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Original Article

Investigating the Lethal Effects of Carvone on the Protoscoleces of Hydatid Cyst, *In Vitro* and *Ex Vivo*

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Abstract

Background: Medicinal plants and their derivatives due to having high availability, low cost, low toxicity, and high efficacy are recognized as significant reservoirs of a diverse array of valuable therapeutic compounds. We aimed to investigate the lethal effects of carvone (CV) on protoscoleces of hydatid cyst, in laboratory and *ex vivo* conditions.

Methods: The protoscolicidal effects of CV were investigated both *in vitro* and *ex vivo* on hydatid cyst protoscoleces using the eosin exclusion assay. Furthermore, the impact of CV on the induction of apoptosis and DNA damage in the protoscoleces was assessed through a colorimetric protease assay and Real-time PCR analysis, respectively.

Results: CV, particularly at a concentration of 150 µg/ml, effectively eradicated protoscoleces of hydatid cysts within a 20-minute exposure period. Furthermore, CV exhibited sustained anti-parasitic effects in the *ex vivo*, leading to the complete elimination of hydatid cyst protoscoleces after a 30-minute exposure, in contrast to its immediate lethal effects observed in vitro. CV concentrations of 1/3 IC_{50}, 1/2 IC_{50}, and IC_{50} ($P < 0.001$) resulted in caspase-3 activation levels of 11.3%, 19.8%, and 28.4%, respectively. The expression levels of the *EgATM* and *EgP53* genes significantly upregulated after treatment with CV ($P<0.001$).

Conclusion: The findings demonstrated the potential of CV to eliminate protoscoleces by inducing apoptosis and causing DNA damage. However, further studies are required to clarify the specific mechanisms underlying its action and to assess its efficacy in clinical trials, which may facilitate the application of CV in the context of hydatid cyst surgical procedures.

Introduction

Echinococcus granulosus, a zoonotic tapeworm, is a cestode parasite responsible for cystic echinococcosis (CE), also known as hydatid disease (1). The incidence of human CE infection is widespread globally, with the majority of cases occurring in low- and middle-income countries (1). The life cycle of *E. granulosus* is characterized by canid species serving as definitive hosts and herbivorous or omnivorous species acting as intermediate hosts. Human infection typically occurs through the inadvertent consumption of eggs found in soil, water, or vegetables contaminated with feces from infected canines (2). Hydatid cysts, representing the larval stage or metacestode, form within the internal organs, predominantly the liver and lungs, of intermediate hosts (2). The larval stages of *E. granulosus* are characterized by a cyst wall composed of two distinct layers, along with cyst fluid and protoscoleces (2). As the sole infectious form of the larval stages, PSCs possess the capability to engage with both definitive and intermediate hosts (2).

In humans, hydatid cysts primarily found in the liver and lungs; however, they can also be found in the abdominal cavity and even nervous system, resulting in a wide range of clinical presentations (2). In Iran, analyses indicated that the prevalence of cystic echinococcosis (CE) was markedly elevated in the northern (9%) and western regions (6%) of the country, particularly among patients under 40 years of age (7%), as well as among rural and nomadic populations (6%). Furthermore, studies employing a combination of serological, clinical, and imaging diagnostic techniques reported higher prevalence rates (2).

Presently, there are some main treatment options for CE: surgical resection, PAIR (puncture, aspiration, injection, and re-aspiration), and chemotherapy (3). Surgery, often combined with adjuvant chemotherapy, is the primary treatment modality employed worldwide; however, this approach is not universally applicable to all stages of cysts (4). To enhance the safety of cyst surgery, the use of appropriate scolicidal agents is essential. Various materials and techniques have been explored in this context, many of which are associated with adverse effects. For instance, the injection of hypertonic saline, formalin, silver nitrate, and cetrimide into cysts has been documented, yet these substances often lead to complications such as leakage and necrosis in surrounding healthy tissues (5,6). Consequently, selecting an effective pharmacological agent to mitigate the risk of recurrence is of utmost importance.

Medicinal plants and their derivatives due to having high availability, low cost, low toxicity, and high efficacy are recognized as significant reservoirs of a diverse array of valuable therapeutic compounds (6). Carvone (CV, $C_{10}H_{14}O$), a monoterpene ketone, is found in the essential oils of numerous plant species, including those from the genera *Mentha*, *Thymus*, and *Rosmarinus* (7). CV is produced and released as a secondary metabolite of essential oils, although its primary function within the plant remains unclear (7). CV possesses significant medicinal properties, particularly its neuroprotective effects, suggesting its potential development as a therapeutic agent for various conditions, including depression, sedation, pain management, and seizure disorders (8). Furthermore, contemporary medical studies have validated the efficacy of carvone as an antifungal, antiparasitic, antibacterial, anticancer, anti-inflammatory, and antioxidant agent (8,9). In recent years, the antiparasitic properties of specific medicinal plants and their derivatives, such as *Zataria* spp., *Curcuma* spp., *Mentha* spp., and *Allium* spp., have been evaluated through both *in vitro* and *in vivo* studies targeting *E. granulosus* protoscoleces and hydatid cysts (10, 11). Nevertheless, the findings of these investigations are constrained by

various factors, including insufficient comprehension of the underlying mechanisms of action and apprehensions regarding their potential toxicity.

Taking into account the explanations provided, as well as the biological activities of CV, we aimed to investigate the anthelminthic, apoptosis induction, and DNA damage effects of carvone, a natural monoterpene against hydatid cyst protoscoleces *in vitro* and *ex vivo*.

Materials and Methods

Ethics

This project was approved by the Ethics Committee of Lorestan University of Medical Sciences and with ethics code IR.LUMS.REC.1402.217.

Preparation of Carvone

Carvone ($C_{10}H_{14}O$) with a purity of 98% was purchased from Sigma-Aldrich, Germany.

Protoscoleces preparation

Initially, livers harboring cysts are procured from infected sheep at the Khorramabad slaughterhouse and subsequently transported to the laboratory of the Department of Parasitology. Under sterile conditions, the necessary protoscoleces are extracted from the cysts within the liver and placed into sterile tubes and washed with sterile normal saline at least three times (12).

In vitro investigating the protoscolicidal effect

Following the acquisition of living protoscoleces, we assess their viability percentage through the observation of flame cell movement and staining with 0.1% eosin (prepared by dissolving one gram of eosin in 1000 cc of distilled water). A total of 100 protoscoleces are examined, with those that do not exhibit eosin staining classified as viable. Only cysts demonstrating a viability rate exceeding 90% are selected for subsequent testing. First, 20% NaCl (as a positive control) is placed alongside a suspension of protoscoleces (1×10^3 per mL) and their viability is evaluated at intervals of 5, 10, 20, 30, and 60 minutes. Concurrently, sterile normal saline supplemented with Tween 20 serves as a negative control. It is important to note that all procedures are conducted in triplicate using 48-well plates (13). The 50% inhibitory concentration (IC_{50}) is determined through Probit analysis utilizing SPSS software version 25.0.

Ex vivo investigating the protoscolicidal effect

In this phase of the procedure, the liver of sheep that are naturally infected with hydatid cysts is utilized. Initially, over 50% of the hydatid fluid is aspirated to isolate protoscoleces, and their viability is subsequently verified using a 0.1% eosin staining test. For each target concentration of Carvone, three hydatid cysts will be utilized. Carvone will be administered until the entire inner surface of each cyst is saturated. Subsequently, a small volume of cyst fluid will be extracted at specified time intervals (7, 10, 12, 15, 20, 25, and 30 minutes). In the following step, this fluid will be mixed with 0.1% eosin. After a duration of 10 minutes, a smear will be prepared from the remaining protoscoleces and placed on a glass slide for examination under a light microscope, with the objective of assessing the viability of the protoscoleces (14). The IC_{50} value was determined through Probit analysis using SPSS software version 25.0.

Evaluating the Caspase-3-Like Activity

This was accomplished using a commercially available caspase-3 activity kit (Sigma-Aldrich, Germany), following the manufacturer's instructions. The assay was conducted by assessing the spectrophotometric changes in color resulting from the release of a molecule (pNA linked to the substrate) due to the enzymatic action of caspase 3. Following a 2-day exposure of protoscoleces to FMEO, the samples were centrifuged at 600 rpm for 5 minutes at 4 °C. The resulting sedimented

protoscoleces were subsequently lysed and subjected to a second centrifugation at 20,000 rpm for 10 minutes. In the subsequent step, 5 µg of the supernatant was combined with 85 µL of buffer and 10 µL of the caspase-3 substrate (pNA-DEVD-Ac), and the mixture was incubated for 2 hours at 37 °C. The final absorption of the samples was measured at 405 nm using an ELISA reader (15).

Effect on the expression of DNA damage related genes by qRT-PCR

The effect of CV on the expression levels of DNA damage related genes of *E. granulosus* ataxia-telangiectasia mutated (EgATM) and EgP53 in the protoscoleces after treatment with CV was examined utilizing real-time PCR methodologies. Total RNA was extracted using a commercial kit (Parstous, Iran) in accordance with the manufacturer's guidelines. The conversion of RNA to complementary DNA (cDNA) was performed with a kit from Sinaclon Company, Iran, following the provided instructions. The primers used for *EgATM* (F': GTTCCTACAGTCCATCCTAAT and R': CTCCATCAAGCCAGCATT) and *EgP53* (F': AACCACCGAACTCACAAC and R': AACCGACACAACTCATCAA) in this study were derived from previous research by Lu et al (16). The real-time PCR protocol commenced with an initial denaturation phase at 92 °C for a duration of 10 minutes. This was succeeded by 40 cycles, each consisting of a denaturation step at 97 °C for 10 seconds, followed by an annealing and extension phase at 55 °C for 40 seconds. The expression levels of the *EgATM* and *EgP53* genes were measured utilizing the optical system software (iQTM5 model, Bio-Rad, Hercules, CA) by employing the $2^{-\Delta\Delta CT}$ method, with β-actin designated as the housekeeping gene.

Data analysis

After data collection, descriptive statistics, calculation of central and dispersion indices were used to describe the data. ANOVA, Tukey's and Post-hoc tests were used to analyze the data. All statistical tests were performed using SPSS version 26 software and two-tailed, and $P<0.05$ was considered as the significance level.

Results

In vitro investigation of the protoscolicidal effect

The findings indicate that CV, at a concentration of 150 µg/ml effectively killed protoscoleces of hydatid cysts within a 20-minute exposure period. In contrast, a concentration of 75 µg/ml achieved complete destruction of protoscoleces after 60 minutes of exposure. Among the concentrations evaluated, 32.5 µg/ml exhibited the lowest level of protoscoleces viability, resulting in an 89.4% reduction after 60 minutes of exposure. Overall, the data demonstrate that CV at varying concentrations (32.5, 75, and 150 µg/ml) significantly diminished the viability of hydatid cyst protoscoleces ($P < 0.001$) when compared to the control group (normal saline), as shown in Fig. 1. The IC_{50} values for CV at time intervals of 5, 10, 20, 30, and 60 minutes were found to be 111.6, 94.4, 47.4, 16.4, and 12.7 µg/mL, respectively.

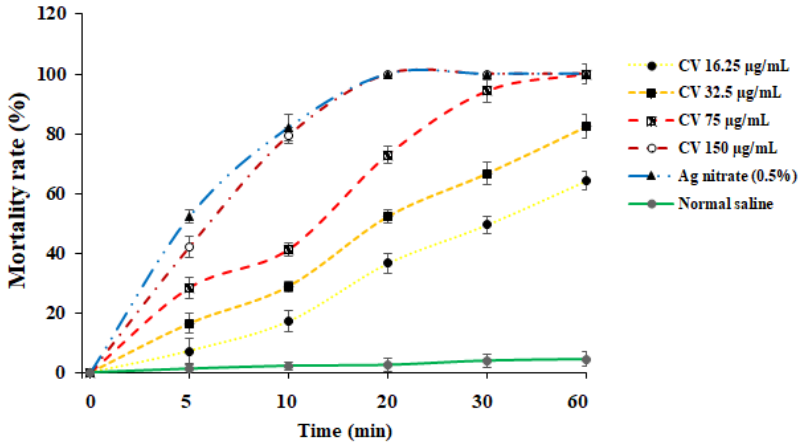

Fig. 1: In vitro lethal effects of various concentrations of carvone (CV) on hydatid cyst protoscoleces after 5, 10-, 20-, 30-, and 60-minutes exposure time by eosin exclusion assay. (Mean±SD). n=3

Ex vivo investigation of the protoscolicidal effect

CV demonstrates extended anti-parasitic activity in an ex vivo context, in contrast to its lethal effects observed in vitro. Notably, at a concentration of 150 µg/ml, CV effectively eradicated hydatid cyst protoscoleces following a 30-minute exposure period. Conversely, a concentration of 75 µg/ml led to the destruction of 93.1% of protoscoleces after a 60-minute exposure duration. Consistent with the *in vitro* results, CV demonstrated the least protoscolicidal activity among the concentrations tested, achieving a 66.9% reduction in protoscoleces after 60 minutes. Moreover, the data demonstrated that CV, at concentrations of 75, 150, and 300 µg/ml, significantly ($P<0.001$) increased the mortality of protoscoleces obtained from hydatid cysts in an *ex vivo* setting when compared to the control group (Fig. 2). The IC_{50} values for CV at time intervals of 5, 10, 20, 30, and 60 minutes were found to be >150, 133.2, 59.7, 29.6, and 20.6 µg/mL, respectively.

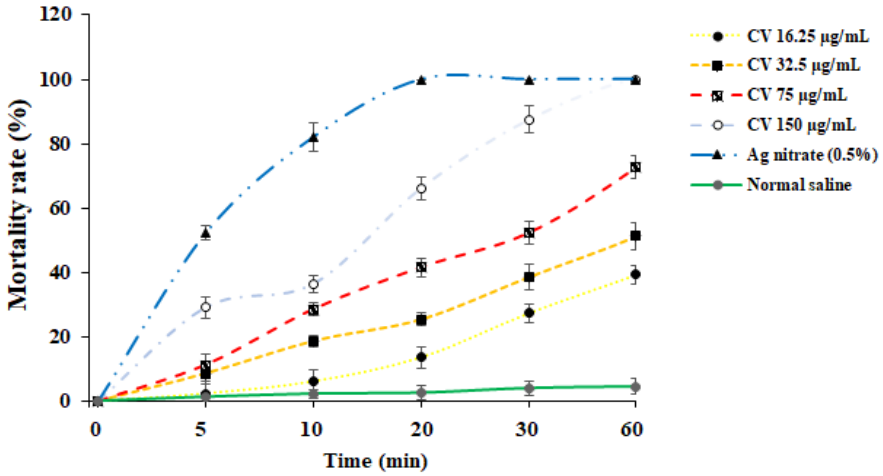

Fig. 2: Ex vivo lethal effects of various concentrations of carvone (CV) on hydatid cyst protoscoleces after 5, 10, 20, 30, and 60 minutes exposure time by eosin exclusion assay. (Mean±SD). n=3

Evaluating the Caspase-3-Like Activity

Fig. 3 illustrates the effects of various concentrations of CV on caspase-3-like activity in hydatid cyst protoscoleces. CV concentrations of 1/3 IC50, 1/2 IC50, and IC50 ($P < 0.001$) resulted in caspase-3 activation levels of 11.3%, 19.8%, and 28.4%, respectively.

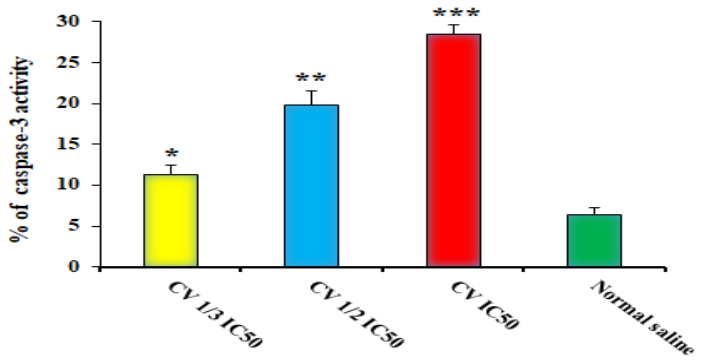

Fig. 3: Evaluating the caspase-3-like activity of hydatid cyst protoscoleces after exposure to various concentrations of carvone (CV) by the colorimetric protease assay. The IC50 value, representing the concentration required to inhibit 50% of the target (N = 3)

Effect on the expression of DNA damage related genes

The findings from the Real-time PCR analysis showed that the expression levels of the *EgATM* and *EgP53* genes were significantly upregulated (Fig. 4), with increases of 7.3, 6.3, 10.6, and 7.9 times, respectively, after treatment with CV at concentrations of 1/3 IC50, 1/2 IC50, and IC50. These differences were statistically significant compared to the control group ($P<0.001$).

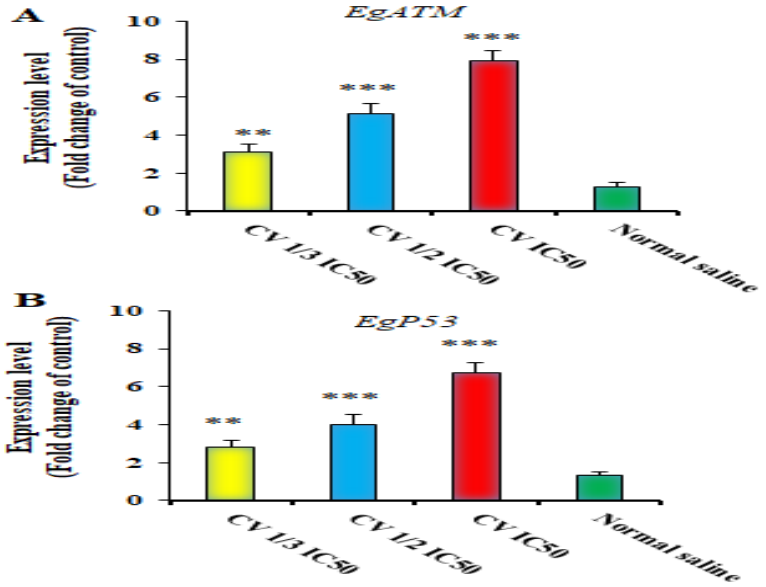

Fig. 4: Effect of various concentrations of carvone (CV) on the expression of DNA damage-related genes of *EgATM* (A) and *EgP53* (B) by Real-time PCR. The results are presented as mean ± standard deviation (SD). The IC50 value, representing the concentration required to inhibit 50% of the target (N = 3)

Discussion

Surgery, frequently accompanied by adjuvant chemotherapy, represents the primary treatment strategy for hydatid disease on a global scale for treating the hydatid cyst; however, this method is not universally applicable across all cyst stages (4). During hydatid cyst surgery, to improve the safety of cyst surgeries, the application of suitable scolicidal agents is crucial. A variety of materials and techniques have been investigated in this regard, many of which are linked to adverse effects (5). For example, the administration of hypertonic saline, formalin, silver nitrate, and cetrimide into cysts has been reported, yet these agents frequently result in complications such as leakage and necrosis of adjacent healthy tissues (5). The objective of this study was to evaluate the protoscolicidal properties, induction of apoptosis, and DNA damage caused by CV on hydatid cyst protoscoleces in both *in vitro* and *ex vivo* conditions. The results demonstrated that CV, particularly at a concentration of 150 µg/ml, effectively eradicated protoscoleces of hydatid cysts within a 20-minute exposure period. Furthermore, CV exhibited sustained anti-parasitic effects in the *ex vivo*, leading to the complete elimination of hydatid cyst protoscoleces after a 30-minute exposure, in contrast to its immediate lethal effects observed *in vitro*.

Antimicrobial effects of CV against various fungal (*Candida* spp., *Trichophyton* spp., and *Fusarium* spp.), bacterial (e.g., *Staphylococcus aureus, Escherichia coli, Streptococcus spp,* and *Pseudomonas* spp.) and viral (influenza viruses) pathogenic strains (9). Concerning the potential antibiofilm properties of CV, in a study (17), CV exhibited potent *in vitro* anthelmintic activity against eggs obtained from sheep feces infected with a multidrug-resistant strain of the gastrointestinal nematode *Haemonchus contortus* with a lethal concentration (LC_{50}) value of 0.085 mg/mL. Another study (18), showed that among the tested monoterpenes, the highest antiparasitic effects against *H. contortus* eggs were exhibited by carvacrol with IC50 = 185.9 µg/mL. The strong *in vitro* leishmanicidal effects of CV against *Leishmania amazonensis* promastigotes with IC50 value 25.4 µg/mL (19). The observed variations in results may be attributed to several factors, including the specific type of parasite, the source of provided CV, the concentration applied, and the methodologies adopted in the studies.

The antimicrobial mechanisms of action of CV, while not fully understood, have been the focus of researches indicating that monoterpene compounds, including CV, exert their antimicrobial effects by disrupting the lipid composition of microbial plasma membranes (20). This disruption leads to alterations in membrane permeability, resulting in the leakage of intracellular substances, as well as changes in pH and ATP levels. Furthermore, these compounds affect the integrity of lipids within bacterial membranes, interfere with DNA, and disrupt cell motility (20, 21). They also generate reactive oxygen species (ROS), induce apoptosis, and influence the localization of electrons (20, 21).

Apoptosis is recognized for its dual function in the relationship between the host and hydatid cysts, facilitating both survival and suppression mechanisms (22). Essential enzymes that mediate apoptosis, including caspase-3 and caspase-9, are integral to the advancement of this process, especially concerning DNA fragmentation and the morphological changes that characterize cellular death (22). Our results indicate that CV concentrations of 1/3 IC50, 1/2 IC50, and IC50 ($P < 0.001$) resulted in caspase-3 activation levels of 11.3%, 19.8%, and 28.4%, respectively. Consequently, it is reasonable to propose that the utilization of CV may successfully trigger apoptosis, thereby leading to the removal of PS.

DNA damage is detected by proteins that possess both signaling and repair functions

(23). A key component of the DNA damage response is the ataxia-telangiectasia mutated (ATM) protein (24). ATM is capable of sensing DNA damage and can induce G1/S cell cycle arrest and apoptosis through the stabilization of P53 (24). RAD54 serves as a fundamental element of the enzymatic machinery involved in the repair of DNA double-strand breaks (25). Monoterpene compounds can induce DNA damage in microbial pathogens (9). We hypothesize that the CV can cause DNA damage in hydatid cyst protoscoleces. Consequently, we employed Real-time PCR to assess the expression levels of *EgATM* and EgP53, in protoscoleces following treatment with various concentrations of CV. The expression levels of the *EgATM* and *EgP53* genes showed significant upregulation after treatment with CV. CV has been shown to induce DNA damage in protoscoleces and activate the ATM-P53-Topo2a signaling pathway. Concerning the cytotoxicity effects of CV, previous studies showed despite CV and its derivatives did not show cytotoxicity up to 200 µg/mL on normal cells such as H9C2 heart cells and MRC-5 lung cells, however, cytotoxic activity of CV against prostate and breast cancer cell lines, human chronic myeloid leukemia, human hepatocellular liver carcinoma and acute lymphoblastoid leukemia T (26, 27).

The important limitations of this study include the absence of animal studies and the lack of a systematic toxicity assessment for this compound.

Conclusion

CV possesses significant anthelminthic activity against hydatid cyst PS in controlled laboratory conditions. Additionally, the findings underscored the potential of CV to eliminate protoscoleces by inducing apoptosis and causing DNA damage. However, further studies are required to clarify the specific mechanisms underlying its action and to assess its efficacy in clinical trials, which may facilitate the application of CV in the context of hydatid cyst surgical procedures.

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Declaration of interest

The authors declare that there is no conflict of interest.

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