Nanoemulsion of clove essential oil in the experimental cystic hydatid disease in mice, evidence on the high importance of in vivo models in nano drug delivery systems studies

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Abstract

Cystic hydatid disease (CHD) is a global zoonotic infection caused by *Echinococcus granulosus*. Several *in vitro* researches have demonstrated high efficacy of nanoformulations against protoscoleces of *E. granulosus*, but only a limited number of them evaluated their safety on CHD animal models. Based on our previous report on the *in vitro* effectiveness of clove essential oil (CEO) and the developed nanoemulsion (N-CEO) against *E. granulosus*, herein we evaluated the therapeutic and side effects of CEO and N-CEO on CHD-mice. Number of 48 mice were infected to CHD by intraperitoneal injection of 10^3 protoscoleces, and 8-week post injection received the treatments, CEO-20 and -50 and N-CEO-20 and -50 (20 and 50 mg/kg), and albendazole (ALB) as the standard treatment (50 mg/kg), by oral gavage for a 6-week period. D-CON served as the control

and were infected to CHD but received only saline. All the tested treatments resulted in a significant reduction in the average number and size of cysts. Treatment with ALB, CEO-20 and CEO-50 had no increasing effect on serum activity of liver enzymes. However, the highest alkaline phosphatase and alanine transferase activities have been observed in N-CEO-50. The level of antioxidant enzymes, and the gluthathione content were lower in N-CEO-50 compared to the D-CON mice. The most significant histopathological damages were noted in N-CEO-50 including infiltration of edematous cells, inflammation, hyperemia and degeneration. Further studies to find the mechanism of liver injury despite the slight *in vitro* cytotoxicity can be a step forward in reevaluating the safety of nanoformulations.

Key words: herbal preparation, nanomedicine, safety, toxicity, zoonoses

1. Introduction

Cystic hydatid disease (CHD) is characterized as a zoonotic infection caused by the cestode species of the genus *Echinococcus granulosus*. The parasite needs two mammalian hosts for completing its life cycle. The adult cestodes inhabit the small intestine of a carnivore (definitive host) and produce eggs containing infective oncospheres (1). After oral intake of infective eggs by an intermediate host, the metacestode develops into a unilocular fluid-filled cyst in the internal organs, most commonly the liver (2). By contacting to an infected animal or ingestion of infective eggs, humans can become accidental intermediate hosts. The clinical features of CHD in the human beings depend on the involved organ, the site of involvement, stage of cyst development and viability of the cyst contents (3).

The perfect treatment for CHD is based on complete elimination of the parasite and prevention of recurrence of the disease. There are three available methods for the treatment of CHD including systemic chemotherapy, surgery, and “puncture, aspiration, injection, re-aspiration” known as PAIR. Chemotherapy and PAIR are recommended as alternatives to surgery, especially for patients who are not optimal candidates of the surgery (4). Chemotherapy is particularly appropriate for inoperable

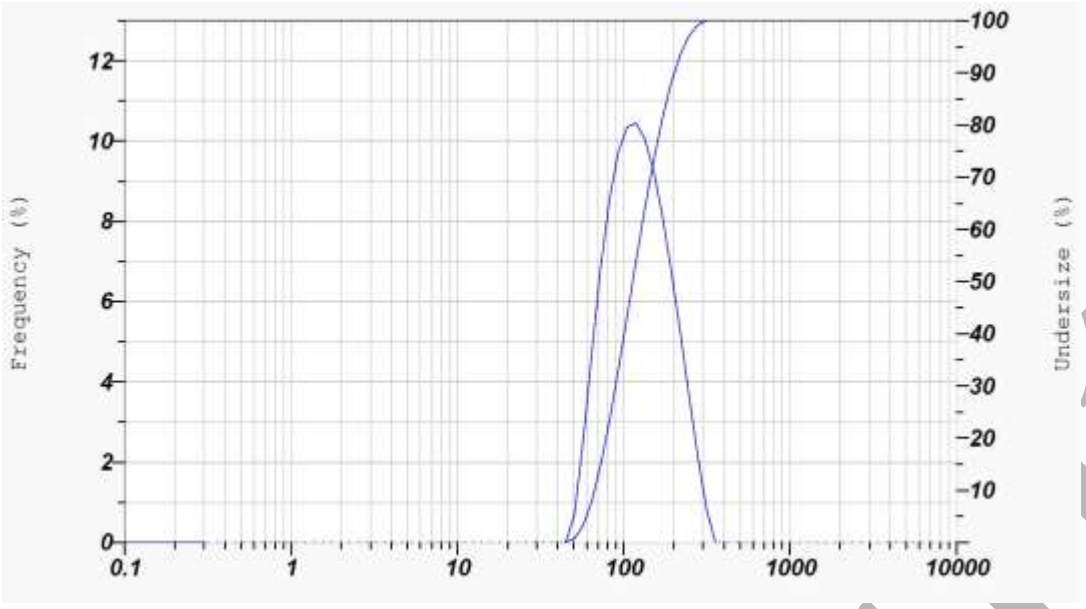
patients having deep cysts or peritoneal cysts and comprises the benzimidazoles namely mebendazole and albendazole. Administration of benzimidazoles is accompanied by several side effects such as nausea, hepatotoxicity, neutropenia, and occasionally alopecia (5). These considerations have resulted in an indispensable need for finding novel therapeutics for CHD with lower side effects.

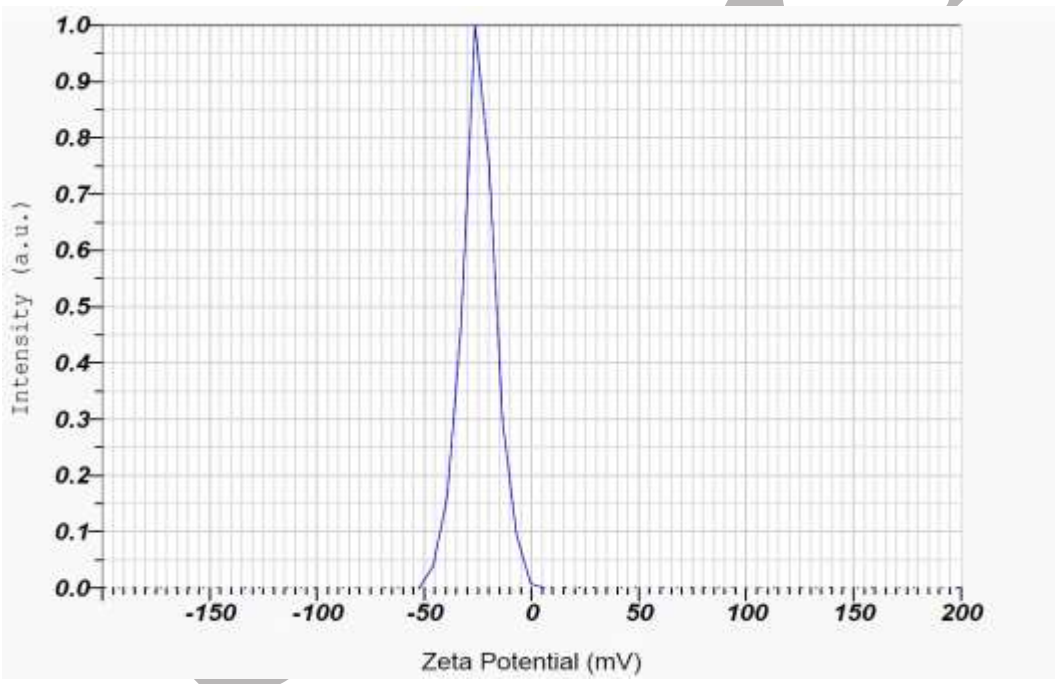
In recent years, great progress has been achieved through nanotechnological approaches in designing drug delivery systems possessing the advantages of targeted or controlled release (6, 7). Nanoformulation improves the bioavailability and target specificity of phytochemicals, thereby maximizing their therapeutic potential (8). Several *in vitro* studies have demonstrated the high efficacy of nanoformulations against protoscoleces of *E. granulosus* (9-14), but only a limited number of studies evaluated therapeutic potential or safety of nanoformulated therapies on CHD(11, 15). Hereby, based on the findings of our previous report on the *in vitro* effectiveness of clove essential oil (CEO) and its nanoemulsion (N-CEO) against *E. granulosus* (Naser et al. 2022), we decided to evaluate the therapeutic efficacy and probable adverse effects of CEO and N-CEO on experimental CHD in mice

2. Material and methods

2,1. Chemicals

Glyceryl monooleate (Anmol Chemicals, Indai), polyoxyl 40 hydrogenated castor oil (BASF, Germany), and polyethylene glycol 400 (Sigma, Germany) according to a previously described method were used for the synthesis of N-CEO (Naser et al. 2022). Characterization of the developed formulation was confirmed by a Nano-ZS ZEN 3600 particle size analyzer (Malvern Instruments, UK) (Supplementary Figure 1).





Supplementary Figure 1. Particle size and polydispersity index distribution (A); zeta potential for nanoemulsion of clove essential oil (N-CEO) (B)

2,2. Study design

Number of 56 male mice were purchased from Pasteur Institute of Iran, North Research Center, Amol, Iran. Mice with average weight of 25 to 30 grams were divided into 7 groups as follows: CEO-20, mice receiving 20 mg/kg of CEO; CEO-50, mice receiving 50 mg/kg of CEO; N-CEO-20, mice receiving 20 mg/kg of N-CEO; N-CEO-50, mice receiving 50 mg/kg of N-CEO; ALB, mice receiving 50 mg/kg of the standard drug albendazole; and D-CON and H-CON, mice received normal saline as controls.

All the groups except H-CON after acclimatization period were infected to CHD by intraperitoneal injection of 10^3 protoscoleces, recovered from infected sheep livers collected from an abattoir in Babol, Iran (Dr. Keshavarzi abattoir). All of the groups were kept for 8 weeks at the standard conditions with free access to water and feed. 8-week post injection of protoscoleces, the mice received the treatments as described above for different experimental groups in the form of oral gavage for a 6-week period. During the study, mice were monitored regarding their health conditions and weigh gaining.

At the end of the sixth week of treatments (14-week post injection), all groups were weighed, and after taking blood samples were euthanized. Autopsy and determination of the number and size of cysts in different groups were done. The study was conducted in accordance to animal welfare law and approved by the institutional ethics committee of animal care and use, according to ARRIVE guidelines (approval number IR.IAU.SRB.REC.1399.189).

2,3. Biochemical and histopathological analysis

At the end of the study, blood samples were taken from all groups. Sera were separated from blood samples for measurement of some biochemical factors such as serum activity of liver enzymes alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST), using a spectrophotometer and commercial kits (Pars Azmoun, Iran). Antioxidant enzymes levels, glutathione peroxidase (GPx) and superoxide dismutase (SOD), also liver gluthatione content (GSH) were measured in the liver samples of different groups of mice by commercial kits (Navand Salamat, Iran) according to the manufacture's described protocol. To prepare tissue sections, the liver samples of different

experimental groups were placed in a 10% buffered formalin. After blocking with paraffin, 5µm thick sections were prepared and stained with hematoxylin and eosin stain (H&E). Stained sections were examined microscopically, at ×100 and ×400 magnification. Evaluation of the sections was done based on the severity of the histopathological alterations including immune cell infiltration or degenerations. The following scores were given to the severity of histopathological lesions: 0: none, 1: mild, 2: moderate and 3: severe.

2,4. Statistical analysis

Data were analyzed by using SPSS version 23.0 (Chicago, IL, USA). Differences between the mean number and size of cysts, serum liver enzymes activity, and liver antioxidant enzymes in different groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The histopathological alterations data were analyzed by Kruskal-Wallis's and Dunn's as the *post hoc* test. P values <0.05 were considered statistically significant.

3. Results

3.1.Mean number and size of cysts

The results of treating infected mice with CEO and N-CEO on the number and size of cysts compared to the albendazole and the control groups are shown in Table 1.

Table 1. Mean number and size of cysts in mice infected to cystic hydatid disease and received clove essential oil (CEO), nanoemulsion of clove essential oil (N-CEO) or the standard drug albendazole (ALB) as treatments in comparison to the infected control (D-CON) and non-infected control (H-CON) mice.

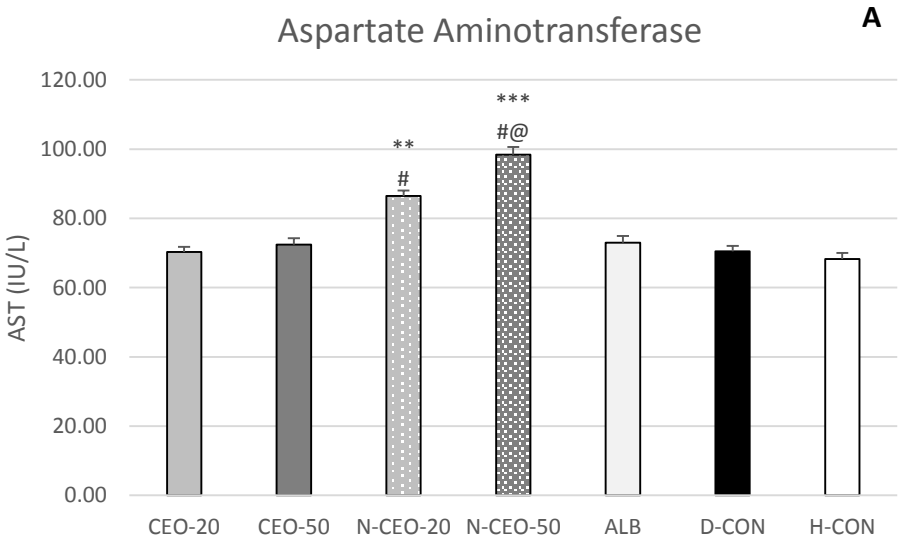
Groups	Mean number of cysts	Mean size of cysts (mm)
CEO-20	30.12 ± 0.90[a]	24.73 ± 1.31[a]
CEO-50	29.62 ± 0.53[a]	23.31 ± 0.97[a]

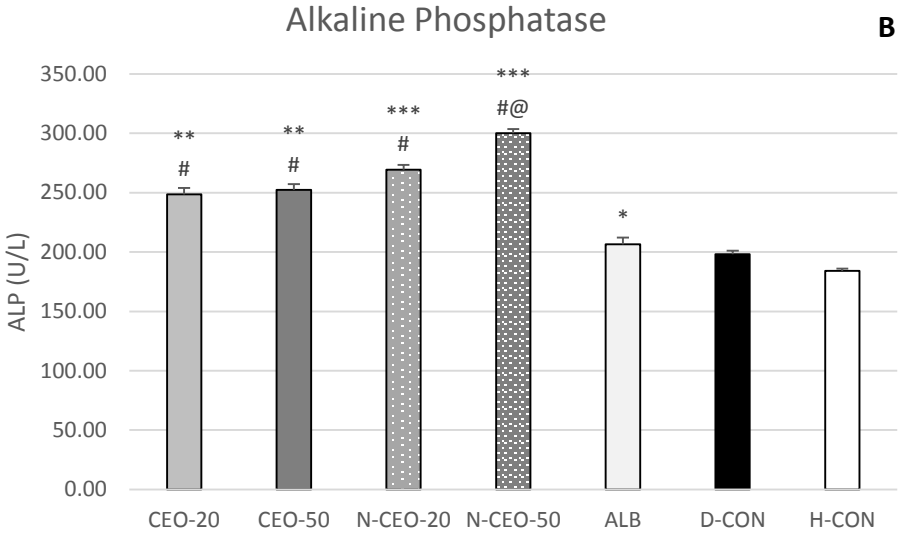
N-CEO-20	27± 0.84[a]	23.03± 0.84[a]
N-CEO-50	26.62 ± 0.88[a]	21.84 ± 0.69[a]
ALB	30.5 ± 2.44[a]	25.14 ± 1.59[a]
D-CON	44.75 ± 2.24[b]	39.61 ± 1.34[b]
H-CON	0[c]	0[c]

Induction of CHD by intraperitoneal injection of *E. granulosus* protoscoleces resulted in the successful induction of experimental infection in mice as can be seen by formation of cyst in all groups except the H-CON group. All of the treatments resulted in a significant reduction in the average number and size of cysts compared to the D-CON ($P<0.05$); however, no significant difference was noted between different treatments comparing nanoformulated or standard drug ($P> 0.05$).

3.2.Liver enzymes activity

The serum liver enzymes activity after treatment of CHD mice with CEO, N-CEO, and ALB in comparison to the control mice are shown in Figure 1.





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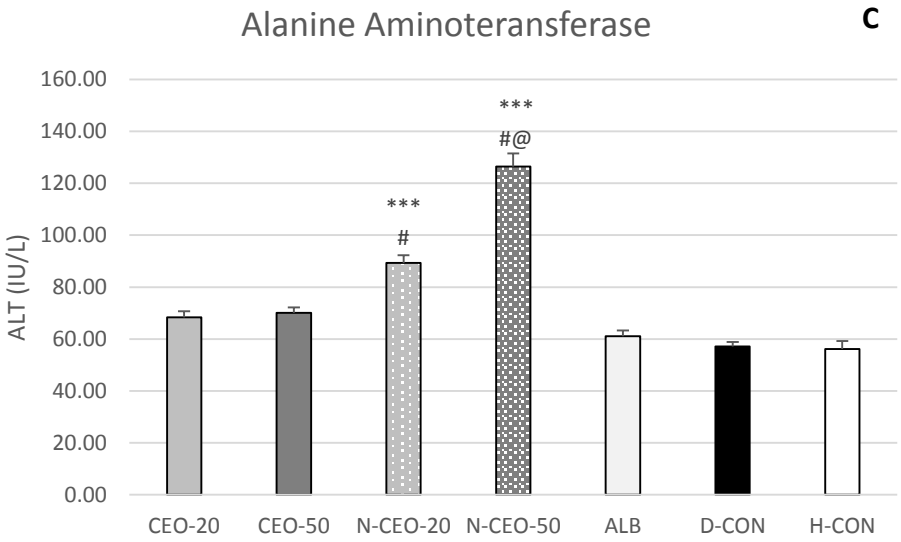

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173 **Figure 1.** Serum liver enzymes activities of aspartate aminoteransferase (A), Alanine aminoteransferase (B), alkaline
174 phosphatase (C) in mice infected to cystic hydatid disease and received clove essential oil (CEO), nanoemulsion of clove
175 essential oil (N-CEO) or the standard drug albendazole (ALB) as treatments in comparison to the infected control (D-CON)
176 and non-infected control (H-CON) mice.

177

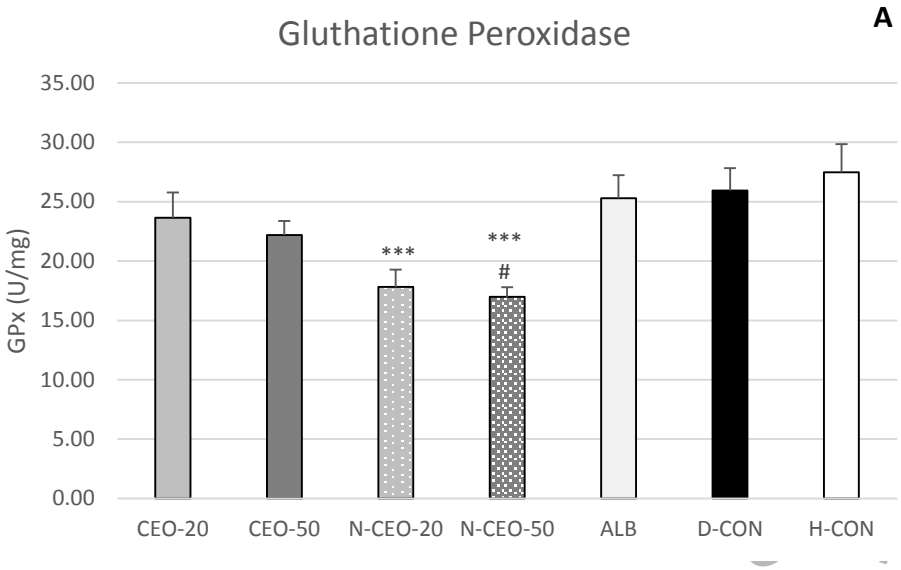
178 Infection to CHD did not increase the AST activity in D-CON mice in comparison to the

179 H-CON mice. In addition, treatment with ALB, CEO-20 and CEO-50 had no increasing effect on serum
180 levels of AST. On the other hand, AST activity increased in mice treated with N-CEO at both doses of
181 20 and 50 mg/kg (Figure 1A). Infected CHD mice showed higher serum activity of ALP in comparison
182 to the non-infected mice. As can be seen in Fig. 1B, groups of ALB and D-CON ($P < 0.05$), CEO-20 and

CEO-50 ($P< 0.01$) had higher ALP activity relative to H-CON. Among different treatments, highest ALP activity has been observed in N-CEO-50 which was significant in comparison to all other treatments ($P< 0.05$). It should be noted that no significant difference was resulted by induction of CHD or treatment with CEO and ALB in ALT activity of CEO-20, CEO-50, ALB, D-CON mice; however, N-CEO, at both of the treated doses led to an increase in ALT activity in comparison to all other groups. Significant difference was also noted between N-CEO-20 and N-CEO-50 ($P< 0.05$) (Figure 1C).

3.3.Liver Antioxidant enzymes

Measurement of antioxidant enzyme, GPx, in mice infected to CHD and treated with N-CEO showed a significant decrease in comparison to the H-CON ($P<0.001$); GPx in N-CEO-50 was lower in comparison to the D-CON ($P<0.05$) (Figure 2A). SOD was significantly deceased in D-CON relative to the H-CON ($P<0.05$), treatment with N-CEO, not CEO or ALB caused decrease in SOD levels in the livers of treated mice compared to the H-CON mice ($P<0.001$). SOD in livers of N-CEO-50 mice was also significantly lower than the D-CON mice ($P< 0.05$) (Figure 2B). Regrading GSH contents of livers of treated mice, significant lower amounts of GSH were noted in CEO-50, N-CEO-20, and N-CEO-50 groups relative to the H-CON. The lowest GSH content has been detected in the N-CON-50 mice, which was lower than the D-CON ($P< 0.05$) (Figure 2C).


A
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GPx (U/mg)
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ALB
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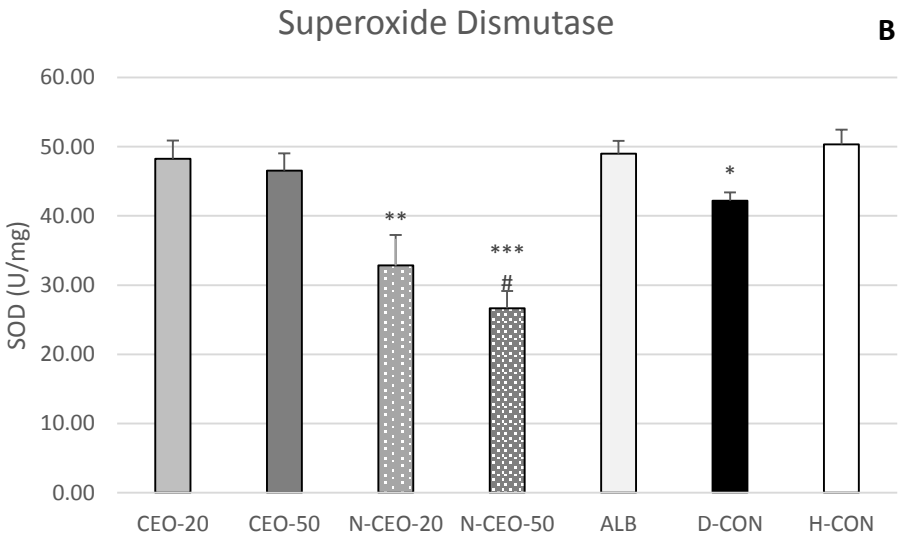
H-CON

200


B
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SOD (U/mg)
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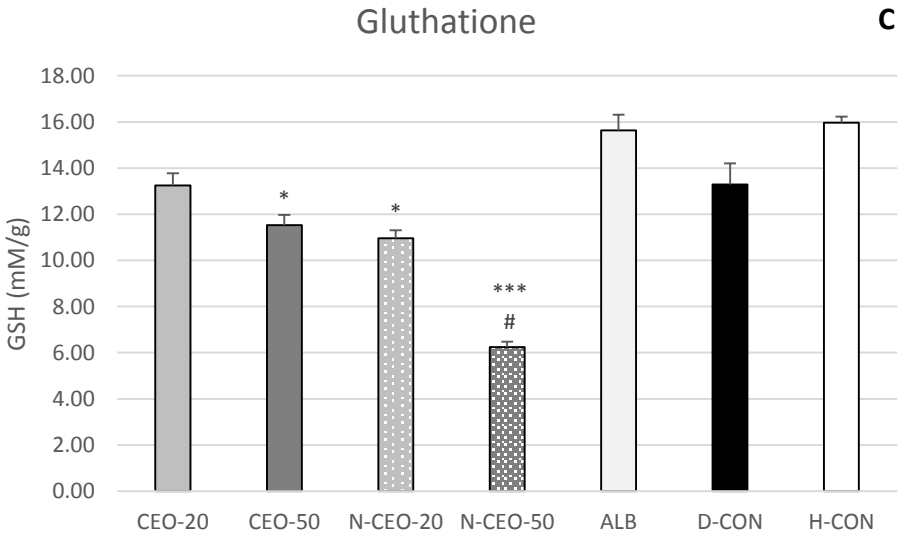
C
Gluthatione
GSH (mM/g)
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CEO-20
CEO-50
N-CEO-20
N-CEO-50
ALB
D-CON
H-CON

202

Figure 2. Antioxidant enzymes gluthatione peroxidase (A), superoxide dismutase (B), and the liver gluthatione content (C) in mice infected to cystic hydatid disease and received clove essential oil (CEO), nanoemulsion of clove essential oil (N-CEO) or the standard drug albendazole (ALB) as treatments in comparison to the infected control (D-CON) and non-infected control (H-CON) mice.

3.4. **Results of histopathological evaluation**

The most significant scores were in N-CEO-50 mice in comparison to the D-CON. Apparently, treatment with N-CEO, especially at the dose of 50 mg/kg, caused pathological alterations in liver of treated mice. Infection of mice to CHD in D-CON mice resulted in the formation of cysts in liver of animals. In the histopathological evaluation of cystic livers, the cystic space was visible. Connective and fibrotic tissues could be seen around the cyst. In the liver tissue, hematuria and infiltration of edematous cells were evident. Moderate hyperplasia of bile ducts also could be observed in the samples. In the CEO treated mice, especially CEO-50, the cystic space was significantly smaller than D-CON. Hyperemia was mild and inflammation was less visible. In the liver samples of N-CEO treated mice, particularly N-CEO-50 mice, infiltration of edematous cells was more prominent. Hyperemia was severe and liver cell necrosis was noted. Also, vacuolar degeneration and considerable inflammation was evident in this group. In the ALB treated mice, mild hyperemia, and inflammation was observed (Figure 3).

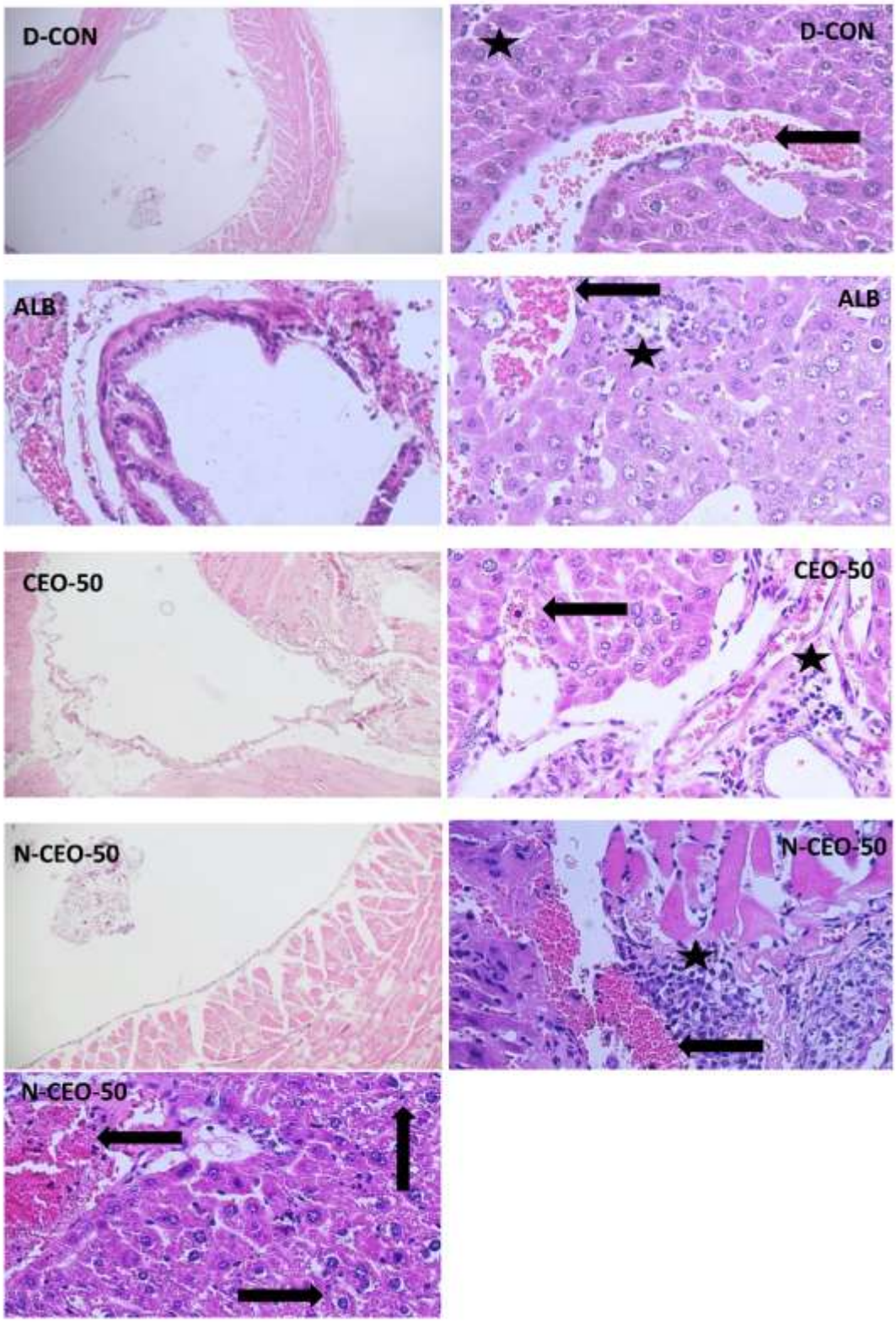

Figure 3. Histopathological changes in liver tissue samples including: hyperemia (arrow to the left), infiltration of mast cells (star), vacuolar degeneration (arrow to the right), necrosis (arrow up), in mice infected to cystic hydatid disease and received clove essential oil at the dose of 50 mg/kg (CEO-50), nanoemulsion of clove essential oil at the dose of 50 mg/kg (N-CEO-50) or the standard drug albendazole (ALB) as treatments in comparison to the infected control (D-CON) mice; magnification 10X, 40X, H&E staining

4. Discussion

Advances in the development of nanostructures, through control of their size and shape and their unique properties, have created broad potential applications, making nanodrug delivery systems an attractive field in biological sciences. However, translation of nanostructures into clinical applications has raised serious concerns regarding their potential toxicity (16). Most recent research on nanostructure toxicity has focused on cell culture systems. Nevertheless, data from in vitro studies can be misleading, highlighting the need for additional animal studies. In vivo systems are far more complex, and interactions between nanostructures and biological components can result in unique biodistribution, clearance, immune responses, and metabolism .(17)

In our previous study, we reported promising in vitro scolicidal activity of a developed nanoformulation of clove essential oil (CEO) against E. granulosus protoscoleces, with only minimal cytotoxicity in human primary fibroblasts (Naser et al., 2022). In the present in vivo study, the same nanoformulation in CHD-infected mice effectively reduced the number and size of cysts. However, unlike the in vitro findings, no significant differences were observed between CEO and N-CEO. By contrast, Moazeni et al. (2017) reported higher in vivo activity of a nanoemulsion of Zataria multiflora essential oil in reducing both the size and number of cysts in treated mice ($P < 0.05$) .(15)

CHD infection elevated serum liver enzyme activities of ALP and ALT, but not AST. These enzymes are indicators of liver function and damage, and their elevation has also been reported in previous studies on CHD-infected mice (18, 19). Moreover, treatment with N-CEO, especially at 50 mg/kg, further increased serum liver enzyme activities, decreased antioxidant enzyme levels, depleted liver glutathione content, and caused histopathological alterations. Hepatotoxicity has been identified as a major concern in the clinical translation of nanomedicines (20). It is known that the hepatotoxicity of nanomaterials may result from their high distribution and excessive accumulation in the liver (21). In the present study, we did not measure the concentration of N-CEO or its metabolites in the liver; therefore,

we cannot provide direct evidence that the observed liver toxicity of N-CEO-50 was due to accumulation. Nevertheless, histopathological findings and measurements of antioxidant enzymes and glutathione suggest that oxidative damage and inflammation were likely the underlying mechanisms. Nanomaterials can induce reactive oxygen species (ROS) generation, leading to an oxidative stress. ROS induction is considered the primary cause of nanotoxicity, and has been attributed to the presence of pro-oxidant groups on the surface of nanomaterial or the nanomaterial-cell interactions (22, 23). Probably, in the present study interaction of N-CEO with some biological process in hepatocytes including oxidative balance led to the pathological alterations in the liver of treated mice.

On the other hand, a previous study showed that nanoformulation of several hepatotoxic compounds was associated with lower hepatotoxicity than their small-molecule counterparts (17). However, in our study, N-CEO was toxic on liver and induced oxidative damages and inflammation in the treated mice which was significantly higher than the CEO. Nano-induced oxidative stress and liver injury have also been reported for titanium dioxide- and silica-nanoparticles (24, 25). Moreover, in spite of several studies on the hepatoprotective effect of CEO, a report demonstrated a case of hepatic failure after ingesting 10 ml of clove oil in a 15-moth boy (24). Although many herbal products may be relatively safe, their possible adverse effects should not be ignored or underestimated (24,25). Herein, in spite of using a nature derived herbal preparation for the development of N-CEO, its synthesis in the form of nanoemulsion, most probably due to the nanometric size scale or the effect of cosolvent and surfactants (25), increased the adverse effects and caused sever liver damages in animals.

Altogether, data on nanomaterial safety and toxicity remain controversial. Results from in vitro or cell culture studies may differ greatly from those of animal studies, emphasizing the pivotal role of in vivo models in the development and evaluation of nanodrug delivery systems. CEO is generally recognized as safe for humans; however, in the synthesized N-CEO, the nano-dimensional scale and interactions with biological processes likely resulted in adverse effects in CHD-infected mice. Further studies

investigating the mechanisms of liver injury, despite the minimal in vitro cytotoxicity, are necessary to reevaluate the safety of nanoformulations.

Conflict of interest

The authors declare that they have no conflict of interest.

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Data availability statement

Data of the present study will be available upon request from the corresponding author.

Ethics approval

The experimental procedure was approved by the Institutional Animal Care and Use Committee of Amol University of Special Modern Technologies (AUSMT) in accordance with the ARRIVE guide for care and use of the laboratory animals.

Consent for publication

All the authors make substantial contributions to conception and design, acquisition of data, analysis, interpretation of data, drafting the paper, approval of the submitted version, and agreed to be accountable for all aspects of the work.

Authors' contribution

Nasser, A, Shirali, S, Yousefi, MR, Shemshadi, B, Abouhosseini tabari, M, contributions in conception and design of the study. Yousefi, MR and Abouhosseini tabari, M have contributed in acquisition of data, analysis and interpretation of data. Abouhosseini tabari, M, Nasser, A, and Yousefi, MR drafted the paper and Shirali, S and Abouhosseini tabari, M revised it. All authors approved of the submitted version.

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