

In vitro scolical activity of clove oil and its nano-emulsion on hydatid cyst protoscoleces

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ABSTRACT. Surgical management of cystic echinococcosis needs injection of a scolical agent into the cyst cavity. Considering side effects of available scolicals, growing interest for the green drugs, and advantages of novel drug delivery systems, the present study aimed to find out the scolical activity of clove oil and its nano-emulsion on protoscoleces of *Echinococcus granulosus*. The nano-emulsions of clove oil was developed in a spontaneous oil phase and characterized by dynamic light scattering method (DLS). The scolical effect of clove oil and its nano-emulsion at different concentrations of 1, 2.5, 5, 10, 25, and 50 µg/ml were measured at 1, 2, 6, 12, and 24 hours of incubation. Mortality rates were recorded by eosin exclusion test with an optical microscope. The particle size in the developed nano-emulsion was 74.8 nm. After 1 hour of incubation, the EO and its nano-emulsion at 50 µg/ml, killed 64 and 94% of the protoscoleces, respectively. Based on 50% lethal concentration, nano-emulsion of clove EO (LC₅₀ of 1.54 µg/ml) was significantly more active than clove EO (LC₅₀ of 8.14 µg/ml). In scanning electron microscopy (SEM), ultrastructural alterations were evident. Most likely, tegumental disruption is the main cause of scolical activity of clove nano-emulsion. Development of nano-emulsion resulted in increased toxicity of clove oil on protoscoleces. Further studies are needed to assess *in vivo* efficacy and safety of this formulations.

Keywords: cystic echinococcosis, *Echinococcus granulosus*, drug delivery, green drugs

Introduction

Hydatidosis, also known as echinococcosis or hydatid disease, is a parasitic infection caused by the larval stages of taeniid cestodes of the genus *Echinococcus* [1]. Cystic echinococcosis (CE) caused by *E. granulosus* and alveolar echinococcosis (AE) caused by *E. multilocularis* both have been reported in several countries of the Mediterranean region and the Middle East. *E. granulosus* is the most common species which is present in the Mediterranean region, while *E. multilocularis* has been reported only sporadically in limited areas of France, Italy, Serbia and Montenegro, Tunisia, and Turkey [2].

Hydatidosis is endemic across the Mediterranean basin, especially in the south bank and in all the west Asian countries of the Middle East. The annual

incidence of hydatidosis in these countries varies between 1 and 27 cases per 100,000 inhabitants, with most being in the 5–10 cases per 100,000 ranges. It is estimated that nearly half a million people across this endemic area are living with CE [3]. CE is highly prevalent in Iran, especially in sheep grazing, areas of East Azerbaijan and Kurdistan [4]. Cases of human hydatidosis are regularly reported from medical centers in different parts of the nation. Indeed, CE is responsible for approximately 1% of admissions to surgical wards and the rate of human infection is 0.6–1.2% [5]. CNS involvement is seen in 2–3% of patients and affects mainly young adults [4].

Treatment of this disease depends on the stage, size, location, and complications of the cysts. At present, four treatment modalities are in practice for CE: surgery (the only treatment until the 1980s),

chemotherapy with synthetic drugs like benzimidazole compounds, puncture aspiration injection and re-aspiration (PAIR), and the watch and wait method for clinically silent and inactive cysts [6]. Benzimidazole derivatives, such as albendazole (ABZ) and mebendazole, are widely used in CE treatment as an alternative or adjuvant to PAIR technique or surgery according to the site and stage of cysts [7]. However, the reported efficacy of ABZ is only ~50%, with an overall cure rate not exceeding 30% to 48% [8]. Novel drugs or new methods of formulation are needed for better pharmacotherapy of CE.

Clove oil is an essential oil (EO) which is derived from clove trees. The clove tree, known as *Syzygium aromaticum*, is native to Southeast Asia, but nowadays can be found in many parts of the world. This EO has been used for centuries in a variety of applications including antimicrobial, pain killer, and anti-inflammatory effects [9]. The clove EO has been classified generally regarded as safe and many studies have reported evidences on its antimicrobial, antioxidant and antiparasitic activities [10,11].

The recent advances in drug delivery system have increased drug's efficacy in diseases control and management. Nano-emulsions serve as suitable carriers of active EOs due to their easy preparation, simple structure, high thermodynamic stability, and cost-effectiveness for production on an industrial scale [12]. Newly, the use of various compounds for scolicidal has received much attention. Among them, herbal compounds due to their natural origin and general safety have a distinctive position.

The aim of this study was to compare the scolicidal effect of clove EO and nano-emulsion of clove EO in comparison to albendazole under *in vitro* condition.

Materials and Methods

Gas chromatography analysis of the clove EO

The chemical analysis of the EO was carried out on an Agilent 7890A gas chromatograph equipped with 5975C mass spectrometer. The oil sample was diluted to 1% with n-hexane, and 2 µl of the solution was injected into the GC-MS system. The carrier gas was at a flow rate of 1 ml/min. The injector and detector temperatures were 230 and 250°C, respectively. The identification of the compounds was based on the comparison of the retention indices and mass spectra with those contained in the commercial libraries.

Preparation of nano-emulsion

A nano-emulsion of clove EO was formed spontaneously in an oil phase of GMO, Cremophor RH40 and PEG 400 (1:8:1 ratio). Clove EO, surfactant and co-surfactant were stirred at 100 rpm for 2 h. Further sonication for 1 h by using a bath-sonicator was applied to complete the mixing process. Deionized water was added to the oil phase at a ratio of 5:1 to obtain nano-emulsion.

Particle size, zeta potential and polydispersity index analysis

The particle size, the surface charge (zeta potential), and polydispersity index (PDI) for the prepared nano-emulsion of clove oil were determined by a Nano-ZS ZEN 3600 particle size analyzer (Malvern Instruments, UK). The scattering intensity was assessed at an angle of 90° and at 25°C.

Collection of protoscoleces

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

Protoscoleces viability test

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

In vitro scolicidal activity

Clove EO and nano-emulsion of clove EO at six different concentrations in 10% DMSO, corresponding to 1, 2.5, 5, 10, 25, and 50 µg/ml, were added to test tubes containing 1000 protoscoleces in medium 199 (Gibco®). Albendazole at concentrations

Table 1. Chemical composition of clove essential oil

| No | Component ^a | Calculated RI ^b | Abundance (%) ^c |
|----|------------------------------|----------------------------|----------------------------|
| 1 | Limonene | 1025 | 0.2 |
| 2 | 1,8-Cineole | 1032 | 0.1 |
| 3 | <i>cis</i> -Limonene oxide | 1136 | tr |
| 4 | <i>trans</i> -Limonene oxide | 1147 | tr |
| 5 | Methyl salicylate | 1190 | 0.1 |
| 6 | Chavicol | 1251 | 0.2 |
| 7 | Eugenol | 1358 | 82.2 |
| 8 | β -Caryophyllene | 1415 | 3.3 |
| 9 | α -Humulene | 1452 | 1.2 |
| 10 | Eugenol acetate | 1520 | 12.2 |
| 11 | Caryophyllene oxide | 1580 | 0.2 |
| | Total identified (%) | | 99.7 |

Explanations: ^a compounds are listed in order of their elution from a HP-5MS column; ^b linear retention index on HP-5MS column, experimentally determined using homologous series of C₈-C₃₀ alkanes; ^c relative percentage values are means of three determinations with a RSD% in all cases below 10%

of 10 and 25 μ g/ml was used as the control. Tubes were kept for 1, 2, 6, 12, and 24 hours at 37°C. After these times, the supernatant was detached, and protoscoleces were mixed with 50 μ l of 0.1% eosin stain. After 10 minutes, the smeared protoscoleces were checked under a light microscope. The number of dead protoscoleces was counted, and mortality rates were recorded [13].

Scanning Electron Microscopy (SEM)

For ultrastructure studies, samples were fixed with 3% glutaraldehyde in sodium cacodylate buffer for 24 h at 4°C. Then several washes in cacodylate buffer were made. For SEM analysis, the specimens were dehydrated by sequential incubations in increasing concentrations of ethanol (50–100%) and were finally immersed in hexamethyl-disilazane for 5 minutes, 1 hour, and then overnight. They were then sputter coated with gold (100 Å thick) and inspected on a SNE-4500M (SEC, South Korea) scanning electron microscope operating at 15 kV.

MTT cytotoxicity assay

To assess the cytotoxicity of clove EO and nano-emulsion of clove EO, an MTT assay was used.

Primary fibroblast cells (10⁴) were seeded on a 96-well plate and cultivated for 24 h at 37°C with 5% carbon dioxide. Then cells were exposed to clove EO and nano-emulsion at different concentrations ranging 0 to 50 μ g/ml, and incubated for 24 h. After 1 day, 5 μ l of MTT reagent, isopropanol 50% and 10% sodium dodecyl sulphate were added and incubated for 5 h at 37°C. Samples were measured spectrophotometrically at 540 nm. All experiments were run in triplicate.

Statistical analysis

Data was analyzed by using SPSS version 23.0 (Chicago, IL, USA). Differences between the means of mortality rate in different tested compounds at each time of exposure (1, 2, 6, and 12, 24 hour) were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Differences between the means of mortality rate at different exposure times for each concentration of tested compounds were analyzed by repeated measures ANOVA followed by Bonferroni *post hoc* test. *P*-values < 0.05 were considered statistically significant. Fifty and ninety percent lethal concentrations (LC₅₀ and LC₉₀) values were calculated by Probit regression analysis.

Results

Chemical composition of the EO

The chemical constituents of clove EO were identified by GC-MS analysis and are shown in table 1. The EO composition was dominated by eugenol (82.2%) followed by eugenol acetate (12.2%).

Particle size, and zeta potential of NanoCAV

Mean size of droplets in prepared nano-emulsion of clove EO was 74.8 nm. Poly dispersity indices were equal to 0.61 (Fig. 1A). The zeta potential of the nano-emulsion was -24.2 mv (Fig. 1B). These results indicate that the formulated emulsion was on the nanometric scale and the particles were homogenous in the dispersion.

Scolicidal activity

Figure 1 shows the mortality rates of *E. granulosus* protoscoleces over different times of exposure to EO of clove, and nano-emulsion of clove EO in comparison to two doses of albendazole and DMSO as the control group. In the present study, a significant effect of the concentration, the

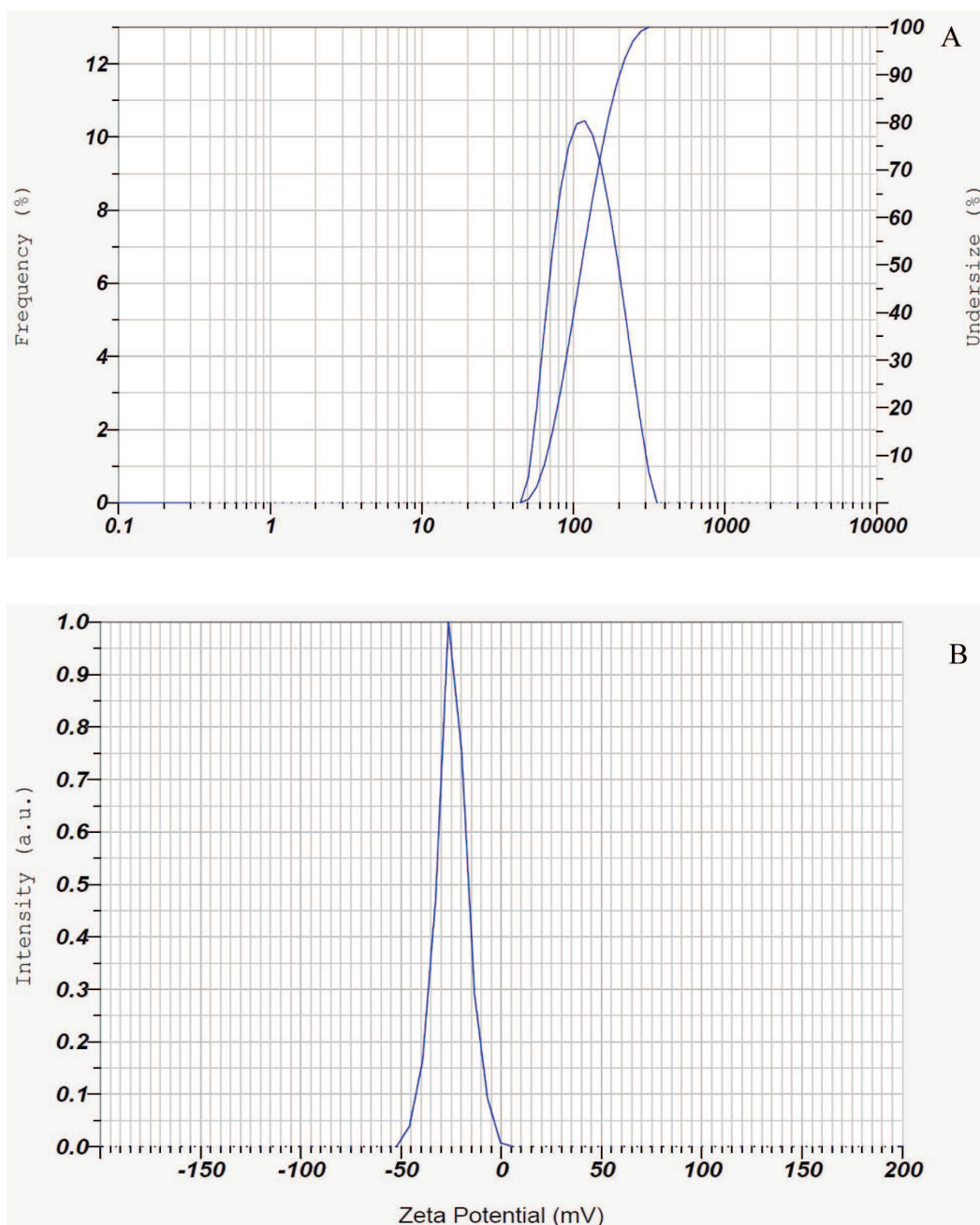


Figure 1. Particle size and polydispersity index distribution (A); zeta potential for nano-emulsion of clove oil (B)

time of exposure, and their interaction were noted.

After 1 hour of exposure, the EO and its nano-emulsion at 50 $\mu\text{g/ml}$, killed 64 and 94% of the protozoa, respectively. Scolicidal activity of these tested compounds after 2 hours of exposure reached to 95 and 100% mortality rates, respectively. On the other hand, albendazole was less toxic to protozoa, and at the highest concentration even after 24 hours of exposure, just led to 32% mortality in protozoa. After 24 h treatment, total mortality rates were achieved for nano-emulsion of clove EO at all tested concentrations. However, 100% mortality was recorded for concentrations of 5, 10, 25 and 50

$\mu\text{g/ml}$ of EO of clove. The mean mortality rates of protozoa at 25 and 50 $\mu\text{g/ml}$ concentrations of clove EO at 12 and 24 h time of exposure did not show any significant difference ($P>0.05$), while at 1, 2.5, and 5 $\mu\text{g/ml}$ between 1, 2, and 6 hour exposure time, a significant difference was noted ($P<0.05$). Nanoemulsion of clove at the concentrations of 1, 2.5, 5, and 10 $\mu\text{g/ml}$ showed a significant difference between 1, 2, 6 and 12 hour exposure times ($P<0.05$).

Surprisingly, albendazole did not show any promising scolicidal activity at the tested concentrations, however, at the concentration of 25 $\mu\text{g/ml}$, albendazole caused significantly higher

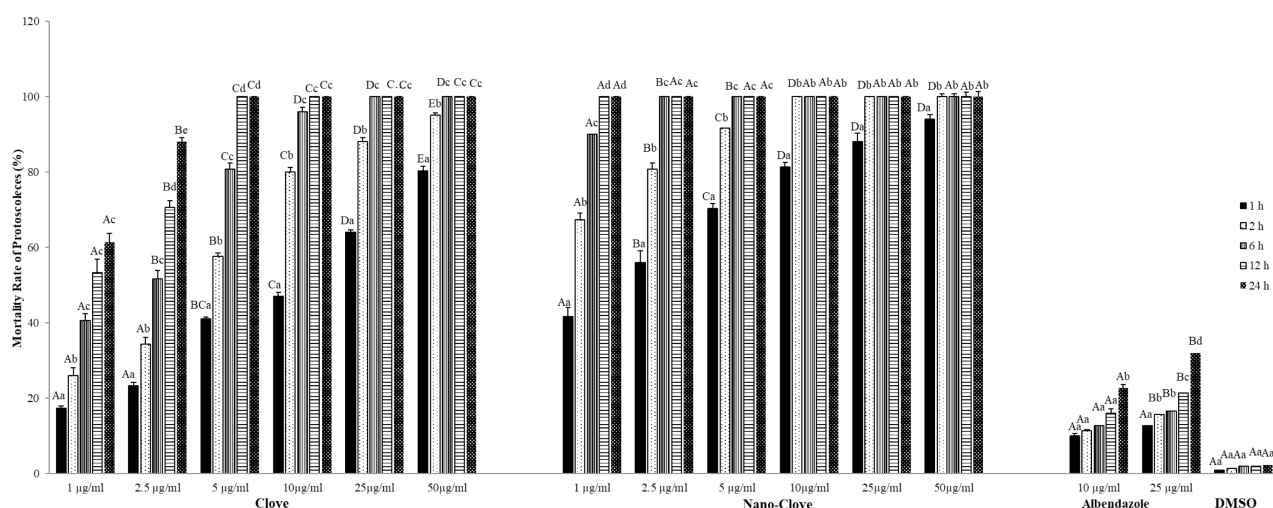


Figure 2. Mortality (%) of *Echinococcus granulosus* protoscolices over different times of exposure to clove and nano-clove, in comparison to albendazole and DMSO. T-bars represent SE. Within each concentration of tested compounds, columns marked with different letters (lowercase) are significantly different 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 (1, 2, 6, 12, and 24 h, separately) are significantly different between concentrations (ANOVA, Tukey’s test, $P < 0.05$)

toxicity in comparison to the 10 µg/ml, it was not as efficient as the tested clove EO and clove EO nanoemulsion. No significant effect of time has been observed for DMSO as the solvent of the

tested compounds ($P > 0.05$).

EO of clove showed scolicidal activity with 1 h LC₅₀ value of 8.14 µg/ml. After 1 hour of exposure, nano-emulsion of clove EO showed promising

Table 2. Lethal concentration values of clove essential oil and nano-emulsion of clove essential oil on *Echinococcus granulosus* protoscolices

| Components | Concentration (µg/ml) | 1-hour mortality ^a (%) | LC ₅₀ (µg/ml) (LCL-UCL) | LC ₉₀ (µg/ml) (LCL-UCL) | χ^2 (df) ^b |
|------------|-----------------------|-----------------------------------|------------------------------------|------------------------------------|----------------------------|
| Clove | 1 | 17.33±0.66 | 8.14(6.53–10.25) | 112.71(70.31–218.57) | 2.74(4) n.s. |
| | 2.5 | 23.33±0.88 | | | |
| | 5 | 41.00±0.57 | | | |
| | 10 | 47.00±1.00 | | | |
| | 25 | 64.66±0.57 | | | |
| | 50 | 80.33±1.20 | | | |
| Nano-clove | 1 | 41.66±2.02 | 1.54(1.03–2.08) | 22.45(15.87–36.55) | 1.49(4) n.s. |
| | 2.5 | 56.00±2.30 | | | |
| | 5 | 70.33±3.17 | | | |
| | 10 | 81.33±1.33 | | | |
| | 25 | 88.00±1.15 | | | |
| | 50 | 94.00±2.30 | | | |

Explanations: SE standard error; LCL 95% lower confidence limit; UCL 95% upper confidence limit; n.s. not significant ($P > 0.05$);

^a values are mean ± SE of three replicates; ^b Chi-square, df: degrees of freedom

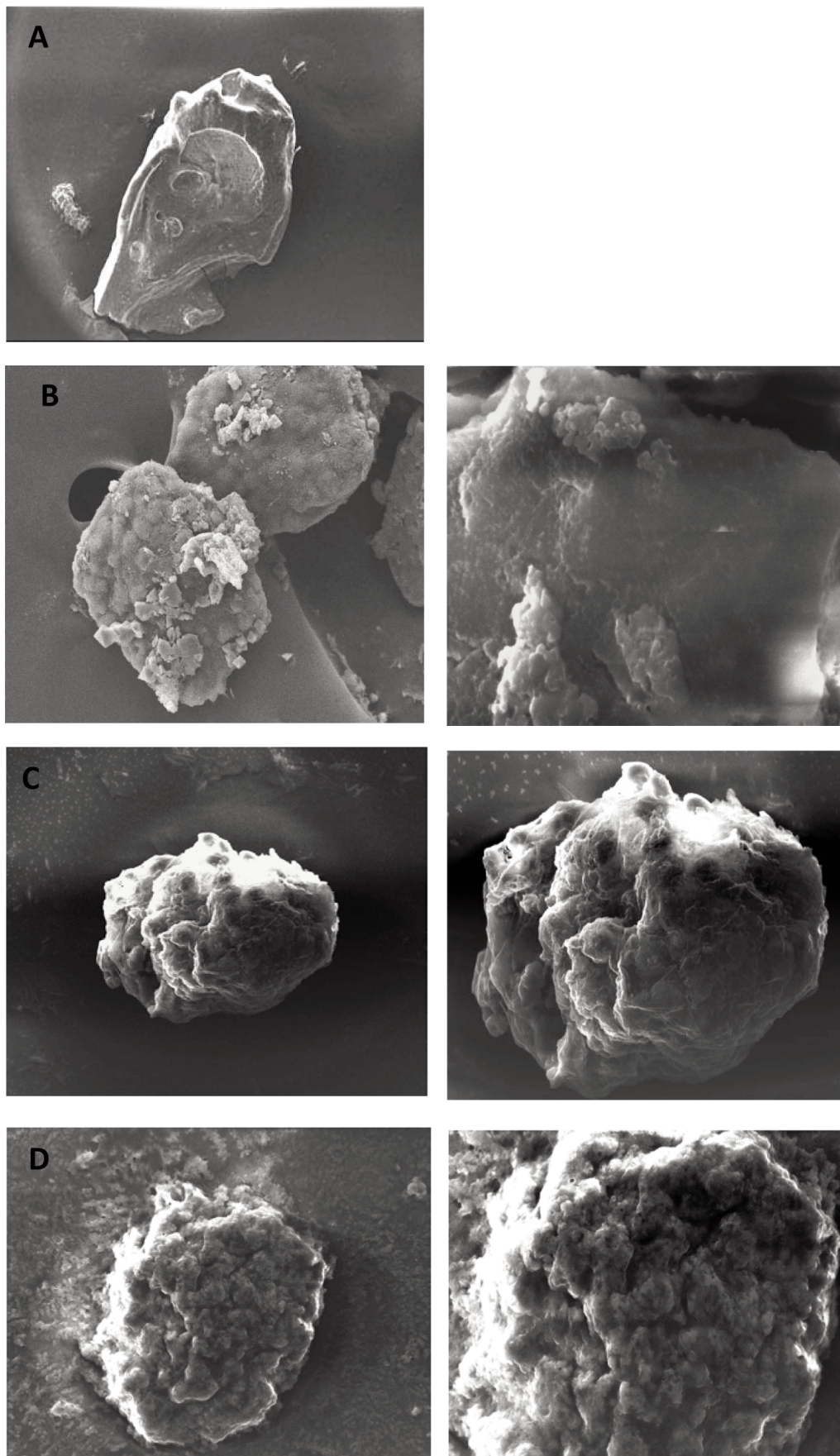


Figure 3. Evaluation of the effect of clove and nano-clove on the tegument of protozoa in 30 and 60 minutes. A: clove 30 min, B: clove 60 min, C: Nano-clove 30 min, D: Nano-clove 60 min

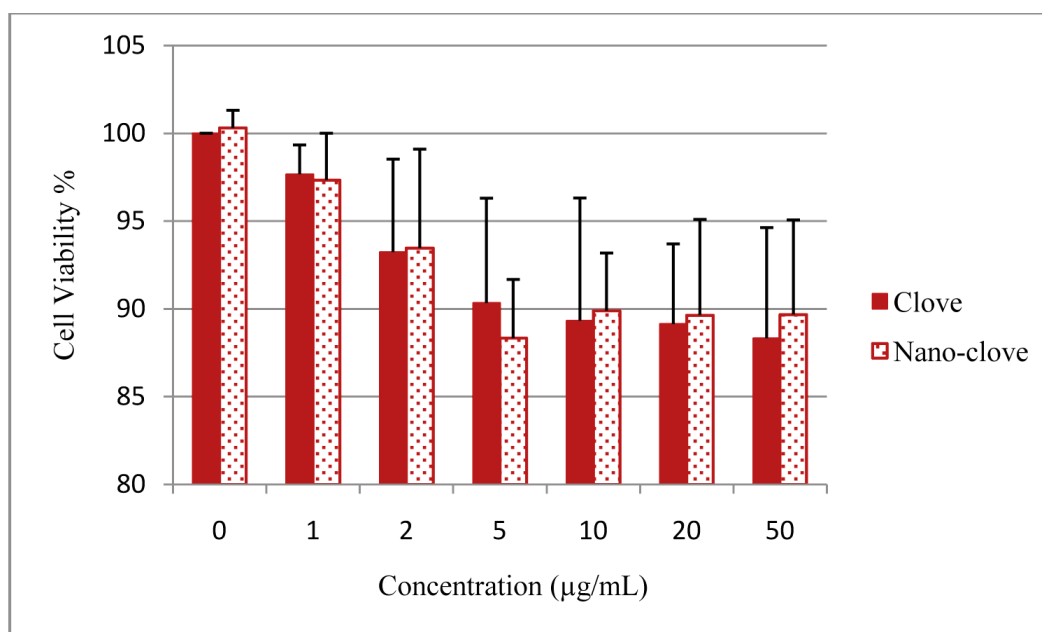


Figure 4. Mean viability of cells exposed to clove essential oil and nano-emulsion of clove essential oil for 24 h

activity with LC_{50} value of $1.54 \mu\text{g/ml}$ (Tab. 2). Based on the obtained LC_{50} and LC_{90} values, nano-emulsion of clove EO (LC_{90} of $22.45 \mu\text{g/ml}$) was significantly more active than clove EO (LC_{90} of $112.71 \mu\text{g/ml}$).

SEM ultrastructural changes

SEM demonstrated the morphological and structural damages in treated protoscolices. Ultrastructural alterations rostellar disorganization, loss of hooks and shedding of microtriches of the scolex region were evident. Loss of morphology and marked tegumental alterations were observed in protoscolices (Fig. 3).

MTT cytotoxicity results

Viability of primary fibroblast cells incubated with different concentrations of clove EO and its nano-emulsion (0 – $50 \mu\text{g/ml}$) was determined after 24 h (Fig. 4). Clove EO and nano-emulsion especially at higher concentrations induced a slight cytotoxicity but the effect was not significant.

Discussion

Hydatid cyst is one of the oldest common diseases of humans and animals that attacks various organs, especially the host liver. For drug prophylaxis of hydatidosis, benzimidazoles such as albendazole are used today; however, lack of proper absorption from the gastrointestinal tract, lack of

proper solubility in hydatid cyst fluid and many other side effects have limited the use of chemotherapy and continued surgery as the most efficacious approach for management of hydatidosis. Considering surgery as the most effective measure in the treatment of this disease, and also the recurrence of disease and spread of infection during surgery, special attention is necessary to be paid on the use of scolicidal as an important factor in surgical treatments [14].

The criteria for selecting a promising scolicidal compound are suitable efficacy with low concentration, non-toxicity or low toxicity, desired ability in term of time-response and good solubility in hydatid cyst fluid [14]. Therefore, there are increasing number of studies on herbal compounds scolicidal activity during recent years. In this trend, the present study evaluated toxic effects of clove essential oil, nano-emulsion of clove EO relative to albendazole on hydatid cyst protoscolices. The results showed that nano-emulsion of clove EO possessed promising scolicidal effect which was significantly different in comparison to clove EO and albendazole.

Elisando et al. [15] evaluated the toxic effects of thymol on hydatid cyst protoscolices at concentrations of 1, 5 and $10 \mu\text{g/ml}$ and showed that after 1 to 4 days thymol showed excellent scolicide effects. Comparing to the results of the present study, clove EO showed higher efficacy relative to thymol with 1 h LC_{50} value of $8.14 \mu\text{g/ml}$. Eugenol

has been reported as the main constituent of clove comprising about 70% of chemical constituents of the clove essential oil [16]. It has been reported that eugenol showed high scolicedal activity with LC₅₀ of 0.298 µl/ml after 30 min incubation with protoscoleces of *E. granulosus* [17]. By comparing the results with the present study, it can be concluded that eugenol is one of the effective protoscolicedal compounds in the EO of clove, and probably some of clove scolicedal effects is due to this compound. Eugenol has also shown excellent antimicrobial activity, being active against a wide range of bacteria and fungi. From mechanistic point of view, it has been demonstrated that eugenol could disrupt cytoplasmatic membrane and increases membrane nonspecific permeability, affects the transport of ions and ATP [18]. The same can be attributed to the scolicedal activity of this compound in the clove essential oil. β-caryophyllene, the second highest chemical constituent of clove EO, possesses antimicrobial effects via affecting cell wall integration [19]. This component also can exert toxic effects on protoscoleces of *E. granulosus*. It is worth to note that compounds in EOs may interact to cause synergistic or additive effects. Using EOs and their components in combination are new approaches toward increasing the efficiency of EOs and postponing the emergence of resistance in microbial pathogens due to various pathways of actions.

Maurice et al. [17] have reported that there was no significant difference between eugenol and its nano-emulsion regarding their scolicedal activity; interestingly, nano-emulsion of eugenol did not cause higher toxicity on protoscoleces of *E. granulosus*. On the other hand, in our study, nano-emulsion of clove EO was more toxic on protoscoleces than clove EO. Nanometric scale of clove EO in nano-emulsion which was confirmed by DLS and consequently better penetration in to the protoscoleces, most likely are the main causes of higher scolicedal effects of nano-emulsion of clove EO.

Since no *in vivo* study was done for nano-emulsion of clove EO, predicting efficacy of this formulation in the infected patient surgery will be speculative and further *in vivo* studies are needed to warrant efficacy and safety of this formulation in CE.

Considering side effects of available scolicedals and emergence of CE even after surgical interventions, there is a need to find novel scolicedal

agents. The clove oil nano-emulsion based on *in vitro* activity could be further studied for its efficacy and safety profile in the CE management.

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