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Original article

IN VITRO TOXIC EFFECT OF ORDINARY AND MICRO-EMULSIFIED A-PINENE AND B-CARYOPHYLLENE ON HYDATID CYST PROTOSCOLECES

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Summary

Tabari, M. A., C. Zizzadoro, M. Moeini, S. Z. Tabatabaei & M. R. Youssefi, 2023. *In vitro* toxic effect of ordinary and micro-emulsified α-pinene and β-caryophyllene on hydatid cyst protoscoleces. *Bulg. J. Vet. Med.* (online first).

Due to the side effect of commonly used chemical scolicidals, advantages of micro drug delivery system and growing interest for the phytoceuticals, the present study aimed to find out the scolicidal activity of two volatile terpenes (α-pinene and β-caryophyllene) and their microemulsions on protoscoleces of *Echinococcus granulosus*. The ordinary and microemulsions of α-pinene and β-caryophyllene and their combination at three different ratios (2:1, 1:1, 1:2) were developed and characterised by dynamic light scattering to confirm micro-dimensions. The scolicidal effect of the developed formulations at the concentrations of 0.5, 1, 2, 5 and 10 µg/mL were measured at 10, 30, and 60-min time points. Mortality rates were recorded by eosin exclusion test. At the 10-min time point, ordinary and micro α-pinene at the concentration of 10 µg/mL killed 94.66% and 100% of the protoscoleces, respectively. At the same time point and concentration, ordinary and micro β-caryophyllene caused 44.33% and 68.66% mortality rates in protoscoleces, respectively. Based on the 50% lethal concentration (LC_{50}) values, there was no significant difference between ordinary and micro-emulsified α-pinene. For the micro β-caryophyllene, a LC_{50} value of 2.1 µg/mL was obtained which was significantly lower than that of ordinary β-caryophyllene (49.85 µg/mL). No synergistic interaction existed between β-caryophyllene and α-pinene regarding their activity on *E. granulosus*. Development of microemulsions increased the toxicity of these terpenoids on protoscoleces, especially that of β-caryophyllene, probably due to improved penetration into the parasite. Further studies are needed to assess *in vivo* efficacy and safety of this specific preparation.

Key words: echinococcosis, formulation, herbal drugs, hydatid cyst, terpene

INTRODUCTION

Echinococcosis is a parasitic zoonosis disease caused by infection with adult or larval tapeworms of the genus *Echinococcus*. This disease is a major public health issue and classified as either cystic or alveolar echinococcosis. Liver is the most common site of cystic echinococcosis (accounting for 75% of cases) and treatment ranges from surgical intervention including ordinary or laparoscopic approach to percutaneous drainage or pharmacotherapy (Youssefi *et al.,* 2020). Because the results of pharmacotherapy and percutaneous treatment are controversial, surgery still remains the treatment of choice for liver hydatid cysts. In the surgical treatment of this disease, neutralisation of the parasites, evacuation of the cyst content and management of the residual cavity are the pivotal steps. In order to minimise chances of leakage and re-infection, injecting a scolicidal agent into the unopened cyst is one of the commonest practices during hydatid cyst surgery. Formalin, hypertonic saline, cetrimide, chlorhexidine, and hydrogen peroxide are some of the commonly used scolicidals (Babaei *et al.,* 2018). However, current scolicidal agents are associated with risk of anaphylactic shock, chemical cholangitis or damage, and if the cyst communicates with the biliary system, spillage of the cyst contents may lead to secondary hydatidosis after surgery. Mortality and relapse rates of liver cystic echinococosis surgery are 8% and 20%, respectively (Junghanss *et al.,* 2008).

Currently, the use of medicinal plants as alternatives to chemical substances has gained much attention, and there have been several reports on the scolicidal bioactivity of plant derived preparations (Rostami *et al.,* 2016; Tabari *et al.,* 2019). Alpha-pinene is an organic compound of the monoterpene class, occurring in nature with two enantiomeric forms. This alkene contains a reactive four-membered ring and is found in the essential oils of many species of the coniferous trees, notably the pine. It is also found in the essential oil of rosemary (*Rosmarinus officinalis*) and bean herb (*Satureja myrtifolia*) (da Franca Rodrigues *et al.,* 2015). Beta-caryophyllene is a natural bicyclic sesquiterpene that is a constituent of many essential oils, especially clove oil (*Syzygium aromaticum*), hemp, rosemary, and hops (Aguilar-Ávila *et al.,* 2019). Studies have reported the inhibitory activity of α-pinene and β-caryophyllene against some parasite species (Amaral *et al.,* 2016; Estevam *et al.,* 2017; dos Santos Sales *et al.,* 2018). Moreover, it has been reported that α-pinene and β-caryophyllene combination resulted in synergistic interaction and selectively enhanced their toxicity on parasites without increasing cytotoxicity (Amaral *et al.,* 2016).

In the recent years, application of novel drug delivery systems including nano and micro-particles or emulsions has contributed significantly to improve the therapeutic potential of bioactive compounds including plant-derived extracts and their constituents. Nano- and micro-drug delivery systems possess the advantages of increased solubility, stability, targeted delivery, and reduced toxicity (Patra *et al.,* 2018). Existing evidence suggests that microemulsions are superior in increasing the permeabilisation of the microbial membranes allowing better integration of the phytophenols, direct improvement of the interaction with cell wall components, and increase of the concentration of phytophenol dispersed in the interface (Abouhosseini Tabari *et al.*, 2022).

Due to the side effects of commonly used chemical scolicidals (Eryilmaz & Bulbuloglu, 2004) and the growing interest for natural pharmaceuticals, especially phytoceuticals, the present study aimed to find out the scolicidal activity of α-pinene and β-caryophyllene *per se* and at different combination ratios. Also, single and combined microemulsions of compounds were developed, characterised and evaluated for their toxicity on *E. granulosus* protoscoleces.

MATERIAL AND METHODS

Chemicals

Alpha-pinene [(+/-)-2-pinene 98%] and β-caryophyllene (98%) were purchased from Sigma (Germany). Glycerol monooleate, polyoxyl hydrogenated 40 castor oil, and polyethylene glycol (PEG) 400 were obtained from Anmol Chemicals (India). All other chemicals were analytical grade and commercially available.

Hydatid cyst protoscoleces

Protoscoleces of *E. granulosus* were collected at a slaughterhouse in Amol (Mazandaran, Iran) from sheep livers infected with hydatid cysts, then transferred to the parasitology laboratory of the I. A. U. Faculty of Veterinary Medicine, Babol, Iran. The surface of livers was disinfected with 70% alcohol-impregnated cotton swab. Afterwards, the cyst contents (consisting of protoscoleces and fluids), were drained into sterile Erlenmeyer flask and left still to settle at the bottom of the flask. After complete sedimentation, protoscoleces were washed three times with normal saline. Trypan blue staining was used to determine the viability percentage of the protoscoleces. Identifying the live protoscoleces as the non-stained ones, and the dead protoscoleces as the stained ones, a viability of 96±0.57% was recorded for the obtained samples.

Development and characterisation of terpenes and their microemulsions

The ordinary α-pinene and β-caryophyllene emulsions were prepared by adding these terpenoids (0.5% w/v) to glycerol monooleate (15% w/v) and polyoxyl hydrogenated 40 castor oil (5% w/v). The mixture was then dispersed in water and stirred for 30 min to form an emulsion. Development of microemulsions was done spontaneously in an oil phase glycerol monooleate, polyoxyl hydrogenated castor oil as surfactant, and PEG 400 as cosurfactant (1:8:1). Various amounts of α-pinene and β-caryophyllene were added to 10 g of oil phase and were stirred at 100 rpm for 2 h. One hour of sonication in a bath sonicator was done to complete the mixing process. Afterwards, deionised water at the ratio of 5:1 was added to the oil phase and stirred gently. Dynamic light scattering (DLS) was used to confirm the micro-dimensions of the developed formulations. The scattering intensity was assessed at an angle of 90° and at 25 °C (Rachmawati *et al.*, 2020).

In vitro scolicidal activity

The scolicidal activity of both ordinary and micro-emulsions of α-pinene, β-caryophyllene and their combinations at three different ratios (2:1, 1:1, and 1:2) was assessed after 10, 30, and 60 min of parasite exposure to a total terpene concentration of 0.5, 1, 2, 5 and 10 μg/mL. More specifically, one mL of each prepared ordinary and micro-terpene emulsion along with an equal volume of M199 medium (Biowest, France) containing about 10^3 viable protoscoleces of *E. granulosus* were added in a test tube and

mixed gently. All test tubes were then incubated at 37 °C and, from each of them, at each pre-established timepoint, 100 µL were taken for measuring mortality rates in the treated protoscoleces. Mortality rates were evaluated by eosin exclusion test using an optical microscope (Youssefi *et al.,* 2020). Protoscoleces that did not receive any treatment except the emulsion and microemulsion carriers were used as controls.

Statistical analysis

Data were analysed by using SPSS statistical package (version 25.0) (SPSS Inc., IL, USA). Differences between the means of mortality rate in different tested compounds at each time of exposure (10, 30, 60, and 120 min) were analysed by one-way analysis of variance (ANOVA), followed by Tukey-HSD *post hoc* test. Differences between the means of mortality rate in different exposure times in each concentration of tested compounds were analysed by Repeated measures ANOVA, followed by Bonferroni *post hoc* test. Fifty percent lethal concentration (LC_{50}) and ninety percent lethal concentration (LC_{90}) values were determined by subjecting mean mortality rates to Probit regression analysis. P values < 0.05 were considered statistically significant.

RESULTS

Microemulsions characterisation

Fig. 1 shows that all formulations were successfully developed at micrometric dimensions. Size distribution was significantly different in microemulsion of α-pinene and combination of α-pinene and β-caryophyllene at 1:1 ratio in comparison to all other microemulsions. Microemulsions of α-pinene and 1:1 α-pinene+β-caryophyllene presented larger distributions and particles relative to all other microemulsions that showed similar droplet size distributions.

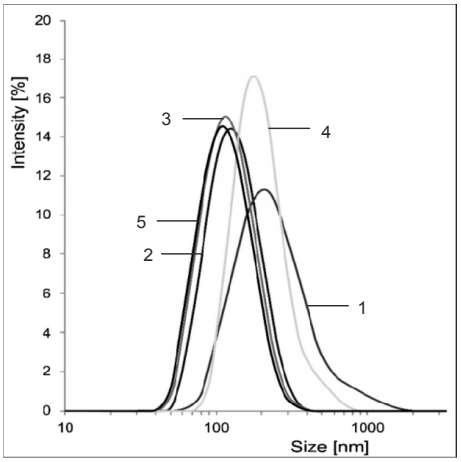

Fig. 1. Dynamic light scattering (DLS) of developed microemulsions of α-pinene (1), β-caryophyllene (2), 2:1 α-pinene+β-caryophyllene (3), 1:1 α-pinene+β-caryophyllene (4), and 1:2 α-pinene+β-caryophyllene (5).

Scolicidal activity

In the present study, a significant effect of concentration ($F_{3, 176}=130.5$, $P<0.001$) was noticed for all of the tested materials. Fig. 2 shows the mortality rates of *E. granulosus* protoscoleces over different times of exposure to ordinary and micro-formulated emulsions of α-pinene. At all of the time points, ordinary and micro-formulated α-pinene at all tested concentrations resulted in a significant mortality in comparison to the control group ($P<0.05$). At the 10, 30, and 60-minute time points, micro-formulated α-pinene at the concentration of 5 and 10 µg/mL killed 100% of the protoscoleces, while the ordinary α-pinene at the concentration of 5 µg/mL resulted in 86.66%, 94.66%, and 100% mortality rates, respectively. At

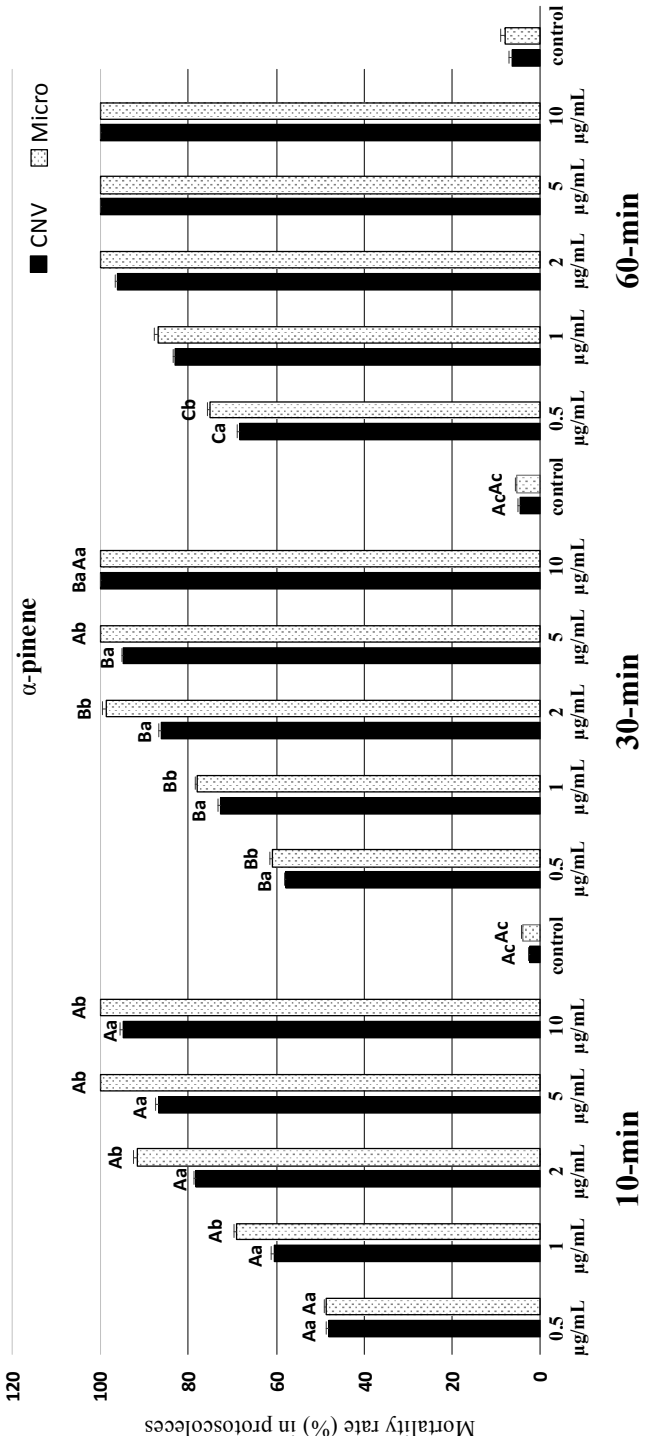

Fig. 2. Mortality (%) of *Echinococcus granulosus* protoscoleces over different times of exposure (10, 30, and 60 min) to ordinary (CNV) and micro-formulated (Micro) emulsions of α-pinene in comparison to control (without treatment). Data are presented as mean ± SEM (standard error of the mean). Within each concentration of tested compounds, columns with different letters (lowercase) are significantly different (ANOVA, Tukey's HSD test, $P<0.05$). Within each tested compound, columns marked with different letters (uppercase) are significantly different between time points (repeated measures ANOVA, Bonferroni test, $P<0.05$).

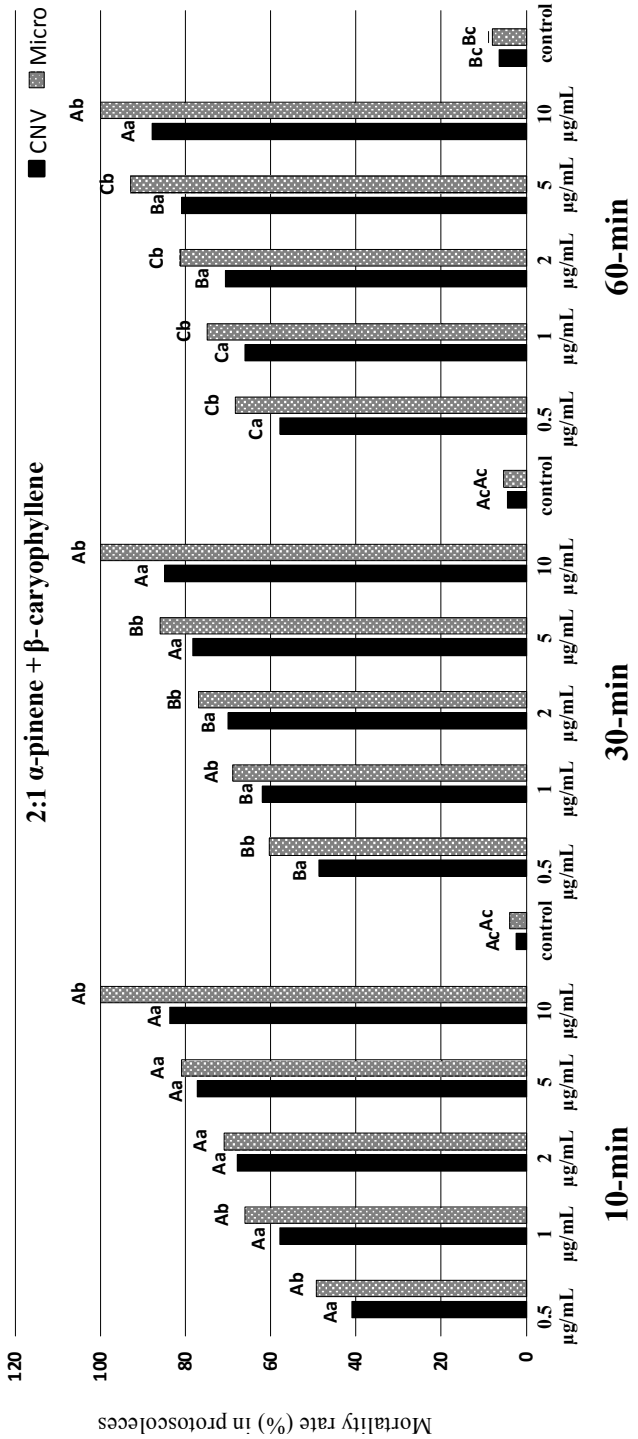

Fig. 3. Mortality (%) of *Echinococcus granulosus* protoscoleces over different times of exposure (10, 30, and 60 min) to ordinary (CNV) and micro-formulated (Micro) emulsions of 2:1 α-pinene+β-caryophyllene in comparison to control (without treatment). Data are presented as mean ± SEM (standard error of the mean). Within each concentration of tested compounds, columns with different letters (lowercase) are significantly different (ANOVA, Tukey's HSD test, $P<0.05$). Within each tested compound, columns marked with different letters (uppercase) are significantly different between time points (repeated measures ANOVA, Bonferroni test, $P<0.05$).

all of the tested concentrations and time points, except 10 µg/mL after 30-minute and 5 and 10 µg/mL after 60-minute exposure, there was a significant difference between ordinary and micro-formulated α-pinene ($P<0.05$).

Combination of α-pinene and β-caryophyllene at 2:1 ratio caused significant mortality in protoscoleces in comparison to the control at all of the tested concentrations ($P<0.05$) (Fig. 3). In the form of ordinary and micro-emulsions at concentration of 10 µg/mL 2:1 α-pinene+β-caryophyllene caused 83.66% and 100% mortality rates, respectively after 10 min of exposure. Ordinary 2:1 α-pinene+β-caryophyllene emulsions could not cause 100% mortality rates in protoscoleces even at the highest concentration and longest duration of treatment. Moreover, at 30 and 60-minute exposure to 2:1 α-pinene+β-caryophyllene at all of the tested concentrations, a significant difference was noted between ordinary and micro-emulsions ($P<0.05$).

After 10 min of treatment with combination of α-pinene and β-caryophyllene at 1:1 ratio, the ordinary and micro-emulsions resulted in significant mortality rates in comparison to the control treatment ($P<0.05$) (Fig. 4). At this time point, all of the tested concentrations, except 2 µg/mL resulted in mortality rates which were significantly different between ordinary and micro-emulsions ($P<0.05$). Longer duration of treatment caused higher mortality rates; however, mortality rates in ordinary emulsions were not different between 30 and 60-minute at the concentrations of 0.5, 1, and 5 µg/mL ($P>0.05$). The 60-minute exposure to 10 µg/mL of the ordinary and micro-emulsion of 1:1 α-pinene+β-caryophyllene resulted in the protoscoleces mortality rates of 79% and 93%, respectively.

The combination of 1:2 α-pinene+β-caryophyllene (10 µg/mL) after 10 minutes of exposure reached the mortality rates of 59 and 70.33%, respectively in the forms of ordinary and micro-formulated emulsions (Fig. 5). Longer exposure times increased the killing effect of ordinary and micro-emulsions of 1:2 α-pinene+β-caryophyllene; however, no significant difference was noted in comparison of mortality rates at consecutive exposure times for ordinary emulsion ($P>0.05$). There has been a significant difference between ordinary and micro emulsions at all tested time points and concentrations ($P<0.05$).

The ordinary and micro-emulsion of β-caryophyllene at all tested concentrations and time points led to significant mortality rates in protoscoleces ($P<0.05$) (Fig. 6). Neither the ordinary emulsion nor the microemulsion of β-caryophyllene could achieve total eradication of protoscoleces even at the highest concentration and longest duration of exposure. The killing rates of 57.33% and 77.66% were recorded at 10 µg/mL and 60-minute exposure for β-caryophyllene ordinary and micro-emulsion, respectively.

Table 1 shows 50% lethal concentration (LC_{50}) and 90% lethal concentration (LC_{90}) values of the tested compounds against *E. granulosus* protoscoleces. The microemulsion of α-pinene achieved the LC_{50} and LC_{90} values of 0.56 and 1.80 µg/mL, respectively. Considering LC_{50}, there was no significant difference between the ordinary and micro-formulated α-pinene; however, by LC_{90} values, the microemulsion of α-pinene was significantly more potent than the ordinary one (1.80 vs 5.89 µg/mL). Due to the overlapping 95% confidence limits, no significant difference was noted between LC_{50} and LC_{90} values of ordinary and micro-emulsi-

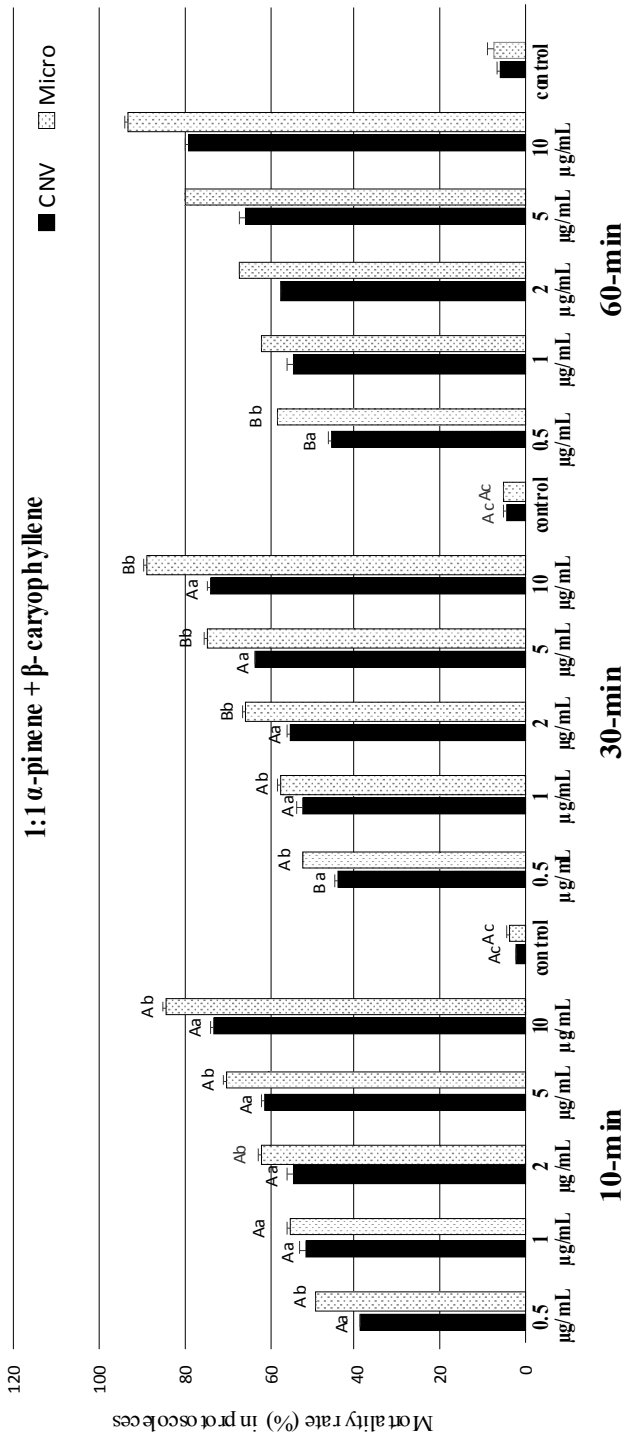

Fig. 4. Mortality (%) of *Echinococcus granulosus* protoscoleces over different times of exposure (10, 30, and 60 min) to ordinary (CNV) and micro-formulated (Micro) emulsions of 1:1 α-pinene+β-caryophyllene in comparison to control (without treatment). Data are presented as mean ± SEM (standard error of the mean). Within each concentration of tested compounds, columns with different letters (lowercase) are significantly different (ANOVA, Tukey's HSD test, $P<0.05$). Within each tested compound, columns marked with different letters (uppercase) are significantly different between time points (repeated measures ANOVA, Bonferroni test, $P<0.05$).

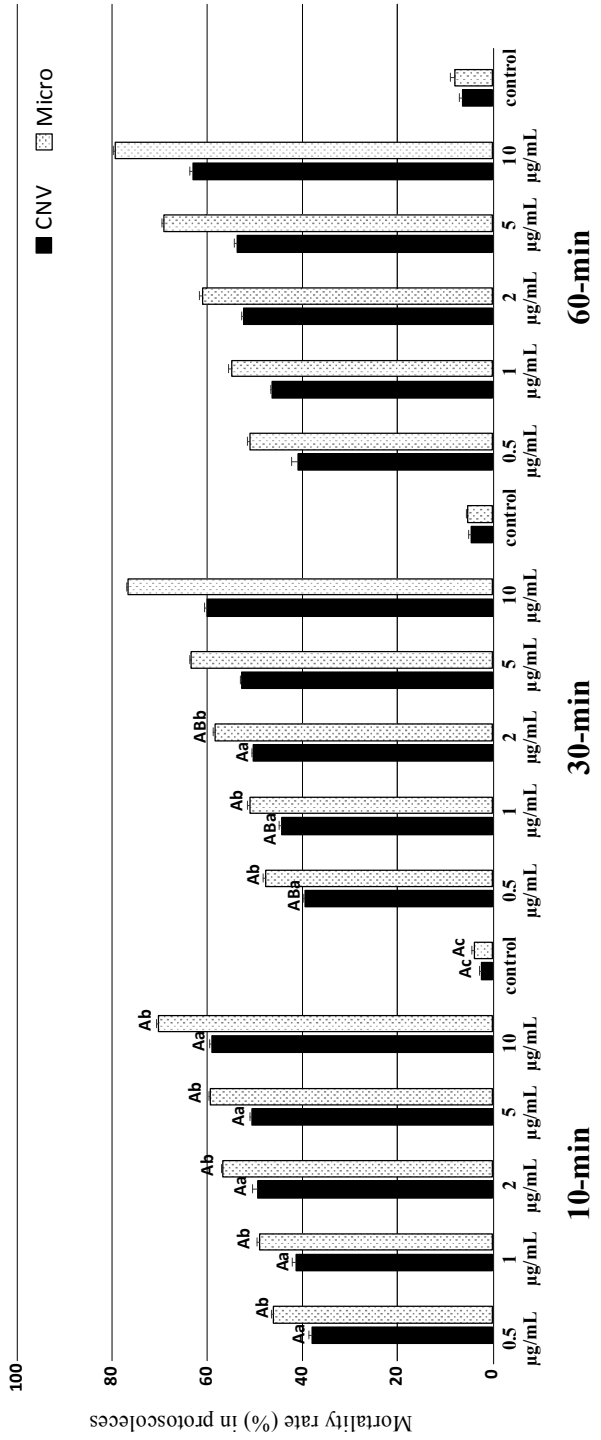

Fig. 5. Mortality (%) of *Echinococcus granulosus* protoscoleces over different times of exposure (10, 30, and 60 min) to ordinary (CNV) and micro-formulated (Micro) emulsions of 1:2 α-pinene+β-caryophyllene in comparison to control (without treatment). Data are presented as mean ± SEM (standard error of the mean). Within each concentration of tested compounds, columns with different letters (lowercase) are significantly different (ANOVA, Tukey's HSD test, $P<0.05$). Within each tested compound, columns marked with different letters (uppercase) are significantly different between time points (repeated measures ANOVA, Bonferroni test, $P<0.05$).

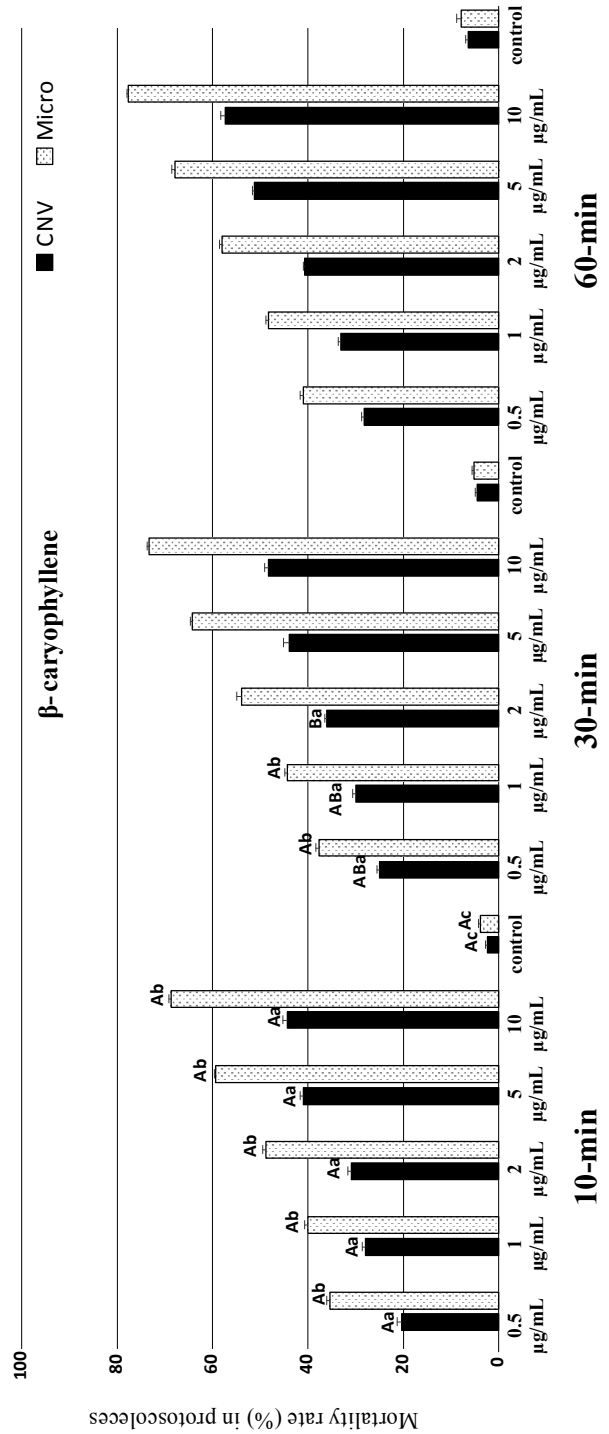

Fig. 6. Mortality (%) of *Echinococcus granulosus* protoscoleces over different times of exposure (10, 30, and 60 min) to ordinary (CNV) and micro-formulated (Micro) emulsions of β-caryophyllene in comparison to control (without treatment). Data are presented as mean ± SEM (standard error of the mean). Within each concentration of tested compounds, columns with different letters (lowercase) are significantly different (ANOVA, Tukey's HSD test, $P<0.05$). Within each tested compound, columns marked with different letters (uppercase) are significantly different between time points (repeated measures ANOVA, Bonferroni test, $P<0.05$).

Table 1. Fifty percent lethal concentration (LC_{50}) and ninety percent lethal concentration (LC_{90}) values of ordinary (CNV) and micro-formulated (Micro) emulsions of α-pinene and β-caryophyllene at different combination ratios at 10-minute time interval against *Echinococcus granulosus* protoscoleces

Tested compound	10 min LC_{50} (µg/mL) (LCL-UCL)	10 min LC_{90} (µg/mL) (LCL-UCL)	χ^2 (df)[a]
CNV			
α-pinene	0.56 (0.37–0.75)	5.89 (4.24–9.55)	0.91 (3) [ns]
2α + 1β	0.71 (0.42–1.01)	18.7 (10.58–50.03)	1.16 (3) [ns]
1α + 1β	1.22 (0.65–1.88)	158.34 (48.89–293.1)	1.13 (3) [ns]
1α + 2β	16.32 (7.67–87.47)	> 1000	0.57 (3) [ns]
β-caryophyllene	49.58 (45.17–62.17)	> 1000	0.48 (3) [ns]
Micro			
α-pinene	0.56 (0.45–0.65)	1.80(1.56–2.32)	2.59 (3) [ns]
2α + 1β	0.72 (0.47–0.96)	9.68 (5.72–15.45)	1.45 (2) [ns]
1α + 1β	0.64 (0.30–1.00)	39.39 (17.02–111.1)	1.93 (3) [ns]
1α + 2β	0.97 (0.30–1.71)	609 (500–894.62)	0.94 (3) [ns]
β-caryophyllene	2.10 (1.38–3.16)	> 1000	0.29 (3) [ns]

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

ons of α-pinene+β-caryophyllene at ratios of 2:1 and 1:1 (P>0.05). Unlike other compounds, development of microemulsions for 1:2 α-pinene+β-caryophyllene and β-caryophyllene significantly increased their scolicidal activity. Micro 2:1 α-pinene+β-caryophyllene showed LC_{50} value of 0.97 µg/mL which was significantly lower than that of the ordinary emulsion (16.32 µg/mL). For β-caryophyllene, LC_{50} value of the microemulsion (2.1 µg/mL) was significantly reduced relative to the ordinary emulsion (49.85 µg/mL) (P<0.05).

DISCUSSION

Cystic echinococcosis is one of the most important neglected tropical diseases with estimated annual economic loss of 194 million USD in infected human cases (Elissondo *et al.,* 2013). The global burden of this disease intensifies the urgent need for novel and safe scolicidal agents. Drug delivery systems can be used as an approach for increasing efficacy of biologically active compounds by enhancing their absorption, penetration to the site of action, and duration of their maintenance in the therapeutic window (Safari & Zarnegar, 2014). In the recent decade, several studies have focused on using novel drug delivery systems for the control and cure of cystic echinococcosis (Cheng *et al.*, 2009; Ahmadnia *et al.,* 2013; Rahimi *et al*., 2015; Farhadi *et al.,* 2018); however, only a limited number has evaluated plant derived bioactive compounds as the functional constituent of their formulations (Moazeni *et al.,* 2017).

Moazeni *et al.* (2017) have reported the *Zataria multiflora* essential oil nanoemulsion as a potent scolicidal agent. The nanoemulsion at the concentration of 1 mg/mL resulted in 88.01% mortality

rate after 10 min, and 100% after 20 min. Considering clinically relevant threshold of scolicidal activity, 100% killing activity at concentrations as high as 1 mg/mL and exposure time of as long as 20 minutes cannot be an effective treatment during surgery. However, it seems that α-pinene, β-caryophyllene and their combination especially in the form of micro-emulsions possess higher anticoccidial efficacy in comparison to the nanoemulsion of *Z. multiflora* and could be considered as possible scolicidal agents for perioperative applications, since rapid acting is a privilege for scolicidals which are injected to neutralise the cysts content. In line with the present study, Amaral *et al.* (2016) evaluated the effect of α-pinene and β-caryophyllene on *Trypanosoma evansi* in both under *in vitro* and *in vivo* conditions and demonstrated that each of these two compounds had promising *in vitro* anti-trypanosomal effects. The authors also reported that by combining α-pinene and β-caryophyllene, the *in vitro* lethal effects increased, and the combination caused significantly higher antitripanosomal activity in the animal model than the standard treatment (diminazene aceturate). However, in the present study, combining α-pinene and β-caryophyllene did not increase their scolicidal activity. It seems that at least regarding the bioactivity against *E. granulosus*, no synergistic interaction existed between these two terpenoids as in the combinational formulations adding to the proportion of β-caryophyllene relative to α-pinene resulted in some lower scolicidal effects. On the other hand, synergistic effect of β-caryophyllene in combination with other terpenes have been shown on *Trypanosoma cruzi* and it was reported that combinations of β-caryophyllene and capolic acid due to their high activity may lead to new alternative treatments for trypanosomiasis in the future (Izumi *et al.,* 2012). In line with the findings of our study, da Franca Rodrigues *et al.* (2015) demonstrated that α-pinene as the main constituent of *Syzygium cumini* essential oil was more effective (IC_{50} values of 15.6 µg/ mL) than the whole essential oil (IC_{50} values of 43.9 µg/mL) against intracellular amastigotes of *Leishmania amazonensis*.

From a mechanistic point of view, it has been reported that α-pinene exerts its antimicrobial activity through decreasing membrane integrity and enhancing microbial influx (Kovač *et al.,* 2015). Also, the increase in the nitric oxide production, lysosomal and phagocytic activity through modulation of immunity responses are reported as pathways of α-pinene biological toxicity on parasites (da Franca Rodrigues *et al.,* 2015). For β-caryophyllene, antimicrobial activity through alteration of the membrane permeability and integrity, membrane damage, and intracellular content leakage by affecting the expression of responsible genes have been previously described (Francomano *et al.,* 2019; Moo *et al.,* 2020). The two tested terpenoids, β-caryophyllene and especially α-pinene, may cause mortality in microbial pathogens by affecting the membrane integrity. Development of microemulsions in the present study especially for β-caryophyllene resulted in increased toxicity on *E. granulosus*, probably due to the higher penetration of the bioactive compounds into the protoscoleces.

CONCLUSIONS

The present study demonstrated that α-pinene, β-caryophyllene and their combinations, formulated as both ordinary and microemulsions can exert *in vitro* scoli-

cidal activity on *E. granulosus* after just 10 min of contact at concentrations ranging from 0.5 to 10 µg/mL. However, only some of the tested treatments produced a potentially clinically relevant mortality (90–100%) of the protoscoleces. Further studies are warranted to assess the *in vivo* efficacy and safety of these formulations.

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