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Effect of Some Plants Extracts on the Viability of Protoscoleces in Vitro

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Abstract

This study was conducted to evaluate the protoscolicidal effect of four plants alcoholic extracts on protoscoleces of the parasite *Echinococcus granulosus* in vitro. The plants that had been used in this study were: *Viscum album* (Mistletoe) and *Prunus armeniaca (*Apricot bitter kernels) with concentrations of 375, 750 and 1500 mg/ml, also *Syzygium aromaticum* (Clove flower buds) and *Capsicum frutescence* (Chili pepper) with concentrations of 375, 750 and 1500 µg/ml. All extracts were treated at four periods (15, 30, 45 and 60 min.).

In vitro, the high percentage of protoscoleces killing was observed in *V. album* alcoholic extract (80.7%) in the high concentration 1500 mg/ml at 60 min., and the lowest percentage of protoscoleces killing (62%) was in the concentration 375 mg/ml at 15 min.

P. armeniaca alcoholic extract high percentage of killing (70.2%) was also at the high concentration 1500 mg/ml at 60 min., and the lowest percentage of protoscoleces killing (50.4 %) at the concentration 375 mg/ml at 15 min.

The high percentage of protoscoleces killing of alcoholic extract of *S. aromaticum* (73.7%) was at the concentration 1500 µg/ml at 60 min. and the lowest percentage (58.7%) was at the concentration 375 µg/ml at 15 min.

C. frutescence alcoholic extract high percentage of protoscoleces killing (71.4 %) was at the concentration 1500 µg/ml at 60 min. and the lowest percentage (40.6 %) was at the concentration 375 µg/ml at 15 min.

Introduction

Hydatidosis or hydatid cyst disease is a chronic infection of medical and veterinary importance caused by the metacestode or larval stage of the cosmopolitan cestode *Echinococcus granulosus* [1]. The stray dogs, as a definitive host of the adult *Echinococcus granulosus*, play the most important role in spread of infection in the Middle East countries via contamination of the environment with its eggs [2]. On the other hand, sheep, goats, cattle, camels, buffaloes, pigs and donkeys play as suitable intermediate hosts have been repeated the infection with hydatidosis in Iraq, Iran, Jordan, Lebanon, Syria, Kuwait and Saudi Arabia [3]. It grows inside host viscera (mainly liver and lungs) as a fluid-filled cyst, which can reach several centimeters in diameter (4). *Viscum album* (European mistletoe), *Prunus armeniaca* (Apricot bitter kernels), *Syzygium aromaticum* (Clove flower buds) and *Capsicum frutescence* (chili pepper) alcohol extracts have been chosen in this study. The aims of this study were to clarify the effect of alcohol plants extracts on protoscolices of *Echinococcus granulosus* in vitro.

Materials and Methods

Hydatid cysts were obtained from the infected livers and lungs of infected sheep after slaughter. The samples were collected from Erbil abettor from 1/8/2015 to 1/6/2016. The hydatid cysts were transported to the laboratory at the same day in insulated containers at about 4˚C.

The isolation and preparation procedure was applied according to Smyth [5]. The exposed surfaces of the cysts were sterilized with 70% ethanol and the fluid was aspirated by using large size syringe with needle (20 G x 1). The aspirated fluid left to settle for 10 minutes and the protoscoleces were settled down at the bottom of the tubes. The supernatant was removed by Pasture's pipette and the yielded protoscoleces were washed three times with phosphate buffered saline (PBS) using centrifuge at 3000 rpm for 10 minutes.

The viability of protoscolices was confirmed from their flame cells motility under the light microscopy. In this study, eosin stain with 0.1% concentration (1g of eosin powder in 1000 mL of distilled water) was used for the viability test of protoscolices. After 10 minutes of exposure, the protoscolices that appear in green color considered alive and the dead appear with red color [6]. The viability of the protoscoleces in this study was about 96%.

The plants of this study were obtained from spicier market in Erbil province to prepare the alcoholic extract for each plant. The extracts were prepared according to Abbas [7]. The plant cleaned with distilled water and left to dry in shade at room temperature. 20 grams of dried seeds were added to 200 mL of ethanol 70% for 24 hours at room temperature on magnetic stirrer 300 rpm. Then it was filtered by using 5 layers of gauze to separate the solid materials and the solution was filtered again with Whattman No.1 filter paper. The solvent was removed by using rotary evaporator (95 rpm in water path at 45◦C). The resulted semisolid material then was totally dried by using incubator at 40-45◦C. The obtained residues were placed in a sterile glass container and stored in the refrigerator at 4ºC for until use [8]. The concentrations were prepared by dissolving 1.5 g in 10 ml PBS (1500 mg/ml) for *V. album* and *P. armeniaca*; and dissolving 1.5 mg in 10 ml (1500 µg/ml) for *S. aromaticum* and *C. frutescence*. The diluting method had been used to prepare other concentrations for each extract. The last solutions were filtered by Zyze Filters (0.45µ) then in the refrigerator (4 C) until use [9].

The scolicidal tests in this study we examined three concentrations (375 mg, 750 mg and 1500 mg /ml) of the *V. album*, *P. armeniaca* and extracts and (1500

µg, 750 µg and 375 µg) of *C. aromaticum* and *C. frutescense* of for 15, 30, 45 and 60 minutes.

In a test tube, 0.5 ml of each concentration was placed and 10 µl was removed and replaced by 10 µl of protoscoleces to the tube of the extract and mixed gently. The tube was then incubated at 37°C for 15, 30, 45 and 60 min. At the end of each incubation time, the tube of the solution was centrifuged for 5 minutes then the supernatant was removed with a pipette avoiding settled protoscolices. Then 5 µl of 0.1% eosin stain was added to 5 µl settled protoscoleces and mixed gently and waited for 15 minutes. The settled protoscolices were then smeared on a manual scaled glass slide, covered with a cover slide and examined microscopically for viability. The live protoscolices after each test were calculated by using this formula [10]:

$$\textbf{Live protoscoleces \%} = \frac{\textbf{No. of the live protoscoleces in 1ml}}{\textbf{No. of total sample protoscoleces in 1 ml}} \times 100$$

Results

The results of this study had been showed the effect of alcoholic extract of *V. album* on protoscoleces in vitro using concentrations (1500, 750 and 375 mg/ml) at different incubation times (15, 30, 45 and 60 minutes). The high percentage of killing effect observed in *V. album* alcoholic extract in the high concentration (1500 mg/ml) was (80.7%) at (60 min.) and the killing percentage decreased when the time of exposure was less. It was (62%) at 15 minutes (Table 1).

Table-1- In Vitro Protoscolicidal Effect of Alcoholic Extract of *V. album*.

Concentration mg/ml	Timing %			
	15 min.	*30 min.*	*45 min.*	*60 min.*
1500	72.7 cd	75.6 bc	76 b	80.7 a
750	67.8 e	72.1 cd	73.2 bcd	74.9 bc
375	62 f	70.5 de	72.7 cd	73.6 bcd

*Similar letters mean there is no significant difference according to Dunkan's test.

The results of this study had been showed the effect of alcoholic extract of *P. armeniaca* on protoscoleces in vitro using concentrations (1500, 750 and 375 mg/ml) at four different incubation times (15, 30, 45 and 60 minutes). The high rate of protoscolicidal effect was at 1500 mg/ml (70.2%) at 60 minutes and the low rate was at the concentration 357 mg/ml (50.4 %) at 15 minutes, see Table (2).

Table-2- In Vitro Protoscolicidal Effect of Alcoholic Extract of *P. armeniaca*.

Concentration mg/ml	Timing %			
	15 min.	*30 min.*	*45 min.*	*60 min.*
1500	63.2 cd	64 bc	66.7 b	70.2 a
750	51.6 gh	53.9 efg	60.6 d	61.2 d
375	50.4 h	53 fgh	55.6 ef	56.9 e

*Similar letters mean there is no significant difference according to Dunkan's test.

The results of this study had been showed the effect of alcoholic extract of *S. aromaticum* on protoscoleces in vitro using concentrations (1500, 750 and 375 µg/ml) at different incubation times (15, 30, 45 and 60 minutes). The high rate of protoscolicidal effect was at 1500 µg/ml (73.7%) at 60 minutes and the low rate was at the concentration 357 µg/ml (58.7 %) at 15 minutes, see Table (3).

Table-3- In Vitro Protoscolicidal Effect of Alcoholic Extract of *S. aromaticum*

Concentration µg/ml	Timing %			
	15 min.	*30 min.*	*45 min.*	*60 min.*
1500	69.6 bc	71 ab	71.2 ab	73.7 a
750	69.1 bc	70.8 bc	71 ab	71.5 ab
375	58.7 d	67.7 c	68.4 bc	69.1 bc

*Similar letters mean there is no significant difference according to Dunkan's test.

The results of this study had been showed the effect of alcoholic extract of *C. frutescence* on protoscoleces in vitro using concentrations (150, 75 and 37.5 µg/ml) at different incubation times (15, 30, 45 and 60 minutes). The high rate of protoscolicidal effect was at 1500 µg/ml (71.4%) at 60 minutes and the low rate was at the concentration 375 µg/ml (40.6 %) at 15 minutes, see Table (4).

Table-4- In Vitro Protoscolicidal Effect of Alcoholic Extract of *C. frutescence*.

Concentration µg/ml	Timing %			
	15 min.	*30 min.*	*45 min.*	*60 min.*
1500	50.9 f	55.5 de	64.2 b	71.4 a
750	53.2 e	58.5 c	64.9 b	69.6 a
375	40.6 g	53.2 e	55.8 de	59.6 c

*Similar letters mean there is no significant difference according to Denkan's test.

Discussion

Due to the inability of the researcher to find any other studies to compare with them these results, the discussion will take place with a study that had tried the effect of *V. album* alcoholic extract on *Cryptosporidium parvum* oocysts [8]. The inner layer of the sporozoites and oocysts of *C. parvum* have multiple mucin-like glycoproteins (e.g., gp40 and gp900) which have the similar structure to that covering the *E. granulosus* protoscoleces outer membranes [11,12,13]. The mucin is the composition structure of *E. granulosus* adult, metacestode and protoscolices[13, 14]. Table (1) showed the high concentration of alcohol extract of *V. album* (80.7%) as a protoscolicidal effect in 60 minutes showed higher percentage of killing than Obaid [8] results on *C. parvum* which was (76%) of (1000 mg/kg) at the day 7th. This might be related to the site of the mucin-like glycoproteins on the inner membranes of the sporozoites and oocysts and the rigid outer layer that resist more than the protoscoleces which had this layer in direct exposure with the concentration [13].

Alcohol extract of the *P. armeniaca* showed high effect at (1500 mg/ml) in 60 minutes (70.2%) and the low rate was at the (357 mg/ml) concentration at 15 minutes was (50.4 %). Table 2 showed that there was significant difference among values which ensure that the time of exposure and the concentration play an important role in the death of protoscoleces. It seems that hydrogen cyanide (HCN), one of amygdalin decomposition products, had played an important role in the inhibition of the tested bacterial growth; the toxicity of hydrogen cyanide is caused by cyanide ion, a result of partial ionization of hydrogen cyanide [15].

Clove (*S. aromaticum*) results showed high rate of protoscoleces death at the high concentration (1500µg/ml) at 60 minutes was (73.2%) and the low rate was at the lower concentration (375 µg /ml) at the minute 15. There were significant differences showed by table (3) considered with time of exposure and concentration. According to the study about in vitro effect of *S. aromaticum* on the trematode *Cotylophoron cotylophorum* and inhibition of the carbohydrate metabolism enzymes, investigations on the effect of ethanol extracts of *S. aromaticum* on the cytosolic and mitochondrial fraction of *C. cotylophorum* revealed a significant inhibition of the key regulatory enzymes involved in carbohydrate metabolic pathway [16]. On the other hand, in vitro anthelmintic activity of *S. aromaticum* ethanolic extract were also dependent on time and concentration, Kumar and Singh [17] found that the ethanol extract was more toxic at 2h LC50, and the eugenol in *Fasciola gigentica* body could change the different enzymes activity. This may explain the permeability of the extract color into the protoscoleces in addition to eosin 0.1% staining.

C. frutescence protoscolicidal effect in (1500µg/ml) as showed in table (4) was (71.4%) in 60 minutes and (40.6%) at 15 minutes in concentration of (375 µg/ml. This result was compared with the research of Abdulla [5]; they used *C. annum* alcoholic extract on protoscoleces at the same time intervals with concentrations of (1, 1.25, 1.5, 1.75, 2, 2.25 mg/ml). The results of this study showed higher percentages than their result which was (11.00%) in 60 minutes. The reason is due to the amount of capsaicin in the *C. frutescence* sp. is more than found in *C. annum* and this is the cause of hot taste of the *C. frutescens*.

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تاثير بعض المستخلصات النباتية على حيوية الرؤوس الأولية في الزجاج

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الملخص

أجريت هذه الدراسة لتقييم التأثيرالقاتل للمستخلصات الكحولية لأربع نباتات على الرؤوس الأولية لطفيلي المشوكة الحبيبية خارج الجسم الحي وهي:حب الدبق *Viscum album* ، لوز المشمش المر *Prunus armeniaca*، براعم زهرة القرنفل *Syzygium aromaticum* ,*والفلفل الحار Capsicum frutescence*. كان تراكيز النباتين الأولين (375، 750 و1500 (ملغم/ مل) وتراكيز النباتين الأخيرين كانت (375، 750 و1500 ميكروغرام / مل) في 4 فترات زمنية (15، 30، 45، 60) دقيقة.

في تجربة المستخلصات خارج الجسم الحي لوحظ أن أعلى نسبة قتل للرؤوس الأولية كانت للمستخلص الكحولي لنبات حب الدبق بنسبة (80.7٪) وبالتركيز الأعلى (1500 ملغم / مل) في (60 دقيقة)، وكان أدنى نسبة قتل لنفس النبات (62٪) في تركيز (375 ملغم / مل) في (15 دقيقة).

كما اظهر المستخلص الكحولي لنبات لوز المشمش المر نسبة عالية من القتل (70.2٪) أيضا في التركيز العالي 1500 ملغ / مل في 60 دقيقة، وأدنى نسبة قتل للرؤوس الأولية (50.4٪) في تركيز 375 ملغ / مل في 15 دقيقة.

وكانت اعلى نسبة قتل للمستخلص الكحولي من بذور زهرة القرنفل (73.7٪) في تركيز 1500 ميكروغرام / مل في 60 دقيقة. وأقل نسبة (58.7٪) كانت عند التركيز 375 ميكروغرام / مل في 15 دقيقة.

وأظهر مستخلص الفلفل الحار الكحولي اعلى نسبة قتل (71.4٪) في تركيز 1500 ميكروغرام / مل في 60 دقيقة. وأقل نسبة (40.6٪) كانت عند التركيز 375 ميكروغرام / مل في 15 دقيقة.