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LETHAL EFFECTS OF *PISTACIA ATLANTICA* LEAVES EXTRACT AGAINST PROTO-SCOLECES OF HYDATID CYSTS

Mohammad Reza Aflatoonian [1, 2], Mojgan Saki [3], Farnaz Kheirandish [4], Hossein Mahmoudvand [3] and Mohammad Reza Nazer[*4]

Bam University of Medical Sciences [1], Bam, Iran.
Research Center for Tropical and Infectious Diseases [2], Kerman University of Medical Sciences, Kerman, Iran.
Student Research Committee [3], Razi Herbal Medicines Research Center [4], Lorestan University of Medical Sciences, Khorramabad, Iran.

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Correspondence to Author:
Dr. Mohammad Reza Nazer

Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran.

E-mail: Dr_nazer1@yahoo.com

ABSTRACT: Surgery is one of the best options to treat cystic echinococcosis (CE, hydatid cyst). To date, surgeon used different protoscolicidal drugs to eliminate of protoscoleces during CE surgery, but most of these agents have some serious side effects. The current study aims to explore the *in-vitro* protoscolicidal effects of *Pistacia atlantica* leave extract against hydatid cyst proto-scolices. Proto-scolices were collected from livers of infected sheep and were exposed with different concentrations of *P. atlantica* extract (8.75 - 70 mg/ml) for 10 - 60 min. Finally, to explore the viability of proto-scolices eosin exclusion examination was applied. The major components of leave extract of *P. atlantica* in gas chromatography/ mass spectrometry were trans-caryophyllene (15.18%), α-amorphene (8.1%), and neoalloocimene (6.21%), respectively. The obtained results revealed that *P. atlantica* leave extract had remarkable proto-scolicidal effects against proto-scoleces of hydatid cysts; so that killed completely (100%) all of proto-scoleces at the dose of 70 mg/ml after 10 min of treatment. Also, *P. atlantica* leave extract at the concentration of 35 mg/ml was eliminated 100% of proto-scoleces after 20 min of incubation. These findings indicated that *P. atlantica* leave extract had remarkable protoscolicidal properties versus *hydatid cyst* proto-scoleces which indicated the ability of *P. atlantica* as a natural resource to produce of novel proto-scolicidal drug for CE surgery.

INTRODUCTION: Historically, the use of medicinal plants to treat illness and keep public health is highly prevalent around the world. Plant materials and their derivates show a central role in the field of novel drugs investigation for treatment or prevention of a wide range of diseases such as infectious ones [1].

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One of these interesting plants is *Pistacia atlantica* Desf. (Anacardiaceae), which commonly observed in various parts of the world such as Iran. In folk medicine, some parts of the *P. atlantica* have been extensively applied to treat several disease and illness such as diarrhea, asthma, anemia and infectious diseases [2]. Reviews have also reported anti-inflammatory, anticancer, antitumor, antialergic and antimicrobial properties of *P. atlantica* [2].

Cystic echinococcosis (CE) or hydatidosis consider as one of the main parasitic diseases in human and animals which caused by a small dog tapeworm, *Echinococcus granulosus* [3].

Normally, humans acquire infection *via* consumption of vegetables, food or water contaminated with *E. granulosus* eggs. CE in human because of its effects on vital organs including liver and lung, and livestock due to considerable economic problems in stock breeding has attracted World Health Organization (WHO) attention to itself [4]. From last decades, surgery is the most common choice in CE treatment. Surgeons during open cyst removal use some chemical scolicidal agents to decrease the risk linkage of proto-scoleces [5].

However, it has been proven that these common proto-scolicidal agents have some side effects such as necrosis of liver [6, 7]. Therefore, search for a proper protoscolicidal drug with potent efficacy and lowest side effects is necessary for surgeons. The present investigation aims to evaluate the protoscolicidal effects *P. atlantica* on protoscoleces of hydatid cyst on *in - vitro.*

MATERIALS AND METHODS:

Plant Materials: Leaves of *P. atlantica* were collected from mountains of Lorestan province, West of Iran, in September 2014. The materials were determined by a botanist of the Botany Department of Lorestan University, Lorestan, Iran. A voucher sample also has been recorded in the herbarium of Research Center for Agriculture Sciences, Khorramabad, Iran (LF 2522).

Preparation of Extract: One hundred g of powdered dried leaves were isolated using percolation method with methanol (80%) for 72 h. in 21 °C. The extract was crossing through filter paper (Whatman No. 3, Sigma, Germany) to delete artifacts. Finally, the extract was concentrated at 50 °C using a rotary evaporator (Heidolph, Germany) and kept at -20 °C, until use [8, 9].

Gas Chromatography/Mass Spectrometry (GC/ MS) Analysis of Extracts: Chemical compounds of *P. atlantica* extract were identified by extracting them using a solid phase micro extraction (SPME) technique. Initially, the samples were first ground into a fine powder using a household mill. Two grams of the sample was weighed and transferred to a 20 ml vial. Then the vial containing the sample was transferred to the ultrasonic device to extract the volatile substances. The temperature of the ultrasonic device was set on 50 °C for 15 min. In the next step, the SPME fiber was placed on the upper space of the sample for 40 min to extract the volatile compounds. Immediately after the extraction, the SPME fiber was injected into the GC-Mass device for desorbing and identifying the compositions of sample. Desorbing was performed in the GC column for 2 min.

The SPME fiber holder for manual use and the 100 µm Polydimethyl siloxane (PDMS) fibers were obtained from Supelco (Bellefonte, PA, USA) [10]. GC/MS was carried out by an Agilent 6890N coupled with a HP-5MS column (30m × 0.25 mm, film thickness 0.25 mm). The column temperature was maintained at 40 °C for 12 min and programmed to 180 °C at a rate of 10 °C per min, and kept constant at 180 °C for 4 min. Injector and interface temperatures were 250 and 280 °C, respectively. The flow rate of Helium as carrier gas was (1 mL/min C.F). The percentages were calculated by electronic integration of FID peak areas without the use of response factors correction. Linear retention indices for all components were determined by co-injection of the samples with a solution containing homologous series of C8-C24 *n*-alkanes.

Identification of Components: The constituents of the extracts were determined in comparison with their standards Wiley 2001 library data of the GC/MS system or compared with those of reported in the previous studies [11].

Collection of Proto-scoleces: *E. granulosus* proto-scolexes were obtained from the livers of naturally infected livestock slaughtered at Kerman abattoir, Iran. The cyst fluid was aseptically aspirated and was left to set for 30 min. After washing of proto-scoleces for two times with PBS (pH 7.2) solution the number of protoscoleces/ml was adjusted as 2×10^3 protoscoleces in 0.9 % NaCl solution with at least 90 % viability rate [16].

Proto-scolicidal Effects: To determine the lethal effects *P. atlantica* on hydatid cyst protoscoleces, various doses of *P. atlantica* extract were applied for 10, 20, 30 and 60 min. The protocol was performed according to the method described by Mahmoudvand *et al*., (2014). Initially, 0.5 ml of the protoscoleces (2×10^3/ml) and 0.5 ml of different doses of tested extract was added to each test tube. Test tubes were slowly mixed and then kept at

37°C for 10 - 60 min. After the time of incubation, the supernatant was discarded and 50 µl of 0.1 % eosin stain was added to protoscoleces and smeared on a glass slide and tested under a light microscope [17]. The percentages of dead proto-scoleces were counted by counting 100 proto-scoleces [18, 19]. Moreover, normal saline and hypertonic saline of 20% were applied as negative and positive controls.

Evaluation of Viability of Protoscoleces: In this study, eosin exclusion assay (eosin solution 0.1%) was applied to determine the viability of proto-scoleces of hydatid cysts [14]. The live proto-scoleces are colorless and displayed characteristic muscular movements and flame cell activity after exposure to the stain, whereas dead proto-scoleces are red color.

Statistical Analysis: Statistical analysis was performed using SPSS software (17.0). Moreover, *t*-test was applied to determine the differences between groups. $P<0.05$ was also considered statistically significant [15].

RESULTS:

GC/MS Analysis of Extracts: Table 1 shows the identified compounds of leave extract of *P. atlantica* using GC/MS.

TABLE 1: LEAVE EXTRACT COMPOSITION OF *P. ATLANTICA* IDENTIFIED BY GC/MS

S. no.	Compound	Retention time	% of components
1	α-Pinene	6.67	3.345
2	β-Pinene	7.89	0.801
3	β-Myrcene	8.30	2.097
4	Tetradecane	10.36	0.480
5	γ-Terpinene	10.48	0.715
6	Nonanal	12.03	0.554
7	L-camphor	13.44	0.134
8	Menthone	13.76	1.298
9	Borneol	14.30	0.662
10	L-Menthol	14.54	1.725
11	Dodecane	15.19	0.369
12	Bornyl acetate	18.18	0.725
13	Thymol	19.04	0.180
14	Dodecane	19.41	0.633
15	Camphene	20.30	0.837
15	Aromadendrene	20.98	3.137
17	Copaene	21.12	1.818
18	α-Cadinene	21.55	0.417
19	Tetradecane	21.77	1.113
20	α-Gurjunene	22.15	1.826
21	trans-Caryophyllene	22.48	15.18
22	Calarene	22.70	1.290
23	Germacrene D	22.91	1.149
24	Neoalloocimene	23.05	6.21
25	β-Selinene	23.15	1.493
26	α-Humulene	23.47	3.000
27	Heptacosane	23.56	1.100
28	Valencene	23.65	1.537
29	α-Amorphene	24.11	8.1
30	Dodecane	24.48	2.451
31	Isoledene	24.61	2.989
32	α-Muurolene	24.76	2.803
33	β-Cadinene	25.16	3.669
34	delta.-Cadinene	25.38	5.915
35	β-Cadinene	25.69	0.607
36	α-Muurolene	25.77	1.143
37	α-calacorene	25.95	1.118
38	Nerolidol	26.47	1.422
39	Caryophyllene oxide	27.00	1.620
40	1,2-Dihydro-2,2,3-trimethyl-1-quinoxalinol-4-dioxide	28.35	3.436
41	Eudesmol	28.79	1.718
42	Tritetracontane	30.68	0.901
43	Tetracosane	32.75	1.699
	Total		85.7

The major components of leave extract of *P. atlantica* were trans-caryophyllene (15.18%), α-amorphene (8.1%), and neoalloocimene (6.21%), respectively.

Protoscolicidal Effects: As shown in **Table 2** *P. atlantica* leave extract demonstrated protoscolicidal activity against hydatid cysts protoscoleces. The current finding revealed that *P. atlantica* leave extract had remarkable protoscolicidal effects against protoscoleces of hydatid cysts; so that killed completely (100%) all of proto-scoleces at the dose of 70 mg/ml after 10 min of treatment. Also, *P. atlantica* leave extract at the concentration of 35 mg/ml was eliminated 100% of protoscoleces after 20 min of incubation. The protoscolicidal effect of *P. atlantica* leave extract at the concentration of 17.5 mg/ml was 14.3, 37.6, 63.3 and 100 % after 10, 20, 30 and 60 min of incubation, respectively.

The mean dead of proto-scoleces in the normal saline control group was 5.3 % after 60 min of treatment, while the proto-scolicidal effects of 20% hypertonic saline as the positive controls was 100% after and 10 min of application. These findings exhibited that the scolicidal activity of *P. atlantica* leaves extract was significant higher (p<0.05) in comparison with the control group at all exposure times.

TABLE 2: SCOLICIDAL EFFECTS OF *P. ATLANTICA* LEAVE EXTRACT AGAINST PROTO-SCOLECES OF HYDATID CYST AT VARIOUS CONCENTRATIONS FOLLOWING VARIOUS EXPOSURE TIMES

Concentration (mg/mL)	Exposure time (min)	Mean of mortality rate (%)
70	10	100
	20	100
	30	100
	60	100
35	10	59.6
	20	100
	30	100
	60	100
17.5	10	14.3
	20	37.6
	30	63.3
	60	100
8.75	10	6.6
	20	29.3
	30	52.3
	60	93.3
Normal saline	10	1.3
	20	2.6
	30	4.3
	60	7.1
2 0 % Hypertonic saline	10	100
	20	100
	30	100
	60	100

DISCUSSION: Medicinal plants and their pure compounds show proper opportunities for new drug searches in order to the having many benefits such as higher efficacy and availability as well as lower toxicity [16]. Here, we found that *P. atlantica* leave extract had remarkable protoscolicidal effects against proto-scoleces of hydatid cysts; so that killed completely (100%) all of protoscoleces at the dose of 70 mg/ml after 10 min of treatment. Also, *P. atlantica* leave extract at the concentration of 35 mg/ml was eliminated 100 % of protoscoleces after 20 min of incubation. This scolicidal effects of *P. atlantica* is comparable with scolicidal activity of hypertonic saline, silver nitrate and mannitol, cetrimide, ethyl alcohol (95%), H_2O_2 and 10% providone iodine, albendazole, chlorhexidine gluconate, Selenium NPs and some plant extracts [17- 24]. Reviews have reported antibacterial, antiviral, antifungal and antiparasitic effects of *P. atlantica* [3].

We found that the major components of leave extract of *P. atlantica* were trans-caryophyllene (15.18%), α-amorphene (8.1%), and neoalloo-cimene (6.21%), respectively. However, according

to the previous investigations chemical composition of extracts depend on species, climate, and time of collection along with growth stage, thereby altering the biological activities studied [25].

Previous studies also revealed the presence of terpenoids, phenols, flavonoids, fatty acids and sterols in phytochemical screening of the crude extract *P. atlantica* [3]. In several investigations biological especially antimicrobial activities of these components have been proven [24 - 27]. Thus, we can suggest that these components are responsible for the scolicidal activity of *P. atlantica*; however their exact action mechanism is not clearly understood. Regarding the some mechanism of antimicrobial terpenoids compounds such as monoterpens, investigations have demonstrated that these compounds diffuse into pathogen and destroy cell membrane. Other studies also reported that anti-microbial effects of these components interfering with vital intracellular sites when cross the cell membranes and penetrate into the cell [28, 29].

CONCLUSION: These findings indicated that *P. atlantica* leave extract had remarkable proto-scolicidal properties versus hydatid cyst protos-coleces which indicated the ability of *P. atlantica* as a natural resource to produce of novel proto-scolicidal drug for CE surgery.

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