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Effect of Ethanolic Extract and Crude Alkaloides of *Peganum harmala* Seeds on The Viability of *Echinococcus granulosus* Protoscolices *in vitro*

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ABSTRACT

The crude ethanolic extract and crude alkaloids of *Peganum harmala*_seeds were prepared, the scolicidal effect of these extracts on the protoscolices of *Echinococcus granulosus in vitro* were determined at 4°C and 37°C at different time intervals.

Both extracts exerted higher effect at 37°C, and the crude alkaloid extract was more effective than the ethanolic extract as it gave a scolicidal effect at a concentration of 31 mg/ml and it i also exerted a complete scolicidal effect at (48-72) hrs. which was similar to the effect of mebendazole.

تأثير المستخلص الكحولي والقلويدات الخام لبذور الحرمل على حيوية الرؤيسات الأولية للمشوكة الحبيبية في الزجاج

الملخص

تم في هذه الدراسة تحضير المستخلص الأيثانولي والقلويدات الخام لبذور نبات الحرمل،حيث حـدد التأثير القاتل لهذه المستخلصات على الرؤيسات الأوليـة لطفيلـي المـشوكة الحبيبيـة *Echinococcus granulosus* في الزجاج، في الدرجتين الحراريتين°4م و°37م وضمن فترات زمنية مختلفة.

اظهر كلا المستخلصين تأثيرا أقوى في درجة°37م، كما تبين ان القلويد الخام لنبات الحرمل كـان اكثر تأثيرا من المستخلص الكحولي في قتل الرؤيسات الأولية للأكياس المائية،إذ أعطـى تـأثيرا قـاتلا بتركيز 31 ملغم/مل. كما أنها أحدثت قتلا كاملا للرؤيسات الأولية خلال فترة (48-72) ساعة،وهو تأثير مشابه لتأثير عقار الميبندازول.

INTRODUCTION

Hydatid disease was considered as one of the major health problem in Iraq and all over the world (Andersen *et al*., 1997 and Al-Hammo, 1999). It is caused by the larval stage (metacestodes) of *Echinococcus granulosus*. Adult worm inhabits the small intestine of carnivores, humanget the infection by ingestion of ova that discharged to the environment by the carnivores (Zeibig, 1997).

Currently there are three traditional methods for treatment of hydatid disease: 1-Surgery. 2-Puncture, aspiration, injection and reaspiration (PAIR). 3-Chemotherapy. Chemotherapy is still considered as the last resort for the treatment, and is only indicated to prevent secondary echinococcosis and for inoperable cases (WHO, 1996). Success of treatment with mebendazole depends highly on the localization of hydatid cyst, and using the drug only 21% of pulmonary and 7% of hepatic hydatidosis were healed (Akhan *et al.*, 1994). While Aktan and Yalin (1996) showed that the removed cysts, appeared to be completely non viable in only 35% of patients, treated with mebendazole before surgery for hepatic hydatidosis. Kuro *et al*. (1997) also concluded that the treatment with benzinadozole, albendazole, and mebendazole given successful results in only 30% of hepatic cysts. Hence, great efforts are being spent to find an effective medical cure for the disease.

Recently, many workers look forward for the use of natural products in the treatment of many diseases, including echinococcosis. Al-Tae (1996) and Ali (1999) reported that the extracellular polysaccharides extracted from some species of bacteria and fungi act as immunomodulators in experimental infection with hydatid disease in mice while Al-Hammo(2002) showed that *Matricaria chamomilla* Linn. and *Cyperus rotundus* Linn. Posses inhibitory effect on the viability of hydatid cyst protoscolices *in vitro*.Mahmoud (2002) showed that the alkaloidal extract of *peganum harmala* seeds caused reduction in the viability of protoscolices of human and sheep origion.

In the present study the seeds of *Peganum harmala* Linn. which belongs to the family Rutaceae were used. *Peganum harmala* seeds have been used in India for treatment of chronic malaria, it also have antibacterial, protozoacidal and anthilminthic effect, and were used for tapeworm infection (Kotb, 1985). It is found to be useful as antirheumatic, diuretic and lactagogues drug (Hilal *et al*., 1978).

Principle constitutes: *Peganum harmala* seeds contain 2.5-4% of alkaloids, mainly: harmine, harmaline and harmalol together with other exogenous compounds like flavinoides, resins and fatty acids. (Kotb, 1985 and Khorsheed, 1998).

MATERIALS AND METHODS

Seeds of *Peganum harmala* were collected from local markets in Mosul city and classified in the herbarium of Biology Department/College of Science/University of Mosul.

Preparation of ethanolic extract: The seeds were milled into coarse powder, then soaked in 80% ethanol, kept at 4C° for 24hrs,filtered by Buschner funnel, ethanol then evaporated at 40C° and the extract was lyophilized to get dry powder of the extract (Verporte et al., 1988).

Preparation of crude alkaloids: The powdered seeds were extracted with ethanolic alcohol (80%) that contained diluted HCl (1N), pigments and unwanted materials were

removed by shaking with chloroform. The free alkaloids were then precipitated by the addition of excess ammonia and separated by filtration (Evans, 1997).

Protoscolices collection and suspension:

In this study hepatic hydatid cysts of infected sheep were obtained from the municipal abattoir in Mosul city, Iraq. Protoscolices were removed from the cysts by aseptic techniques, washed in several changes of sterile phosphate buffer saline (pH 7.2). Protoscolices were then suspended in sterile hydatid fluid that contain 2% organic solvent, Dimethyl sulfoxide (Farjou and Al-Hussainawi, 1984).

The suspension then put in siliconized test tubes, 2ml\tube, 500 Protoscolices\ml of the suspension.

All used hydatid cysts possessed a mean viability of at least 90%, this was determined by peristaltic movements of protoscolices, negative staining with 0.1% aqueous eosin and flame cell movement. (Smyth and Barrett, 1980).

The effect of *Peganum harmala* ethanolic extract:

Two groups of protoscolices suspensions were used, each group consist of nine test tubes, the first tube was considered as a control, the second tube was treated with 1mg mebendazole/ml of the suspension, the remaining tubes (3-9) were treated with different concentrations of the ethanolic extract (250, 125,62.5,31.25,15.6,7.81,3.91) mg of the extract/ml of the protoscolices suspension. The first group was incubated at 4°C, and the second group was incubated at 37°C. The viability of potoscolices was examined for each group and after each exposure time (each treatment was carried out in triplicates).

The effect of crude alkaloids of *Peganum harmala* on protoscolices:

To prepare different concentrations of crude alkaloid extract, the same procedure in preparing ethanolic extract treatments was used as mentioned before.

Statistical analysis used in this study included: F-test using analysis of variance (ANOVA table) and Duncan`s multiple range test. All results were significant with $P<0.05$ and $P<0.01$ levels. (Al-Rawi and Kalaf Allah, 1980).

RESULTS AND DISCUSSION

The survival percentage of protoscolices treated with crude ethanolic and alkaloid extracts of *Peganum harmala* seeds at 4°C and 37°C in time intervals tested was given in table (1) and table (2) respectively.

Combined ANOVA Table (3) indicates that there are a high significant effect of the treatments, temperature and exposure time, used in this study on the of viability protoscolices *in vitro.* In table (4) the results arranged from highest inhibitory effects on the viability of the protoscolices to the lowest effect at 4°C (using capital letters), Duncan`s test shows that the protoscolices treated with both extracts give a progressive decline in survival which was marked since the first hour of incubation for both extracts and give a complete scolicidal effect between (48-72) hrs. especially at higher concentrations 250 and 125 mg/ml, which is comparable with the effect of mebendazole , table (4) also shows that the crude alkaloids is more effective than the ethanolic extract as lower concentrations 62.5 and 31.2 mg/ml respectively gave a complete scolicidal activity between (48-72)hr.

This result indicates that the alkaloides are the active components that may inhibit viability of the protoscolices. Adday et al.(1989) reported that the seeds of *Peganum*

harmala possessed antimicrobial activity; Feng and Kang (1994) showed that the ethanolic extract of *Peganum harmala* seeds gave a stronger scolicidal effect at 2% concentration, as the mortality rate of Protoscolices were reaches 90%, and they conclude that the scolicidal activity of this extract may be due to the presence of alkaloids in the seeds of *P. harmala* and this is consistent with the present results.Mahmoud (2002) recorded good scolicidal activity of the alkaloidal extract which appeared within minutes of incubation specially at high concentration (50 mg/ml) and she mentioned that this effect may be due to it's effect on the metabolic pathway and enzymes activity of protoscolices .

Table 1: The effect of ethanolic extracts and crude alkaloids of *Peganum harmala* seeds on the viability% of *E. granulosus* protoscolices *in vitro*, at 4°C compared with mebendazole and control groups.

Treatment	**Concentration mg/ml**	**Mean viability% of Protoscolices after:**							
		1hr	**3hr**	**6hr**	**24hr**	**48hr**	**72hr**	**96hr**	**120hr**
*Control	0	100	100	99	98.7	88.7	91.3	88.3	76.7
mebendazole	0.1	87	67.3	11.7	1	0.3	0	0	0
Ethanolic extract	250	6.7	1.3	0.7	0.3	0.3	0	0	0
	125	32.3	14	3.7	0.3	1.3	2.7	0.3	0.3
	62.5	81.7	67.3	11.7	1	3	2	0.3	0
	31.2	70.3	30.7	30.0	17.7	5	0	0	0
	15.6	99	97.3	87.7	38.7	28	17	4	0
	7.8	91.7	91.8	90	87	18	0.3	0	0
	3.9	95	92	73.7	33	5.7	2	0	0
Crude alkaloides	250	7	1	0.3	0	0	0	0	0
	125	11	5	2	1.7	0.3	0	0	0
	62.5	20.3	17	16	3	2	0	0	0
	31.2	42	25	26	3	1.3	0	0	0
	15.6	56	33.3	28	6	1.3	0.7	0	0
	7.8	85	42	37	25.7	3	4	0.3	0
	3.9	98	97	96.3	88.3	35	12	8	6

N=3

*Control group=500 potoscolices/ml of hydatid fluid.

Viability percentage at zero time considered as 100%

It is evident from table (2) that both alkaloides and ethanolic extracts were more effective at 37°C and showed more than 90% inhibition for the 250, 125 and 62.5 mg/ml concentrations within the first three hours of incubation, most of the examined crude alkaloids concentrations showed a complete scolicidal effect after 48hrs. of incubation, comparing to the effect of mebendazole (table 5). Since the living cells were metabolically active at 37°C, many of the inhibitory substances were more effective at this temperature (Al-Habib, 1991). Simultaneously, that survival period for the protoscolices that incubated at 4°C is longer than incubated at 37°C, because of the faster autolytic activity of protoscolices at 37°C. (Andersen and Loveless, 1978).

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Table 2: The effect of ethanolic extracts and crude alkaloids of *Peganum harmala* seeds on the viability% of Protoscolices of *Echinococcus granulosus in vitro*, at 37°C compared with mebendazole and control groups.

Treatment	Concentration mg/ml	Mean viability% of Protoscolices after:							
		1hr	3hr	6hr	24hr	48hr	72hr	96hr	120hr
*Control	0	100	100	100	99	93.7	87.3	35.3	0.3
Mebendazole	0.1	23.3	23.7	23.7	10.7	0	0	0	0
Ethanolic extract	250	3	1	1.3	0.7	0.3	0.3	0	0
	125	1	1.3	1	0.7	0.3	0.3	0	0
	62.5	10	10	1.3	0.3	0.3	0	0	0
	31.2	24	24.3	0.3	0	0.3	0	0	0
	15.6	63.3	39.7	15.3	5.3	0.3	0	0	0
	7.8	85	52.7	32	26.7	13.7	1	0.3	0
	3.9	87.7	86.7	88.7	36.3	15.7	3	0	0
Crude alkaloids	250	3	0.3	0	0	0	0	0	0
	125	2.3	0	0	0	0	0	0	0
	62.5	15	10.3	1.3	0.3	0	0	0	0
	31.2	32.7	18	6.7	1.3	0.7	0	0	0
	15.6	72.3	39.3	28	5.3	3	0.3	0	0
	7.8	59	62.7	62.3	22.3	4.3	0	0	0
	3.9	99	97.3	96	75	42.7	0.3	0.3	0

N=3

*Control group=500 Protoscolices/ml of hydatid fluid.

Viability percentage at zero time considered as 100%.

Table 3: Combined ANOVA table for the effect of treatments (A), temperatures (B) and exposure times (P) on the viability of *E. granulosus* Protoscolices *in vitro*.

Source of variance	Degree of freedom	Sum of square	Mean square	Calculated F
A	15	256197.35	17079.87	101.48**
B	1	6880.66	6880.66	40.88**
AxB	15	9178.45	611.80	3.64**
Error a	64	10771.54	168.31	
P	7	194434.93	27776.42	1530.38**
AP	105	76193.55	725.653	39.98**
BP	7	62576.35	8939.48	492.53**
AbP	105	6670.21	63.53	3.53**
Error b	448	8129.06	18.15	
Total	767	631032.1		

** P<0.01

Table 4: Levels of the inhibitory effects of ethanolic and alkaloidal extracts on the viability of *E. granulosus* Protoscolices at 4°C compared with mebendazole and control group (Duncan`s test at P>0.05).

Treatment	Concentration Mg/ml	Viability mean of Protoscolices	Level of effect
control	0	1729.49	j
Mebendazole	0.1	549.85	E
Ethanolic extract	250	90.57	A
	125	187.03	B
	62.5	340.73	D
	31.2	549.85	F
	15.6	961.8	H
	7.8	1171.24	I
	3.9	788.26	G
Crude alkaloids	250	54.98	A
	125	106.27	A
	62.5	254.58	C
	31.2	320.37	D
	15.6	447.97	E
	7.8	649.34	F
	3.9	759.20	G

Table 5: Levels of the inhibitory effects of ethnolic and alkaloid extracts on the viability of *E. granulosus* Protoscolices at 37°C compared with mebendazole and the control group (Duncan`s test at P>0.05).

Treatment	Concentration mg/ml	Viability mean of Protoscolices	Level of effect
Control	0	1649.52	H
Mebendazole	0.1	359.0	C
Ethanolic extract	250	70.57	A
	125	58.57	A
	62.5	142.41	B
	31.2	191.85	B
	15.6	383.63	C
	7.8	630.25	E
	3.9	1145.38	G
Crude alkaloids	250	11.5	A
	125	30.62	A
	62.5	142.71	B
	31.2	248.95	B
	15.6	468.72	D
	7.8	611.92	E
	3.9	1088.09	F

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