Evaluating the effect of Myrtus communis on programmed cell death in hydatid cyst protoscolices

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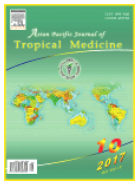

Original research

Evaluating the effect of *Myrtus communis* on programmed cell death in hydatid cyst protoscolices

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Abstract

Objective

To evaluate the possible involvement of programmed cell death strategy in hydatid cyst protoscolices following treatment with *Myrtus communis* (*M. communis*) as an herbal medicine.

Methods

Protoscolices were aseptically collected from sheep liver hydatid cysts. Evaluating the effect of *M. communis* extract on programmed cell death and increased activity of caspases 3, 8, and 9 in hydatid cyst protoscolices was conducted by treating the protoscolices with different concentration (5, 50, and 100 mg/mL) of *M. communis* extract at 37 °C and 5% CO_2 for 4 h by using the Bradford test and ELISA commercial kits.

Results

The extract of *M. communis* at all concentrations led to initiation of programmed cell death in protoscolices and this effect, was only significant at 50 and 100 mg/mL concentrations, compared to the negative control ($P < 0.05$). Also, the activity of caspases 3, 8, and 9 in hydatid cyst protoscolices, was shown that the extract at all 3 concentrations could only increase the activity of caspases 3 and 9. Moreover, a significant increase in the activity of caspase 3 was only observed at concentrations 50 and 100 mg/mL by 37.00% and 66.19% while a significant increase in the activity of caspase 9 at the same concentrations was observed by 20.89% and 63.67%, respectively ($P < 0.05$).

Conclusion

The extract of *M. communis* at different concentrations could increase the activity of caspases 3 and 9 and caused programmed cell death in hydatid cyst protoscolices however, this effect was significant at high concentrations of the extract.

Keywords

Echinococcus granulosus; Protoscolex; *Myrtus communis*; Programmed cell death; Caspase

1. Introduction

Hydatidosis is a zoonotic disease caused by larval stage of *Echinococcus* granulosus and its final localization in different organs of the human body such as liver, lung, and brain. The major hosts of this parasite are dogs and carnivores; herbivores as intermediate hosts; and human as accidental intermediate host. This disease is widely distributed and remains a health concern globally and has important economic consequences [1–4]. Surgery is the most effective therapeutic approach and the choice of less harmful but potent protoscolicidal agents before surgery and the injection of such compounds into the cysts decrease the risk of leakage by viable protoscolices and is of vital importance for the surgeons [5–7]. At present, the application of herbal medicines as substitutions for chemical compounds has gained growing acceptance and credibility [8] and some of these herbal plants have been reported to demonstrate protoscolicidal activity [9]; however, the mechanism of their action is not yet fully uncovered. The human natural immune response against hydatid cyst and its various layers in particular the germinal layer, is regarded as one of the possible mechanisms in suppressing hydatid cyst [10]. Recently, the occurrence of apoptosis in disabling the *E granulosus* parasites is considered as an important part of host's innate immunity and this approach could be among the ideal measures in destroying these parasites [11,12]. Apoptosis or in other words “the programmed cell death” is an energy-dependent active process that in comparison with necrosis is a well-organized signaling defense mechanism. During the appearance of apoptosis phenomenon, cells are smaller in size with other changes such as cell shrinkage and breakdown of cytoplasmic membrane into smaller apoptotic bodies. The evident morphological changes in apoptosis include nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation and contrary to necrosis, no inflammation occurs in apoptosis. The process of apoptosis is principally activated through two pathways; one is an extrinsic route (death receptor-mediated pathway) and the other is an intrinsic route (mitochondrial pathway) [13,14]. The stimulation of death receptors by their appropriate ligands leads to activation of caspases cascade and induction of apoptosis [15]. Apoptosis is reported to occur in many cestodes including hydatid cyst [10,12,16]. Despite obvious scolicidal activity of a number of herbal drugs [9] such as *Myrtuscommunis* (*M. communis*) (identified by the authors of the present study but not yet published), the mechanism of its action is not yet understood. The medicinal plant *M. communis* is a genus of family Myrtaceae and widely distributed in the Mediterranean regions. This plant is also scattered in various parts of Iran and is one of the oldest and most commonly used herbal drug in traditional medicine. Different parts of *M. communis* plant contain numerous compositions such as flavonoid, tannin, and phenolic acid compounds for which antibacterial, antiviral, antifungal, anti-parasitic, and anti-inflammatory activities have been reported [17,18]. Considering the scolicidal activity of the herbal plant *M. communis* and its unknown mode of action, the present study aimed at investigating the possible engagement of the programmed cell death strategy in protoscolices of hydatid cyst when treated with *M. communis* extract.

2. Material and methods

2.1. Preparation of hydatid cyst protoscolices

The contents of hydatid cysts of sheep liver, obtained from the major slaughterhouse in Qazvin (Iran), were aseptically aspirated and protoscolices were collected. Viability test was performed by staining the protoscolices with 0.1% eosin solution and the organisms with viability higher than 90% were collected and stored at 4 °C in a refrigerator [19,20].

2.2. Preparation of *M. communis* extract

The medicinal plant *M. communis* was obtained from a local grocery for herbal plants. The authenticity of *M. communis* was confirmed by a botanist who meticulously supervised the extraction process. A tiny amount of this plant was kept, as an official document for the study, in the Central Herbarium of Medicinal Plants (ACECR) and the remainder was left at room temperature and away from direct sunlight to be air-dried. Extraction process was accomplished following drying and grinding the plant sample using a percolator apparatus and ethanol. Later, the extract was collected and concentrated in a distillation system [21]. In total three different dilutions of the extract required for the experiments (5, 50, and 100 mg/mL) were prepared using a sterile gentamycin-containing PBS solution.

2.3. Reagents

The commercial kits including Cell Death Detection ELISA^{PLUS} Kit (BioteK Company), Caspases 3, 8, and 9 Detection Kits (Abcam), and Bradford protein determination Kit (Roche) were used in our experiments. Also, the tissue culture medium RPMI 1640 was purchased from Gibco and used in the study.

2.4. Determination of appropriate number of protoscolices

Based on the instructions provided by the manufacturers of Cell Death Detection ELISA^{PLUS} Kit and caspases kits, the protein content of the supernatant following protoscolices lysis was recommended to be between 50 and 200 µg. To reach such protein concentration, different dilutions of protoscolices (500–64 000/mL) in RPMI 1640 tissue culture medium were prepared and later the extract of *M. communis* plant, at a final concentration of 25 mg/mL, was added and incubated at 37 °C in 5% CO_2 for 4 h. This concentration of *M. communis* extract was previously identified to have produced scolicidal activity. Following incubation and lysis of protoscolices, the protein content of supernatant was determined by Bradford method. A protein content of 200 µg was found in the tube containing 1000 protoscolices in 1 mL of RPMI 1640 culture medium.

2.5. Preparation of supernatant following the lysis of protoscolices treated with *M. communis* extract

Three different concentrations (5, 50, and 100 mg/mL) of *M. communis* extract were added to 1 mL of RPMI culture medium containing 1000 protoscolices and the mixture incubated at 37 °C in 5% CO_2 for 4 h. After incubation, 100 µL of lysis buffer was added to 100 µL of extract-treated protoscolices and the tubes were left on ice for 30 min. Later the tubes were centrifuged

at 10 000 × *g* for 10 min and the supernatants removed and used in experiments associated with caspase activity and apoptosis studies. Tubes with no *M. communis* extract (protoscolices alone) were regarded as negative control in our experiments.

2.6. Investigating the programmed cell death in the protoscolices treated with different concentrations of *M. communis* extract

According to the Cell Death Detection ELISA^{PLUS} kit instructions, 20 µL of each supernatant (obtained following lysis of protoscolices), positive, and negative controls (included in the kit) were added to each well of an ELISA plate, mixed with the reactant solutions present in the kit, and incubated at 37 °C for 2 h. After incubation, the optical density of samples in all wells was measured at 405 nm using an ELISA reader spectrophotometer (Biotek Epoch). All experiments were performed in duplicate and repeated three times.

2.7. Measurement of caspases activity

Activity of caspases was determined by the commercial kits purchased from Abcam Company. Based on the Kit instructions, 50 µL of each supernatant obtained following protoscolices lysis plus the negative control was separately added to each well marked for a specific caspase and later the reacting solutions present in the kit were added to each well, thoroughly mixed, and incubated at 37 °C for 2 h. Finally, the optical density of all wells was measured at 405 nm using an ELISA reader spectrophotometer (Biotek Epoch). All experiments were performed in duplicate and repeated three times.

2.8. Statistical analysis

Data were analyzed by SPSS version 19 and presented in the form of appropriate figures and tables. Statistical tests including *t*-test, one-way analysis of variance (ANOVA), and the post-hoc Tukey test were used for statistical analysis. A P value <0.05 was considered as significant.

3. Results

3.1. Programmed cell death effect of *M. communis* extract on protoscolices

The extract of *M. communis* plant induced programmed cell death in hydatid cyst protoscolices at all concentrations (5, 50, and 100 mg/mL) used in the present study however, this effect, compared to the negative control group, was only significant by 46.65% and 55.26% at two concentrations of 50 and 100 mg/mL, respectively (P < 0.05) (Table 1).

Table 1. Apoptotic effect of different concentrations of *M. communis* extract on protoscolices (n = 6).

Concentration	Optical density (mean ± SD)	Apoptosis (%)
5 mg/mL *M. communis* extract	0.062 7 ± 0.004 8	1.31

50 mg/mL *M. communis* extract	0.090 7 ± 0.004 9	46.65*
100 mg/mL *M. communis* extract	0.096 1 ± 0.005 2	55.26*
Negative control	0.006 2 ± 0.004 7	1.00
Positive control	0.115 0 ± 0.009 6	100.00

*: Compared with negative control ($P < 0.05$).

3.2. The effect of *M. communis* extract on activity of caspases 3, 8, and 9 in hydatid cyst protoscolices

In assessing the effect of different concentrations of *M. communis* extract on increasing the activity of caspases 3, 8, and 9 in the extract-treated protoscolices, it was demonstrated that the activity of caspases 3 and 9 was increased in the presence of all concentrations of the extract used in the experiments nevertheless, this increase was only significant at concentrations 50 and 100 mg/mL of the extract for caspase 3 by 37.00% and 66.19% and caspase 9 by 20.89% and 63.67%, respectively ($P < 0.05$) (Table 2). There was no significant increase in the activity of caspase 8 when different concentrations of *M. communis* extract were used (Table 2).

Table 2. Effect of different concentrations of *M. communis* extract on hydatid cyst protoscolices, in the increased activity of Caspases 3, 8 and 9 ($n = 6$).

Concentration	**Optical density (mean ± SD)**			**Increased activity (%)**		
	Caspase 3	**Caspase 9**	**Caspase 8**	**Caspase 3**	**Caspase 9**	**Caspase 8**
5 mg/mL *M. communis* extract	0.055 9 ± 0.001 2	0.055 6 ± 0.002 8	0.055 7 ± 0.001 3	1.24	1.49	1.64
50 mg/mL *M. communis* extract	0.075 7 ± 0.001 4	0.066 3 ± 0.005 1	0.055 8 ± 0.001 1	37.00*	20.89*	1.95
100 mg/mL *M. communis* extract	0.091 9 ± 0.002 4	0.089 7 ± 0.004 8	0.055 9 ± 0.001 2	66.19*	63.67*	2.01
Negative control	0.055 3 ± 0.001 1	0.054 8 ± 0.002 8	0.054 7 ± 0.001 2	1.00	1.00	1.00

*: Compared with negative control ($P < 0.05$).

4. Discussion

The choice of potent protoscolicidal agents but with less harmful effect in hydatid cyst surgery is of essential importance for many surgeons [5–7]. Currently, the use of medicinal plants as replacements for chemical substances has gained so much attention but despite the scolicidal activity of a number of medicinal plants such as *M. communis*[8,9,22], the mechanism of their action is not yet understood. Among the new pathways of natural immunity, apoptosis or programmed cell death is considered as one of the possible mechanisms in suppressing hydatid cyst [23,24]. The programmed cell death is a well-organized process in eliminating the damaged cells and in contrast to necrosis, causes no inflammatory response [13,14].

In the present study, the effect of the herbal plant *M. communis* on the process of programmed cell death in hydatid cyst protoscolices was investigated and it was revealed that the extract of this plant at high concentrations (50 and 100 mg/mL) could increase the activity of caspases 3 and 9 and eventually caused programmed cell death in the protoscolices of hydatid cyst which was a dose-dependent event. Also, our study showed that no considerable increase in the activity of caspase 8 was observed when treated with different concentrations of the extract. There are numerous numbers of studies regarding the occurrence of programmed cell death in the plants of Mirtaceae family including the plant *M. communis*, which is a genus within this family, and their different compounds. Tretiakova *et al* in 2008 investigated the activity of Myrtucommulone (a substance derived from *M. communis* leaves) on the induction of apoptosis in different cancer cell lines. Their results indicated that Myrtucommulone could increase the activity of caspases 3 and 9 and cause programmed cell death through mitochondrial pathway in different cancer cell lines used in the study whereas no change in the activity of caspase 8 was observed [25]. In the current study, consistent with the results mentioned above, the induction of apoptosis in hydatid cyst protoscolices was observed through increased activity of caspase 3 and 9 while no significant rise in the level of caspase 8 was revealed. This analogy between the results of the two studies could be attributed to the presence of a common substance, i.e., Myrtucommulone, in the previous study and the extract of this study. In another study, the cytotoxic and apoptotic effect of Myrtucommulone-A (an active ingredient among the derivatives of *M. communis* herbal plant) on cancer cells was examined and it was reported that Myrtucommulone-A could induce the programmed cell death in cancer cells through increased expression of genes associated with apoptosis and the effect was also a dose-dependent response [26].

In a study reported in 2016, the effects of two chemical substances (Epirubicin and cisplatin) plus Myrtucommulone-A (an active ingredient of *M. communis* plant) on murine breast cancer cells were investigated and it was claimed that the apoptotic effect of each single chemical in combination with Myrtucommulone-A was higher on cancer cells compared to results obtained when this chemicals were used alone. The authors attributed their results to the substantial effect of Myrtucommulone-A compound derived from *M. communis* plant in increasing the

apoptotic activity of epirubicin and cisplatin used in chemotherapy [27]. This finding could also be comparable to the presence of similar compounds in the *M. communis* extract used in our study and its action on induction of programmed cell death in hydatid cyst protoscolices.

In some studies, the effect of several extracts, obtained from the family Myrtaceae (*Eucalyptus camaldulensis* and *Eucalyptus citridora* resin) and a bioactive substance from one of these herbal plants (a flavonoid compound), on inhibiting the growth and apoptosis in cancer cells, were evaluated. Their results, in harmony with our findings, showed that the apoptotic effects of aforementioned plants and the bioactive substance were accompanied with increased level of caspase 3 in cancer cells but with no change in the activity of caspase 8 [28,29]. In our study, it was also demonstrated that the extract of *M. communis* could induce programmed cell death in protoscolices of hydatid cyst through increased activity of caspase 3. Considering the fact that the genus *M. communis* is a member in the same family as the plants mentioned earlier, it could denote the presence of similar compositions including flavonoid compounds in the extracts of two studies and similar to the data found in the previous study, the extract used in our study failed to increase the activity of caspase 8.

Also, a number of studies reported the anti-cancer and apoptotic actions of *Psidium guajava* L. extract and a flavonoid derived from the plant *Eucalyptus occidentalis* (both within the family Myrtaceae) on myeloid cells. The results of these studies, similar to our findings, demonstrated increased levels of caspases 3 and 9 which eventually led to induction of apoptosis in the cells used in the these studies. In the above-mentioned studies, inconsistent with the result found in our study, caspase 8 activity also increased in the presence of the extract [30,31], a discrepancy which could possibly be attributed to the differences in the type of plants, the presence of various compounds, and the type of cells used.

In a study by Hsieh *et al* in 2007, the possible apoptotic effect of aqueous extract of *P.guajava*, a member of the family Myrtaceae, on endothelial cells of umbilical cord was examined. The results found in their study demonstrated the aqueous extract of mentioned plant could induce apoptosis in epithelial cells through the activation of nuclear factors and this effect was claimed to be associated with the polyphenolic compounds derived from this plant [32]. In our study, the extract of *M. communis* plant also caused the induction of apoptosis in protoscolices of hydatid cyst and again being the members of the same family and the presence of similar active compounds (polyphenols) used in both studies could explain the apoptotic action of *M. communis* extract on the protoscolices of hydatid cyst; however, this effect, according to the variations in the compositions of above-mentioned plants and their synergistic or antagonistic interactions, needs further investigations.

A number of Chinese researches in 2016 evaluated the effect of *Zanthoxylum bungeanum* compounds on induction of apoptosis in skin cancer cells. These authors showed that the compounds of this herbal plant could inhibit the cellular growth in a dose-dependent manner. They also reported that the extract of aforementioned plant could stop the cell cycle at phase S,

increase the activity of caspases 3, 8, and 9, increase the expression of *Bax*, and decrease the level of *Bcl-2* in cancer cells, leading to induction of apoptosis through both intrinsic and extrinsic pathways. The authors attributed these effects to the presence of analyzed substances of *Z.bungeanum* plant such as limonene and terpinen [33–37]. In our present study, the extract of *M. communis* plant could also increase the activity of caspases 3 and 9 and kill the hydatid cyst protoscolices in a dose-dependent manner. Considering the presence of limonene and terpinen in both plants, the consistency in induction of apoptosis could be attributed to the existence of above-mentioned active compounds in two herbal plants. Contrary to the study mentioned above, the extract of *M. communis* plant used in our study, had no effect on caspase 8 and this dissimilarity could be due to the differences in type of plant and cells used in two studies and also the pathways investigated nevertheless, more detailed and comprehensive studies are required.

In addition to the studies mentioned above, there are other studies investigating the different mechanisms of apoptosis in protoscolices. In this respect, Spotin *et al* examined the expression of apoptosis-inducing ligands *TRAIL* and *Fas-L* on the surface of the germinal layer of infertile and fertile hydatid cyst as well as the human intact tissue surrounding the cyst. The authors observed relatively high level expression of apoptosis-inducing ligands in infertile cysts, compared to fertile and the normal tissue and concluded that the occurrence of apoptosis in germinal layer may be one of the important pathways to cause infertile cysts [38]. In another study by Parades *et al* in 2007, the possible association of apoptosis with fertility and infertility of hydatid cysts was investigated using DNA fragments analysis and observed higher level of apoptosis in infertile cysts [10]. The effects of different substances including dexamethasone, H_2O_2, and Praziquantel, as agents to induce apoptosis in hydatid cyst protoscolices, were also investigated [12,16]. Therefore, considering the protoscolicidal activity of chemical agents and the herbal drugs mentioned above and their various compounds plus the scolicidal effect of *M. communis* plant identified in our previous study, the programmed cell death induced in protoscolices by the *M. communis* extract of this study could be better justified although more future in-depth studies are needed.

Conflict of interest statement

The authors declare no conflict of interests.

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