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Original Article

Synergistic Effects of Combined Caryophyllene and Albendazole against Invaginated and Evaginated Protoscoleces of *Echinococcus granulosus*

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Abstract

Background: Natural compounds have garnered increasing attention as candidate antiparasitic therapeutics. We aimed to evaluate the protoscolicidal efficacy of caryophyllene (CAP), alone and in combination with albendazole (ALZ), against hydatid cyst protoscoleces.

Methods: Suspensions of 10,000 invaginated and evaginated protoscoleces (400 µL normal saline) were exposed to CAP (62.5–500 µg/mL), ALZ (250 µg/mL), or CAP+ALZ combinations in 24-well culture plates. Protoscolex viability was assessed by eosin exclusion test. Reactive oxygen species (ROS) induction was quantified using the DCFH-DA assay, and pre-apoptotic gene expression was analyzed by real-time PCR.

Results: CAP, particularly in combination with ALZ, significantly reduced the viability of both invaginated and evaginated protoscoleces in a concentration- and time-dependent manner. Combination therapy achieved 100% mortality of invaginated protoscoleces within 10–30 minutes, demonstrating significant synergism over either monotherapy. Evaginated protoscolex viability was also markedly diminished. The observed scolicidal activity was mechanistically correlated with apoptosis induction, evidenced by elevated ROS production and significant upregulation of caspase-3 and P53 gene expression ($P<0.001$).

Conclusion: CAP, particularly in combination with ALZ, exhibits potent *in vitro* protoscolicidal activity against hydatid cyst protoscoleces, with apoptosis induction and ROS generation implicated as underlying mechanisms. Further pharmacodynamic investigations and clinical trials are warranted to elucidate the precise mechanisms of action and evaluate the therapeutic potential of CAP-based combination therapy in the management of cystic echinococcosis.

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Introduction

Hydatid disease, recognized as a "neglected parasitic infection," is caused by the larval stage of the cestode *Echinococcus granulosus* (1). This zoonotic infection is transmitted from animals to humans and is prevalent in numerous regions worldwide, particularly in areas with extensive livestock farming (1). Human infection occurs incidentally through the ingestion of water or vegetables contaminated with *E. granulosus* eggs shed by dogs, or via direct contact with infected dogs (2). Although hydatid cysts are typically asymptomatic during the initial stages, progressive cyst growth exerts mechanical pressure on the involved organ, leading to complications (3). The disease severity and clinical presentation depend on the organ affected, the infection intensity, and the organ's susceptibility to cyst development (3).

Pharmacological treatment with benzimidazoles, such as albendazole or mebendazole, is advised for patients who are not candidates for surgery, those with cysts in multiple organs, and for postoperative management (4). Surgical intervention, while a primary treatment modality, is complicated by frequent recurrences due to the potential spillage of viable protoscoleces during cyst excision, underscoring the complexity of hydatid cyst surgery (4). To mitigate recurrence risk, surgeons administer scolicidal agents into the cyst cavity intraoperatively. Various chemical scolicides have been employed, including 2% formalin, 20%-30% hypertonic saline, 0.54% silver nitrate, and betadine solutions (5). However, the use of these agents is constrained by their associated adverse effects, such as hepatic and biliary fibrosis, allergic and hypersensitivity reactions, and damage or necrosis of adjacent tissues.

Now, compounds derived from natural supplies are acknowledged for their promising potential as applicants for antiparasitic therapeutics. Likewise, phytomedicines are progressively recognized as economical and safe alternatives to conventional treatment modalities (6). Caryophyllene ($C_{15}H_{24}$, CAP) is a prominent natural compound within the terpene class, particularly classified as a sesquiterpene, and is commonly present in the essential oils of various plants including cloves, cinnamon, rosemary, and species within the cannabis genus (6). Its distinctive chemical structure, notably the incorporation of a cyclobutane ring, underpins a range of biological activities such as anti-inflammatory, analgesic, antioxidant, and antimicrobial effects (7). In addition, CAP-rich herbs displayed efficacy in inhibiting the growth and proliferation of parasites like *Leishmania* spp., and *Trypanosoma* spp. (8). The proposed mechanisms underlying these effects involve disruption of parasite cell membranes, impairment of cellular energy production via mitochondrial inhibition, and enhancement of host immune responses targeting the parasites (9). Additionally, the antioxidant capacity of caryophyllene may contribute to mitigating oxidative stress-induced damage commonly associated with parasitic infections (10).

We aimed to evaluate the protoscolicidal effects of caryophyllene (CAP) alone and along with albendazole (ALZ) against protoscoleces of hydatid cysts.

Materials and Methods

Ethics

This work was approved the by the ethical committee of the Shahrekord University of Medical Sciences, Shahrekord, Iran (No. IR.SKUMS.REC.1403.019).

Chemicals

Caryophyllene (≥98.0 purity), albendazole (≥98.0 purity), and eosin dye were purchased from Sigma-Aldrich, Germany.

Collecting the protoscoleces

Sheep livers harboring hydatid cysts were obtained from Junaqan slaughterhouse (Iran)

and transported to the Parasitology Laboratory, Shahrekord Medical School, Iran. Large, unilocular cysts containing adequate protoscoleces were selected for analysis. Under sterile conditions, cyst surfaces were decontaminated sequentially with 70% ethanol and betadine solution. Cyst fluid was aspirated via syringe into 50 mL Falcon tubes; cysts were then incised with a sterile scalpel, and protoscoleces were aspirated, sedimented, and washed three times with phosphate-buffered saline (PBS) (11).

Viability examination

Protoscolex viability was assessed by mixing a drop of sediment with 0.1% eosin solution (Sigma-Aldrich, Germany) and examining under a 10× objective; protoscoleces absorbing the stain (orange-to-pink coloration) were classified as non-viable (Fig. 1). Only cysts with >90% viability, determined by evaluating 100 protoscoleces, were selected for further analysis. The protoscolex suspension was subsequently standardized to 2×10^3/mL (12).

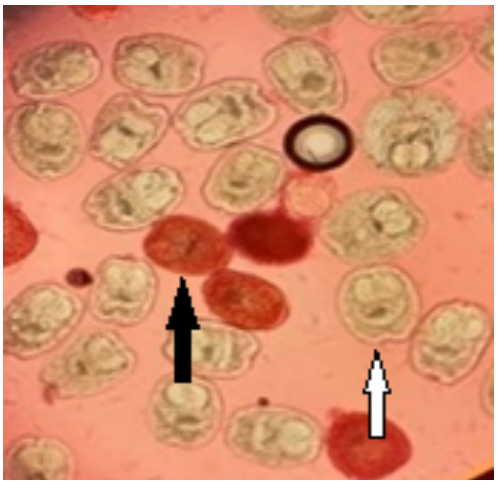

Fig. 1: Hydatid cyst protoscoleces stained with eosin dye (present study). Viable and non-viable protoscoleces are indicated by white and black arrow, respectively.

Antiparasitic Efficacy on invaginated protoscoleces

Following previously established protocols (13), suspensions of 10,000 protoscoleces in 400 µL normal saline were exposed to CAP (62.5–500 µg/mL), ALZ (250 µg/mL), or CAP+ALZ combinations in 24-well culture plates. Viability was assessed at 5–60 min intervals by eosin exclusion test: protoscoleces absorbing 0.01% eosin solution with orange-to-pink uniform staining of cellular contents were classified as non-viable. All experiments were performed in triplicate, and IC50 values were calculated using GraphPad Prism v8.4.3.

Antiparasitic Efficacy on evaginated protoscoleces

Biphasic culture

The biphasic culture medium consisted of a liquid phase comprising CMRL 1066 (Gibco), yeast extract, penicillin/streptomycin (Biosera), and 0.5% dog bile in PBS, and a solid phase of fetal bovine serum (Gibco). Approximately 10,000 protoscoleces (100 µL sediment) were initially transferred into CMRL 1066 supplemented with FBS and dog bile for 48-hour acclimatization, then transferred to solid-phase flasks with ~45 mL liquid medium and incubated at 37°C in a CO_2 incubator. To prevent aggregation, flasks were gently agitated every 4–6 hours, followed by continuous shaking via an in-incubator device. The liquid phase was renewed every 3–5 days, guided by protoscolex density, developmental stage, and medium color change. Only evaginated protoscoleces were employed for pharmacological assessment (22).

Effects on the viability of evaginated protoscoleces

Protoscolex viability was monitored daily by inverted microscopy throughout culture. Following 48-hour incubation, evaginated protoscoleces were exposed to CN concentrations of 25–200 µg/mL, with viability assessed at 5–60 minute intervals. All experiments were performed in triplicate, and IC50 values were determined using GraphPad Prism v8.4.3.

Inducing reactive oxygen species (ROS) generation

The induction of ROS by CAP alone and in combination with ALZ was evaluated employing the dichlorodihydrofluorescein diacetate (DCFH-DA) assay. Specifically, protoscoleces were incubated with 10 µM DCFH-DA (Sigma-Aldrich, Germany) at 37°C for one hour. Following this incubation, the protoscoleces were exposed to CAP alone and in combination with ALZ for 180 minutes. Fluorescence intensity, corresponding to ROS levels, was measured at excitation/emission wavelengths of 488/525 nm over a four-hour timeframe using a microplate reader (14).

Expression level of the pre-apoptotic genes

mRNA extraction

Total RNA was extracted from CAP-, ALZ-, and combination-treated protoscoleces using the SinaPure™ RNA kit (SinaClon, Iran), followed by DNase I treatment to eliminate DNA contamination. RNA quantity and integrity were assessed by Nanodrop spectrophotometry and agarose gel electrophoresis, respectively.

Complementary DNA (cDNA) synthesis

cDNA synthesis was performed using the Yekta Tehiz kit (Iran) per manufacturer's instructions, with successful synthesis confirmed by target gene amplification and gel visualization; cDNA was stored at −80°C until use.

Primer design

Primers for caspase-3, P53, and β-actin were designed based on Ensembl sequence data using Gene Runner software, with secondary structure and primer-dimer analysis conducted via Oligo Analyzer, in silico PCR validation using the UCSC tool, and specificity confirmed by NCBI Primer-BLAST (Table 1) (15, 16).

Table 1: Primer sequences utilized for real-time polymerase chain reaction (PCR) analysis

Primer	Sequence (5'–3')
P53	F: AACCACCGAACTCACAAC R: AACCGACACAACTCATCAA
Caspase-3	F: TGGTTCATCCAGTCGCTTTG R: CATTCTGTTGCCACCTTTCG
β-actin	F: GTGACGTTGACATCCGTAAAGA R: GCCGGACTCATCGTACTCC

qRT-PCR analysis

qRT-PCR was performed on a Rotor-Gene system (Qiagen, Germany) using SYBR Green master mix (Yekta Tajhiz Azma) in a 25 µL reaction containing 7 µL master mix, 1 µL each primer (10 pM), 3 µL cDNA, and 13 µL sterile water. Thermal cycling comprised initial denaturation at 96°C for 10 min, followed by 39 cycles of 96°C denaturation, 61°C annealing (30 sec), and 73°C extension (30 sec). Relative gene expression was quantified using the $2^{-}\Delta\Delta Ct$ method with β-actin as the reference gene (15, 16).

Statistical analysis

Following data collection, the dataset was input into the statistical software SPSS version 26 (IBM Corp., Armonk, NY, USA) for analysis, primarily utilizing one-way ANOVA. Additionally, post hoc tests were conducted to evaluate comparative effects among groups. The findings were presented through relevant tables and statistical graphs. Where correlation analyses were warranted, suitable statistical tests, including the chi-square test with a significance threshold of $P < 0.05$, were employed.

Results

In vitro Scolicidal Activity Against Invaginated Protoscoleces

CAP significantly reduced invaginated protoscolex viability in a concentration- and time-dependent manner (Fig. 2). At 60 min, mortality

rates of 39.7%, 46.8%, 78.9%, and 100% were recorded at 62.5, 125, 250, and 500 µg/mL, respectively, with IC50 values of >500, >500, 370.9, 260.9, and 181.4 µg/mL at 5, 10, 20, 30, and 60 min, respectively. CAP+ALZ (250 µg/mL) combination therapy achieved 100% mortality at 62.5, 125, 250, and 500 µg/mL after 30, 20, 10, and 10 min, respectively, with IC50 values of 171.4, 75.9, 34.8, <30, and <30 µg/mL at 5, 10, 20, 30, and 60 min, respectively.

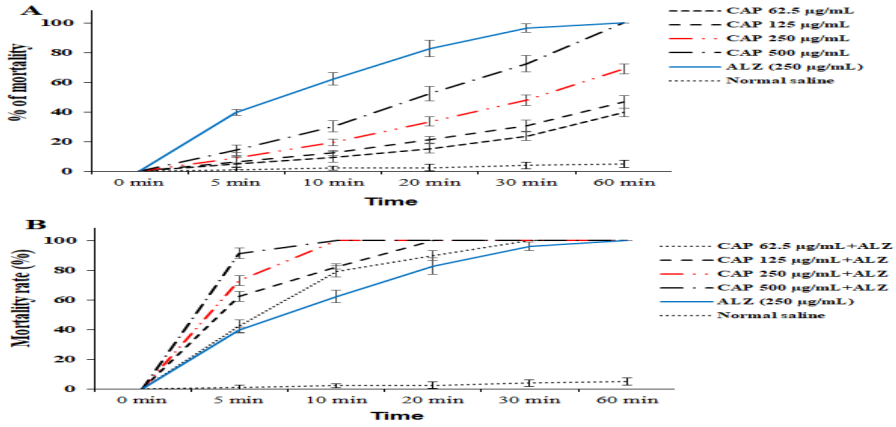

Fig. 2: The effect of caryophyllene (CAP) and albendazole (ALZ) alone (A) and in combination (B) on the viability of hydatid cyst invaginated protoscoleces was evaluated using the eosin exclusion test. Results are presented as mean ± standard deviation (N=3). Statistical significance was determined via one-way ANOVA with Tukey's post-hoc analysis

In vitro Scolicidal Activity Against Evaginated Protoscoleces

Microscopic analysis revealed that CAP reduced evaginated protoscolex viability in a dose- and time-dependent manner (Fig. 3). After 60 min, mortality rates of 30.2%, 41.3%, 59.6%, and 100% were recorded at 62.5, 125, 250, and 500 µg/mL, respectively, with IC50 values of >500, >500, 486.4, 376.5, and 209.7 µg/mL at 5, 10, 20, 30, and 60 min, respectively. CAP+ALZ (250 µg/mL) combination therapy markedly enhanced scolicidal efficacy, achieving mortality rates of 38.6%, 66.6%, 79.6%, and 100% at 62.5, 125, 250, and 500 µg/mL CAP after only 10 min of exposure, with corresponding IC50 values of 207.9, 93.8, 37.4, 65.3, and 33.7 µg/mL at 5, 10, 20, 30, and 60 min, respectively.

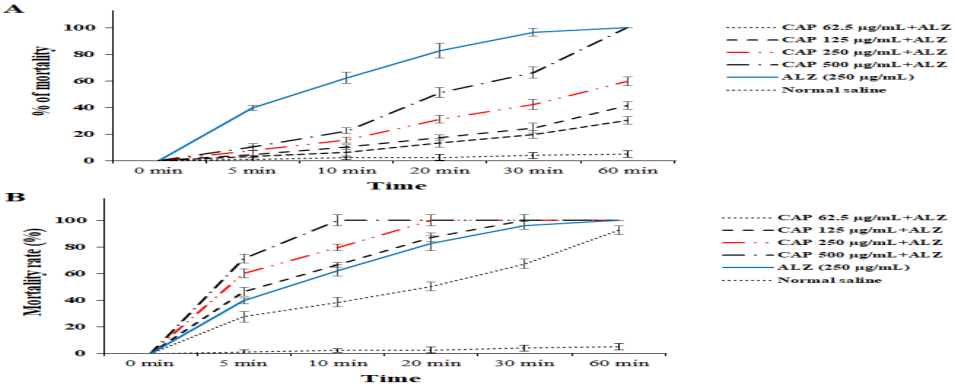

Fig. 3: The effect of caryophyllene (CAP) and albendazole (ALZ) alone (A) and in combination (B) on the viability of hydatid cyst evaginated protoscoleces was evaluated using the eosin exclusion test. Results are presented as mean ± standard deviation (N=3). Statistical significance was determined via one-way ANOVA with Tukey's post-hoc analysis

Inducing ROS generation

The findings from the DCFH-DA assay demonstrated that the mean fluorescence intensities were 7.3, 13.9, 25.3, and 29.3 arbitrary units following exposure of protoscoleces to CAP at half the IC_{50} concentration, CAP at the IC_{50} concentration, a combination of ALZ and CAP at half the IC_{50} concentration, and a combination of ALZ and CAP at the IC_{50} concentration, respectively. ANOVA with Tukey's post-hoc analysis revealed that these increases in fluorescence intensity were statistically significant (P<0.001) when compared to the control group treated with normal saline (Fig. 4).

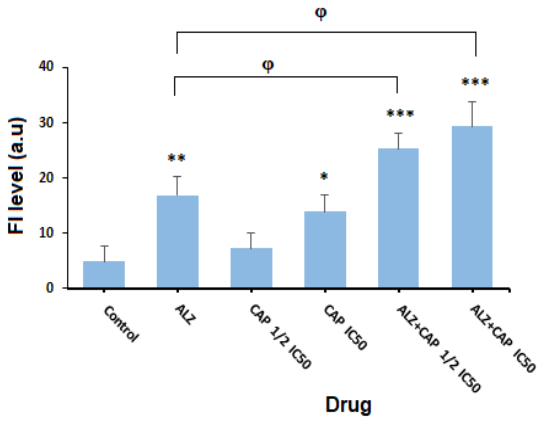

Fig. 4: The impact of caryophyllene (CAP) alone, as well as in combination with albendazole (ALZ, 250 µg/mL), on the induction of reactive oxygen species (ROS) in hydatid cyst protoscoleces was evaluated using the dichlorodihydrofluorescein diacetate (DCFH-DA) assay. Results are presented as mean ± standard deviation (N=3). *P<0.05; ** P<0.01; and *** P<0.001 compared to normal saline. φ P<0.05 compared to AL; Statistical significance was determined via one-way ANOVA with Tukey's post-hoc analysis

Expression level of the pre-apoptotic genes

Real-time PCR analysis demonstrated that following exposure of protoscoleces to CAP and the combination of CAP+ALZ, the expression levels of the *caspase-3* and *P53* genes were significantly (P<0.001) elevated (Fig. 5). ANOVA followed by Tukey's post-hoc test revealed that the expression levels of the caspase-3 gene were significantly (P<0.05) up-regulated in protoscoleces exposed to CAP at half the IC50 concentration, CAP at the IC50 concentration, the combination of ALZ and CAP at half the IC50 concentration, and the combination of ALZ and CAP at the IC50 concentration. Specifically, the fold changes in gene expression were -1.78, -2.12, -3.11, and -4.32, respectively, relative to the control group. Conversely, the expression levels of the P53 gene after exposure to CAP at 1/2 IC50, CAP at IC50, ALZ+CAP at 1/2 IC50, and ALZ+CAP at IC50 were 1.56, -1.98, -4.11, and -5.39-fold changes, respectively, compared to the control group.

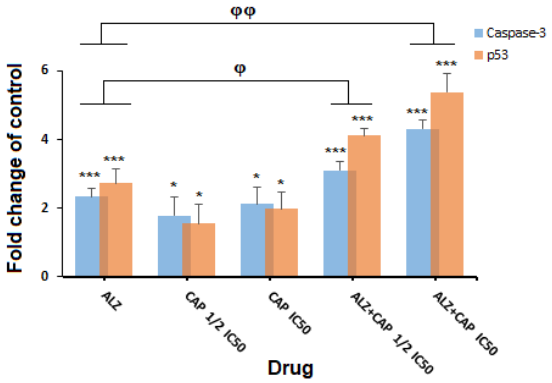

Fig. 5: The impact of caryophyllene (CAP) alone, as well as in combination with albendazole (ALZ, 250 µg/mL), on the expression of pre-apoptotic genes in hydatid cyst protoscoleces was evaluated using Real-time PCR. Results are presented as mean ± standard deviation (N=3). *P<0.05; ** P<0.01; and *** P<0.001 compared to normal saline. φ P<0.05; φφ P<0.001 compared to ALZ. Statistical significance was determined via one-way ANOVA with Tukey's post-hoc analysis

Discussion

The present study sought to assess the protoscolicidal efficacy of CAP and to investigate its underlying cellular mechanisms, both independently and in conjunction with ALZ,

against hydatid cyst protoscoleces. Our results showed the potent protoscolicidal effects of CAP mainly combined with ALZ against invaginated and evaginated hydatid cyst protoscoleces. Recent studies have demonstrated that combination therapy involving synthetic pharmaceuticals and natural compounds has garnered significant interest as an innovative strategy for addressing parasitic diseases (17). Natural products, through their enhancing, synergistic, or complementary effects, have the potential to augment the efficacy of antiparasitic agents, thereby enabling dose reduction of synthetic drugs and minimizing associated adverse effects (18). By targeting distinct metabolic pathways or modulating the host immune response, these natural compounds not only enhance therapeutic outcomes but also contribute to the prevention of drug resistance development in parasites (19). Consequently, the integration of chemical drugs with herbal constituents is regarded as a promising approach for the advancement of more effective and sustainable antiparasitic treatments (19).

Regarding the antiparasitic properties of CAP, investigations involving leaf extracts of *Pelargonium quercetorum*, which contain CAP, have demonstrated inhibitory effects against *Leishmania major* parasites; however, these effects were not observed to be dose-dependent (20). Additionally, another study reported that the essential oil derived from *Protium ovatum* leaves, also containing CAP, exhibited notable activity against the trypomastigote forms of *Trypanosoma cruzi* (21). The presence of CAP in certain plant extracts has been associated with their traditional application in the treatment of parasitic infections, suggesting a potential synergistic antiparasitic effect when combined with other bioactive compounds (22). The antimicrobial activity of caryophyllene is primarily attributed to its ability to disrupt the cell membrane, thereby enhancing membrane permeability and inducing the leakage of intracellular constituents. This compromise of membrane integrity constitutes a fundamental mechanism underlying its antibacterial and antifungal properties (23,24).

Concerning the involvement of ROS in the regulation and death of hydatid cyst protoscolices, research indicates that ROS play a significant role in eliminating these protoscolices by inducing cellular damage and death, thereby representing a potential therapeutic target (24). Nonetheless, *Echinococcus granulosus* parasites possess intrinsic antioxidant defense mechanisms that mitigate host-generated ROS, helping to maintain the host-parasite equilibrium (24). When ROS concentrations exceed the parasite's antioxidant capacity, oxidative damage occurs to the parasite's DNA, lipids, and proteins, ultimately resulting in loss of viability and cell death (24). It has been proven that CAP can activate MAPK signaling pathways, leading to an increase in reactive oxygen species (ROS) in eukaryotic cells (25). Our results reported the CAP mainly combined with ALZ markedly induced the ROS generation against hydatid cyst protoscoleces, proposed that CAP primarily showed its protoscolicidal effects by increasing the generation of ROS.

Apoptosis serves a dual function in the inhibition of parasites: it operates as a host defense strategy by eliminating infected cells, thereby restricting the propagation of the parasite (27). The results of the present study indicate that the induction of apoptosis serves as an effective mechanism for the elimination of hydatid cyst protoscoleces. Apoptosis offers several advantages over necrosis, notably the mitigation of inflammation and the avoidance of extensive immune responses (28). These characteristics render apoptosis a viable therapeutic target for managing hydatid cysts (28). In this investigation, molecular markers indicative of apoptosis was significantly uplregulated in protoscoleces following treatment with CAP alone or in combination with ALZ. Specifically, the activation of caspases, which are critical apoptotic markers, was prominently observed in treated specimens. These findings

align with those reported in prior research. One hypothesized mechanism underlying apoptosis induction in protoscoleces involves the elevated generation of ROS and consequent mitochondrial damage. This pathway may facilitate rapid and effective cell death, particularly in parasitic cells that lack sophisticated antioxidant defense mechanisms (29). Collectively, the data suggest that apoptosis induction represents a promising and targeted approach for hydatidosis therapy. These insights contribute to the advancement of novel pharmacological agents and non-surgical interventions characterized by enhanced efficacy and reduced adverse effects. Previous studies have demonstrated that ROS generation induced by CAP can initiate apoptosis, a regulated cell death mechanism, as indicated by elevated Annexin V binding, disruption of mitochondrial membrane potential, and activation of caspases (30). Therefore, it is reasonable to propose that the use of CAP can effectively trigger apoptosis, thus facilitating the removal of protoscoleces.

Notwithstanding the encouraging results obtained, this study is subject to certain limitations. Primarily, its exclusively *in vitro* design does not account for the physiological complexities inherent to living organisms, nor does it consider pharmacokinetic factors such as absorption, distribution, metabolism, and excretion (ADME). Additionally, the elevated concentrations of CAP employed in the experiments prompt concerns regarding the therapeutic viability and potential toxicity of the treatment. Therefore, it is imperative to conduct preclinical investigations using in vivo models to validate the efficacy, establish a safe dosage range, and thoroughly assess the toxicity profile of this therapeutic regimen.

Conclusion

Recent investigation revealed promising *in vitro* effects of CAP, particularly when combined with ALZ, on invaginated and evaginated hydatid cyst protoscoleces. Furthermore, the study highlighted potential underlying mechanisms, including the induction of apoptosis and the generation of ROS, in the control of hydatid cyst protoscoleces. Nonetheless, additional research is necessary to elucidate the precise mechanisms of action and to evaluate the efficacy of this approach in clinical trials, which could support the integration of CAP into therapeutic strategies for the treatment and management of cystic echinococcosis.

Declaration of interest

The authors declare that there is no conflict of interest.

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