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Original article

Hydatid Cyst Killing Mechanism of *Ziziphora tenuior* by Inducing Apoptosis via Mitochondrial Intrinsic Pathway

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Abstract

Background and objectives: *Ziziphora tenuior* is considered as an appropriate protoscolicidal agent yet the mechanism of such effect is not known so far. The aim of this study was to evaluate the apoptotic effect of *Z. tenuior* extract on protoscolices of hydatid cyst. **Methods:** Protoscolices were collected aseptically and the Bradford test was employed to determine the number required for the experiments. Various concentrations of *Z. tenuior* extract (5, 50, and 100 mg/mL) was incubated with hydatid cyst protoscolices at 37 ^{o}C and 5% Co_2 for 4 h. The apoptotic effect of *Z. tenuior* extract on hydatid cyst protoscolices and the evaluation of caspases 3, 8, and 9 activities were ditermined using ELISA-based commercial diagnostic kits. **Results:** In the present study, 50 and 100 mg/mL of *Z. tenuior* extract produced apoptosis in the protoscolices of hydatid cyst significantly ($p<0.05$). Also, the activity of caspase 3 at 50 and 100 mg/mL significantly increased by 29.99% and 36.01%, respectively ($p<0.05$). Similarly, caspase 9 also demonstrated a significant increased activity up to 15.23%, and 45.31% at the same concentrations used for caspase 3, respectively ($p<0.05$). **Conclusion:** Our findings in this study indicated that, *Z. tenuior* extract can induce apoptotic cell death on hydatid cyst protoscolices by increasing the activity of caspases 3 and 9 via the internal apoptotic pathway.

Keywords: Apoptosis; caspase; hydatid cyst; protoscolices; *Ziziphora tenuior*

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Introduction

Hidatidosis or Echinococcosis is a zoonotic parasitic disease and still remains as an important concern for both public health system and economy. Hidatidosis has a widespread distribution worldwide [1-3] and is also present endemically in Iran [4]. The therapeutic protocols could be different depending on various factors such as type of cyst, size, location, and the

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number of cysts. Surgery is the best treatment for hydatidosis but in patients with several cysts in various organs or in the metastatic form of the disease, the non-surgical approaches could be more appropriate methods to be replaced for surgery [5]. Therefore, the application of safe protoscolicidal agents that produce no inflammatory reactions and with the potential to be effectively used during surgery and also post-surgery drug treatment is of prime importance [1-3,6]. Currently, the use of chemical protoscolicidal compounds is limited due to their detrimental side effects and herbal medicines as alternatives have raised the focus of attention [6-8]. So far, a number of such herbal drugs, as protoscolicidal agents, have been reported but the mechanism of their action is still unknown [9,10]. A mechanism suggested for suppression of hydatid cyst is the human innate immunity and its different layers, mainly the germinal layer. Apoptosis phenomenon is a newly recognized pathway among the host's innate and unknown immunity against hydatid cyst [11-13]. In addition, the apoptosis pathway in living organism is a useful approach to destroy the damaged and infected cells which does not usually lead to the occurrence of inflammatory responses [14,15].

Considering the results of authors' previous study on *Z. tenuior* scolicidal effect [9,10] and its unknown mechanism of action, the present study was aimed at investigating the *Z.tenuior* extract apoptotic effect on hydatid cyst protoscolices.

Materials and Methods

Ethical considerations

Ethical approval for this study was granted by the Ethical Committee of Qazvin University of Medical Sciences, Qazvin, Iran (ethics committee reference number: IR.qums.rec.1394.153, 2016).

Chemicals

The commercial kits used in the present study were the Bradford protein determination Kit purchased from Biotek Company; Cell Death Detection ELISA^{PLUS} (Apoptosis Kit) Roche (Germany) (Cat. No. 11 774 425 001); Caspases 3, 8, and 9 assay Kit by Abcam (USA) (cat no: ab39401 Caspase 3, ab39700 Caspase 8 and ab65608 Caspase 9); tissue culture medium RPMI 1640 from Gibco (USA).

Preparation of *Z. tenuior* extract

Ziziphora tenuior was purchased from a medicinal plants shop in Karaj, in May 2014 and its authenticity was confirmed by a botanist. A small sample of this plant was kept at the Central Herbarium of Medicinal Plants (ACECR) as a document (Code No. 93-70). The plant shoots were dried at room temperature (RT). Fifty g of ground plant was extracted using percolation method by 80% ethanol. The extract was concentrated in a vacuumed distillation system and was kept at 4 °C until use [16].

Preparation of different concentrations of *Z. tenuior* extract

Initially, a stock solution containing 400 mg/mL of the extract was prepared in sterile PBS and later 5, 50, and 100 mg/mL dilutions were obtained.

Preparation of hydatid cyst and removal of protoscolices

The livers and lungs infected with hydatid cyst were obtained from an abattoir in Qazvin, Iran. The external surfaces of cysts were disinfected and the cysts contents were aseptically removed with syringe and transferred to sterile Falcon tubes [17-20]. The protoscolices viability was examined by staining with 0.1% eosin; then, protoscolices >90% viability was chosen and following washing with gentamycin-containing PBS were kept in a refrigerator at 4 ºC [21].

Determination of appropriate number of protoscolices to evaluate the level of protein required for the experiments

According to the instructions made by the manufacturers of apoptosis and caspases 3, 8, and 9 kits, to lyse protoscolices, the supernatant protein level should be about 50-200 µg. To achieve such protein content, different dilutions of protoscolices (500-64000 per mL) in RPMI 1640 tissue culture medium were prepared and later the extract of *Z. tenuior* plant, at a final concentration of 100 mg/mL, was added and incubated at 37 °C in 5% CO_2 for 4 hours. This concentration of *Z. tenuior* extract was previously reported to produce 100% scolicidal activity [9,10]. Then, the supernatant protein level was measured by Bradford method as soon as the protoscolices were lysed. The protein level was measured using 1000 protoscolices in 1 mL of RPMI 1640 medium.

Preparation of cell lysate

Each tube containing 1 mL PRIM medium+1000 protoscolices received *Z. tenuior* extract at 5, 50, and 100 mg/mL concentrations; the mixture was incubated at 37 °C in 5% CO_2 for 4 h. Then, a 100-µL lysis buffer was poured into 100 µL of protoscolices treated with the extract; the tubes were then kept on ice for 30 min. They were likewise centrifuged at RCF equal to 10^4 g for ten min; the supernatants were collected and used for apoptosis and caspase activity studies. The tubes with untreated protoscolices (no extract added) were used as the negative control in our experiments.

Performance of apoptosis study

Based on the Cell Death Detection ELISA^{PLUS} kit instructions in addition to a 20-µL supernatant (collected after protoscolices lysis) both the positive control including DNA-Histone-complex (ready to use in the kit) and negative control (culture supernatant and cell lysate after centrifugation of untreated protoscolices) were added to wells of the ELISA plate; it was then mixed with the reactant solution provided with the kit, and incubated at 37 °C for two h. Then, using an ELISA reader spectrophotometer (Biotek Epoch, USA), both the samples and controls absorbance values were read at 405 nm. Experiments were carried out in duplicate and repeated three times [13,22].

Measurement of caspases activity

According to the instructions recommended by the caspase kit manufacturers the negative control plus 50 µL of cell lysis supernatant were added to a well allocated to a specific caspase; afterwards, the reacting solutions provided with the kit were added to the wells, mixed thoroughly, and incubated at 37 °C for two h. Following incubation, using an ELISA reader spectrophotometer (Biotek Epoch, USA), absorbances of the wells were measured at 405 nm. The experiments were carried out in duplicate and repeated three times. The specific substrates used for caspases 3, 8, and 9 were DEVD-pNA, IETD-pNA, and LEHD-pNA, respectively [13,23].

Statistical analysis

SPSS version 19 was used to analyze the data. One-way ANOVA and the post-hoc Turkey test were employed for this purpose. The level of significance was set to <0.05.

Results and Discussion

The apoptotic effect of *Z. tenuior* extract (yield 6%) on protoscolices at 5, 50, and 100 mg/mL concentrations were assessed in the study and the results were compared to that of the negative controls; the results were significant by 32.85% and 40.25% at 50 and 100 mg/mL, respectively ($p < 0.05$). Also, figure 1 confirmed a significant increase in the apoptotic rate in protoscolices treated with 50 and 100 mg/mL of *Z. tenuior* extract ($p < 0.05$).

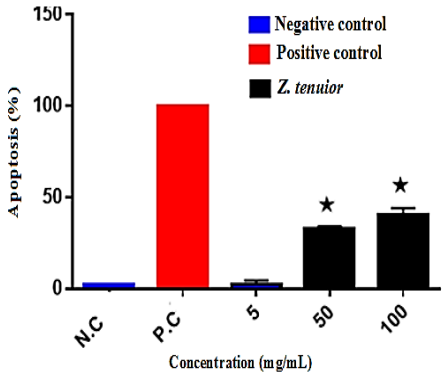

Figure 1. Apoptotic effect of *Ziziphora tenuior* extract on hydatid cyst protoscolices. Various concentrations of *Z. tenuior* extract (5, 50, and 100 mg/mL) were incubated with hydatid cyst protoscolices for 4 h. N.C: Negative control, culture supernatant and cell lysate after centrifugation of untreated protoscolices; P.C: positive control, DNA-Histone-complex (ready to use in the kit); the values are mean of triplicates, and the error bars represent Mean ± SD; p values less than 0.05 was considered significant

Evaluating the effect of *Z.tenuior* extract at different concentrations on caspases 3, 8, and 9 activity in protoscolices treated with *Z. tenuior* extract revealed that caspases 3 and 9 activities increased; however, the increase was only significant at 50 and 100 mg/mL concentrations of the extract for caspase 3 by 29.99% and 36.01%, and caspase 9 by 15.23% and 45.31%, respectively ($p < 0.05$). However, applying *Z. tenuior* extract at different concentrations showed no significant increase in terms of caspase 8 activity (figure 2). In our study, the apoptotic effects of *Z. tenuior* extract on hydatid cyst protoscolex was assessed and results showed that *Z. tenuior* extract at higher concentrations (50 and 100 mg/mL) increased caspases 3 and 9 activity in protoscolices and produced apoptosis which was a concentration-dependent effect.

In the present study, the activity of caspase 8 was not considerably increased following treatment of protoscolices with different concentrations of *Z. tenuior* extract.

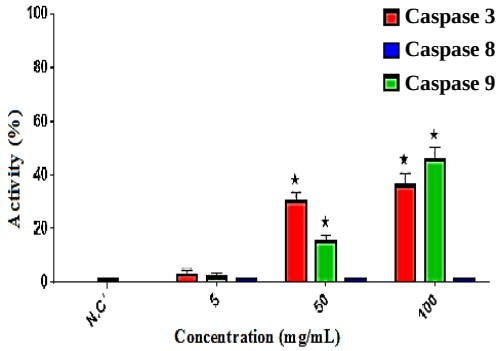

Figure 2. After treatment of hydatid cyst protoscolices with various concentrations of *Ziziphora tenuior* extract (5, 50, and 100 mg/mL), cell lysis supernatant were used for caspase activity regarding to company instructions; N.C: Negative control, culture supernatant and cell lysate after centrifugation of untreated protoscolices; the values are mean of triplicates, and the error bars represent Mean ± SD; p values less than 0.05 was considered significant.

Although the number of published studies on the apoptotic effect of *Z. tenuior* plant is limited [24], there are considerable numbers of reports regarding the apoptotic effect of plants of the Lamiaceae family in which *Z. tenuior* is a genus among the members of this family [25,26]. In this respect, the effect of essential oil and extract of the plant *Lavandula angustifolia*, Lamiaceae, on cancer and healthy cells was investigated and the authors demonstrated that both the essential oil and extract of *L. angustifolia* could inhibit the growth of cancer cells; this apoptotic effect is in association with substances including thymol and pulegone [25]. In our study, consistent with the data found in the report mentioned above, the extract of *Z. tenuior* could produce apoptosis in protoscolices and it may be similarly associated with the presence of thymol and pulegone in the plant. In another study and again comparable with ours, the apoptotic effect of the *L. angustifolia* on both cancer and normal cells was accompanied with increased activity of caspase 9 and this effect showed direct relationship with increased concentrations of the extract [27]. Again, this could be associated with the fact that both *L. angustifolia* and *Z. tenuior* are two genera within the family Lamiaceae and similar compounds in a family are highly expected.

The effect of thymol on colorectal carcinoma was the target of a study in which thymol increased the activity of caspases 8 and 9 and this led to initiation and induction of apoptosis [28]. In a similar study, the effect of thymol on growth of bladder cancer cells was examined and the authors concluded that the induction of apoptosis and raised caspases 3 and 9 activity were dose- and time-dependent effect [29]. The current study also indicated caspases 3 and 9 activities in hydatid cyst protoscolices; however, there was no change in caspase 8 activity. As thymol is one of the important components in the extract of *Z. tenuior*, it was considered as a factor contributing caspase 8 increased activity in our study however, the lack of increase in caspase 8 activity in the present study could be due to the in vitro condition of the experiments used in our study as caspase 8 usually needs the presence of human immune mediators for its activation [30].

In addition to the studies mentioned earlier, the apoptotic effect of drugs and different substances such as H_2O_2, dexamethasone, ATP, and praziquantel on hydatid cyst protoscolices has been reported and in most cases, caspase 3 activity increased following the occurrence of apoptosis in the present study [13,22-23,31]. Pensel et al in 2014, investigated *Thymus vulgaris* and *Origanum vulgare* essential oils effects (Lamiaceae) on hydatid cyst protoscolices and showed that these two plants could induce apoptosis in protoscolices of hydatid cyst after an incubation time of 16 h. The concentration of thymol in the essential oil of the two herbal plants was adjusted to 10 µg/mL and in the same time thymol was individually tested on hydatid cyst protoscolices. The thymol-induced apoptosis in hydatid cyst protoscolices was observed after 8 h in their experiments [32]. In our study, the extract of *Z. tenuior* could significantly increase the activity of caspase 3 and induce apoptosis in hydatid cyst protoscolices. Considering the apoptotic, immunomodulatory, anti-tumor and protoscolicidal effect of chemical compounds, medicinal plants, and their different constituents reported by various authors [13,22,23, 31-38] and also *Z. tenuior* extract scolicidal effect indicated in our previous studies [9,10], the current study findings about *Z. tenuior* extract apoptotic effect on hydatid cyst protoscolices could be justified; therefore, this medicinal plant with its apoptotic effect could be considered as an appropriate and useful substitution for common and routinely used agents in surgical and drug treatment of hydatid cyst.

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Author contributions

Mojtaba Shahnazi and Abbas Azadmehr designed, supervised the study, prepared and revised the manuscript; Hamidreza Aghaei and Reza Norian performed the experiments; Reza Hajiaghaee supervised the study, prepared the plant extract and contributed to the revision of the manuscript; Mehrzad Saraei advised the project; Morteza Oladnabi contributed in the preparation and revision of the manuscript, Mahmood Alipour performed statistical analysis. All authors approved the final version of the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

PBS: phosphate-buffered saline; ELISA: enzyme-linked immunosorbent assay; RCF: relative centrifugal force; ANOVA: analysis of variance