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Original article

TOXICITY OF GERANIUM OIL, GERANIOL AND THEIR NANOEMULSIONS ON PROTOSCOLECES OF HYDATID CYST UNDER *IN VITRO* CONDITIONS

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Summary

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Surgery is the main efficacious treatment for many cases of cystic echinococcosis by removing *Echinococcus granulosus* cysts. However, to reduce risk of cyst spillage and insemination of the content, using a scolicidal agent is crucial. Considering side effects of available scolicidals, and growing nanotechnological approaches in novel pharmaceuticals, the present study aimed to find out the scolicidal activity of geraniol (GL), geranium oil (GM), and their developed nanoemulsions (Nano-GL and Nano-GM) on the protoscoleces of *E. granulosus*. Nanoemulsions were developed by ultrasonication emulsification and characterised by dynamic light scattering method (DLS). The scolicidal effect of GL, GM, Nano-GL, and Nano-GM at different concentrations of 1, 2.5, 5, 10, 25, and 50 µg/mL were determined after 0.5, 1, and 2 hours of incubation. Mortality rates were measured by eosin exclusion test. The average droplet size for Nano-GM and Nano-GL were 124.8 and 88.59 nm, respectively. After one hour of exposure, all tested concentrations of GL and Nano-GL resulted in higher than 90% mortality rates, while GM and Nano-GM killed 70.66% and 90.33% of protoscoleces, respectively. Based on 50% lethal concentration, Nano-GL was significantly more potent than Nano-GM (LC_{50}: 4.52 *vs* 102.95 µg/mL). Results of scanning electron microscopy revealed tegumental disruption in the treated protoscoleces. This study described an easily applicable and eco-friendly procedure of nano-formulating functional phytochemicals, showing promising scolicidal activity *in vitro*. The developed formulations, especially Nano-GL, showed the characteristics of an ideal scolicidal agent. Further studies are needed to assess *in vivo* efficacy and safety of this formulation.

Key words: cystic echinococcosis, green drugs, nanotechnology, phytochemicals

INTRODUCTION

Echinococcosis is a zoonotic disease caused by larval stages of cestodes belonging to the genus *Echinococcus* and the family Taeniidae. The life cycle of this parasite implies two mammalian hosts. The intermediate hosts and humans acquire the infection after ingesting eggs from the faeces of definitive carnivorous hosts, which harbour the adult egg-producing stage in the intestine (Youssefi *et al.*, 2013; Craig *et al.*, 2015). This disease is becoming an important public health problem in many parts of the world, where dogs are used for cattle breeding (Kademvatan *et al.*, 2019; Ali *et al.*, 2020). Primary echinococcosis is established when metacestodes develop in various sites of the human body from oncospheres liberated from ingested *Echinococcus* spp. eggs. Secondary echinococcosis occurs when metacestode material spreads from the primary site to adjacent or distant organs and proliferates. Secondary cystic echinococcosis (CE) may also occur after release of viable parasite material during invasive treatment procedures (Cucher *et al.*, 2016; Pourseif *et al.*, 2021).

Surgery is still the main treatment for many cases of CE that can potentially remove *E. granulosus* cysts and lead to the complete eradication of this disease. It has been reported that up to 90% of the patients can be treated surgically if a cyst does not have a risky localisation or if the disease is not too far advanced (Ran *et al.*, 2016). However, surgery may be impractical in patients with multiple cysts localised in several organs and if surgical facilities are inadequate. Chemotherapy and punctuation, aspiration, injection, re-aspiration (PAIR) are other options, especially in inoperable patients and cases with high surgical risk (Patkowski *et al.*, 2017).

Geranium oil (GM) is extracted through a steam distillation process from *Pelargonium graveolens* leaves. This essential oil contains citronellol, geraniol, and linalool as the main constituents (Al-Jumaili *et al.*, 2019). GM was attributed to several bioactivities, including antimicrobial, antiviral, and antioxidant properties (Androutsopoulou *et al.*, 2021). Geraniol (GL) is an acyclic terpenoid isolated from the essential oils of several aromatic plants. Recent studies have shown various pharmacological properties of GL, including antioxidant, anti-inflammatory, and antimicrobial, suggesting the promising potential of GL as a drug candidate (Lira *et al.*, 2020; Mączka *et al.*, 2020).

Nanometric delivery systems are interesting strategies for bioactive compounds which provide better stability to the volatile compounds and protect them against environmental factors causing chemical degradation. Moreover, nanosystems can enhance the bioavailability and efficacy of formulations as a result of higher absorption or controlled release of the bioactive compounds (Bilia *et al.*, 2018; Abouhosseini Tabari *et al.*, 2022). In recent years, interest in nanotechnological approaches and the use of natural products has risen, so the present research aimed to develop nanotechnology-based formulations of GM and GL and evaluate their scolicidal activity compared to conventional formulations of the standard antiscolicidal drug, albendazole, under *in vitro* conditions.

MATERIALS AND METHODS

Chemicals

The geraniol and geranium oils were acquired by Sigma-Aldrich® (St. Louis,

MO, USA); nonionic surfactants polyethylene sorbitan monooleate (822187, Tween® 80) synthetic grade and sorbitan monooleate (840123, Span® 80) synthetic grade were procured by Merck-Millipore® (Darmstadt, Germany); all experiments were carried out with MilliQ® water (Darmstadt, Germany).

Preparation of nanoemulsion

The ultrasonication emulsification method has been used to prepare a variety of essential oil nanoemulsions (Hosseini *et al.*, 2017; Tabari *et al.*, 2021; 2022; Shahavi *et al.*, 2022). The formulation of the nanoemulsions included 15% (w/w) GM and GL essential oils, as well as 5% (w/w) of the mixture of surfactants with hydrophilic-lipophilic balance (HLB) = 9, including Span 80 and Tween 80 surfactants at a weight ratio of 56:44%, the remainder of the MilliQ water, were mixed at room temperature (25 ^{o}C) and agitated for 3 min on the vortex mixer to obtain the initial emulsion. Afterward, the sample was placed in an ultrasonic processor model UP400S (Dr. Hielscher GmbH, Germany) and ultrasonically irradiated for 10 min (24 kHz and 0.8 W/cm^2). Ultrasonic waves were used in this device to mix the phases dispersing the GM and GL essential oils in the continuous phase more uniformly and in smaller droplet sizes.

Particle size, polydispersity index, and zeta potential analysis

Photon correlation spectroscopy (PCS) was used to analyse nanoemulsions for particle size and polydispersity index (PDI), monitoring the variation in light scattering from the Brownian motion of droplet oils over time. The particle size, the surface charge (zeta potential), and PDI for the prepared nanoemulsion of GM and GL essential oils were determined by a Nano-ZS ZEN 3600 particle size analyser (Malvern Instruments, UK). The scattering intensity was assessed at an angle of 90° and 25 °C.

Collection of protoscoleces

Protoscoleces were recovered from the liver of sheep infected with hydatidosis, slaughtered in Babol, Mazandaran Province, Iran, and transferred to the parasitology laboratory of the Faculty of Veterinary Medicine, Azad University, Babol, Iran. Under sterile conditions, the contents of cysts, fluids, and protoscoleces were drained into a sterile flask, and the protoscoleces were allowed to settle down for 30 min. Afterward, the protoscoleces were washed twice with a PBS solution (pH 7.2), and the their viability was determined by the eosin (0.1%) exclusion test.

Protoscoleces viability test

To determine the ratio of viable protoscoleces before the experiments, 50 µL of pooled protoscoleces were transferred over a slide and mixed with the same amount of 0.1% aqueous eosin stain (Sigma-Aldrich, Germany); viability was evaluated after 10 min by an optical microscope. Stained protoscoleces were considered dead, while unstained ones were deemed viable. Protoscoleces with higher than 95% viability were considered appropriate for further scolicidal experiments.

In vitro scolicidal bioassay

GM, GL, and the developed nanoemulsions (Nano-GM and Nano-GL) at six different concentrations, corresponding to 1, 2.5, 5, 10, 25, and 50 µg/mL, were added to test tubes containing 1000 protoscoleces in Medium 199 (Gibco®, USA). Albendazole (ALB) was used as positive control at concentrations of 10 and 25

µg/ml. Tubes were kept for 0.5, 1, and 2 hours at 37 ^{o}C. After these time points, the supernatant was recovered, and protoscoleces were mixed with 50 µL of 0.1% eosin stain. The smeared protoscoleces were checked under a light microscope after 10 min. The number of dead protoscoleces was counted, and mortality rates were recorded (Hosseini *et al.,* 2017).

Scanning electron microscopy (SEM)

For ultrastructure studies, samples were fixed with 3% glutaraldehyde in sodium cacodylate buffer for 24 h at 4 °C. The samples were then washed with cacodylate buffer three times. For SEM analysis, the specimens were dehydrated by sequential incubations in increasing ethanol concentrations (50–100%) and were finally immersed in hexamethyldisilazane for 5 min, 1 hour, and then overnight. The samples were then sputter-coated with gold (100 Å thick) and inspected on an SNE-4500M (SEC, South Korea) scanning electron microscope operating at 15 kV.

Statistical analysis

Data were analysed by using SPSS version 26 (Chicago, USA). Differences between the means of mortality rate in tested compounds at each time of exposure (0.5, 1, and 2 hours) were analysed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Differences between the mean mortality rates at different exposure times for each concentration of tested compounds were analysed by Repeated measures ANOVA followed by the Bonferroni *post hoc* test. P values <0.05 were considered statistically significant. Fifty and ninety percent lethal concentrations (LC_{50} and LC_{90}) values were calculated by Probit regression analysis.

RESULTS

Particle size, polydispersity index, and zeta potential of Nano-GM and Nano-GL

Fig. 1 shows graphs of the size distributions and zeta potentials of Nano-GM and Nano-GL. As a result of the experiments, Fig. 1A indicates one peak of Nano-GM with 87.92 nm with 35.89 nm width. The results also showed that the average droplet size and PDI for Nano-GM were 124.8 ± 0.040 nm, and 0.210 ± 0.020, respectively. A zeta potential of about −21.0 ± 1.650 mV was obtained, indicating the perfect size and very stable and separate oil droplets (Fig. 1C). In addition, Fig. 1B shows one peak of Nano-GL with 73.26 nm with 21.89 nm width. Nano-GL's average droplet size and PDI were 88.590 ± 0.100 nm and 0.094 ± 0.001, respectively. Fig. 1D illustrates a Nano-GL's zeta potential at about −24.8 ± 0.040 mV. These results indicated successful development of Nano-GM and Nano-GL at nanometric size and their good stability.

Scolicidal activity

Fig. 2 shows the mortality rates of *E. granulosus* protoscoleces to GM, Nano-GM, GL, and Nano-GL compared to albendazole as the standard drug and the control group over different exposure times. GM and Nano-GM at the lowest tested concentration (0.5 µg/mL) after 0.5 h of exposure resulted in 9±2.66% and 16±3.78% mortality in protoscoleces, respectively. Scolicidal activity of GL and Nano-GL at the concentration of 0.5 µg/mL after half hour of exposure reached 42.66±4.08% and 54.66±6.35%, respectively. After one hour of exposure, all tested GL and Nano-GL concentrations resulted in higher than 90% mortality rates in protoscolices. However, GM and Nano-GM, after one hour at the highest concen-

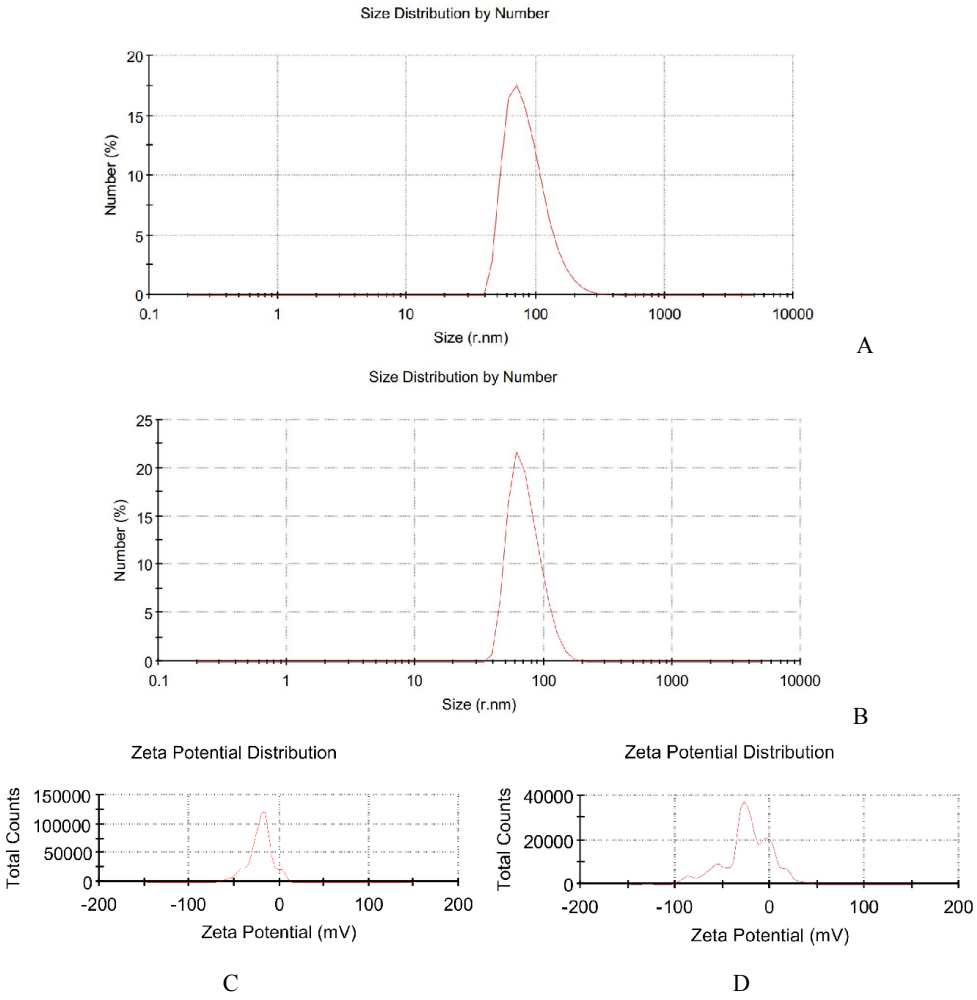

Fig. 1. Size distribution of Nano geranium (A) and Nano geraniol (B) and zeta potential of Nano geranium (C) and Nano geraniol (D).

tration, killed 70.66±5.88% and 90.33± 4.9% of hydatid cyst protoscoleces, respectively. After two hours of exposure, GL and Nano-GL at all tested concentrations led to total eradication of protoscoleces. The mean mortality rates of protoscoleces at 20 and 50 µg/mL concentrations of GM and Nano-GM and after 1 and 2 h time of exposure showed no significant difference (P>0.05); yet significant difference was noted (P<0.05) for GL and Nano-GL between 0.5, 1, and 2 h exposure at all concentrations.

Conversely, albendazole was less toxic to protoscoleces and, after 2 h of exposure, led only to 22.66±1.45 % mortality. No significant time effect was observed for DMSO as the solvent of the tested compounds (P>0.05).

GM and Nano-GM showed scolicidal activity with 0.5 h LC_{50} values of 102.95 and 28.67 µg/mL, respectively. GL and

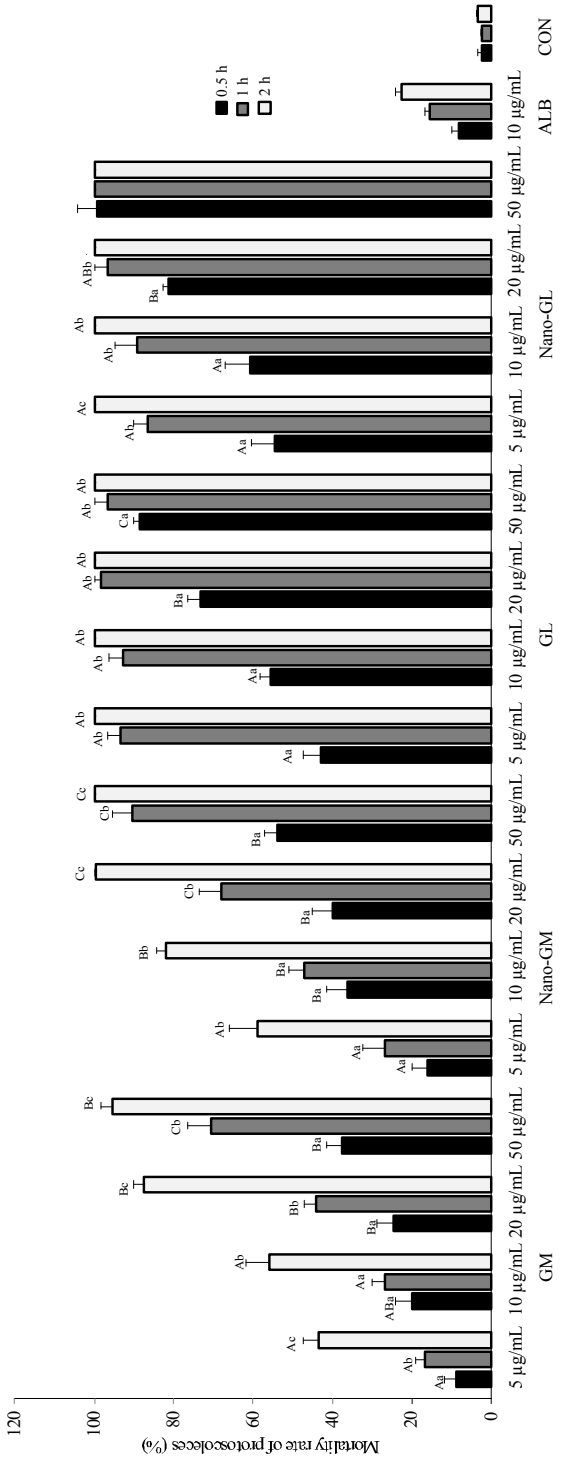

Fig. 2. Mortality rates of *Echinococcus granulosus* protoscoleces over different times of exposure (0.5, 1, and 2 h) to geranium oil (GM), nano-formulated geranium oil (Nano-GM), geraniol (GL), and nano-formulated geraniol (Nano-GL), in comparison to ALB (albendazole) and CON (control). T-bars represent 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 compound, columns marked with different letters (uppercase) in each time of exposure (0.5, 1, and 2 h, separately) are significantly different between concentrations (ANOVA, Tukey's test, $P<0.05$).

Table 1. Lethal concentration values of geranium oil, nanoemulsion of geranium oil, geraniol and nanoemulsion of geraniol on *Echinococcus granulosus* protoscoleces. Values are mean±SE of three replicates

Concentration (µg/mL)	30 min mortality (%)	LC_{50} (µg/mL) (LCL-UCL)	LC_{90} (µg/mL) (LCL-UCL)	χ^2 (df)[a]
Geranium oil				
5	9.00±2.64	102.95	> 2000	1.03
10	20.00±4.16	(57.19-374.06)		(3)
20	24.66±4.05			n.s.
50	37.66±3.74			
Nanoemulsion of geranium oil				
5	16.00±3.78	28.67	373.73	2.92
10	36.33±5.04	(21.78-42.66)	(301.00-454.04)	(3)
20	40.00±5.03			n.s.
50	54.00±3.21			
Geraniol				
5	42.66±4.08	7.22	60.67	0.29
10	55.66±2.33	(5.18-9.18)	(41.21-114.88)	(3)
20	73.00±3.51			n.s.
50	88.33±1.66			
Nanoemulsion of geraniol				
5	54.66±5.69	4.52	52.08	2.77
10	60.66±6.35	(2.54-6.34)	(34.38-109.7)	(3)
25	81.33±1.33			n.s.
50	99.33±4.66			

SE: standard error, LCL: 95% lower confidence limit, UCL: 95% upper confidence limit, n.s.: not significant (P>0.05); [a] Chi-square, df: degrees of freedom.

Nano-GL at 0.5 h time point showed promising activity with LC_{50} values of 7.22, and 4.52 µg/mL (Table 1). Based on the obtained LC_{90} values (LC_{90} of 52.08 µg/mL) Nano-GL was significantly more active than Nano-GM (LC_{90} of 373.73 µg/mL). Considering overlapping confidence limits, no statistically significant difference was noted between GL and Nano-GL; however, Nano-GM was significantly more active than GM.

SEM ultrastructural changes

SEM revealed that structural damages in treated protoscoleces were higher in GL and Nano-GL treated scolices relative to GM and Nano-GM. Ultrastructural changes, including rostellar disorganisation, loss of hooks, and shedding of microtriches of the scolex region, were more evident in Nano-GL and GL groups which can justify the higher lethality of these compounds in comparison to GM and Nano-GM (Fig. 3).

DISCUSSION

To date, the more advisable practice for CE treatment is surgery, though spillage of hydatid fluid rich in protoscoleces du-

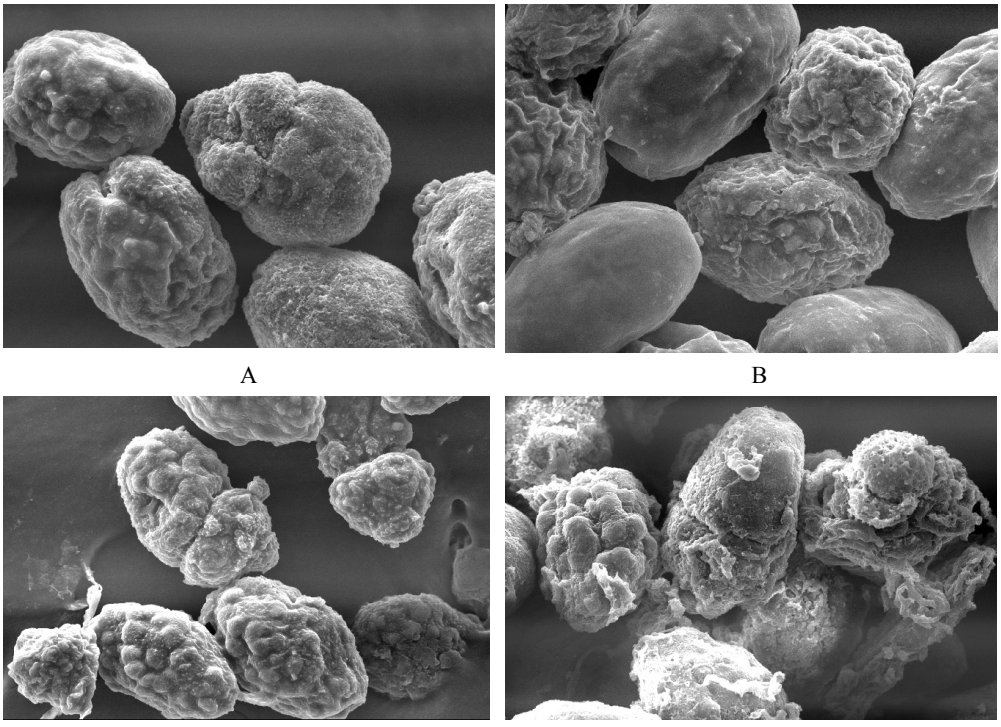

Fig. 3. Scanning electron microscopic analysis of the ultrastructural effects of geranium (A), nano-formulated geranium (B), geraniol (C), and nano-formulated geraniol (D) on the tegument of protoscoleces after 2 h of exposure.

ring surgical operation may lead to CE recurrence as well as an anaphylactic reaction which could cause death. Therefore, the application of a scolicidal agent is an essential part of CE surgery (Shnawa *et al.*, 2021). It has been recognised that phytochemicals are non-toxic, low-cost, and environmentally friendly sources of a bioactive compound that can be manipulated by nanotechnological approaches to increase their unique features of therapeutic potential (Kim *et al.*, 2021). This study describes for the first time the *in vitro* toxic effect of GM, GL, and their nano-formulated preparations on the protoscoleces of *E. granulosus*. All of the tested compounds showed significant mortality in protoscoleces compared to the control.

Several studies have reported scolicidal activity of plant-derived nanoemulsions on protoscoleces of *E. granulosus*. Moazeni and coworkers reported that nanoemulsion of *Zataria multiflora* essential oil at concentration of 1 mg/mL killed 88.01 and 100% of protoscoleces after 10 and 20 min, respectively (Moazeni *et al.*, 2017). Compared with the results of our study, it seems that Nano-GM can exert its scolicidal activity at lower concentrations, and with its 30 min-LC_{50} value of 28.67 μg/mL, was more toxic for protoscoleces than *Zataria multiflora* nanoemulsion. In another study, eugenol and nanoemulsion

of eugenol have been introduced as promising alternative scolicidal agents. It was shown that eugenol and its formulated nanoemulsion were significantly more active against hydatid protoscoleces compared to ALB ($P<0.05$) (Maurice *et al.*, 2021). The same findings have been observed in our study, where all tested compounds showed higher toxicity relative to ALB. Part of this lower *in vitro* activity of ALB is due to the fact that ALB, after oral administration, is oxidised to a sulfoxide, which is further oxidised to a sulfone, and ALB sulfoxide is the main *in vivo* metabolite. The biological scolicidal activity of ALB primarily depends on its metabolites (Redondo *et al.*, 1999). It has been reported that under *in vitro* conditions, 50 µg/mL ALB sulfone killed 97.3% of the scolices, ALB sulfoxide killed 98.4% of the scolices, and the combined solution (sulfone+sulfoxide) killed 98.6% of the scolices in 5 minutes (Adas *et al.*, 2009). In spite of the fact that ALB metabolites are rapid and potent scolicidals, oral administration of ALB is accompanied by severe side effects caused by both metabolites, such as hepatic function abnormalities, leukopaenia, and alopecia (Dervenis *et al.*, 2005). Considering teratogenic effects in animal models, albendazole is contraindicated in pregnancy and lactation (Akhan *et al.*, 2014).

Moreover, ALB has been reported to be ineffective in the vast majority of patients with hepatic hydatidosis and should not be used as the first-line therapy for surgical candidates (Kapan *et al.*, 2008). So, an opportunity could be given to novel formulations based on natural bioactive compounds to be evaluated as safe promising alternatives to ALB and its derivatives for treating CE. Based on the results of the present study, tested compounds, especially Nano-GL, can be studied more extensively for possible *in vivo* efficacy and safety.

As a rule, particles of smaller size travel faster than particles of larger size. Laser scattering intensity showed rapid fluctuations at a fixed angle due to oil droplets diffusion around a mean value. The calculated photoelectron time correlation function generated a histogram of the oil droplets' size distribution. PDI can range from 0 to 1, where 0 (zero) represents monodisperse, and 1 represents a polydisperse system (Ahmad *et al.*, 2021).

In liquids, zeta potential is used to determine the surface charge of particles. The zeta potential predicts dispersion stability by using physicochemical properties of oil droplets on the nanoscale. The electrophoretic mobility of oil droplets is used to estimate the zeta potential of nanoemulsions. A zeta potential of ±30 mV is thought to be sufficient to ensure nanoemulsions' stability. Generally, oil in water nanoemulsions is very stable and can only be separated into two phases quickly using unique methods such as electric fields (Hosseini *et al.*, 2012). Based on the results of the present study, nanoemulsions were successfully formulated at desired droplet size and represented promising stability.

From a mechanical point of view, ultrastructural studies allowed us to examine the toxic effects induced by GM, GL, and their nanoemulsions on scolices. All treatments induced ultrastructural changes after 2 hours of incubation. However, the changes were more evident in GL and Nano-GL, including the formation of blebs on the tegument, rostellar disorganisation, and wrinkling of the surface of protoscoleces. It is well documented that tegument is a metabolically active surface that plays a pivotal role in the physiology of cestodes and is important in absorption,

excretion, ionic exchange, and defense against a host. Alteration in the tegument will hamper nutrition in protoscoleces and lead to death (Mousavi *et al.*, 2020).

Altogether, the present study described an easily-applicable and eco-friendly procedure of nano-formulating functional phytochemicals, showing promising *in vitro* scolicidal activity. The developed formulations, especially Nano-GL, possess the characteristics of ideal scolicidal agents that are potent at low concentrations, act in a short period, are readily available, easily prepared, and inexpensive. Based on these considerations, Nano-GL can be suggested for further *in vivo* efficacy and safety studies.

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