

In vitro and in vivo Anti-*Toxoplasma* Effects of *Allium sativum* Essential Oil Against *Toxoplasma gondii* RH Strain

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Background: Since no effective vaccine has been developed for toxoplasmosis, prophylaxis in seronegative pregnant women and immunocompromised patients with a CD4 <100 cells/ μ L is highly recommended as an ideal strategy to prevent this disease. This study aimed to assess the chemical composition, in vitro, and in vivo effects of *Allium sativum* essential oil (ASEO) against *Toxoplasma gondii* RH strain.

Methods: The in vitro anti-*Toxoplasma* effects of different concentrations of ASEO (32.5, 75, 150 μ g/mL) were measured by MTT assay for 0.5, 1, 2, and 3 h. Male Balb/c mice were orally administrated ASEO at the doses of 200, 400, and 600 μ g/kg/day for 14 days. One day after the completion of oral drug administration, the mice in all groups were infected intraperitoneally with 1×10^4 tachyzoites. They were checked daily and the rate of survival was recorded. The peritoneal fluids of the mice were collected and the mean number of tachyzoites was calculated via a light microscope. The level of liver lipid peroxidation (LPO) and nitric oxide (NO), toxicity effects on the liver and kidney, and the mRNA expression levels of some pro-inflammatory cytokines such as IL-1 β and IFN- γ were determined by quantitative real-time PCR.

Results: Different concentrations of ASEO showed a significant ($p < 0.001$) anti-*Toxoplasma* activity against *T. gondii* tachyzoites, and the highest efficacy was observed at the concentration of 150 μ g/mL. Fourteen days of pre-treatment of infected mice with ASEO at the doses of 200, 400, and 600 μ g/kg/day significantly ($p < 0.001$) decreased the mean number of tachyzoites and mortality rate by the 6th, 7th, and 8th days after infection, respectively. ASEO at the doses of 200, 400, and 600 μ g/kg/day significantly ($p < 0.05$) improved the increase in the LPO and NO. Pre-treatment of mice with different doses of ASEO provoked a considerable ($P < 0.001$) down-regulation of IL-1 β and IFN- γ mRNA gene expression levels, but it had no significant toxicity on the serum levels of some liver and kidney enzymes.

Conclusion: The present study demonstrated the considerable prophylactic effects of ASEO that increased the survival rate of mice and reduced the parasite load in them. Our findings also showed that ASEO promotes the innate immune system, pro-inflammatory cytokines, inhibition of hepatic injury, etc. in the mice with acute toxoplasmosis. However, additional investigations are mandatory to clarify the accurate prophylactic and therapeutic anti-*Toxoplasma* mechanisms of ASEO as well as all its toxicity aspects, especially in clinical settings.

Keywords: toxoplasmosis, RH strain, prophylaxis, herbal medicines, tachyzoites

Introduction

Toxoplasma gondii is a forced intracellular protozoan that causes toxoplasmosis in humans and animals worldwide.¹ Acquired infections usually occur by eating raw and undercooked meat containing tissue cysts of *T. gondii* or consuming vegetables

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and water contaminated with oocysts excreted in cat feces. Congenital *Toxoplasma* infections occur with the placental transmission of tachyzoites in early infections during pregnancy.²

Acquired toxoplasmosis in immunocompetent individuals occurs mainly as benign and self-limiting lymphadenopathy and rarely causes severe cerebral and ocular manifestations.³ However, the disease is an important opportunistic agent in people with impaired immune function (immunocompromised), especially in patients with AIDS and organ recipients, and is the most common cause of encephalitis in them.^{3,4} Toxoplasmosis is also a major congenital infection with a wide clinical spectrum, varying from neonatal asymptomatic birth to abortion, stillbirth, and birth of infants with severe cerebral and ocular complications (hydrocephalus or microcephaly, calcification and calcification).^{3,5}

Toxoplasmosis is commonly treated with a combination of pyrimethamine and sulfadiazine and some other chemical drugs including spiramycin and atovaquone.⁶ Pyrimethamine is an inhibitor of the enzyme dihydrofolate reductase, which is a key enzyme in the folate synthesis pathway. Sulfadiazine is an inhibitor of the enzyme dihydropteroate, another enzyme in this pathway.⁷ Although the synthetic anti-*Toxoplasma* drugs have a good inhibitory effect on *Toxoplasma*, their side effects (eg, hematopoiesis disruption due to its bone marrow suppressant effect, teratogenic effects, and osteoporosis) are their main limitations.^{7,8}

Because no effective vaccine is available to prevent toxoplasmosis, prophylaxis mainly in seronegative pregnant women and immunocompromised patients with a CD4 <100 cells/ μ L is highly recommended as the ideal strategy to prevent the disease.^{9,10} Therefore, preparing an anti-*Toxoplasma* agent with optimal efficacy and minimal side effects is a *Toxoplasma* research priority.

In recent years, there has been a growing trend of research on the effectiveness of herbal products on various diseases, including parasitic diseases.¹¹ Laboratory and experimental studies in recent years have also shown that some plant extracts and fractions exert significant antiparasitic effects against several protozoan and helminthic parasites such as *Leishmania* spp, *Trypanosoma* spp, *Toxoplasma*, *Echinococcus* spp.^{12,13} *Allium sativum* L. or garlic (Alliaceae family) has long been an important plant widely used in folk medicine worldwide.¹⁴ Garlic possesses many beneficial and pharmacological properties in traditional and modern medicine, such as anticancer,

antioxidant, antidiabetic, immunomodulatory, antithrombotic, anti-fungal, antiviral, and anti-bacterial characteristics.¹⁵ The compounds in garlic are divided into two main groups of lipid-soluble allyl sulfur and water-soluble components.¹⁶ The medicinal and therapeutic properties of garlic are mainly due to the presence of sulfur-containing compounds such as allicin and other thiosulfonates.¹⁶ Garlic extract at the doses of 100, 200, 400, and 500 mg/kg/day displayed promising therapeutic effects against acute toxoplasmosis in mice and increased the survival time and decreased the appearance of the parasite in the tissues of infected mice.¹⁷ However, the prophylactic potential of this plant's essential oil is not yet understood. Thus, the present study aimed to assess the in vitro and in vivo effects of *A. sativum* essential oil (ASEO) against *T. gondii* RH strain (tachyzoites).

Materials and Methods

Plant Collection

Fresh bulbs of *A. sativum* were procured from a market in Riyadh, Saudi Arabia, and identified by a botanist of the Department of Biological Science, Faculty of Science and Humanities, Shaqra University, Ad-Dawadimi, Saudi Arabia. To prepare the essential oil, 200 g of dried and powdered rhizomes was placed in a hydro-distillation device for 180 min by means of a glass Clevenger-type device. The essential oil was then dehydrated by anhydrous sodium sulfate, and kept in darkness at 4°C in glass tubes until testing.¹⁸

Gas Chromatography–Mass Spectrometry (GC-MS)

To identify the compounds in ASEO, a Hewlett-Packard 6890 (Palo Alto, CA, USA) device equipped with an HP-5MS column (30 m \times 0.25 mm, film thickness 0.25 mm) was employed to perform the GC analysis. To this end, 0.1 μ L of essential oil was injected into the gas chromatography apparatus and the column temperature programming (225–80°C) for the separation of compounds. The percentage of essential oil constituents was also calculated. Normal C28–C8 alkanes were injected under the same conditions to calculate the inhibition index of essential oil components. Helium gas was used at a rate of 1.1 mL/min and ionization energy of 70 electrons was used. Finally, the components of green cardamom essential oil were identified in comparison with the standard mass spectra available in the Willy-Library of the software and

compared with the standard numbers available in references.¹⁹

Parasites and Cell Culture

T. gondii RH strain (tachyzoites) procured from King Saud University of Medical Science, Riyadh, Saudi Arabia, were maintained in BALB/c mice via serialized intraperitoneal (IP) passages. Tachyzoites aspirated from the peritoneal cavity of the mice and were washed with phosphate-buffered saline (pH 7.4), and centrifuging it for 10 min at 200 g at room temperature. This centrifugation removed host cells and debris. Subsequently, the supernatant containing the parasites was collected and centrifuged for 10 min at 1000 g. The pellet was washed in two stages: first with PBS at pH 7.2 and then with RPMI-1640 (Gibco, USA) without bovine fetal serum. In the next step, they were adjusted by a hemocytometer slide into 1×10^4 and 1×10^6 tachyzoites/mL for in vivo and in vitro assays, respectively. Vero cells (ATCC No. CCL-81) were cultivated in the RPMI-1640 medium with the addition of 10% inactivated fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 µg/mL of streptomycin and maintained at 37°C with 5% CO₂.

Animal

Seventy-two male Balb/c mice aged 6 to 8 weeks and weighing between 20 and 25 g were selected for this study. They were kept in appropriate conditions and temperatures with ad libitum access to food and water. The mice were handled based on the protocols for the Care and Use of Laboratory Animals provided by National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Washington, D.C, USA.²⁰ This study received the approval of the Committee on the Ethics of Animal Experiments of College of Applied Medical Sciences in Al-Quwayiyah, Shaqra University, Saudi Arabia (SH-28-2020).

In vitro Anti-Toxoplasma Effects Cell Viability Assay

The in vitro effects of different concentrations (32.5, 75, 150 µg/mL) of ASEO against *T. gondii* tachyzoites were measured by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for 0.5, 1, 2, and 3 h. In brief, 0.3 mL of different concentrations (32.5, 75, 150 µg/mL) of ASEO were added to 0.3 mL of tachyzoites (1×10^6 cells/mL) in sterile glass tubes which were then incubated for 0.5, 1, 2, and 3 h at 37°C. After this time, 0.01 mL of MTT (5 mg/mL) was

put into the tested tubes and incubated at 37°C for 4 h with 5% CO₂. Finally, 0.05 mL of dimethyl sulfoxide solution (Merck, Germany) was added to the combinations to dissolve the formazan crystals. The absorbance and optical density (OD) of each tested tube were calculated at 570 nm by ELISA reader (LX800; Biotec, USA).²¹ The survival rate (SR) of *T. gondii* was calculated as follows: % of SR = (OD ASO-tested wells – OD blank)/(OD control – OD blank) × 100.

Effect on Infection Rate and Intracellular Parasites

Vero cells (1×10^5) were seeded in a 96-well cell plate and incubated at 37°C for 24 h. The cells were then infected with 5×10^5 *T. gondii* tachyzoites per well for 24 h. After the incubation time, the supernatant of the culture was removed, and the wells were washed twice by sterile PBS to remove cell debris. The infected cells were then exposed with ASEO at concentrations of 32.5, 75, 150 µg/mL for 3 h. Finally, the cells were washed and stained with Giemsa, and the prepared slides were examined under a light microscope to determine the *T. gondii* infection rate (% of infected cells/100 examined cells) and parasite intracellular replication (mean number of parasites per cell in 100 infected cells). The 50% inhibitory concentrations (IC₅₀) were also measured by the Probit test in SPSS 22.²¹

In vivo Anti-Toxoplasma Effects

Initially, the mice were divided into five groups, each containing 8 mice: (i) mice receiving ASEO at the dose of 200 µg/kg/day for two weeks; (ii) mice receiving ASEO at the dose of 400 µg/kg/day for two weeks; (iii) mice receiving ASEO at the dose of 600 µg/kg/day for two weeks; (iv) mice receiving atovaquone 100 mg/kg/day for two weeks; (v) mice treated with normal saline. Then, 24 h after the completion of oral drug administration, the mice in all groups were infected intraperitoneally with 1×10^4 tachyzoites/100 µL. Finally, the mice were checked daily and the rate of survival was recorded for mice in each group. In addition, on the 3rd day post-infection, peritoneal fluids of all the mice in each group were collected, and the mean number of tachyzoites was calculated using a light microscopic.²²

Evaluation of Liver Lipid Peroxidation (LPO) and Nitric Oxide (NO)

To evaluate the LPO, liver homogenates of four mice from each group on the 3rd day post-infection were examined by a biodiagnostic analysis kit based on the malondialdehyde (MDA) production using the thiobarbituric acid (TBA) methodology explained by Ohkawa et al.²³ NO

production was also measured via the hepatic suspension method described by Green et al.²⁴

Pro-Inflammatory Cytokines mRNA Expression

The mRNA expression levels of several pro-inflammatory cytokines such as IL-1 β and IFN- γ were determined by quantitative real-time PCR. Briefly, total RNA was extracted from the liver tissue via an RNeasy tissue kit (Qiagen, Germany) in accordance with the manufacturer's instructions. In the next step, cDNA was synthesized using random primers for the complementary DNA (cDNA) synthesis based on the manufacturer's recommendations. Subsequently, cDNA was applied for conventional PCR reaction analysis or real-time PCR via SYBR green. The thermal profile of the reaction was 95°C for 5 min, 40 cycles of 95°C for 10 s and 56°C for 30 s, respectively. Finally, the Δ Ct was calculated via the iQTM5 optical system software (Bio-Rad, Hercules, CA). β -actin was applied as a housekeeping gene and normalization control. Table 1 displays the oligonucleotide primers utilized for real-time PCR.²⁵

Sub-Acute Toxicity Effects of ASEO

To determine the sub-acute toxicity effects of ASEO, 32 healthy mice were divided into four groups, each containing 8 mice: (i) healthy mice receiving ASEO at the dose of 200 μ g/kg/day for two weeks; (ii) healthy mice receiving ASEO at the dose of 400 μ g/kg/day for two weeks; (iii) healthy mice receiving ASEO at the dose of 600 μ g/kg/day for two weeks; (iv) mice treated with normal saline. On the 15th day after the oral administration of ASEO, by cardiac puncture, blood specimens were collected from the mice via sterile syringes with and without anticoagulant (EDTA). The blood samples were then centrifuged at 3500 rpm for 15 min and the separated serums were kept at -20°C until testing.²⁶ To assess the toxicity of ASEO, the serum levels of some biochemical factors related to liver and kidney function, eg, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP),

creatinine (Cr), and blood urea nitrogen (BUN) were measured via commercial diagnostic kits.

Statistical Analysis

All the tests were performed in triplicate and the data were represented as means \pm standard deviation. SPSS 22 (SPSS Inc., Chicago, IL, USA) was employed for data analysis. One-way ANOVA with Turkey's post-hoc test was run to assess differences among the experimental groups.

Results

The findings of GC/MS represented that 20 constituents were recognized, constituting 98.1% of the total essential oil (Table 2). The main components were diallyl disulfide (29.2%), diallyl trisulfide (28.6%), and allyl methyl trisulfide (19.8%), respectively.

Cell Viability Assay

Via the MTT assay, different concentrations of ASEO displayed a significant ($p < 0.001$) anti-*Toxoplasma* activity against *T. gondii* tachyzoites after 0.5, 1, 2, and 3 h of incubation compared with the control group (Figure 1). The highest efficacy was observed at the concentration of 150 μ g/mL where the mortality of tachyzoites was 100%

Table 2 GC/MS Analysis of Chemical Compositions of *A. sativum* Essential Oil

No	Compound	RI ^a	Composition (%)
1.	Dimethyl disulfide	728	3.1
2.	Dithio-cyclopentane	743	0.4
3.	2-Methylene-4-pentenal	764	0.5
4.	1,2-Dithiolane	828	0.4
5.	Diallyl sulfide	860	2.2
6.	Allyl (Z)-1-propenyl sulfide	885	0.8
7.	3 methylthio propanol	894	1.1
8.	3,4-Dimethylthiophene	906	0.81
9.	Allyl methyl disulfide	908	0.6
10.	Methyl propyl disulfide	923	0.7
11.	Dimethyl trisulfide	966	0.4
12.	Diallyl disulfide	1072	29.2
13.	Allyl (Z)-1-propenyl disulfide	1091	0.9
14.	Allyl methyl trisulfide	1128	19.8
15.	Methyl propyl trisulfide	1152	1.3
16.	2-Vinyl-4H-1,3-dithiine	1215	0.7
17.	Diallyl trisulfide	1305	28.6
18.	5-Methyl-1,2,3,4-tetrathiane	1356	0.7
19.	Benzoxathiin	1418	0.6
20.	Diallyl tetrasulfide	1533	1.3
Total			98.1

Table 1 The Primers Applied for Real-Time PCR

Amplicon	Primers	Sequence (5'-3')	Size (bp)
IL-1 β	F R	AACCTGCTGGTGTGTGACGTTTC CAGCACGAGGCTTTTGTGTGT	78
IFN- γ	F R	ATGAACGCTACACACTGCATC CCATCCTTTGCCAGTTCCTC	182
β -actin	F R	GTGACGTTGACATCCGTAAGA GCCGGACTCATCGTACTCC	245

after 2 h of incubation. Complete mortality (100%) was also observed after 3 h of treatment of tachyzoites with ASE0 at the concentration of 75 $\mu\text{g/mL}$.

Effect on Infection Rate and Intracellular Parasites

The highest inhibitory effect on the infection rate was observed at the concentration of 150 $\mu\text{g/mL}$, where the infection rate was significantly reduced ($p < 0.001$) by 32.3% (Figure 2A). In addition, ASE0 at the concentrations of 32.5 and 75 $\mu\text{g/mL}$ significantly reduced ($p < 0.05$) the infection rate in the *T. gondii* infected Vero cells by 74.3% and 53.3%, respectively. Figure 2B illustrates the intracellular replication of *T. gondii* in infected Vero cells after treatment with various concentrations of ASE0. ASE0 significantly decreased the mean number of intracellular parasites with IC_{50} of 66.9 $\mu\text{g/mL}$, whereas this value was 72.11 $\mu\text{g/mL}$ for atovaquone.

In vivo Anti-Toxoplasma Effects

Figure 3 depicts the in vivo anti-Toxoplasma effects of different doses of ASE0. The results represented that 14 days of pre-treatment of infected mice with ASE0 at doses of 200, 400, and 600 $\mu\text{g/kg/day}$ decreased the mortality rate by the 6th, 7th, and 8th day post-infection, respectively. The highest survival rate was observed in the mice pre-treated with ASE0 at the dose of 600 $\mu\text{g/kg/day}$, where the infected mice survived three days longer than the control mice. Figure 4 also depicts the mean number of tachyzoites collected from mice in each tested group. Following pre-treatment of infected mice with ASE0 at the doses of 200, 400, and 600 $\mu\text{g/kg/day}$, the mean number of tachyzoites on the 5th day was significantly

reduced by 64.9%, 79.5%, and 92.4%, respectively, whereas the mean number of tachyzoites in the tested mice of the Atovaquone 100 mg/kg group was decreased by 86.7%.

Effect on LPO and NO

The level of hepatic MDA and NO was significantly elevated in the *T. gondii*-infected mice treated with no drug (Figure 5); conversely, ASE0 at the doses of 200, 400, and 600 $\mu\text{g/kg/day}$ significantly ($p < 0.05$) improved this increase in the LPO and NO.

Pro-Inflammatory Cytokines mRNA Expression

Based on Figure 6, *T. gondii* significantly ($P < 0.001$) promoted the expression level of IFN- γ and IL-1 β mRNA, while pre-treatment with different doses of ASE0 induced a considerable ($P < 0.001$) down-regulation of IL-1 β and IFN- γ mRNA gene expression levels.

Effect on Biochemical Parameters

Based on Figure 7, the toxicity findings revealed that pre-treatment with ASE0 at the doses of 200, 400, and 600 $\mu\text{g/kg/day}$ for two weeks resulted in no mortality among the tested mice. Moreover, the findings of biochemical tests demonstrated no significant difference between these biochemical factors compared with the group treated with the ASE0 and the control group ($P > 0.05$).

Discussion

Since no effective vaccine has been developed to prevent toxoplasmosis, prophylaxis is highly recommended as an ideal strategy to prevent toxoplasmosis, especially in

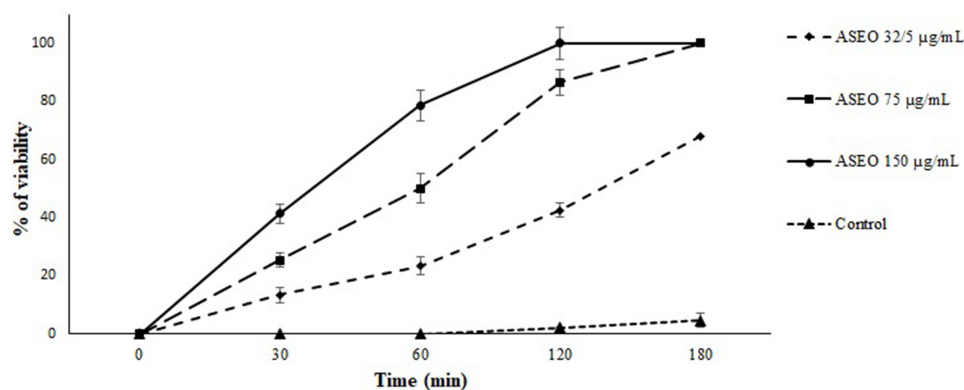


Figure 1 In vitro anti-Toxoplasma effects of different concentration of ASE0 showed against *T. gondii* tachyzoites after 0.5, 1, 2, and 3 h incubation in comparison with the control group. Data are expressed as the mean \pm SD ($n = 3$).

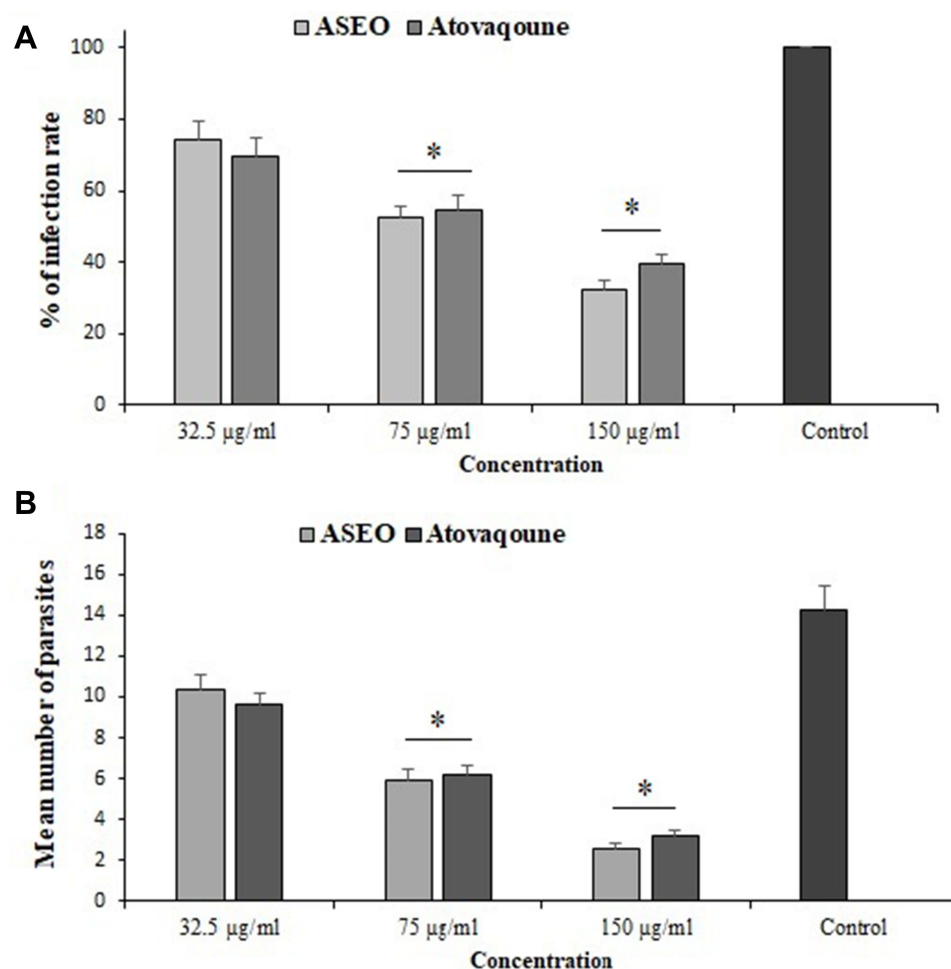


Figure 2 The infection rate of the Vero cells infected with *T. gondii* tachyzoites after exposed with *Allium sativum* essential oil (ASEO) at concentrations of 32.5, 75, 150 µg/ml for 3 h (A). The intracellular replication of *T. gondii* in infected Vero cells after treatment with various concentrations of ASEO (B). Data are expressed as the mean \pm SD (n = 3). * p < 0.05.

seronegative pregnant women and immunocompromised patients with a CD4 < 100 cells/µL.^{9,10} This study assessed the chemical composition, in vitro, and in vivo effects of *A. sativum* essential oil against *T. gondii* RH strain

(tachyzoites). Our findings demonstrated that different concentrations of ASEO showed significant ($p < 0.001$) in vitro anti-*Toxoplasma* activity against *T. gondii* tachyzoites after 0.5, 1, 2, and 3 h of incubation, compared with

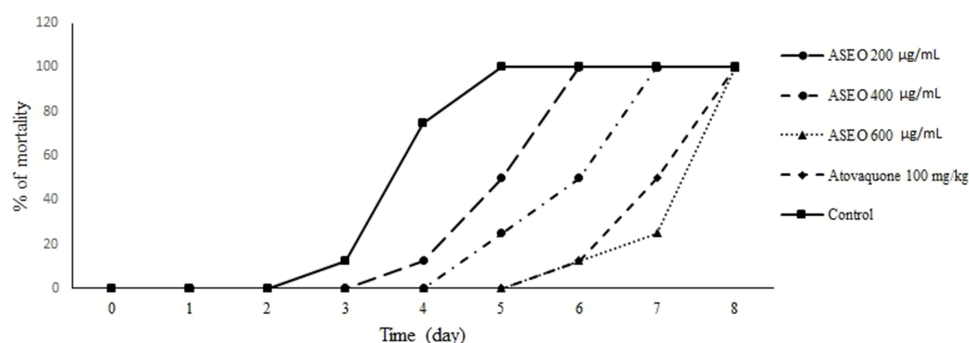


Figure 3 The mortality rate infected mice pre-treated with ASEO at the doses of 200, 400, and 600 µg/kg/day for 14 days in comparison with the control group. Data are expressed as the mean \pm SD (n = 8).

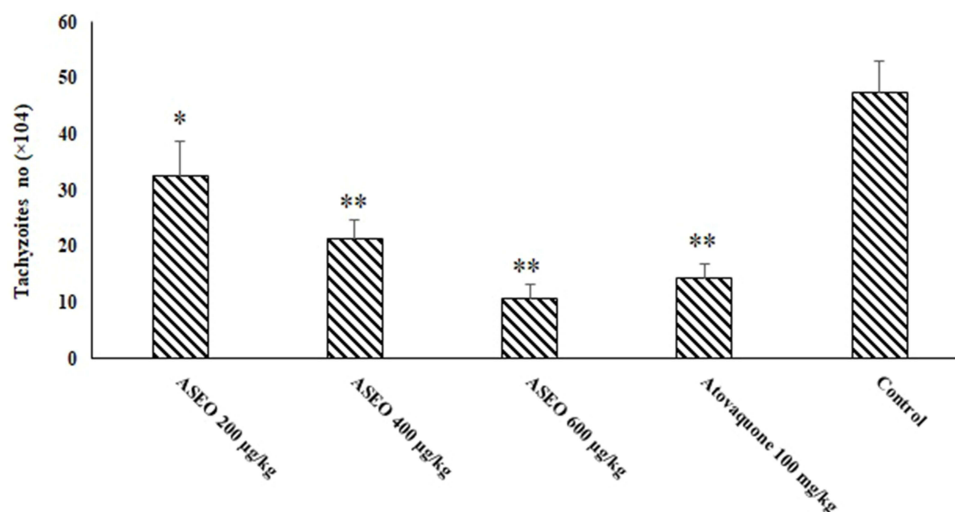


Figure 4 The mean number of tachyzoites in the infected mice pre-treated with ASEO at the doses of 200, 400, and 600 µg/kg/day for 14 days. Data are expressed as the mean ± SD (n = 8). *p < 0.05; **p < 0.001.

the control group. The highest efficacy was observed at the concentration of 150 µg/mL where the mortality of tachyzoites was 100% after 2 h of incubation. Complete mortality (100%) was also observed after 3 h of treatment with ASEO at the concentration of 75 µg/mL. As for the in vivo assay, the results represented that 14 days of pre-treatment of infected mice with ASEO at the doses of 200, 400, and 600 µg/kg/day decreased the mortality rate by the 6th, 7th, and 8th days post-infection, respectively. After pre-treatment of the infected mice with ASEO at the doses of 200, 400, and 600 µg/kg/day, the mean number of tachyzoites on the 3rd day was significantly reduced by 64.9%, 79.5%, and 92.4%, respectively, whereas the mean number of tachyzoites in the tested mice of the atovaquone 100 mg/kg group was reduced by 86.7%.

Considering the biological activities of *A. sativum*, previous studies have reported many beneficial and pharmacological properties for this plant in traditional and modern medicines such as anticancer, antioxidant, antidiabetic, immunomodulatory, antithrombotic, anti-fungal, antiviral, antiparasitic, anti-bacterial effects.^{14–16} Krstin et al have demonstrated the anti-parasitic effects of dichloromethane extract of *A. sativum* against *Trypanosoma brucei brucei* and *Leishmania tarentolae* with an IC₅₀ (50% inhibitory concentrations) value of 0.95 ± 0.04 and 2.89 ± 0.4 µg/mL, respectively.²⁷ Gaafar (2012) has reported the prophylactic and therapeutic efficacy of raw *A. sativum* juice against *Cryptosporidium* infection in mice where raw garlic juice reduced the cryptosporidial oocysts and intestinal lesions

in infected mice.²⁸ In the study conducted by Haji Mohammadi et al, the results showed that *A. sativum* methanolic extract (10 mL/L) alone and combined with albendazole significantly reduced the number, size, and

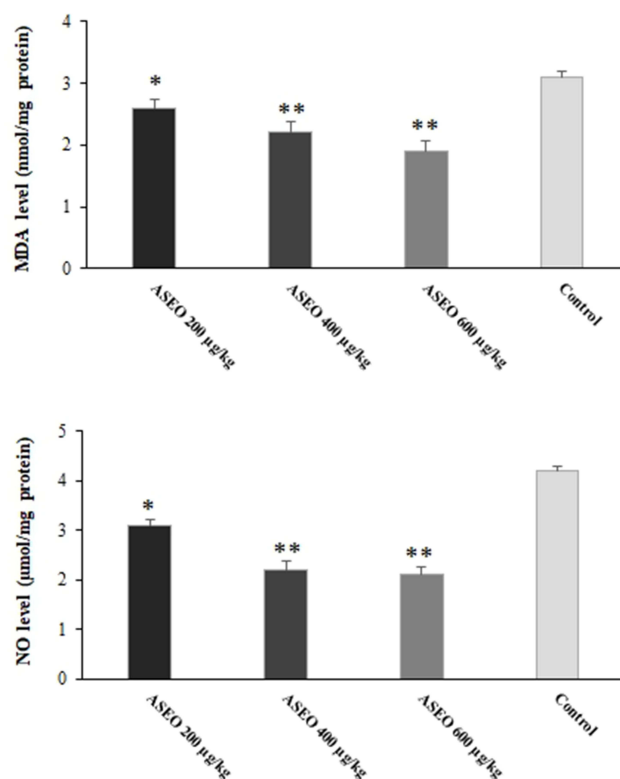


Figure 5 The level of hepatic MDA and NO in the *T. gondii* infected mice pre-treated ASEO at the doses of 200, 400, and 600 µg/kg/day. Data are expressed as the mean ± SD (n = 8). *p < 0.05; **p < 0.001.

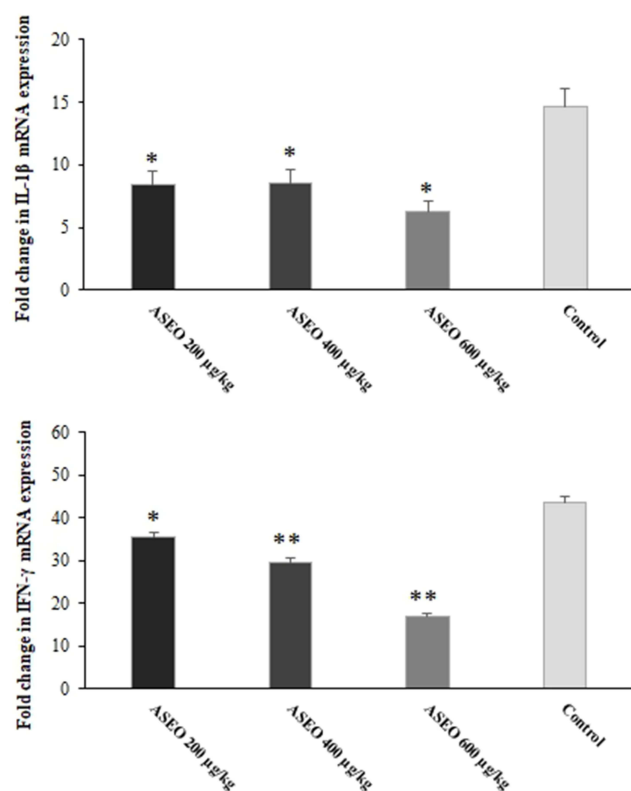


Figure 6 The expression level of IFN- γ and IL-1 β mRNA in the *T. gondii* infected mice pre-treated ASEO at the doses of 200, 400, and 600 μ g/kg/day. Data are expressed as the mean \pm SD (n = 8). *p < 0.05; **p < 0.001.

weight of cysts in mice experimentally infected with hydatid cyst.²⁹ Mahmoudvand et al have demonstrated the in vitro anti-leishmanial effects of the methanolic and aqueous extracts of *A. sativum* in inhibiting the viability of *L. tropica* promastigote with IC₅₀ values of 12.3 and 19.2 μ g/mL, respectively.³⁰ Kinuthia et al also reported that oral administration of *A. sativum* extract significantly decreased lesion size and parasite load in the mice infected with *L. major*.³¹ In addition, Abdel-Ghaffar et al showed the in vitro and in vivo anti-helminthic effects of *A. sativum* against some helminthic pathogens such as *Fasciola hepatica*, *Echinostoma caproni*, *Hymenolepis microstoma*, *Taenia taeniae formis*, and *H. diminuta*.³² Zenner et al also reported the promising anti-parasitic effects of *A. sativum* essential oil against *Tetratrichomonas gallinarum* and *Histomonas meleagridis* with the minimal lethal concentration (MLC) values of 0.125 and 1 μ L/mL, respectively.³³ Another study conducted by Azadbakht et al concluded that *A. sativum* essential oil at the concentration of 0.2 μ g/mL significantly reduced the mortality rate of *Giardia lamblia* cysts and *Entamoeba histolytica* trophozoites by 67.4% and 83.6%,

respectively.³⁴ Recently, Sidiropoulou et al have shown that *A. sativum* essential oil significantly inhibited the *Eimeria tenella* Wisconsin strain sporozoite invasion at the concentration of 100 μ g/mL by 83% or 93% after 2 or 24 h, respectively.³⁵ According to Khoshzaban et al, oral treatment of murine acute toxoplasmosis with the *A. sativum* extract at the doses of 100, 200, 400, and 500 mg/kg/day for seven days led to a survival rate of 100% until the 5th day of the experiment, and the *Toxoplasma* tachyzoites significantly vanished in the liver of the experimented mice.¹⁷

The GC/MS identified 20 constituents, constituting 98.1% of the total essential oil. The main components were diallyl disulfide (allicin, 29.2%), diallyl trisulfide (28.6%), and allyl methyl trisulfide (19.8%), respectively. In consistent with our results, Satyal et al reported that the main constituents of *A. sativum* essential oil were diallyl trisulfide (allitridin) (33.4%), diallyl disulfide (20.8%), allyl methyl trisulfide (19.2%), and allyl (E)-1-propenyl disulfide (5.2%), respectively.³⁶ Similarly, Dziri et al introduced diallyl trisulfide (37.3–45.9%), diallyl disulfide (17.5–35.6%), and methyl allyl trisulfide (7.7–10.4%) as the chief components of *A. sativum* essential oil.³⁷ Despite all these similarities, previous studies showed that the chemical composition of essential oils varies depending on the place where the plant grew, the part of the herbs used, the time of harvesting the herbs, and the technique of isolating the essential oil from the herbs, etc.^{38,39}

The potent antimicrobial effects of allicin against a wide range of bacterial and parasitic pathogens such as *Trypanosoma* sp., *Entamoeba histolytica*, *Giardia lamblia*, Gram-positive and Gram-negative bacteria, *Candida* spp, etc. have been reported.⁴⁰ According to Shan et al, allicin as an organosulfur compound obtained from garlic displayed high efficacy against murine acute toxoplasmosis; after treatment of infected mice with allicin alone (30 mg/kg/day) and combined with sulfamethoxazole, parasite load in the liver tissues and blood samples were significantly reduced.⁴¹

Although the exact antimicrobial mechanisms of these organosulfur compounds are not yet discovered, some studies revealed that these compounds act by disrupting DNA, RNA, and protein synthesis, reacting with sulfhydryl groups of the enzymes and proteins of microbes, damaging the cell wall and membrane, and subsequently degradation of cell membrane integrity, etc.^{42–46} Therefore, the anti-*Toxoplasma* effects of *A. sativum* could be due to the presence of organosulfur compounds

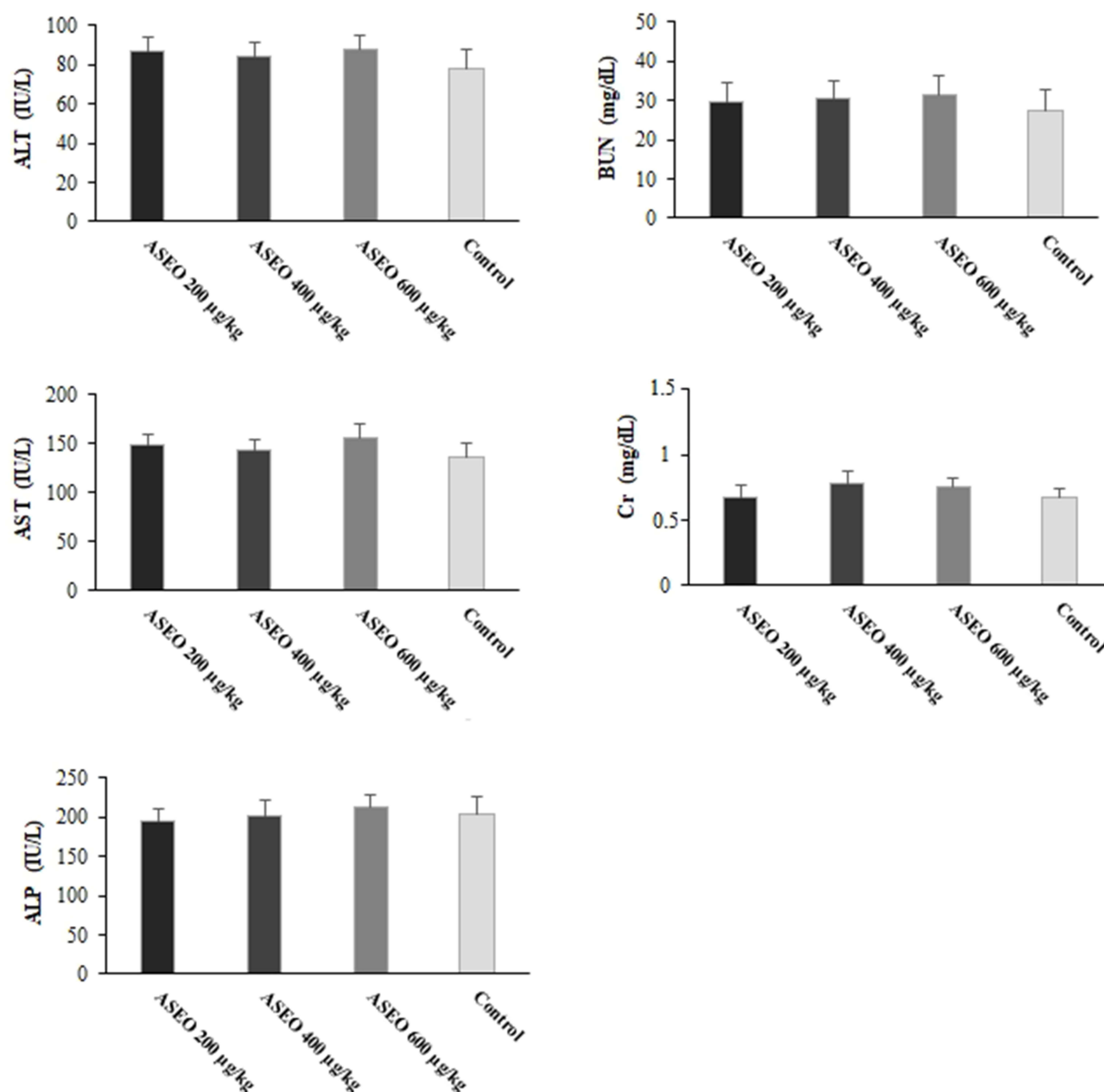


Figure 7 The level of biochemical factors in mice sera after oral administration of ASEO for 14 days. Data are expressed as the mean \pm SD ($n = 8$).

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; Cr, creatinine; BUN, blood urea nitrogen.

in this plant. Another mechanism that contributes to garlic's significant prophylaxis against *Toxoplasma* infection in mice is its immune-boosting effect. Studies confirmed that *A. sativum* strengthens the immune system, especially the cellular immune system, by stimulation of some immune cells (eg, lymphocytes, macrophages, natural killer (NK) cells), modulation of cytokine secretion, activation of macrophages, phagocytosis, production of immunoglobulins, phagocytosis, etc.⁴⁷

The involvement of LPO in the pathogenesis of hepatic damage through the free radical products of *T. gondii* has been confirmed, where it causes cell membrane damage and the release of hepatotoxicity marker enzymes.^{48,49} Malondialdehyde (MDA) as the final product in lipid peroxidation is broadly applied as a marker for the evaluation of LPO.⁴⁷ Our findings revealed that ASEO at the doses of 200, 400, and 600 µg/kg/day significantly ($p < 0.05$) improved the rise in LPO and NO in the

T. gondii infected mice. These findings can therefore show the potential mechanism whereby *A. sativum* acts as an anti-inflammatory drug to protect the liver.

Our real-time PCR results revealed that *T. gondii* significantly ($P < 0.001$) promoted the expression level of IFN- γ and IL-1 β mRNA, while pre-treatment of mice with different doses of ASEO provoked a considerable ($P < 0.001$) down-regulation of IL-1 β and IFN- γ mRNA gene expression levels; this suggests that the pre-treatment of *T. gondii*-infected mice with *A. sativum* can prevent inflammation in them. Previous investigations reported that, in toxoplasmosis, the modulation of the production of pro-inflammatory cytokines IL-1 β , IFN- γ , etc. can prevent hypotension and enhance the host's survival upon sepsis induction.⁴⁹

Nowadays, assessing the function of some vital organs such as the liver and kidneys is the main way to evaluate the toxicity of novel agents in animal experiments.⁵⁰ The most common method for measuring liver and kidney function is calculating the serum levels of some liver (eg, ALT, AST, and ALP) and kidney (Cr, and BUN) enzymes. The toxicity findings revealed that pre-treatment of mice with ASEO at the doses of 200, 400, and 600 $\mu\text{g/kg/day}$ for two weeks resulted in no mortality among the tested mice. Moreover, the findings of biochemical tests demonstrated no significant difference in these biochemical factors between the group treated with ASEO and the control group.

Conclusion

Our results demonstrated the considerable prophylactic effects of ASEO, the increased survival rate of mice, and the reduced parasite load in them. Moreover, ASEO promoted the innate immune system, pro-inflammatory cytokines, hepatic injury inhibition, etc. in the mice with acute toxoplasmosis. However, more investigations are mandatory to clarify the accurate prophylactic and therapeutic anti-*Toxoplasma* mechanisms of ASEO as well as all its toxicity aspects, especially in clinical settings.

Disclosure

The author declares no conflict of interest for this work.

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