

Original paper

In vitro toxicity of cinnamaldehyde and nanoemulsion of cinnamaldehyde on protoscoleces of hydatid cyst

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ABSTRACT. Cystic echinococcosis is a major parasitic and zoonotic disease and surgery is the most common treatment of this disease which carries the risk of intraoperative leakage and recurrence. Using scolicalidal agent to inactivate cyst contents reduces the risk of recurrence. Considering side effects of available scolicalidals and growing interests on natural pharmaceuticals, the present study aimed to evaluate toxicity of cinnamaldehyde (CA), the main component of cinnamon essential oil, and a developed nanoemulsion of cinnamaldehyde (nano-CA) on protoscoleces of hydatid cyst. Nanoemulsion was prepared by the low energy system and characterized by dynamic light scattering to confirm dimensions. For evaluation of scolicalidal effects, serial dilutions of CA and nano-CA were mixed with protoscoleces suspension and mortality were recorded at 10, 30, and 60 minutes by eosin exclusion test. Albendazole was used as the positive control. The mean diameter of nano-CA was characterized as equal to 88.5 nm, and poly dispersity index was 0.09. After 30 min of treatment, nano-CA, at 50 µg/ml, killed 99.33% of protoscoleces. At the same time point and concentration, CA only caused mortality rate of 26.18%. 30 min-LC₅₀ value of 369.39 µg/ml was obtained for CA, while after 30 min of exposure, nano-CA showed promising rapid activity with LC₅₀ value of 3.22 µg/ml. Nano formulation significantly increased scolicalidal activity of CA probably by increasing penetration and tegumental disorganization of protoscoleces. Further *in vivo* safety studies are needed to introduce nano-CA as a clinically applicable scolicalidal agent.

Introduction

Cystic echinococcosis (CE) is a major parasitic and zoonotic disease that affects humans, wild animals, and domestic livestock such as cattle, sheep, pigs, horses, and camels. CE is caused by the intermediate stage of a dog tapeworm, *Echinococcus granulosus*, which invade tissues. Hydatid cysts (larval stages) form in various organs of the host, including liver, lung, heart, brain, spleen, and kidneys, and can lead to death [1]. In numerous endemic areas, the annual incidence rate of CE can range from 1 to 200 per 100,000 inhabitants. Iran is classified as an endemic (in the south) and hyperendemic (in the north) area for CE [2]. At the moment, CE has two treatment options: surgery (conservative and laparoscopic, percutaneous drainage consisting of puncture, aspiration, injection, and re-aspiration (PAIR)) and chemotherapy [3].

Meanwhile, when cysts form in multiple organs or in high-risk areas such as brain and spinal tissues, surgery becomes impractical [4]. Despite many efforts on the development of an effective vaccine, there is no effective vaccine against CE [5]. Benzimidazole derivatives, such as mebendazole and albendazole, are currently the main therapeutics which are used in CE. However, albendazole and mebendazole, when used to treat hydatid cysts, may cause a variety of side effects, including liver function abnormalities, abdominal pain, diarrhea, nausea, dizziness, hepatotoxicity, severe leucopenia, thrombocytopenia, and alopecia [6]. The most common method of treatment for CE is surgery, which carries the risk of intraoperative scoleces leakage, and may result in the recurrence of new hydatid cysts. The use of an effective scolicalidal agent to inactivate cyst contents during surgery may significantly reduce the risk of recurrence [7].

Cinnamaldehyde (CA) is a bioactive compound that is the main component of cinnamon essential oil and is responsible of its distinct aroma. It is widely used in the food industry as a flavoring agent and as a natural preservative against bacteria and fungi [8]. It has been demonstrated that chickens fed CA were less susceptible to infection with the protozoan parasite *Eimeria tenella* and had higher levels of specific antibodies after induction of infection [9]. In addition, the antileishmanial efficacy of CA against promastigote and amastigote of *Leishmania amazonensis* were reported and elucidated that CA possesses good antileishmanial activity, with an IC₅₀ of approximately 212 µM against promastigote of *L. amazonensis* [10].

Nano drug delivery systems have gained considerable interest during recent decade due to the advantages of increasing drugs bioavailability, distribution, and efficacy. Studies have shown that nano drug delivery systems based on bioactive compounds possess the facility of enhancing the permeabilization of the membrane allowing a better penetration, improvement of the interaction with the cell wall components, and increasing the concentration of the active constituents at the interface [11].

Due to the reported antiparasitic effects of CA and advent of nanotechnology as an effective method of drug delivery, the current study was designed to evaluate the scolicidal effect of CA in comparison to a developed nanoemulsion of CA (nano-CA) on protoscoleces of hydatid cysts under *in vitro* conditions.

Materials and Methods

Chemicals

Span 80 and Tween 80 were obtained from Merck (Germany); dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Germany). Albendazole was obtained from ArasBazar Pharmaceutical company (Amol, Iran). All other chemicals were analytical grade and commercially available.

Development and characterization of Nano-CA

For the development of nano-CA, Span 80 and Tween 80 at the 50:50 ratio was applied as the oil phase carrier. CA was added to the oil phase and stirred for 1 h. Deionized water at the ratio of 5:1 was added to the oil phase. Finally, to complete the nanoemulsion preparation, oil and water phases

were mixed by ultrasonic waves with an ultrasonic processor at the intensity of 208 W/cm. Prepared sample was further characterized by using a dynamic light scattering (DLS) equipped with a back scattered light detector (Zetasizer nanoS, Malvern Instrument, UK). The nano-control without the active CA constituent was developed to assess the role of carriers in the nano formulation.

Protoscoleces collection and viability testing

Live *E. granulosus* protoscoleces were collected aseptically from hydatid cysts obtained from the livers of naturally infected sheep slaughtered at Amol abattoir (Amol, Mazandaran). The fluid from hydatid cysts was aspirated and transferred to glass containers, where it was left undisturbed for 30 minutes. The protoscoleces gathered at the dishes' bottoms. The upper hydatid fluid was then removed, and the protoscoleces were washed in sterile normal saline. Protoscoleces viability was confirmed by 0.1% eosin staining and observation under a light microscope (40×).

The scolicidal activities of CA and nano-CA were tested at four different concentrations (5, 10, 25, and 50 µg/ml). Since CA is oily, 0.5% dimethyl sulfoxide (DMSO) was used as an emulsifier. In each experiment, 2 ml of the solution was poured into 24-well plates, followed by a drop of protoscolex-rich sediment (containing at least 1×10^3 *E. granulosus* protoscoleces). Afterwards, the plates were incubated in a shaker incubator at 37°C. Following each test period (30, 60, and 120 minutes), 100 µl of the sample was drawn from each well and poured onto a scaled glass slide. Eosin staining was used to determine the mortality rates [17]. Control group received normal saline solution containing 0.5% DMSO. As a positive control, albendazole was tested at concentrations of 1 and 2 µg/ml in DMSO [12]. All experiments were repeated three times for each tested concentration.

Statistical analysis

Repeated measures ANOVA with the Bonferroni post hoc test was used to examine differences in the means of mortality rate at different exposure times for each concentration of tested compounds. One-way analysis of variance (ANOVA) was used to compare the means of mortality rates in different tested compounds at each time of exposure (30, 60, and 120 minutes), followed by a Tukey-HSD post hoc test. Probit regression analysis was used to calculate the 50 and 90 percent lethal concentrations

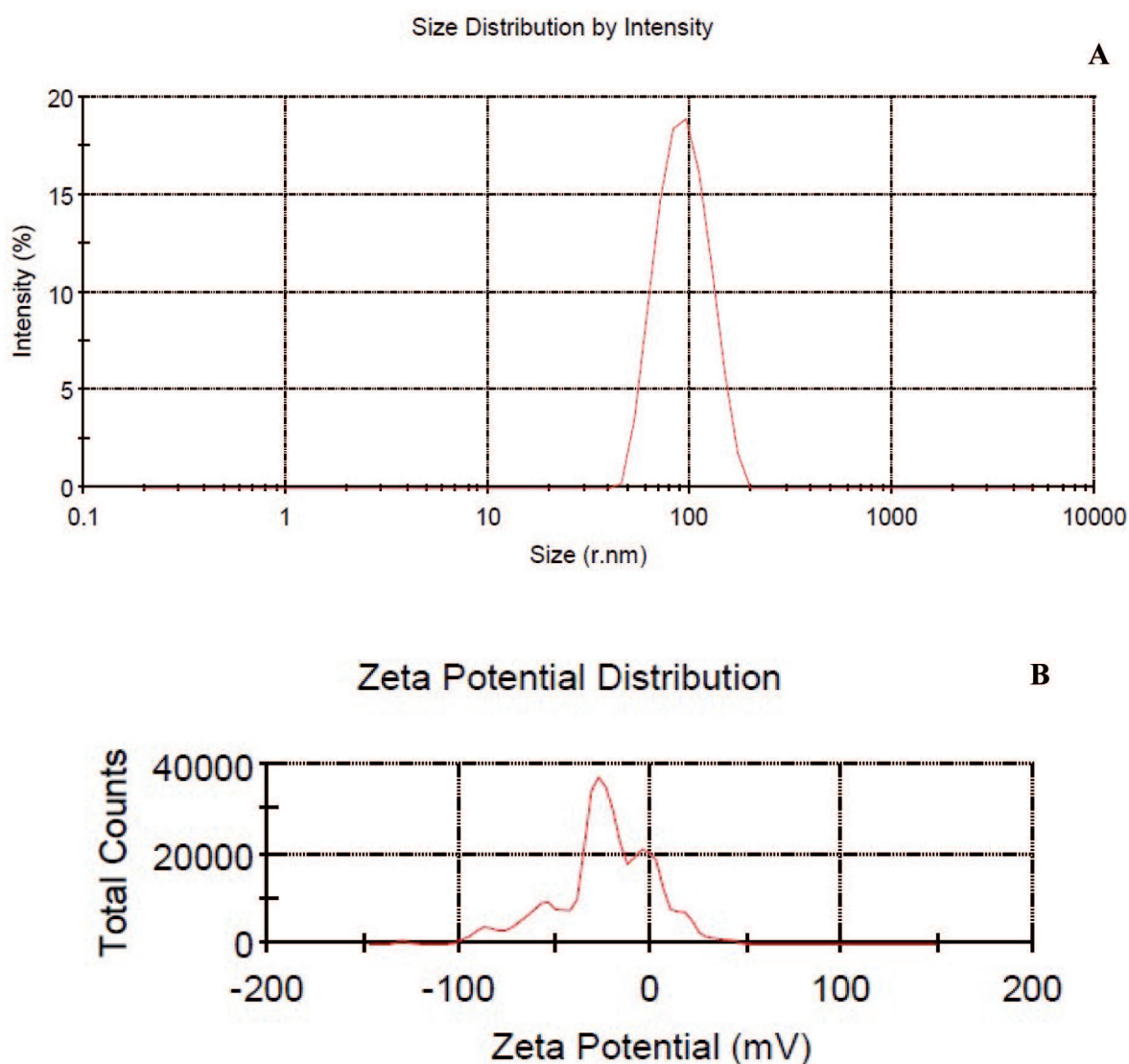


Figure 1. Particle size and polydispersity index (PDI) distribution (A) and zeta potential (B) for nano-cinnamaldehyde

(LC50 and LC90). The SPSS statistical package (version 23.0) (SPSS Inc., Chicago, IL, USA) was used to analyze the data. *P*-values less than 0.05 was considered statistically significant.

Results

Nano-CA particle size and zeta potential

Figure 1A shows the mean diameter of nano-CA which is equal to 88.5 nm, and poly dispersity index is equal to 0.09. The zeta potential of the nano-CA is -24.8 mv (Fig. 1B). Based on these findings, the nano-CA has been successfully formulated in the nanometric scale and nanoparticles are uniform in the dispersion.

In vitro toxicity on protoscolec

Figure 2 shows the mortality rates of *E.*

granulosus protoscolec

over different times of exposure to CA and nano-CA, in comparison to albendazole and the nano and DMSO control. As can be seen in figure 1, nano-CA showed a high scolicidal activity against *E. granulosus*. After 30 min of treatment, nano-CA, at 50 $\mu\text{g/ml}$, killed 99.33% of protoscolec. At the same time point and concentration, CA only caused mortality rate of 26.18%. After 60 min, nano-CA at concentrations of 25 and 50 $\mu\text{g/ml}$ killed 99 and 100% of protoscolec, respectively. On the other hand, at the same time point, CA led to mortality rates of 18.16 and 29.13% at the concentration of 25 and 50 $\mu\text{g/ml}$. After 60 min of exposure, nano-CA at all tested concentrations showed significantly higher toxicity in comparison to all other treatments ($P < 0.05$). At the time point of 120 min, all tested concentrations of nano-CA caused considerable

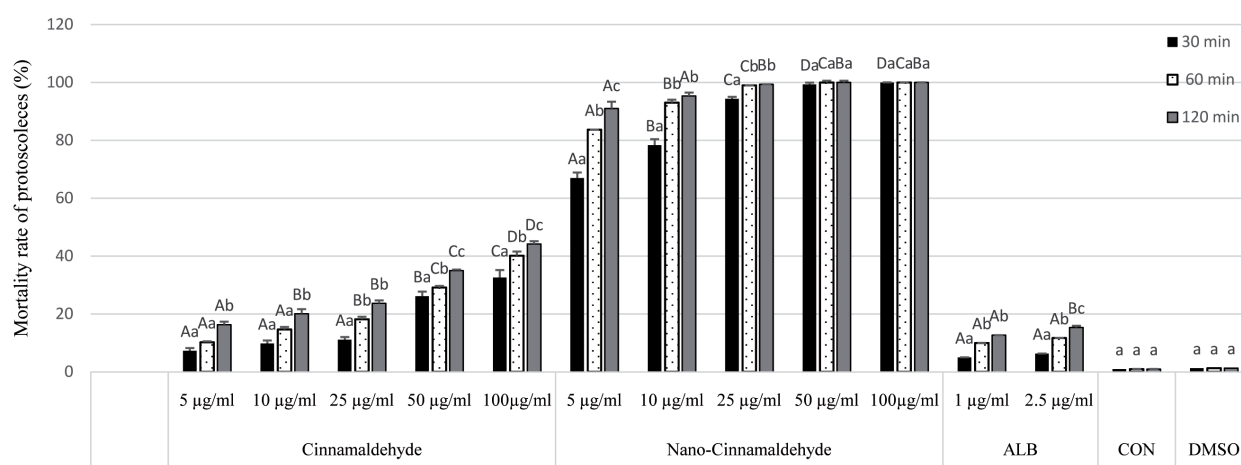


Figure 2. Mortality (%) of *Echinococcus granulosus* protoscoleces over different times of exposure to cinnamaldehyde (CA) and nanoemulsion of cinnamaldehyde (nano-CA), in comparison to albendazole and the nano and DMSO control. Explanations: T-bars represent SE. Within each concentration of tested compounds, columns marked with different letters (lowercase) are significantly different between times of exposure (Repeated measures ANOVA, Bonferroni test, $P < 0.05$). Within each tested compounds, columns marked with different letters (uppercase) in each time of exposure (30, 60, and 120 min separately) are significantly different between concentrations (ANOVA, Tukey's HSD test, $P < 0.05$).

toxicity on *E. granulosus*, with mortality rates higher than 90%. At the 120 min time point, 5, 10, 25 and 50 µg/ml of CA showed mortality rates of 16.33, 20.12, 23.66 and 35%, respectively.

In the nano control group and DMSO, the mortality rate of protoscoleces after 120 min of treatment was 1 and 0.66%, while the mortality for albendazole as the positive control was 12.66 and 15.33%. The nano control group also could not cause significant toxicity on protoscoleces in comparison with DMSO control, and no significant difference was observed for mortality rates between different time points of treatment with nano control. Results also showed that the scolicidal activity of all compounds was significant ($P < 0.05$) compared to the control groups at all exposure times.

Lethal concentration values of CA and nano-CA on protoscoleces is shown in table 1. As can be seen, CA showed some scolicidal activity with 30-min LC_{50} value of 369.39 µg/ml. After 30 min of exposure, nano-CA showed promising rapid activity with LC_{50} value of 3.22 µg/ml. Moreover, nano-CA, with LC_{90} of 15.84 µg/ml, was significantly more active than CA with LC_{90} of > 10000 µg/ml.

Discussion

For many cases of CE, surgery is still the first-line treatment. It has, however, been linked to the risk of recurrence and secondary dissemination of cyst content into peritoneal cavity. Several chemical

scolicidal agents have been used to reduce this risk and inactivate infective protoscoleces. Many of these scolicidal agents may cause unfavorable side effects which limit their use [3,13]. So, the development of alternative scolicidal agents from natural sources with limited side effects and high potency would be a valuable achievement [14]. For this purpose, several studies have evaluated the *in vitro* and *in vivo* efficacy of some herbal preparations on the larval stage of *E. granulosus* [15–17]. However, only a limited number of studies has been conducted to find out the specific component of the essential oils responsible for scolicidal efficacy [18,19]. Despite numerous researches on plant derived extracts and essential oils, due to the impact of environmental factors on the chemical constituents of the plants, variations in chemical constituents is a common observation in the essential oils recovered from taxonomically same species [20] which limits their clinical efficacy. To solve this issue, finding the bioactive constituents of the plants and further research and development on them is a sound approach.

Mahmoudvand et al. [21] demonstrated that essential oil from the bark of *Cinnamomum zeylanicum* which is a rich source of CA, at the concentrations of 100 and 50 µg/ml completely killed hydatid cyst protoscoleces after 5 min of incubation. In addition, in the study of Fabbri et al. [22] it has been reported that under *in vitro* conditions, CA was able to significantly decrease

Table 1. Lethal concentration values of cinnamaldehyde (CA) and nano-emulsion of cinnamaldehyde (nano-CA) on *Echinococcus granulosus* protoscoleces

Components	Concentration (µg/ml)	30 min mortality ^a (%) ± SE	LC ₅₀ (µg/ml) (LCL-UCL)	LC ₉₀ (µg/ml) (LCL-UCL)	χ ² (df) ^b
CA	5	7.33±0.57	369.39 (177.76–1616.06)	> 10000	2.86 (3)
	10	9.83±0.88			n.s.
	25	11.14±1.00			
	50	26.18±0.88			
	100	32.65±1.52			
Nano-CA	5	67.00±1.85	3.22(2.01–4.34)	15.84 (12.77–21.14)	2.04 (3)
	10	78.33±2.02			n.s.
	25	94.33±0.66			
	50	99.33±0.57			
	100	100.00±0.00			

Explanations: SE standard error; LCL 95% lower confidence limit; UCL 95% upper confidence limit; n.s. not significant ($P>0.05$); ^avalues are mean±SE of three replicates; ^bChi-square; df: degrees of freedom

the viability of protoscoleces in a dose and time-dependent manner. CA at the concentration of 50 µg/ml killed 100% of parasites after 8 days [22]. In line with these findings, herein, we showed that CA possesses some toxicity on protoscoleces of hydatid cyst. However, at the highest concentration and longest time of exposure, CA was not as effective as nano-CA, and the maximum toxicity reached to about 40% mortality in protoscoleces. Comparing this finding with the results of [22], it should be noted that long time of exposure is needed for CA to exert its full scolicidal effect which is a limiting factor for being used as a scolicidal agent during surgery.

Nanotechnology changes physical and chemical properties of pharmacological agents and make them superior to those of the original materials. Nano-based drug delivery and targeted delivery approaches can improve efficacy and reduce systemic toxicity of pharmacological agents including scolicidals [23]. In the present study, nano-CA showed promising scolicidal effect and at the concentration of 25 µg/ml killed more than 80% of hydatid cyst protoscoleces by 30 min of incubation. Comparing to CA, nano formulation significantly increased scolicidal activity of nano-CA. In line with this finding, it has been reported that nano emulsion of *Zattaria multiflora* demonstrated high efficacy and led to 100% scolicidal activity at concentrations of 1 and 2 mg/ml after 20 and 10 min, respectively [24]. In

addition, silver nanoparticles at the concentrations of 0.1 and 0.15 mg/ml after 60 min treatment caused mortality rates of 80% and 79%, respectively. Comparing the results of these two studies with the results obtained in our study, it can be concluded that CA, with LC₉₀ of 15.84 µg/ml, is more potent.

From mechanistic point of view, studies showed that CA may cause death of protoscoleces by alteration in tegument, and loss of hooks and rostellar disorganization [22]. Probably, nano-CA due to higher surface to volume ratio may have more binding sites to membranes [25] and higher chance of tegumental disorganization which leads to higher scolicidal efficacy.

In conclusion, this study showed that the developed nano-CA possesses promising toxicity on *E. granulosus* protoscoleces. Further *in vivo* studies are needed for safety measurements and efficacy of nano-CA in animal models for future development as novel scolicidal agent for CE surgeries.

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