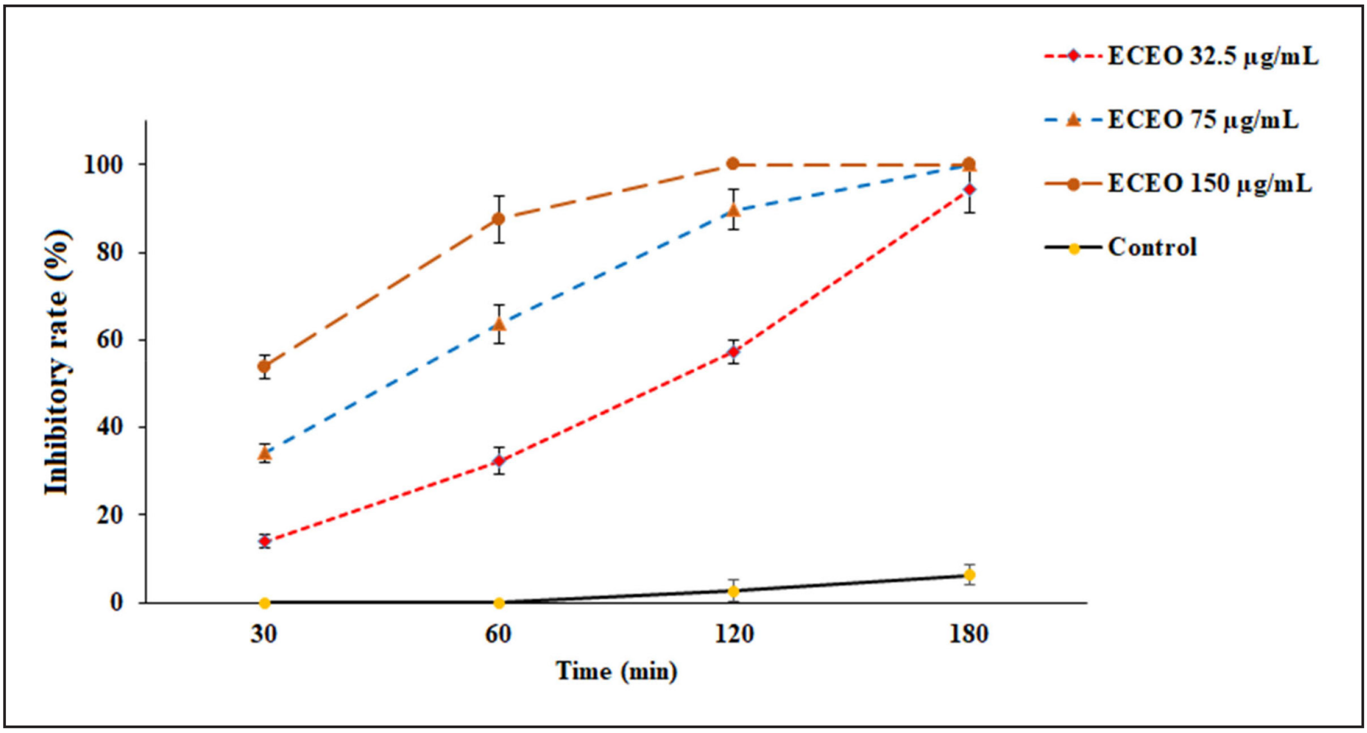

Figure 1. *In vitro* effects *E. cardamomum* essential oil (ECEO) on *T. gondii* tachyzoites. (n = 3) by MTT assay.

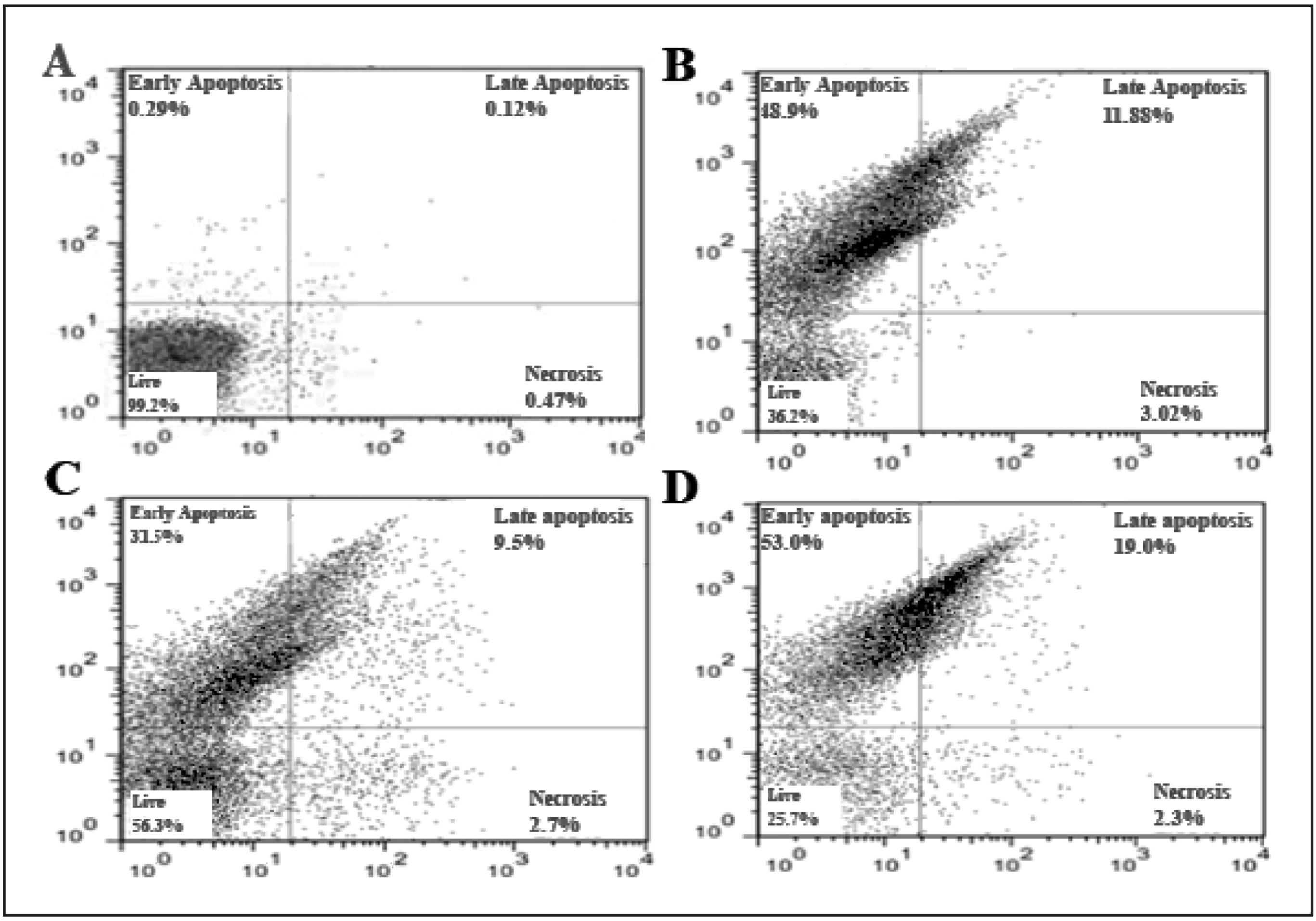

Figure 2. The effect of *E. cardamomum essential oil* (ECEO) at 32.5 (B), 75 (C), and 150 (D) µg/mL on the apoptotic and necrotic cells in tachyzoites of *Toxoplasma gondii* compared with non-treated control (A).

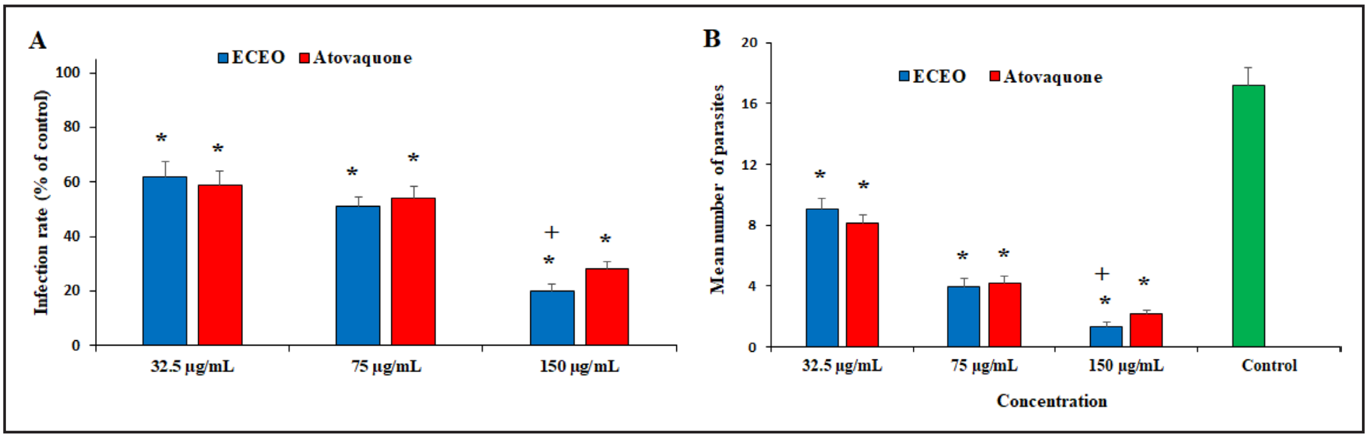

Figure 3. Effect of *E. cardamomum* essential oil (ECEO) and atovaquone (100 µg/mL) on the infection rate of infected cells and intracellular multiplying of *Toxoplasma gondii*. (n = 3). * p<0.05; ** p<0.05. compared to the control. + p<0.05. compared to the atovaqoune.

for ECEO and atovaquone was 343.4 µg/mL and 435.2 µg/mL, respectively.

In vivo effects against acute toxoplasmosis in mice

Based on the results, ECEO at doses of 1, 2, and 4 mg/kg resulted in a 100% delay in mortality on days 7, -8, and -9, respectively, compared to the control group with a 100% mortality rate at day 5. The tachyzoite load was also significantly decreased by 16.7×10^4 (43.2%), 11.4×10^4 (61.2%), and 7.01×10^4 (76.15%), respectively ($p<0.001$) (Figure 4).

Evaluation of oxidant and antioxidant enzymes

Results showed that MDA and NO were higher in the infected mice, but infected mice receiving ECEO at 1-4 mg/kg markedly ($p<0.05$) corrected oxidative stress through reducing the MDA and NO amounts. Infection with *T. gondii* also reduces levels of

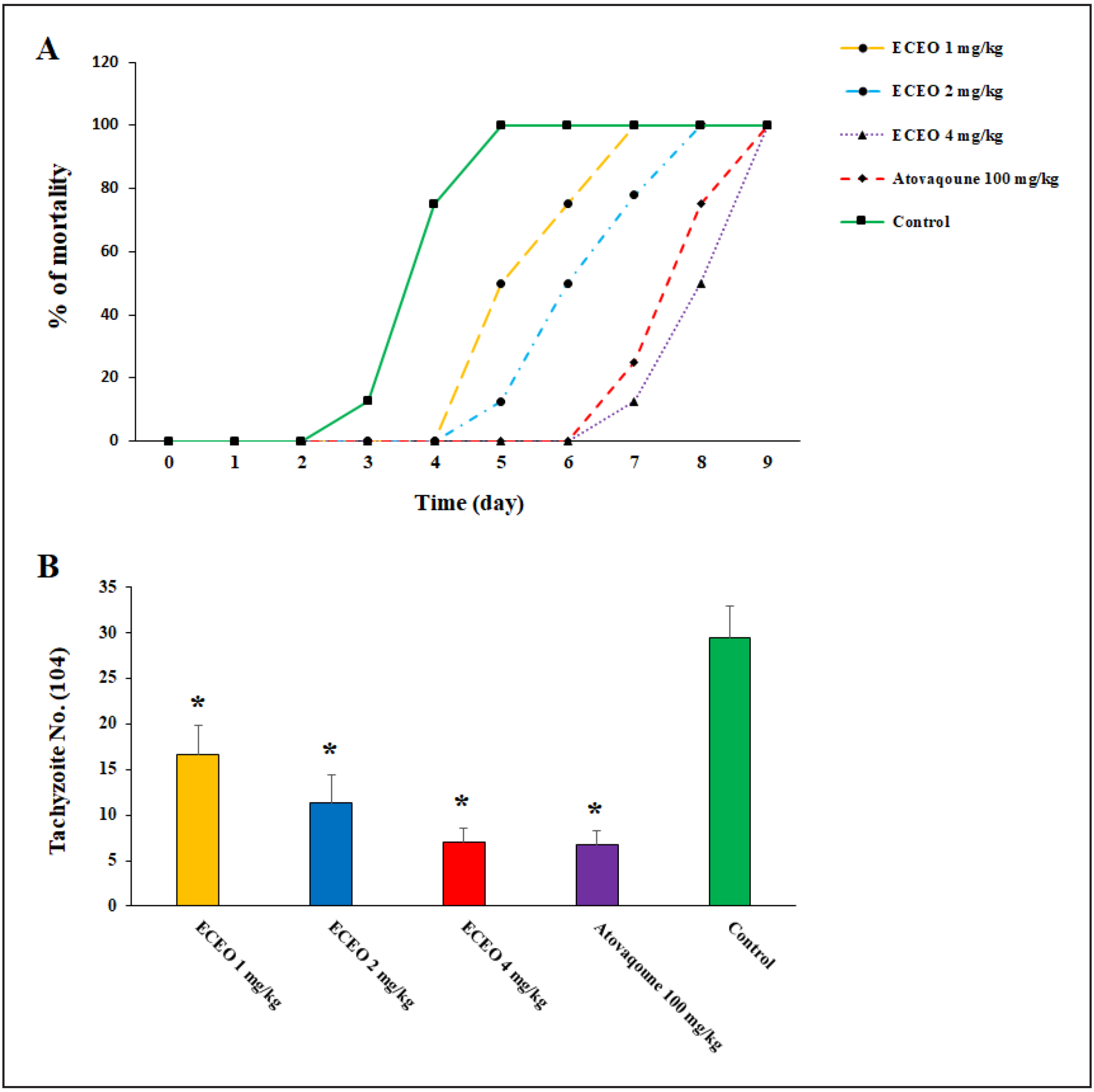

Figure 4. Effect of *E. cardamomum* essential oil (ECEO) and atovaquone on the mortality rate and the mean number of tachyzoites in the infected mice. (n=3) * p<0.05 compared with control.

antioxidant markers such as GPx and SOD; however, infected mice that received ECEO at 1, 2, and 4 mg/kg had significantly (p<0.05) increased levels of these antioxidant enzymes compared with the control mice (Figure 5).

mRNA expression level

According to the results obtained by the $2^{-\Delta\Delta CT}$ method in real-time PCR, pre-treatment with ECEO at 1, 2, and 4 mg/kg caused a significant (P<0.001) increase in the IL-1β and IFN-γ mRNA genes in mice infected with *T. gondii* (Figure 6).

Toxicity effects on healthy mice

The results showed that in healthy mice receiving ECEO at doses of 1, 2, and 4 mg/kg, serum levels of liver and kidney function factors were not significantly changed compared with healthy mice receiving normal saline (P> 0.05) (Figure 7).

DISCUSSION

We demonstrated that ECEO, mainly at a concentration of 150 µg/mL, showed potent effects on tachyzoites and infection rates in Vero cells. We also found that the treatment of infected mice with ECEO at 1, 2, and 4 mg/kg prolongs their lifespan up to day 9. Studies have shown that apoptosis is one of the principal mechanisms by which host cells control and eliminate microbial pathogens (Besteiro, 2015). Flow cytometry analysis showed that ECEO increased (p < 0.01) the level of necrotic and apoptotic cells, suggesting that ECEO exhibits inhibitory activity through the induction of apoptosis.

Farrag *et al*. (2021) reported that ECEO at 1000–2500 µg/mL clearly reduced the parasite load and infection rate in rats infected with *Trypanosoma evansi*. Furthermore, in several studies, the antibacterial activity of ECEO has been demonstrated

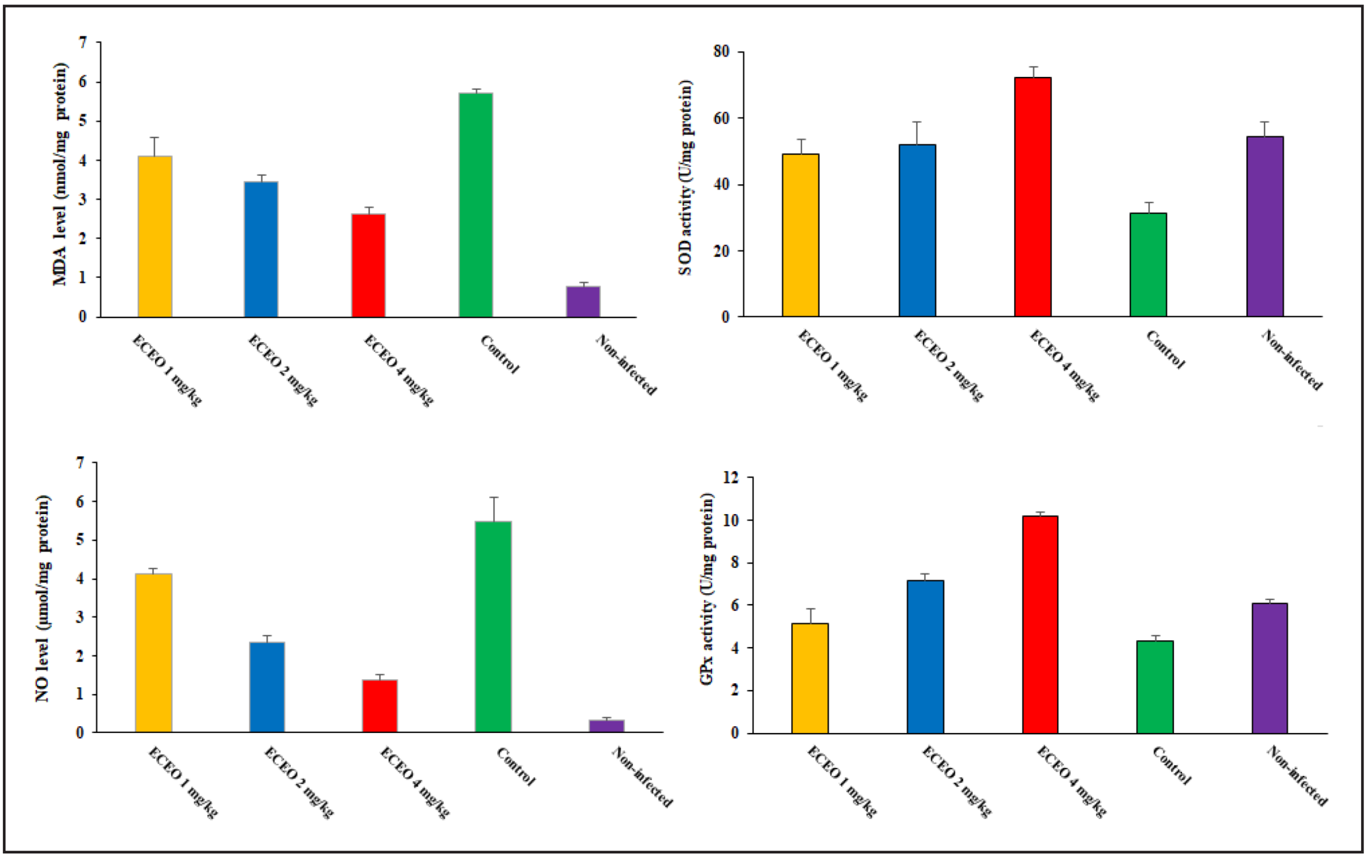

Figure 5. Effects of *E. cardamomum* essential oil (ECEO) on the hepatic malondialdehyde (MDA), nitric oxide (NO), glutathione peroxidase (GPx), and superoxide dismutase enzyme activity (SOD) in infected mice. * p<0.001 compared to the control.

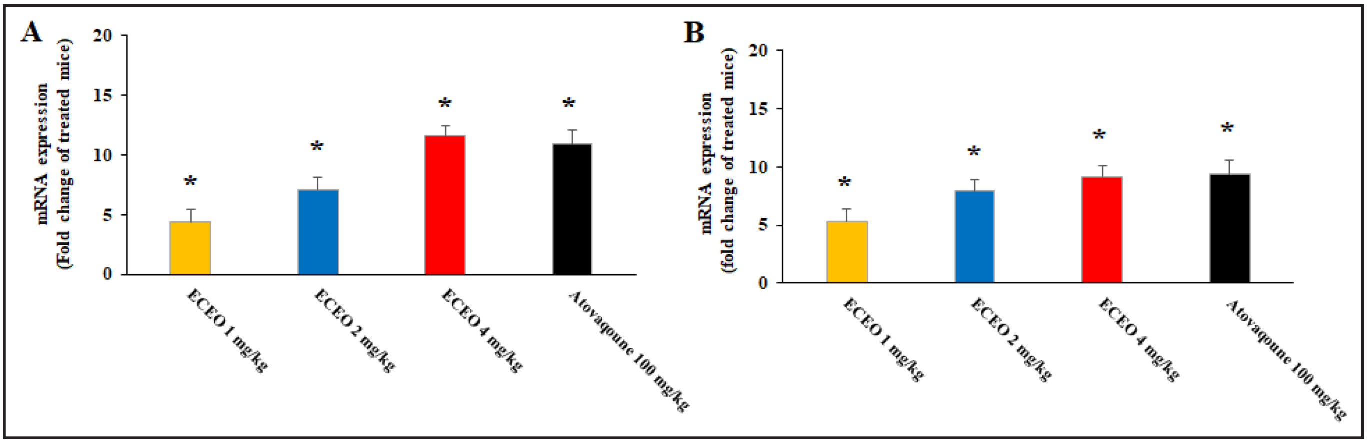

Figure 6. Effects of *E. cardamomum* essential oil (ECEO) on the expression level of IFN-γ (A) and IL-1β (B) mRNA in the infected mice. * p<0.001 compared to the control.

against various bacterial and fungal strains with an inhibition efficacy of 5–12 mm (Singh *et al*., 2008; Asghar *et al*., 2017; Noshad & Behbahani, 2019).

Our results demonstrated that the highest ECEO components were 1,8-cineole, α-terpinyl acetate, and α-pinene, respectively. Several surveys also reported that the highest components of ECEO were 1,8-cineole, terpinenyl acetate, linalyl acetate, and sabinene (Masoumi-Ardakani *et al*., 2016; Goudarzvand Chegini & Abbasipour, 2017; Alam *et al*., 2021).

Today, monoterpene compounds, such as 1,8-cineole, α-terpinyl acetate, and α-pinene are considered essential secondary metabolites of the herb during drug development and discovery (Wojtunik-Kulesza *et al*., 2019). Previous investigations have shown antimicrobial activity of monoterpene combinations against several strains of bacteria, fungi, viruses and parasites (Rodrigues Goulart *et al*., 2004; Marchese *et al*., 2017; Zielińska-Błajet & Feder-Kubis, 2020).

Although the exact mode of antibacterial action of monoterpene composites is still widely understood, previous reports have shown that these compounds demonstrate their effectiveness through the breakdown of the cell wall, limiting oxygen consumption, confounding virulence aspects and modulating the efflux pump (Anand *et al*., 2019; Badawy *et al*., 2019). Therefore, the *in vitro* anti-*Toxoplasma* activity of ECEO may be related to the presence of monoterpene composites.

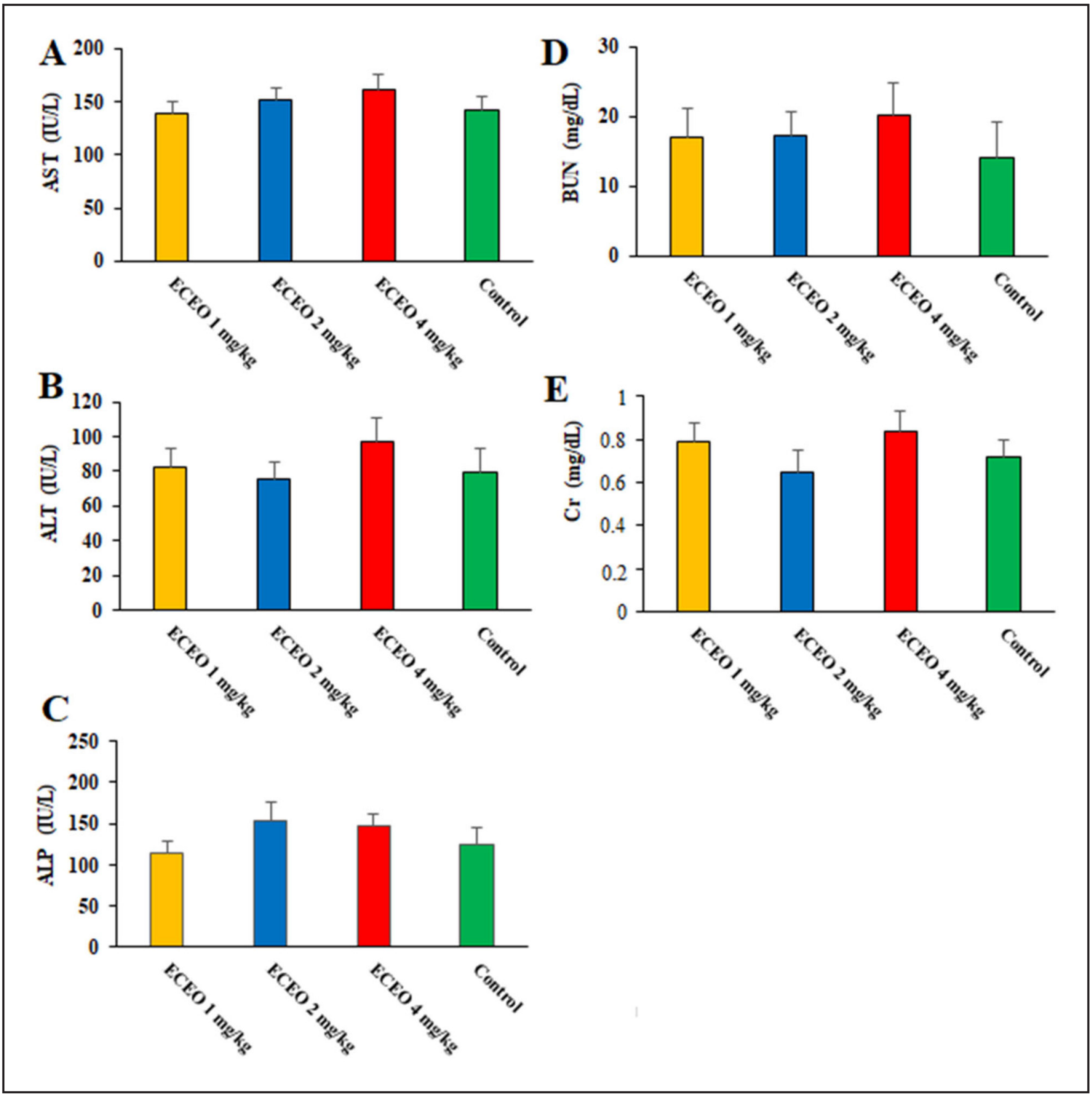

Figure 7. Effects of *E. cardamomum* essential oil (ECEO) on the serum level of (A): aspartate aminotransferase (AST); (B): alanine aminotransferase (ALT); (C): alkaline phosphatase (ALP); (D): blood urea nitrogen (BUN); (E): creatinine (Cr) in the infected mice. mean ± SD.

LPO has been shown to be one of the major contributors to liver damage, acting through free radicals generated by *T. gondii.* LPO is a marker of oxidative stress, which severely disrupts cell membranes and subsequently increases indicators of hepatotoxicity (Souza *et al*., 2017; Nolan *et al*., 2018). Here, we found that ECEO at doses of 1-4 mg/kg ($p < 0.05$) significantly reduced the increase in hepatic lipid peroxidation in toxoplasmosis mice and significantly ($p<0.05$) increased the amount of antioxidant enzymes. Similarly, Elguindy *et al*. (2016) reported that ECEO had promising hepatoprotective effects against mouse-induced hepatocellular carcinoma by decreasing MDA and increasing antioxidant markers. These demonstrate that ECEO, by modulating inflammation and oxidative stress, can delay TOP-induced liver damage.

The release of various cellular immune mediators and proinflammatory cytokines, e.g., IL-1β and IFN-γ was defined as a key factor in prolonging host survival (Souza *et al*., 2017). Administration of IL-1β has been shown to be protective against acute *T. gondii* infection in animal models (Chang *et al*., 1990; Mahmoudvand *et al*., 2011; Ezzatkhah *et al*., 2023). We reported that ECEO treatment at doses of 1-4 mg/kg produced a significant ($P<0.001$) increase of the IL-1β and IFN-γ genes in acute TOP mice, implying that the increase in pro-inflammatory cytokines reduced the parasite load during infection.

Regarding the toxicity of ECEO on liver and kidney function in healthy mice, we showed that ECEO at doses of 1-4 mg/kg showed no significant effect on liver and kidney function compared with normal saline-treated rats. Similarly, Aboubakr &

Abdelazem, (2016) demonstrated that *E. cardamomum* exerts its hepatoprotective effects against gentamicin-induced liver injury in rats by reducing elevated serum levels of AST, ALT, and bilirubin.

CONCLUSION

These results report a significant inhibitory effect of ECEO mainly at a dose of 4 mg/mL, against the *T. gondii* Rh strain through strengthening the immune system and reducing inflammation and oxidative stress; however, further research is needed to verify these results.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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