European Review for Medical and Pharmacological Sciences 2013; 17: 1760-1765

In vitro effect of *Sambucus ebulus* on scolices of Hydatid cysts

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Abstract. – OBJECTIVES: Echinococcosis infection is caused in humans by the larval stage of cestodes belonging to the genus Echinococcus. Hydatid cyst cured by percutaneous aspiration, infusion of scolicidal agents with reaspiration or surgery. Many scolicidal agents have been used for inactivation of the cyst's content, but most of them are not safe due to their unanticipated side effects. In the present study, the scolicidal effect of methanolic extract of *Sambucus (S.) ebulus* fruit is investigated.

MATERIALS AND METHODS: Protoscolices were aseptically aspirated from sheep livers having hydatid cysts. Four concentrations (1, 10, 50 and 100 mg ml^{-1}) of *S. ebulus* extract were used for 5, 10, 30 and 60 min. Viability of protoscolices was confirmed by 0.1% Eosin staining.

RESULTS: The results of our study indicated that methanolic extract of *S. ebulus* fruit showed a high scolicidal activity *in vitro* ($p < 0.0001$).

CONCLUSIONS: Methanolic extract of *S. ebulus* showed high scolicidal activity *in vitro*. It might be used as a scolicidal cause in the surgical treatment of the hydatid cyst. However, further research on the *in vivo* efficacy of *S. ebulus* extract and its potential side effects is recommended.

Key Words:
Sambucus ebulus, Protoscolices, Hydatid cyst.

Introduction

Echinococcosis is a zoonotic disease caused by a parasite of the class Cestodes, genus *Echinococcus*. Adult worm of *Echinococcus (E.) granulosus* is present only in dogs. Adult tapeworms attach to the intestinal epithelium and undergo sexual reproduction, leading to the development of eggs; these eggs are shed into the environment with the faeces. Man, cattle, and sheep, allocation as intermediate hosts, contract the infection by ingesting the eggs. Cystic hydatid disease affects mainly the liver (50-70% of all cysts) but can also develop in lung (20-30%) and, less frequently, in spleen, kidney, bone, brain, and other organs[1,2]. *Echinococcosis* in humans and animals is an economic and public health concern in many parts of the world[3]. The infection is most prevalent in sheep and cattle-raising regions like Australia, South America, the Middle East, South Africa, Eastern Europe, and the Mediterranean areas[4]. In Central Europe and other industrialized countries, measures such as mandatory meat inspection have reduced the incidence of the disease, whereas in countries with lower hygiene standards, *E. granulosus* infections are widespread. Surgery is still the preferred method of treatment, although it increases the risk of intraoperative spillage of scolices[5]. This is the major cause of recurrence, which is seen in approximately 10% of the postoperative cases. Avoiding spillage of the cyst contents and the use of effective scolicidal agents are essential to lowering the recurrence rate[6]. Currently, many scolicidal agents, which have been used for inactivation of the cyst content[7]. Medical physician need less harmful and more effective scolicidal solutions for use in hydatid cyst surgery[8].

Four species of the genus *Sambucus (S.)* are growing in Iran. Of these species, *S. ebulus* extensively growth in the northern regions of Iran. Iranian traditional medicine uses the leaves, fruits and rhizomes of *S. ebulus* in treating some inflammatory cases such as, bee and nettle bites, arthritis, and sore-throat[9,10]. In addition, it has been reported to be an insect repellent, anti-hemorrhoid, anti-protozoa[11], anti-bacterial toward *Helicobacter pylory*, convenient in treatment of burns and infectious wounds, edema, eczema, urticarial, the cold, inflammation and rheumatism[12-16].

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Ebulin1 is a non-toxic novel type 2 ribosome-inactivating protein of *S. ebulus*. Ribosome-inactivating proteins (RIPs) are plant toxins with N-glycosidase activity on the large rRNA of fungal, plant and bacterial ribosomes that irreversibly block protein synthesis[17]. Ebulin1 is not toxic to mice and non-toxic to culture mammalian cells[17]. This allowed us to classify ebulin1 as a new type 2 ribosome-inactivating protein such as ricin, abrin, viscumin, modecin, and volkensin[17], but of a new class, the non-toxic type 2 RIPs.

The present study was conducted to evaluate the *in vitro* scolicidal effect of methanol extract of *S. ebulus* fruit on the scolices of hydatid cysts.

Materials and Methods

Collection of Protoscolices

Protoscolices of *E. granulosus* were aspirated from the infected livers of sheep slaughtered at Mazandaran slaughterhouse in northern Iran. The hydatid fluid was transferred into glass cylinders under sterile condition and left to set for 30 min. The protoscolices settled down at the bottom of the cylinders. The supernatant was removed and the sedimented protoscolices were washed three times with normal saline. The viability of the protoscolices was confirmed by their motility under a light microscope and by eosin 0.1%. The live protoscolices were finally transferred into a dark container containing normal saline solution and stored at 4C for further use.

Preparation of Sambucus ebulus Extract

Fruit of *S. ebulus* was dried under shade, and powdered mechanically using a commercial electrical blender. To obtain the methanolic extract, 70 g of dry powder was added to 350 ml of pure methanol and mixed gradually for 1 h using a magnetic stirrer. The obtained solution was left at room temperature for 24 h. The solution was stirred again and filtered and then the solvent was removed by evaporation in a rotating evaporator. The remaining semisolid material was then freeze-dried. The obtained filtrate (5.5 g) was placed into a sterile glass container and stored at 4°C for further use.

Effect of S. ebulus Extracts on Protoscolices

In this study, four concentrations (1, 10, 50 and 100 mg ml^{-1}) of the *S. ebulus* extract were used for 5, 10, 30 and 60 min. To make the *S. ebulus* extract solution at 1, 10, 50 and 100 mg ml^{-1} concentrations, 0.01, 0.1, 0.5 and 1 g of dried extract was dissolved in 10 ml of normal saline, respectively. Then 2 ml of each solution was placed in test tubes, to which 10,000 washed protoscolex was added. The contents of the tubes were gently mixed. The tubes were then incubated at 37°C for 5, 10, 30 and 60 min. At the end of each incubation time the upper phase was carefully removed so as not to interrupt the protoscolices. Two milliliters of 0.1% eosin stain was then added to the remaining settled protoscolices and mixed gently. The upper portion of the solution was discarded after 15 min of incubation. The remaining pellet of protoscolices was then smeared on a glass slide, covered with a cover glass and examined under a light microscope. The percentages of dead protoscolices were determined by counting a minimum of 250 (usually more than 500) protoscolices. Non-treated protoscolices were considered a control group in each experiment. The experiments were performed in triplicate.

Viability Test

In the present study eosin stain with the concentration of 0.1% (1 g of eosin powder in 1000 ml distilled water) was used to check the viability of the protoscolices. Fifteen minutes after exposure to the stain the protoscolices with no absorbed dye were considered potentially viable (Figure 1); otherwise, they were recorded as dead (Figure 2).

Statistical Analysis

Statistical analysis was performed using GraphPadInStat software (Graph Pad Software, La Jolla, CA, USA). Differences between test and control groups were analyzed by v2 test. A p value less than 0.01 were considered significant.

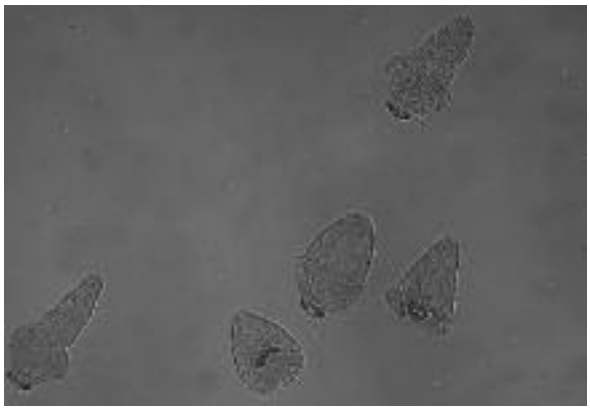

Figure 1. Viable protoscolices with no absorbed dye.

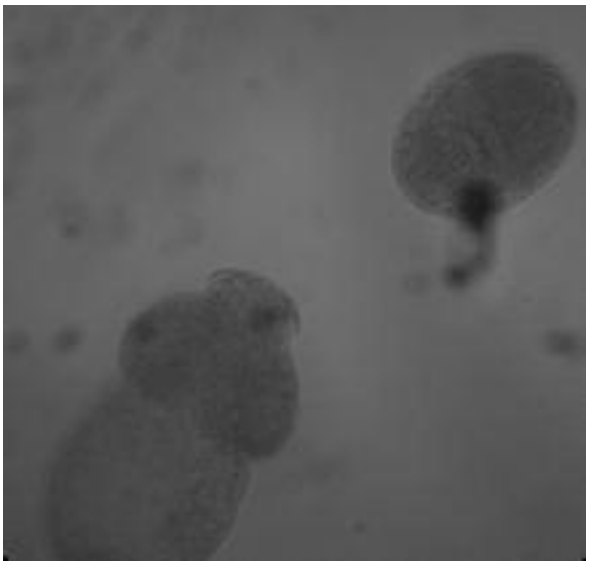

Figure 2. Dead protoscolices that absorbed dye.

Results

The scolicidal effects of *S. ebulus* extract are summarized in Tables I to IV. *S. ebulus* extract at the concentration of 1 mg ml^{-1} killed 38.6, 46.0, 56.6, and 65.6% of the protoscolices, at the concentration of 10 mg ml^{-1} killed 45.3, 50.6, 60.0 and 81.0% of the protoscolices, at the concentration of 50 mg ml^{-1} killed 59.0, 70.0, 79.3 and 88.3% of the protoscolices and at the concentration of 100 mg ml^{-1} killed 58.0, 61.6, 74.6 and 98.6% of the protoscolices after 5, 10, 30, and 60 min of application, respectively. The scolicidal effect of any concentrations of the methanolic extract of *S. ebulus* was extremely significant compared to the control groups at all exposure times ($p < 0.0001$).

Discussion

The results of our study indicated that methanolic extract of *S. ebulus* fruit showed high scolicidal activity *in vitro*. Many studies have investigated the *in vitro* scolicidal activity of hypertonic saline at various concentrations and different exposure times. Various experimental studies have investigated the scolicidal effects of hypertonic saline, silver nitrate, cetrimide, ethyl alcohol (95%) 18, Hot water 19, H_2O_2 and 10% povidone iodine, mannitol, albendazole, chlorhexidine gluconate, honey and *Allium sativum* 20. Many of these scolicidal causes may origin undesirable complications that limit their use. Hypertonic saline is one of the most common scolicidal agents in the world 21. Two percent saline did not have any lethal effect on protoscolices 22. Kayaalp et al[23] (2001) established that 3-6.5% saline to be ineffective, even after 60 min. They found 100% scolicidal activity for 10, 15, 25, and 30% saline at the end of 75, 10, 3, and 3 min. Complete (100%) in vitro scolicidal activity has been reported for 20% hypertonic saline at the end of 6 min 22, 15 min 9, 21,

Table I. Scolicidal effect of *Sambucus ebulus* fruit extract at the concentration of 1 mg ml^{-1} following various exposure times.

Exposure time (min)	Experiments	Protoscolices	Dead protoscolices	Mortality rate (%)
5	1	500	200	40
	2	500	205	41
	3	500	175	35
	Total	1500	580	38.6
10	1	500	225	45
	2	500	225	45
	3	500	240	48
	Total	1500	690	46.0
30	1	500	280	56
	2	500	295	59
	3	500	275	55
	Total	1500	850	56.6
60	1	500	325	65
	2	500	320	64
	3	500	340	68
	Total	1500	985	65.6
Control	1	500	10	2
	2	500	10	2
	3	500	20	4
	Total	1500	40	2.6

Table II. Scolicidal effect of *S. ebulus* fruit extract at the concentration of 10 mg ml^{-1} following various exposure times.

Exposure time (min)	Experiments	Protoscolices	Dead protoscolices	Mortality rate (%)
5	1	500	220	44
	2	500	225	45
	3	500	235	47
	Total	1500	680	45.3
10	1	500	250	50
	2	500	260	52
	3	500	250	50
	Total	1500	760	50.6
30	1	500	300	60
	2	500	295	59
	3	500	305	61
	Total	1500	900	60.0
60	1	500	405	81
	2	500	395	79
	3	500	415	83
	Total	1500	1215	81.0
Control	1	500	10	2
	2	500	10	2
	3	500	15	3
	Total	1500	35	2.3

and 45 min. But Adas et al[24] reported 98.2 and 99.5% scolicidal activity for 20% hypertonic saline at the end of 5 and 10 min, respectively 8. One hundred percent scolicidal activity has been reported for 20% silver nitrate after 20 min. The *in vitro* scolicidal activity of 1.5% cetrimide was found to be 86.9% at the end of 5 min and 92.6% at the end of 10 min 24. Ethyl alcohol (95%) was found to be 100% effective after 15 min only in undiluted form. Further dilutions of 47 and 9.5% ethyl alcohol were ineffective as a scolicidal agent in both 5 and 10 min exposures 21. H_2O_2 (3%) has been found to be 90.3 and 100% effective on scolices after 5 and 15 min, respectively 21,24. Besim et al[8] showed that 10% povidone iodine was not effective as a scolicidal agent at 5 and 10 min *in vitro*. Mannitol (20%) has been found to be 100% effective on

Table III. Scolicidal effect of *S. ebulus* fruit extract at the concentration of 50 mg ml^{-1} following various exposure times.

Exposure time (min)	Experiments	Protoscolices	Dead protoscolices	Mortality rate (%)
5	1	500	295	59
	2	500	290	58
	3	500	300	60
	Total	1500	885	59.0
10	1	500	345	69
	2	500	350	70
	3	500	355	71
	Total	1500	1050	70.0
30	1	500	395	79
	2	500	395	79
	3	500	400	80
	Total	1500	1190	79.3
60	1	500	435	87
	2	500	445	89
	3	500	445	89
	Total	1500	1325	88.3
Control	1	500	15	3
	2	500	10	2
	3	500	10	2
	Total	1500	35	2.3

Table IV. Scolicidal effect of *S. ebulus* fruit extract at the concentration of 100 mg ml^{-1} following various exposure times.

Exposure time (min)	Experiments	Protoscolices	Dead protoscolices	Mortality rate (%)
5	1	500	285	57
	2	500	290	58
	3	500	295	59
	Total	1500	870	58.0
10	1	500	310	62
	2	500	315	63
	3	500	300	60
	Total	1500	925	61.6
30	1	500	375	75
	2	500	375	75
	3	500	370	74
	Total	1500	1120	74.6
60	1	500	495	99
	2	500	490	98
	3	500	495	99
	Total	1500	1480	98.6
Control	1	500	10	2
	2	500	15	3
	3	500	15	3
	Total	1500	40	2.6

scolices after 45 min. The *in vitro* scolicidal activity of albendazole sulfoxide at concentrations of 50 and 100 mg ml^{-1} was 50 and 100% 8. Dalimi et al[23] used low-voltage direct electric current to inactivate the scolices of hydatid cyst *in vitro*. They concluded that current densities of 62.5 mA/cm^2 (11 V), 53.71 mA/cm^2 (10 V), and 18.18 mA/cm^2 (5 V) can kill all protoscolices in the hydatid fluid after 1, 2, and 3 min, respectivel. Moazeni and Larki[24] examined the in vitro scolicidal effect of highly acidic and alkaline solutions. They reported 100% scolicidal effect for solutions with pH 1 and 14 after 5 min. In their study the scolicidal activity of acidic solutions with pH 2, 3, and 4 after 10 min was 100, 100, and 21.5%, respectively. The scolicidal activity for alkaline solutions with pH 13, 12, and 11 was 99.7, 33.44, and 24.5%, respectively. Moazeni and Nazar[20] investigated methanolic extract of *Allium sativum* showed high scolicidal activity *in vitro* and they suggested that methanolic extract of *A. sativum* might be used as a scolicidal agent in the surgical treatment of the hydatid cyst. A rapid and complete scolicidal effect with no local or systemic side effects and also low cost are some properties of an ideal scolicidal solution. From this point of view, no ideal scolicidal agents have been described yet.

In this study, we investigated the efficacy of methanolic S. ebulus fruit extract on the scolices of hydatid cyst. The results of our study showed that *S. ebulus* has a nearly high scolicidal activity at the concentrations of 1, 10, 50 and 100 mg ml^{-1} after 5, 10, 30 and 60 min of application, respectively. *S. ebulus* has been used as herbal medicine for thousands of years for different medical purposes 9. Furthermore, previous studies have revealed the antiprotozoal 12, antibacterial, insect repellent, anti-hemorrhoid activities 12 of *S. ebulus* extract. New effective alternative treatment is extremely important in today's climate where species are becoming resistant and there is resurgence in the use of natural alternative therapies instead of synthetic pharmaceuticals that often have severe side effects 24. Sadjjadi et al[25] investigated the protoscolicidal activity of aqueous, chloroform, and hydro-alcoholic extracts of garlic. They concluded that the chloroform extract of garlic had a 97.9% scolicidal effect at a concentration of 200 mg ml^{-1} after 30 min of exposure.

Conclusions

The results of our study showed that methanolic extract of *S. ebulus* fruits is an effective scolicidal agent and, therefore, may be used in hydatid cyst surgery. However, the *in vivo* efficacy of this extract remains to be explored. Even though *S. ebulus* is edible, its possible side effects when used as a scolicidal agent need more investigation. This is the first report on the scolicidal activity of *S. ebulus*.

Conflict of Interest

None declarer.

Acknowledgements

The Authors would like to express their deep thanks to all Lab Staff of Medical Science, Ehsan Ahmadpour, Abdolsatar Pagheh, Yousef Dadimoghadam in Mazandaran Medical University for help in the study. And special thanks to Mazandaran University of Medical Science and Student Research Committee for financial support.

References

1) Larrieu E, Costa Mt, Cantoni G Alvarez R, Cavagion L. Ovine Echinococcus granulosus transmission dynamics in the province of Rio Negro, Argentina, 1980-1999. Vet Parasitol 2001; 98: 263-272.
2) Ammann RW, Eckert JC. Echinococcus. Gastroenterol. Clin North Am. 1996; 25: 655-689.
3) Lahmar S, Ben Che'hida F, Pe'tavy AF, Hammou A, Lahmar J, Ghannay A. Gharbi HA, Sariron ME. Ultrasonographic screening for cystic echinococcosis in sheep in Tunisia. Vet Parasitol 2007; 143: 42-49.
4) Zulfikaroglu B, Ozalp N, Keskek M. Koc M. Primary echinococcal cyst of the thyroid: report of a case. Surg Today 2008; 38: 833-835.
5) Topcua O, Aydina C, Aricib S, Dumana M, Sena M, Koyuncua A. The effects of various scolicidal agents on the hepatopancreatic biliary system. Chir Gastroenterol 2006; 22: 185-190.
6) Topcu O, Sumer Z, Tuner E, Aydin C, Koyuncu A. Efficacy of chlorhexidine gluconate during surgery for hydatid cyst. World J Surg 2009; 33: 1274-1280.
7) McManus DP, Zhang W, Li J, Bartley PB. Echinococcosis. Lancet 2003; 362: 1295-1304.
8) Adas G, Arikan S, Kemik O, Oner A, Sahio N, Karatepe O. Use of albendazole sulfoxide, albendazole sulfone, and combined solutions as scolicidal agents on hydatid cysts (*in vitro* study). World J Gastroenterol 2009; 15: 112-116.
9) Ahmadiani A, Fereidoni M, Semnanian S, Kamalinejad M, Saremi S. Antinociceptive and antiinflamayory effect of Sambuus ebulus rhizome extract in rats. J Ethnopharmacol 1998; 61: 229-235.
10) Ebrahimzadeh MA, Ehsanifar S, Eslami B. *Sambucus ebulus* elburensis fruits: A good source for antioxidants. Pharmacog Magazine 2009; 4: 213-218.
11) Cenini P, Bolognesi A, Stirpe F. Ribosome-inactivating proteins from plants inhibit ribosom activity of trypanosome and leishmania. J Protozool 1988; 35: 384-387.
12) Ebrahimzadeh MA, Mahmoudi M, Karami M, Saeedi S, Ahmadi AH, Salimi E. Separation of active and toxic portions in *Sambucus ebulus*. Pak J Biol Sci 2007; 10: 4171-4173.
13) Yesilada E, Gurbuz I, Shibata H. Screening of Turkish anti ulcerogeni folk remedies for anti-Helicobacter pylori activity. J Ethnopharmacol 1999; 66: 289-293.
14) Tuzlaci E, Tolon E. Turkish folk medicinal plants, part III: Sile (Istanbul). Fitoterapia 2000; 71: 673-685.
15) Ebrahimzadeh MA, Nabavi SF, Nabavi SM. Antioxidant activities of methanol extract of *Sambucus ebulus* L. Flower. Pak J Biol Sci 2009; 12: 447-450.
16) Ebrahimzadeh MA, Mahmoudi M, Salimi E. Antiinflammatory activity of *Sambucus ebulus* hexane extracts. Fitoterapia 2006; 77: 146-148.
17) Girbes T, Citores L, Iglesias R Ferreras JM, Munoz R, Rojo MA, Arias FJ, Garcia JR, Mendez E, Calonge M. Ebulin1, a non-toxic novel type 2 ribosome-inactivating protein from *Sambucus ebulus*. J Biol Chem 1993; 268:18195-18199.
18) Besim H, Karayalcin K, Hamamci O,Gungor C, Korkmaz A. Scolicidal agents in hydatid cyst surgery. HPB Surg 1998; 10: 347-351.
19) Erzurumlu K, Hokelek M, Baris S, Sahin M, Birinci A. Effect of albendazole sulfoxide solution on the scolices and the hepatobiliary system. Eur Surg Res 1998; 30: 433-438.
20) Moazeni M, Nazer A. *In vitro* effectiveness of Garlic (*Allium sativum*) extract on Scolices of Hydatid Cyst. World J Surg 2010; 34: 2677-2681.
21) Caglar R, Yuzbasioglu MF, Bulbuloglu E, Gul M, Ezberci F, Kale IT. *In vitro* effectiveness of different chemical agents on scolices of hydatid cyst. J Invest Surg 2008; 21: 71-75.
22) Kayaalp C, Balkan M, Aydin C Ozgurtas T, Tanyuksel M, Kirimlioglu V. Hypertonic saline in hydatid disease. World J Surg 2001; 25: 975-979.
23) Dalimi A, Ghasemikhah R, Hashemi Malayeri B. Echinococcus granulosus: lethal effect of low voltage direct electric current on hydatid cyst protoscolices. Exp Parasitol 2005; 109: 237-240.
24) Moazeni M, Larki S. *In vitro* effectiveness of acidic and alkaline solutions on scolices of hydatid cyst. Parasitol Res 2010; 106: 853-856.
25) Sadjjadi SM, Zoharizadeh MR, Panjeshahin MR. *In vitro* screening of different Allium sativum extracts on hydatid cysts protoscoleces. J Invest Surg 2008; 21: 318-322.