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Original Article

High Prophylactic Efficacy of Thymol Loaded Chitosan Nanoparticles for Controlling Acute Toxoplasmosis in Mice

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Abstract

Background: Given the significant role of chitosan nanoparticles in medicine, the present study aimed to assess the *in vivo* efficacy of synthesized chitosan nanoparticles coated with thymol (CNCT) in combating *Toxoplasma gondii* infection.

Methods: Mice were administered CNCT orally at dosages ranging from 0.25 to 0.75 mg/kg/day for a duration of 14 days. Following this treatment, they were infected with *T. gondii* tachyzoites of the Rh strain to induce acute toxoplasmosis. Then, the mortality rate, parasite load, antioxidant activity, and the gene expression level of proinflammatory cytokines were evaluated.

Results: The dimensions of CNTN exhibit variability, with a mean size of 295 nm. The prophylactic administration of CNTN in mice infected with T. gondii resulted in a significant enhancement in survival rates and a considerable decrease in parasite load ($P<0.001$). The CNTN caused a significant decrease in malondialdehyde level, while a notable increase ($P < 0.001$) in the activity of the antioxidant enzymes. The feeding the mice infected with CNTN caused a meaningful elevation in the expression level of TNFα- and IL-1β ($P<0.001$). The biochemical analyses indicated no significant changes in the serum levels of liver and kidney function markers.

Conclusion: The recent study revealed that CNTN demonstrates promising *in vivo* effects against toxoplasmosis in murine models. These effects are attributed to its antioxidant properties and immunomodulatory capabilities, which increase specific pro-inflammatory cytokines without any noticeable signs of toxicity to liver and kidney function.

Introduction

Toxoplasma gondii is an intracellular protozoan parasite that can infect all warm-blooded vertebrates, including humans (1). Approximately 70% of the global population exhibits seropositivity for *T. gondii* (1). Infection primarily occurs through the ingestion of cysts found in raw or undercooked meat, as well as in contaminated fruits, vegetables, and water (1). Additional modes of transmission include congenital transmission, blood transfusions, and organ transplantation (2). Acute toxoplasmosis represents the body's reaction to a primary infection caused by *T. gondii*. While the majority of individuals may remain asymptomatic, some may experience flu-like manifestations, which can include the following symptoms: fever, fatigue, painless lymphadenopathy in the cervical or axillary regions, pharyngitis, hepatosplenomegaly, ocular toxoplasmosis (3, 4).

The conventional therapeutic strategy for the prevention and management of toxoplasmosis in clinical settings entails the use of a combination of pyrimethamine and sulfadiazine (5). Nonetheless, these pharmacological agents are linked to considerable adverse effects, notably the tendency of pyrimethamine to cause bone marrow suppression and hematologic toxicity (6). Moreover, the emergence of resistance to anti-*Toxoplasma* agents has been recorded, mirroring the resistance patterns identified in other apicomplexan parasites, including *Plasmodium* species (6). Despite significant advancements in pharmacological research and safety assessments, there remains a critical shortage of an ideal and effective therapeutic option for chronic toxoplasmosis (7). Consequently, there is an urgent need for the development of a novel therapeutic agent that demonstrates properties such as effective placental penetration, non-toxicity, and efficacy against all developmental stages of the parasite, with a particular emphasis on the cystic form.

Nanoencapsulation represents an innovative and practical field within nanotechnology, especially in relation to the pharmaceutical industry (10). This process involves encapsulating substances at the nanometer scale using biopolymers, films, layers, or nanoparticles. These techniques serve as nanoscale protectors for sensitive compounds, enhancing their bioavailability, facilitating the dissolution of hydrophobic substances in aqueous environments, and enabling the controlled release of drug molecules and their constituents. Chitosan is one of the most significant water-soluble polymers used for creating pharmaceutical nanocarriers (10). Recently, chitosan has gained attention for the encapsulation of various active compounds due to its numerous advantageous properties, including biocompatibility, biodegradability, non-toxicity, the ability to bind to mammalian cells, induction of the immune system, and antimicrobial properties (10). Thymol ($C_{10}H_{14}O$) is a monoterpene phenolic compound, with its primary natural source being the thyme plant (11). This substance possesses antioxidant, anti-inflammatory, disinfectant, antibacterial, and antifungal properties, and its antimicrobial effects against foodborne pathogens have been well-documented (11). Thymol can disrupt the outer membrane of microbial pathogens, leading to the release of lipopolysaccharides (LPS) and an increase in cytoplasmic permeability to adenosine triphosphate (ATP) (12).

Given the significant role of chitosan nanoparticles in medicine (11, 12), we aimed to evaluate the *in vivo* efficacy of synthesized chitosan nanoparticles coated with thymol (CNCT) against acute *T. gondii* infection in mice.

Materials and Methods

Synthesis of chitosan nanoparticles coated with thymol

The formulation of thymol-chitosan nanoparticles was achieved using the ionic gelation method. A measured amount of chitosan (medium molecular weight, >75% deacetylat-

ed, code: 448877, Sigma-Aldrich, Germany) was dissolved in a 0.2% acetic acid solution and placed on a magnetic stirrer for 2 hours. After this period, thymol (1%, Sigma-Aldrich, Germany) and 10 microliters of Tween 20 solution (Sigma-Aldrich, Germany) were added to ensure uniform dispersion. The mixture was then subjected to sonication for half an hour until the particles were completely dispersed within the polymer matrix. A tripolyphosphate (0.1%, Sigma-Aldrich, Germany) cross-linking agent was introduced to create a polymer network. Following 24 hours of stirring, the mixture was dried using a freeze dryer and prepared for subsequent tests (13). The doses of CNCT were prepared in normal saline within test tubes, followed by a series of serial dilutions to achieve the desired concentrations for testing. The selection of these doses was guided by preliminary experiments and toxicity assessments conducted on vital organs, as detailed in the text.

Physical and chemical characteristics of CNCT

The size and distribution of the CNCT were considered using a scanning electron microscope (SEM, Hitachi Model S-4160) and the ZEN3600 Nano Sizer-ZetaSizer device, manufactured by Malvern Instruments in England, respectively.

Fourier transform infrared (FTIR) spectroscopy

The chemical functional groups involved in the synthesis of CNCT were evaluated by using the potassium bromide (KBr) and examined by an FTIR device (Agilent Technologies, Cary 630, Germany).

Parasite

T. gondii RH tachyzoites were obtained through intraperitoneal passages from experimentally infected mice provided by the Department of Parasitology at the Razi Herbal Medicines Research Center in Iran. The tachyzoites were harvested and centrifuged to eliminate waste materials and peritoneal cells. After discarding the supernatant, the remaining tachyzoites were resuspended in phosphate-buffered saline (PBS) and adjusted to a concentration of 1×10^4/mL using a hemocytometer.

Animals

A total of 64 male Balb/C mice, each weighing between 25 and 30 grams and aged 4 to 6 weeks, were utilized for the study. During the course of the experiment, the mice allowing for unrestricted access to both food and water. Furthermore, efforts were made to adhere to the guidelines outlined in the and Use of Laboratory Animals.

Ethics

The study design received approval from the Ethical Committee of Lorestan University of Medical Sciences in Khorramabad, Iran, under the ethics number IR.LUMS.REC. 1401.015.

Study design

Initially, the mice were randomly assigned to five groups, each consisting of eight mice: (i) normal saline (negative control), (ii) pyrimethamine (PMA) at a dosage of 10 mg/kg/day (positive control), and (iii-v) CNCT at dosages of 0.25, 0.5, and 0.75 mg/kg/day. Mice in all groups were orally administered the drugs for 14 consecutive days. Twenty-four hours after the treatment concluded, the animals were infected intraperitoneally with 0.2 mL of tachyzoites (14).

Mortality rate and parasitological tests

Following the infection, mice from all experimental groups underwent daily monitoring, during which the mortality rate was systematically recorded. For parasitological examinations, peritoneal fluids from the tested animals were collected 72 hours post-infection, and the number of tachyzoites was counted using a light microscope (15).

Assessing the antioxidant activity of CNTN

Seventy-two hours following infection, four mice from each experimental group were euthanized utilizing a combination of ketamine and xylazine (15-100 mg/kg) through intraperitoneal injection. Afterward, liver tissues were collected to assess oxidant and antioxidant markers. Liver homogenates were prepared, and the tissue levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were examined using commercial kits Karmania Pars Gene, and Kiazist, Iran (Iran) according to the manufacturer's instructions.

Effects of CNTN on the gene level of pro-inflammatory cytokines

The influence of CNTN on the expression levels of pro-inflammatory cytokine genes in the liver, particularly IL-1β and TNF-α, was examined utilizing real-time PCR methodologies. Total RNA was extracted using a commercial kit (Parstous, Iran) in accordance with the manufacturer's guidelines. The conversion of RNA to complementary DNA (cDNA) was performed with a kit from Sinaclon Company, following the provided instructions. The primers used in this study and the thermal condition were derived from previous research by Baghdadi et al (16) using the optical system software (iQTM5 model, Bio-Rad, Hercules, CA) through the calculation of the $2^{-\Delta\Delta CT}$ method, with β-actin serving as the housekeeping gene.

Toxicity effects of CNTN in mice

The study was performed utilizing a sample of healthy 24 mice, which were categorized into four distinct groups, each comprising six mice. The groups were delineated as follows: (i) a control group of healthy mice administered normal saline orally, (ii) CNTN at a dosage of 0.25 mg/kg orally, (iii) CNTN at a dosage of 0.5 mg/kg, and (iv) CNTN at a dosage of 0.75 mg/kg. Blood samples were collected based on the method described by Mahmoudvand et al (17) and then the serum level of liver enzymes (alanine transaminase (ALT) and aspartate transaminase (AST)), and kidney factors (blood urea nitrogen (BUN) and creatinine (Cr)) using the radioimmunoassay method with the Pars Azmon kits (Tehran, Iran) and an autoanalyzer (RA 1000, Technicon Instruments, USA) (17).

Statistical analysis

Data analysis was performed utilizing SPSS statistical software version 26, (IBM Corp., Armonk, NY, USA). A one-way ANOVA, accompanied by Tukey's post hoc test, was implemented to assess the differences among the experimental groups. Furthermore, a *P*-value exceeding 0.05 was deemed statistically significant.

Results

Characterization of CNTN

Based on the results of SEM analysis, the size of CNTN varied from 100 to 600 nm, while in the analysis of nanoparticles with the nanosize-zeta sizer device, most of the nanoparticles were between 200 and 300 nm with an average size of 295 nm were (Fig. 1). Based on the results of the FTIR analysis, the peaks at 1350 and 3142 cm^{-1} indicated the vibration of hydroxyl (OH) groups, which arise from the more open structures resulting from the crosslinking of chitosan with thymol. The peaks at 2955 and 2752 cm^{-1} correspond to CH_3 groups and fatty acids. The peak at 1649 cm^{-1} signifies the stretching of the amide C=O bond in structural proteins, while the peaks at 1256 and 1171 cm^{-1} indicate the asymmetric stretching of PO_2^- and represent phospholipid groups. Additionally, the peak at 1071 cm^{-1} reflects C-O-C and C-O stretching, representing carbohydrate groups (Fig. 2).

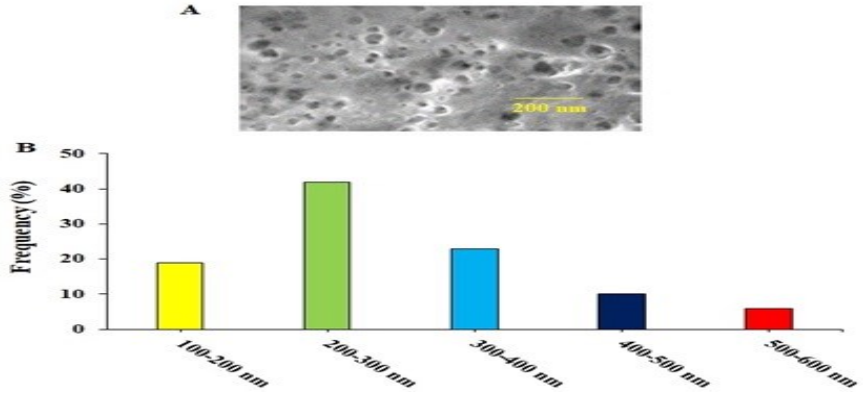

Fig. 1: Scanning electron microscope (A) and size distribution (B) of chitosan nanoparticles coated with thymol

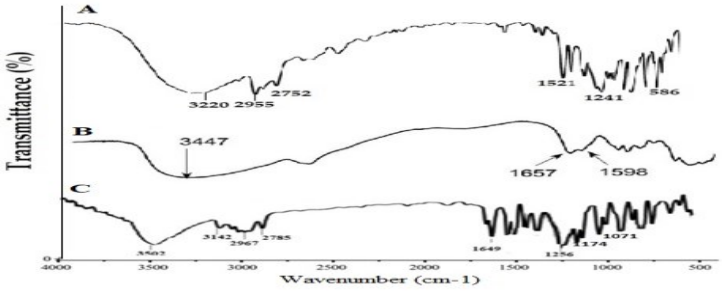

Fig. 2: Fourier transform infrared analysis of thymol (A), chitosan (B), and chitosan nanoparticles coated with thymol (C)

Mortality rate of Infected Mice Treated with CNTN

A 14-day prophylactic treatment with CNTN in *T. gondii*-infected mice, using doses of 0.25 to 0.75 mg/kg per day, resulted in a significant enhancement of survival rates on the seventh, eighth, and ninth days, respectively. Notably, the highest survival rate was recorded in mice administered CNTN at a dosage of 0.75 mg/kg/day, with these infected mice surviving an average of four days longer than those in the control group ($P < 0.001$) (Fig. 3).

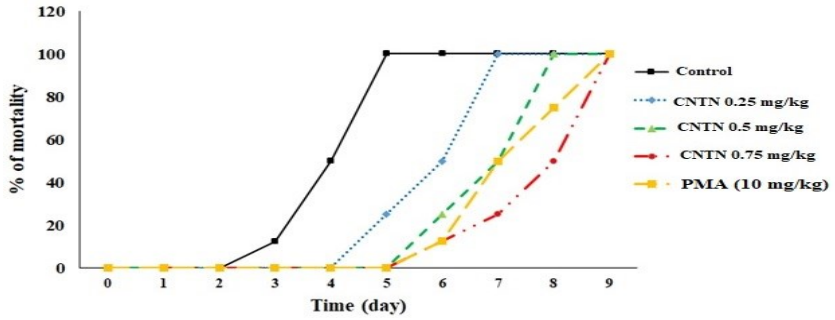

Fig. 3: The mortality rate of infected mice treated with chitosan-based thymol nanoparticles (CNTN) and pyrimethamine (PMA) based on chitosan at doses of 0.25, 0.5 and 0.75 mg/kg per day for 14 days

The quantity of tachyzoites collected from infected mice treated CNTN

Fig. 4 illustrates the mean quantity of tachyzoites extracted from the peritoneal fluid of infected mice that received CNTN treatment on the third day. Following the prophylactic treatment of infected mice with CNTN at doses ranging from 0.25 to 0.75 mg/kg per day, the mean reductions in the number of tachyzoites observed on the third day were 44.3%, 57.6%, and 72.6%, respectively. The most significant decrease in the number of parasites in infected mice occurred in those treated with CNTN at a dose of 0.75 mg/kg ($P < 0.001$).

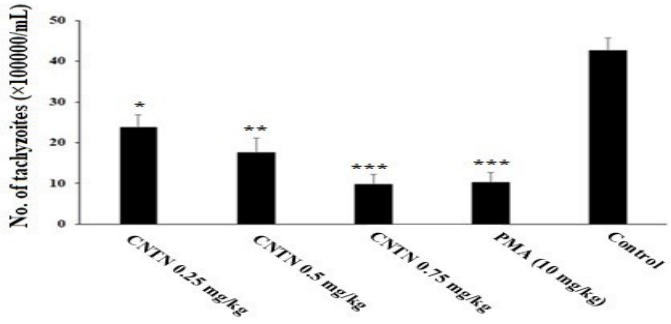

Fig. 4: The number of tachyzoites (parasite load) isolated from infected mice treated with chitosan-based thymol nanoparticles (CNTN) and pyrimethamine (PMA) based on chitosan at doses of 0.25, 0.5 and 0.75 mg/kg/day for 14 days. * P<0.05, ** P<0.01, and *** P<0.001 compared to control

Oxidant/antioxidant factors in infected mice treated CNTN

In Fig. 5, *T. gondii* infection resulted in a significant increase in the oxidative stress marker MDA and a decrease in the tissue concentrations of the antioxidant enzymes SOD and GPx ($P < 0.05$). In contrast, the administration of CNTN to mice infected with *T. gondii* at dosages of 0.25, 0.5, and 0.75 mg/kg for 14 days resulted in a significant reduction in MDA levels. Additionally, a substantial increase ($P < 0.001$) in the activities of GPx and SOD was observed.

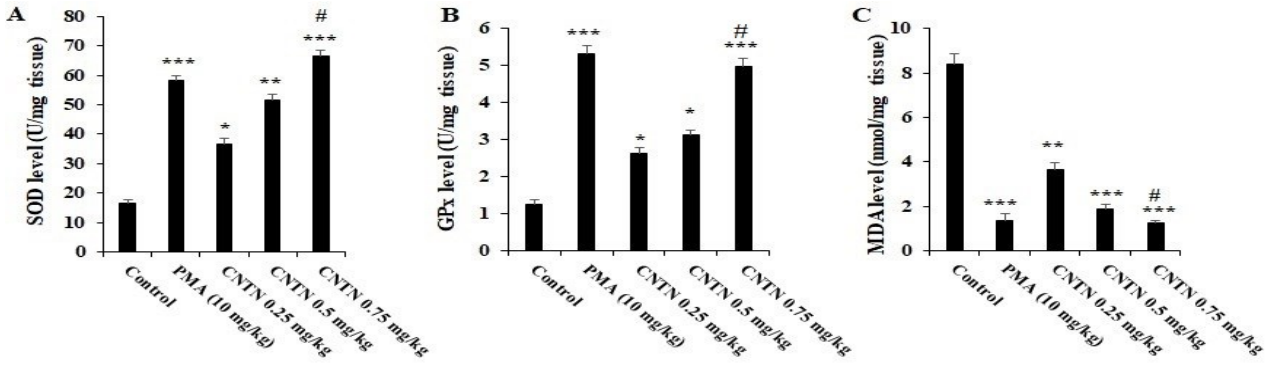

Fig. 5: Effect of chitosan nanoparticles coated with thymol (CNCT) and pyrimethamine (PMA) on oxidant/antioxidant factors (superoxide dismutase (SOD, A), glutathione peroxidase (GPx, B) and malondialdehyde (MDA, C)) in *Toxplasma* infected mice. * P<0.05, ** P<0.01, and *** P<0.001 compared to control; # P<0.05 compared to PMA

Pro-inflammatory factors in Infected Mice treated CNTN

As illustrated in Fig. 6, administering CNTN to mice at dosages of 0.25, 0.5, and 0.75 mg/kg over a period of 14 days displayed a meaningful raise in the mRNA expression levels of the TNFα and IL-1β ($P < 0.001$). Furthermore, the statistical analysis revealed that the 0.75 mg/kg dosage of CNTN led to a notable increase in the expression levels of TNFα and IL-1β ($P < 0.05$) compared with PMA ($P < 0.001$).

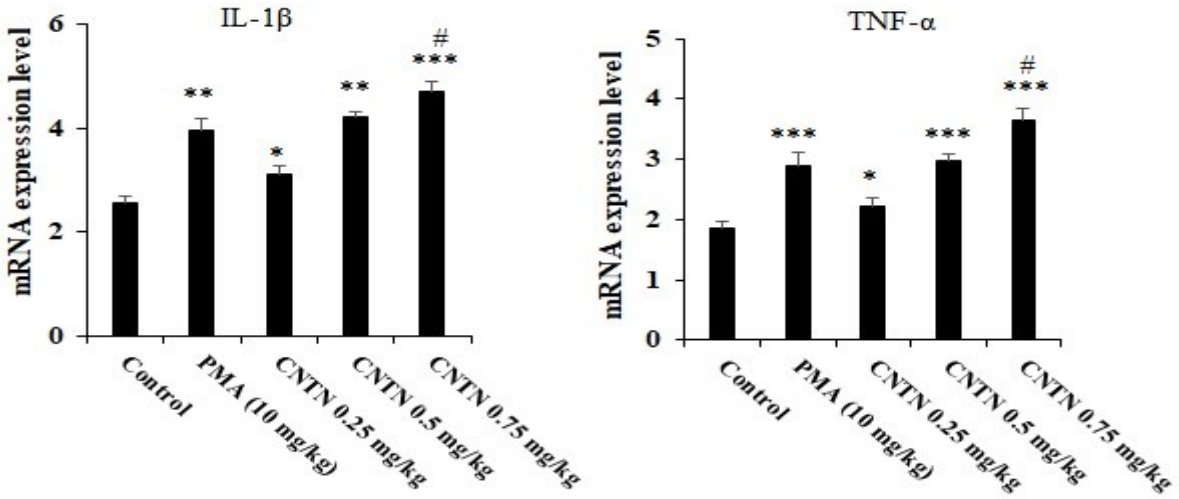

Fig. 6: Effect of chitosan nanoparticles coated with thymol (CNCT) and pyrimethamine (PMA) on tumor necrosis factor (TNF-α), and interleukin 1 (IL-1β) in *Toxplasma* infected mice. * $P<0.05$, ** $P<0.01$, and $P<0.001$ compared to control; # $P<0.05$ compared to PMA

Toxicity effects of CNTN in mice

The biochemical analyses indicated that, although there were changes in several parameters suggestive of increased serum levels of liver and kidney function markers, these alterations did not achieve statistical significance when compared to the control group (Fig. 7).

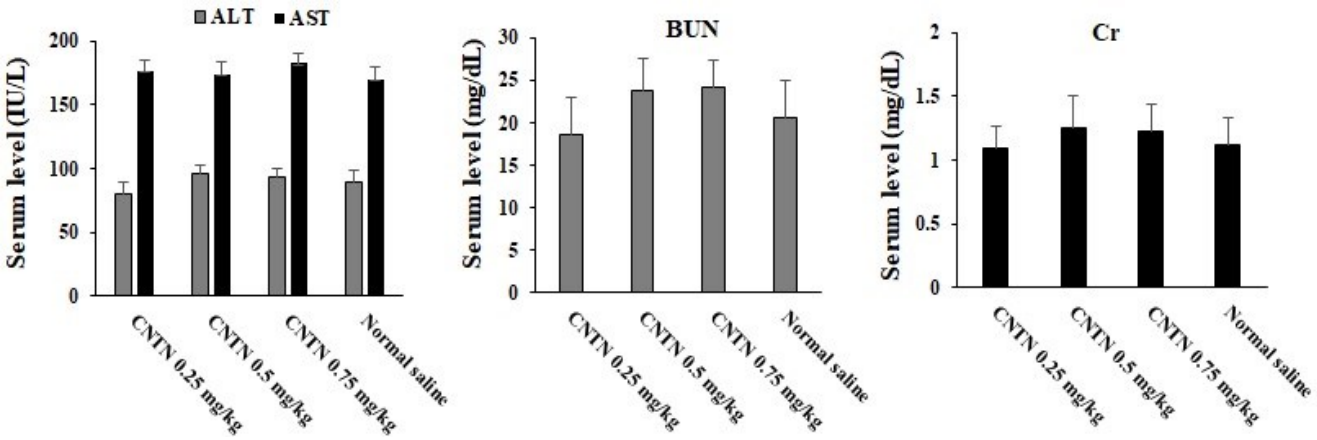

Fig. 7: Effect of chitosan nanoparticles coated with thymol (CNCT) on alanine transaminase (ALT) and aspartate transaminase (AST), blood urea nitrogen (BUN) and creatinine (Cr) in the healthy mice

Discussion

Our findings indicate that the majority of the CNTN measured between 200 and 300 nm, with a mean size of 295 nm. SEM is a widely utilized technique for evaluating the morphology and dimensions of synthesized nanoparticles, both of which are essential parameters that influence fabrication requirements (25). Experimental investigations have demonstrated that the stability and biological functionality of nanoparticles is affected by their size, with smaller nanoparticles typically exhibiting greater stability and reduced accumulation (19). Furthermore, it has been established that the application of specific molecules for coating nanoparticles can lead to an increase in their size; this coating process has the potential to enhance their biocompatibility and biological effects (20).

Our results showed that a 14-day prophylactic treatment with CNTN in *T. gondii* infected mice resulted in a significant enhancement of survival rates on the ninth days post-infection; while, the significantly reduced the parasite load by 72.6%. Considering the antiparasitic effects of these tested compounds, in a study Teimori et al showed that a complete mortality rate of tachyzoites at concentrations of 1,000 and 2,000 ppm of medium molecular weight chitosan nanoparticles, as well as at a concentration of 2,000 ppm of high molecular weight CS NPs, after a duration of 180 minutes. Furthermore, the load of tachyzoites in the mice treated with low, medium, and high molecular weight CS NPs was observed to be 86%-79%, respectively (21). Etewa et al confirmed that spiramycin-loaded chitosan nanoparticles, administered at a dose of 100 mg/kg/day for seven days, significantly decreased the mortality rate and the number of parasites in Swiss albino mice infected with both acute (Rh strain) and chronic (ME49 strain) strains of *T. gondii* (22). Recently, rosuvastatin-loaded chitosan nanoparticles considerably increased survival time while reducing the parasite load in BALB/c mice infected with *T. gondii* Rh strain (23). Thymol, administered at a dose of 80 mg/kg/day for six days, exhibited potent anti-*Toxoplasma* activity in both congenital and non-congenital models of *T. gondii* infection (ME49 strain) in C57Bl/6 mice (24). Tthymol exhibits anti-leishmanial properties, resulting in a reduction in the quantity of liver amastigotes in histopathological samples when compared to a control group. Additionally, thymol was found to exert a more potent inhibitory effect on the growth of *Leishmania infantum*, both *in vitro* and *in vivo*, than carvacrol (25). Further research validated the efficacy of thymol as an antimalarial agent *in vitro*, reporting an IC50 value of 4.5 µg/mL (26). The thymol component present in oregano essential oil demonstrated *in vitro* antimalarial activity against *Plasmodium falciparum*, with an IC50 value of 10 µg/mL (27). Moreover, Tanghort et al. advocated for the use of thymol in combating cryptosporidiosis (specifically *Cryptosporidium baileyi* and *Cryptosporidium galli*), citing its detrimental effects on oocysts at notably low concentrations (28). The discrepancies in outcomes observed across these investigations can be attributed to a variety of factors, including the type of parasite strain, the nature of the infection (whether acute or chronic), the specific strains of the parasite (such as RH, Tehran, and ME49), the techniques employed for nanoparticle synthesis (including chemical, green synthesis, and physical methods), and the animal models selected for experimentation. Phenolic compounds, including thymol, exert their antimicrobial effects through several mechanisms. These mechanisms involve the induction of permeability and depolarization of the cytoplasmic membrane, the promotion of apoptosis, disruption of energy metabolism and membrane transport processes, interference with DNA replication, compromise of cell membrane integrity leading to the efflux of

intracellular components, and inhibition of biofilm formation (29, 30).

The pathogenesis of *T. gondii* is associated with oxidative stress, leading to an increased production of free radicals and a diminished capacity of the endogenous antioxidant defense system (31). The interplay between pro-oxidant and antioxidant mechanisms in the context of toxoplasmosis and its therapeutic strategies has been previously discussed in the scholarly literature (32). Therefore, it is crucial to consider seriously the incorporation of oxidant and/or antioxidant agents in the therapeutic management of this parasitic infection (32). Our study reported that the administration of CNTN to infected mice resulted in a significant decrease in MDA levels, while a notable increase ($P < 0.001$) in the activity of GPx and SOD was observed. These findings demonstrate the antioxidant potential of CNTN in controlling toxoplasmosis in mice.

Pro-inflammatory cytokines are critical components in the initiation and maintenance of both innate and adaptive immune responses, serving to limit the proliferation of *T. gondii* (33). IL-1 acts as a mediator of the acute phase response and works synergistically with tumor TNFα to enhance inflammation during *T. gondii* infection. Furthermore, in an animal model of toxoplasmosis, IFNγ has enhanced anti-toxoplasmic activity by increasing TNF-α production when administered alongside recombinant cytokines (TNF-α and IL-1β) (34). The present results demonstrated that feeding infected mice with CNTN at dosages of 0.25, 0.5, and 0.75 mg/kg over a duration of 14 days resulted in a significant elevation in the mRNA expression levels of the pro-inflammatory cytokines TNFα and IL-1β, indicating the potent immunomodulatory effects of CNTN in controlling toxoplasmosis in mice.

It is evident that the assessment of the toxicological properties of novel pharmaceutical compounds is an essential and imperative undertaking that must be conducted prior to their administration to patients (35). The biochemical analyses conducted in this study revealed that, although there were modifications in various parameters indicative of elevated serum levels of liver and kidney function markers, these alterations did not reach statistical significance when compared to the control group. This finding implies that the administration of CNTN at doses between 0.25 and 0.75 mg/kg did not exhibit any detrimental effects on hepatic and renal functions in healthy murine subjects.

Conclusion

CNTN demonstrates promising *in vivo* effects against toxoplasmosis in murine models. These effects are attributed to its antioxidant properties and immunomodulatory capabilities, which increase specific pro-inflammatory cytokines without any noticeable signs of toxicity to liver and kidney function.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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