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Original Article

Cinnamon (*Cinnamomum zeylanicum*) Oil Loaded Solid Lipid Nano-Particle and Its Protoscolicidal Effects

Zahra Hesari [1], Vida Farahnak [2], Keyhan Ashrafi [2], Zahra Atrkar Roushan [3], Behnaz Rahmati [2], Sara Nemati [4], *Meysam Sharifdini [2]

1. *Department of Pharmaceutics, School of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran*
2. *Department of Medical Parasitology and Mycology, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran*
3. *Department of Biostatistics, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran*
4. *Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

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***Correspondence**
Emails:
sharifdini@gums.ac.ir,
sharifdini5@gmail.com

Abstract

Background: Cystic echinococcosis (CE) is considered as a neglected disease that imposes noticeable medical and economic challenges worldwide. Cinnamon oil (CO) has demonstrated potent antimicrobial effects even on hydatid cysts protoscoleces. We aimed to synthesize cinnamon oil- loaded Solid lipid nanoparticles (CO-SLNs) and to evaluate the protoscolicidal effects of this formulation.

Methods: CO-SLNs were prepared using an emulsification, probe sonication technique, incorporating natural lipids (cholesterol and lecithin). SLNs were evaluated based on particle size, polydispersity index (PDI), zeta potential, electron microscopy, encapsulation efficiency (EE%) and cell compatibility (MTT assay), etc. The scolicidal activity was assessed using the eosin exclusion test (eosin 0.1%) at concentrations of 0.5, 1,2,4 and 8 mg/ml of CO and CO-SLNs for time intervals of 10, 20, 60, 120 and 180 minutes and 24 h.

Results: Characterization of the CO-SLNs showed an average size of 337.6 nm with PDI 0.77 and zeta potential of -26 mV and EE of 83.49% with round morphology. The MTT assay showed a higher cell viability in CO-SLNs compared to CO. A 100% mortality rate of PCs was observed for CO-SLNs at a concentration of 8 mg/ml after 120 minutes and for free CO at a concentration of 8 mg/ml after 30 minutes.

Conclusion: CO-SLNs exhibited a milder scolicidal activity than free CO, which may be due to the sustained release of the oil from SLNs, resulting in a longer effective period and lower toxic effects on normal cells.

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Introduction

Cystic echinococcosis (CE) is a zoonotic parasitic infection caused by accidental ingestion of the ova of *Echinococcus granulosus* Sensu Lato, distributed in different parts of the world (1). CE is a chronic disease with heterogeneous clinical symptoms that depend on the involved organ, cyst number, size and stage; from asymptomatic (mainly on the onset of disease) to mechanical force or fistula on the contiguous tissues or cyst rupture leading to infection or anaphylactic shock (2).

Currently, the main management approaches for CE are drug therapy, surgery, endoscopic interventions, percutaneous methods (punctuations, aspiration, injection, and re-aspiration (PAIR)), as well as observation for inactive cysts (3, 4). The preferred chemotherapeutic agents for the metacestode stage of CE are benzimidazole derivatives (mebendazole and albendazole) as well as praziquantel. However, previous reports have demonstrated several adverse drug reactions such as gastrointestinal discomforts, liver enzymes elevation, hepatotoxicity, alopecia, blood cell disorders, osteoporosis, teratogenicity, etc. (5, 6).

To reduce the risk of cyst rupture or leakage, numerous chemical scolicidal agents have been incorporated for inactivation of protoscoleces in PAIR and surgery such as ethanol, saline 20%, chlorhexidine gluconate, silver nitrate, povidone-iodine, cetrimide, albendazole sulfoxide, and octenidine hydrochloride; but the majority of them are along with hepatobiliary side effects (7). Consequently, investigating an ideal protoscolicidal agent that has minimum side effects with a rapid scolicidal effect seems to be urgent for CE treatment.

Various biological functions have been documented for cinnamon *(Cinnamomum zeylanicum)* oil (CO) including antimicrobial, antioxidant, antipyretic and antidiabetic effects (8, 9); in addition to its applications as a flavoring agent and as an aroma in both the cosmetic and food industries (10). Recently, the antimicrobial characteristics of this oil have attracted growing interest, as investigations have demonstrated its strong activity against parasitic diseases e.g., CE (11, 12). Cinnamaldehyde is one of the principal constituents of cinnamon oil which interacts with the microbial cell membrane by changing the proton motive force and evolving to cell lysis (13, 14). Herbal oils are frequently environmentally sensitive meaning that they can easily decompose when exposed to oxygen, heat, pressure or light. Hence, nano-encapsulation is a feasible strategy to circumvent these problems, in order to preserve and protect their functional properties, while offering controlled release behavior (15).

SLNs are composed of solid biodegradable lipids in aqueous colloidal dispersions, unifying the advantages of liposomes and fat emulsions concomitantly (16). They are approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA). They provide a controlled and continuous drug release. In nature, they are compatible with cell membrane and tissues and properly enhance the bioavailability, tissue distribution and targeting of encapsulated drugs (17, 18).

Although several studies have developed various nano-systems of cinnamon oil and evaluated their antimicrobial effects (8, 19, 20), to the best of our knowledge, scolicidal effects of CO in its nano-form have not yet been evaluated. Hence, by hypothesizing that combining the advantages of cinnamon oil and solid lipid nanoparticles could deactivate CE protoscoleces, this study aimed to design cinnamon oil loaded SLN using an ultrasound homogenization method incorporating natural based lipids (cholesterol and lecithin) aiming to develop a scolicidal agent providing a controlled release of cinnamon oil while maintaining this oil's antimicrobial effectiveness.

Materials and Methods

Ethical statements

The study was approved by Ethics Committee of Guilan University of Medical Sciences, Iran (IR.GUMS.REC.1400.410).

Preparation of solid lipid nanoparticles containing cinnamon oil

SLNs were prepared using emulsification, probe sonication technique, according the previous studies with some modifications (6, 21, 22). Summarily, 1 ml CO (bought commercially and analyzed by GC-MS to confirm its quality) along with 800 µl tween 80 (Merck, Darmstadt, Germany) were dissolved in 10 ml methanol (Merck, Darmstadt, Germany): distilled water (75:25). On the other hand, 100 mg Cholesterol (Sigma-Aldrich) and 200 mg Lecithin (DUKSAN reagents, South Korea) were dissolved in 10 ml dichloromethane (Merck, Darmstadt, Germany). CO solution was mixed manually with dichloromethane solution and this total organic phase was subjected to ultrasound probe sonicator (Hielscher UP400s, Germany) for 8 min at 17000 rpm while adding 10 ml solution of PVA 4% w/v (Merck, Germany) to produce a white cloudy emulsion. Rotary evaporator was used for complete evaporation of the o/w emulsion's organic phase at 45 °C.

Particle size, poly dispersity index (PDI) and zeta potential of CO-SLNs

Two hundred mg of SLN was suspended in 1 ml distilled water and were subjected to a Zetasizer 1033439 (Malvern Instrument, UK) at 25 °C with a count rate of 206.3 kcps and measurement position of 4.65 mm for measurement of average particle size and polydispersity index monodisperse or polydisperse nature of nanoparticles). The zeta potential value of nanoparticles was determined by Zetasizer 1,033,439 (Malvern Instrument, UK) and expressed in mV.

Transmission Electron Microscopy (TEM)

TEM (EM10C-100 KV; Carl Zeiss, Germany) operated at 80 kV was used for investigation of morphology and shape of nanoparticles. Nanoparticle suspensions were subjected to uranyl acetate negative staining, placed on 200–300 mesh grids and coated with Formar (a low absorption resin). Grids were dried by evaporation and analyzed with TEM.

Encapsulation efficiency (EE)

10 mL distilled water was used for dispersion of 500 mg CO-SLNs. Resulted aqueous dispersion was centrifuged with 6000 rpm for 20 min at 25 °C. The amount of free CO was detected in the underlying fluid by UV spectroscopy (PerkinElmer, USA) at 270 nm (23) and the percent of encapsulated oil was calculated using the following equation:

$$EE\ (\%) = (W_{\text{initial CO}} - W_{\text{free CO}}) / W_{\text{initial CO}} \times 100$$

In-vitro release and release kinetic study

Two g of nanoparticle was incorporated into the dialysis bag with a molecular weight cutoff of 14 kDa (Sigma, Steinheim, Germany). The bag was immersed in 100 mL distilled water containing 2% v/v tween 80 as receptor medium. The procedure was performed at 25 °C with constant stirring (100 rpm). Samples (2 ml) were withdrawn at 1, 3, 6, 24, 48, and 72 h time points and substituted with fresh medium. Next, the Content of CO were analyses using UV–Visible spectrophotometry in 270 nm (24).

The release kinetics of CO from SLN was calculated using Zero order, First order, Higuchi, and Korsmeyer-Peppas and Hixson crowell mathematical kinetic models (25), based on CO release data.

Cell compatibility assay

The biocompatibility of the CO-SLNs on HU02 (Foreskin fibroblast) cell lines, obtained from Iranian Biological Resource Center (IRIBC C10309), were evaluated using MTT assay (21). Briefly, cells with a density of 5×10^3 cells/cm^2 were incubated in a 96-well plate in 100 µl of DMEM medium under a humidified atmosphere with 5% CO_2 for 24 hours. Cells were treated with CO-SLNs (25, 50 mg/ml)

and CO (50 mg/ml) while all the sample media contained 2% v/v tween 80. After 24 h of treatment, the cells were incubated with MTT solution (Sigma; 5mg/ml of PBS) for 3h at 37 ºC. Subsequently, the precipitates formed were dissolved in 150 µl DMSO per well and absorbance was recorded using a Biotek Epoch™ microplate reader at 570 nm (n=3).

Evaluations of protoscolicidal effect

Collection of protoscoleces

E. granulosus cysts were obtained from the livers of sheep, slaughtered at Rasht industrial abattoir. A 50 ml sterile syringe was used to aspirate the hydatid fluid from a cyst. Aspirated hydatid fluid transferred into a falcon tube followed by a gentle centrifugation with 1000 rpm for 1 minute to settle down the protoscoleces with minimum time waste. The number of sedimented protoscoleces, with at least 95% primary viability, was adjusted to 2500 protoscoleces per ml. Impermeability to eosin dye (Sigma-aldrich) solution under light microscope (Carl-Zeiss Standard 14) was considered for viability percent determination.

Influence of CO and CO-SLNs on protoscoleces

For evaluation and comparison of scolicidal activity of CO-SLNs and CO, five different concentrations (0.5, 1, 2, 4, 8 mg/ml) were tested against protoscoleces in 10, 30, 60, 120, 180 minutes and in 24 h time points. Normal saline 0.9% solution containing 10% tween 80 was incorporated as control. First, 500 µlit of protoscolex suspension (2500/ml) was placed in 2 ml micro-tubes, then 500 µlit of each concentration was added to micro-tubes. Tube contents were gently mixed and incubated in 37 °C for 10, 30, 60, 120, 180 minutes and 24 h. In each time point, 50 µlit of eosin dye 0.1% was mixed with 50 µlit of settled protoscoleces on a glass slide and covered with a cover glass. The slide's protoscoleces were carefully studied under a light microscope and after total counting of 300 protoscoleces; the percentage of dead protoscoleces was calculated. The procedure was separately performed for CO-SLNs and CO and the experiment was run in triplicate for each concentration and time interval.

Scolicidal assessment

Eosin exclusion test was incorporated, for evaluation of protoscoleces viability. When eosin (0.1%) dye is exposed to protoscolex suspension, live protoscoleces remained impermeable to eosin (colorless) and maintained their flame cell activity and muscular movements, while dead organisms absorbed the dye and appeared with orange-red color.

Statistical analysis

Results are presented as the mean ± standard deviation. Data analysis was performed using SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, USA). For assessment of differences between experimental groups, one-way ANOVA with Tukey's *post hoc* test was used. In addition, $P \leq 0.05$ was considered as statistically significant.

Results

Characterization of CO-SLNs

The reported average particle size of CO-SLNs was 337.6 nm with the PdI of 0.77, however, the size distribution plot reveals a small peak below 100 nm, confirming the polydispersity of the particles (Fig.1a). In addition, the zeta potential of the nanoparticles was around -26 mV (Fig. 1b) which shows a relatively strong electrostatic repulsion between particles that prevents particle aggregation and leads to a better size stability. TEM images provide two-dimensional morphological information including shape, size and other general aspects. Fig. 2a&b presents the negatively stained TEM figures of CO-SLNs revealing the uniformly rounded circular particles with clear edge in 250 nm and 80 nm scale.

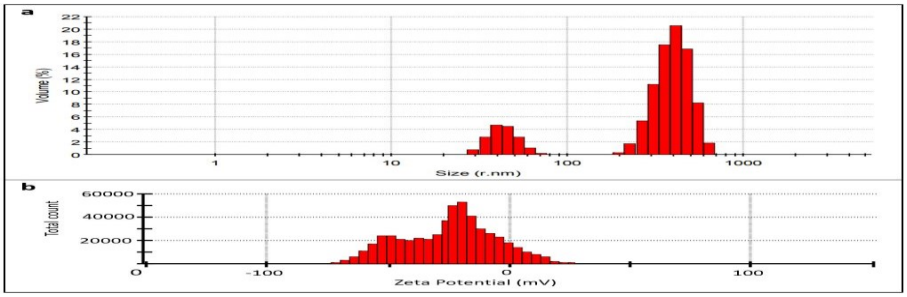

Fig. 1: Cinnamon oil loaded Solid lipid nanoparticles (CO-SLNs) characterization. a) Particle size distribution plot via DLS technique. b) Zeta potential graph obtained using zeta sizer

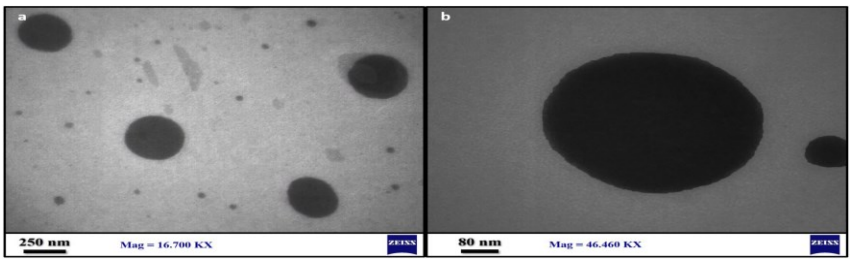

Fig. 2: Morphological characterization of CO-SLNs using transmission electron microscopy. a) The scale bar stands for 250 nm. b) The scale bar stands for 80 nm

Encapsulation efficiency % (EE)

The percentage of encapsulated CO in the lipid matrix was evaluated in comparison with total oil used in reaction. UV spectroscopy and following calculations revealed the encapsulation efficiency around 83.49%. The high EE percent, maybe due to lipophilic character of CO.

Cell viability%

Cell compatibility of the CO and CO-SLNs was initially determined using an MTT method. The CO and CO-SLNs biocompatibility were evaluated with the concentrations: 5 & 10 mg/ml. Results revealed that the cell viability with CO-SLNs (10 mg/ml) was 97.5%, which was significantly higher than three other groups. Cell viability percent for CO 5 and 10 mg/ml were 68.83 and 65.81 respectively and for CO-SLNs 5 mg/ml was 78.19%, which the difference was not considered statistically significant (Fig. 3).

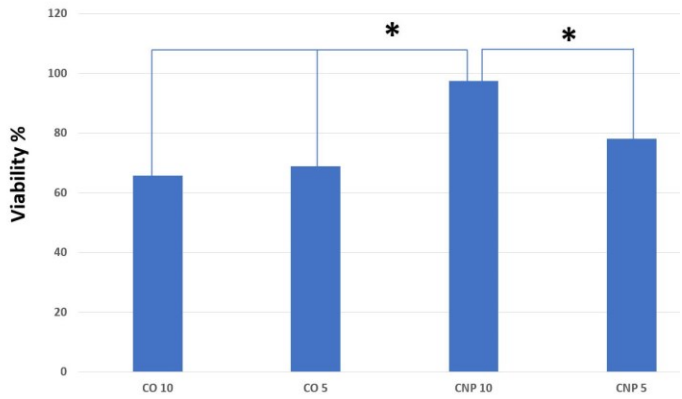

Fig. 3: Normal fibroblast cell viability percent in contact with different concentrations (5 & 10 mg/ml) of CO and CO-SLNs.

In-vitro release and release kinetic study

The *in vitro* release behavior of cinnamon oil from SLNs completely followed the results of previous study (26). Briefly, the maximum cumulative release percent was below 50% in 72h with the release kinetic of Hixson crowell ($R^2 = 0.9856$).

In vitro effects of CO-SLNs and CO on protoscoleces

The scolicidal effect on *E. granulosus* protoscoleces incubated with different concentrations of CO-SLNs as compared with the same concentrations of CO is shown in Fig. 4. Statistical analysis discovered a time and concentration dependent scolicidal effect of CO-SLNs ($P \leq 0.05$) which revealed a considerable scolicidal effect with the concentrations of 4 and 8 mg/ml in 60 minutes (57% and 82% mortality, respectively) and 100% mortality in 120 minutes which was statistically different with concentrations (2, 1 and 0.5 mg/ml) ($P \leq 0.05$). In concentration of 2mg/ml of CO-SLNs, 100% of protoscoleces were killed in 24 h, while in the same time point 37% mortality in 1 mg/ml and 25% mortality in 0.5 mg/ml concentrations was observed (Fig. 4a).

Meanwhile, CO with the concentrations of 4 and 8 mg/ml presented 87% and 95% mortality in 10 minutes in protoscoleces. The 8 mg/ml concentration reached to 100% mortality in 30 minutes, a significantly more rapid effect compared to all other concentrations with a 100% mortality in 60 minutes (P-value ≤ 0.05) (Fig. 4b). Calculated LD_{50} for CO-SLNs and CO were reported as 10.17 and 4.003 mg/ml, respectively. However, based on mentioned results, cinnamon oil showed a stronger scolicidal effect compared to CO-SLNs, its noteworthy that cell viability test revealed a lower toxicity of CO-SLNs in comparison with CO, on normal cell line (HU02) (Fig. 3).

Control PCs remained viable without obvious morphological changes even after 24 h incubation (Fig. 5a). Dead PCs with presence of blebs in the tegument, disorganization of rostellar hooks, and a complete loss of hooks were showed with the addition of CO-SLNs (Fig. 5b-d) after exposure to 8 mg/ml CO-SLNs for 60 minutes. In addition, disorganization of hooks, tegmental destruction with a complete loss of hooks were detected after incubation with 8 mg/ml CO for 60 minutes (Fig. 5e and f).

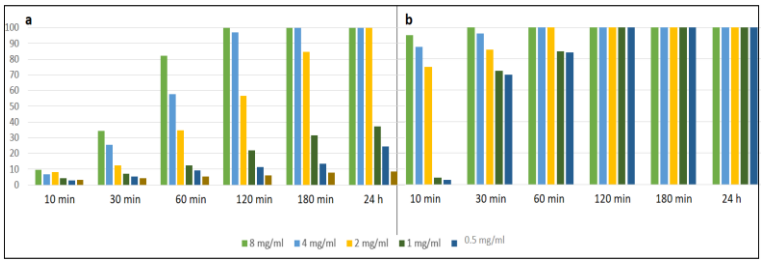

Fig. 4: The in vitro cidal effects of a) CO-SLNs and b) CO against protoscoleces with concentrations of 0.5, 1, 2, 4 and 8 mg/ml in time points of 10, 20, 30, 60, 120 minutes and 24 h

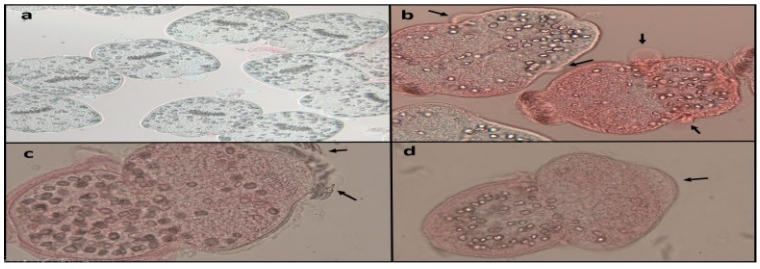

Fig. 5: Light microscope view of E. granulosus (s.s.) protoscoleces (PSC) exposed to CO, CO-SLNs and the non-exposed control after staining with 0.1% eosin. a) Viable colorless PSCs in non-exposed control. b-d) Dead colored PSCs after exposure to 8 mg/ml CO-SLNs for 60 minutes. b) The presence of blebs in the tegument of the evaginated PSCs. c) Tegmental destruction of evaginated PSC with disorganization of rostellar hooks. d) Dead evaginated PSC with a complete loss of hooks. e and f) Dead PCs after incubation with 8 mg/ml CO for 60 minutes e) Invaginated PSC with disorganization of rostellar hooks f) Evaginated PSC with tegmental destruction and a complete loss of hooks

Discussion

Discovering a new more effective scolicidal agent with lower adverse reactions is still of interest for many investigational teams, working on CE. The important criteria for an ideal scolicidal agent are to demonstrate higher efficacy at lower concentrations in shorter exposure time, long-term stability in hydatid fluid, less toxicity, and high accessibility (27, 28). Hence, several studies have incorporated the advantages of nanotechnology in development of scolicidal agents (29-31) including target delivery, controlled release, enhanced drug stability, increased therapeutic efficacy, and reduced erratic absorption. In addition, merits of natural pharmaceutical ingredients such as notable anti-cystic effects, minimal adverse reactions and the ability to boost immunity, have placed them in a high priority for discovery of the ideal CE treatment (32-34).

Recently, development of herbal nanoformulation has been considered to combination of the advantages of both nanotechnology and herbal remedies (27, 35) in CE treatment. For example, eugenol essential oil and its nanoemulsion showed highly significant activity against hydatid PCs compared to albendazole. The 100% mortality rate was recorded at the concentration of 1 µl/ml after 30 min incubation (27). In addition, nano emulsion of *Zataria multiflora* essential oil showed 100% scolicidal power at a concentration of 2mg/mL after 10 minutes (36). However, in another study, nanoemulsion of *Z. multiflora* essential oil achieved 100% mortality of the protoscoleces in 10 min at a concentration of 20 µl/ml (37). Similar results were observed with *Satureja* sp. essential oil nanoemulsion, where a 100% lethal effect was obtained with concentration of 1 mg/ml of *Satureja hortensis* essential oil nanoemulsion (38), and 95.33% mortality was achieved with a concentration of 400 µg/ml of *S. sahendica* essential oil nanoemulsion in 60 minutes (35).

However, cinnamon oil has been converted into several nano systems (39-41), to the best of our knowledge; it has not been incorporated against hydatid cyst PCs in nano-form yet. Mahmoudvand et al. reported *C. zeylanicum* essential oil with the concentrations of 100 and 50 µL/mL killed 100% of protoscoleces after 5 min of exposure (42) while other investigation declared *C. zeylanicum* essential oil at 200 µg/mL reduced the viability of parasites to 7.36 ± 2.4%, 4 days' post-incubation (p.i). In aforementioned study, higher scolicidal activity was observed by cinnamaldehyde as the main constituent of CO. PCs viability decreased to 1.7 ± 0.8% after 4 days p.i with 50 µg/mL concentration (12). In comparison, based on CO in its intact form, our results showed more correlation with Fabbri et al. study. Although, we observed a relatively higher required dose (500 µg/ml) for 100% mortality, the required incubation time was much shorter (60 minutes compared to 4 days). Cinnamon oil in form of SLN revealed a milder scolicidal activity (2 mg/ml in 24 h or 8 mg/ml in 120 minutes revealed a 100% mortality) which may be due to the sustain release of oil from SLNs which leads to a longer effective period of time, along with lower toxic effects on normal cells. However, combining the merits of Fabbri et al. and our study, development of cinnamaldehyde solid lipid nanoparticles can be considered for the next step investigations.

CO and CO-SLNs produced similar evident morphological changes in our study such as presence of blebs in the tegument, disorganization of rostellar hooks, and a complete loss of hooks. Theses alterations have been reported by the authors after incubation of PSCs with praziquantel (43), albendazole (27), tropisetron, granisetron, cyclosporine A (44), eugenol nanoemulsion. It is believed that bleb formation is due to "stress responses" in PSC by any harmful condition (45). It additionally

may due to the fact of a membrane repair mechanism in response to drug caused damage (46).

Conclusion

The LD_{50} for CO-SLNs and CO against protoscoleces were calculated as 10.17 and 4.003 mg/ml, respectively. Based on natural scolicidal activity of cinnamon oil, preparation of CO-SLNs moves forward the development of an ideal scolicial agent, due to longer duration of activity and lower toxicity and adverse reactions for healthy tissue.

Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interest.

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