The effect of ethanolic and methanolic extracts of the Iraqi bee wax in the viability of the protoscolices of *Echinococcusgranulosus* parasite in vitro

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Abstract- The current study was conducted for the period from 1/9/2015 up to 1/3/2016 at the Department of Biology - College of Education for Girls- Kufa University.It aims to study the effectiveness of ethanolic and methanolic extracts for Iraqi honey beewax in the viability of protoscolices of *Echinococcusgranulosus* parasite *In vitro*.The results showed that the ethanolic and methanolic extracts for Iraqi honey beewax are very effective in the destruction of the protoscolices, and that the mortality percentage increases with concentration and duration of exposure to both extracts .The results indicated that the methanolic extract for honey beeswax was more effective than the ethanolic extract in the mortality of protoscolices, where the LC50 for the mortality of the protoscolices was 3.5 mg / ml and 4.9 mg / ml, respectively, after 15 minutes of exposure to the extracts, while the LC50 for the mortality of the protoscolices 1.7 mg / ml and 2.0 mg / ml respectively, after 60 minutes of exposure to the extracts. The results also showed that the LT50 was 39.8 minutes when exposed to the concentration of 2 mg / ml of the methanolic and ethanolic extracts, whilemethanolic extract led to the decimation of theprotoscolices in less time than the ethanolic extract, when exposed to the concentration of 8 mg / ml,where the LT50 of the protoscolices of 11.2 minutes and 20.9 minutes, respectively. We conclude that the methanolic extract of the Iraqi honey bee wax was effective in the destruction of the protoscolicesof the *Echinococcusgranulosus* parasite and for a period of time less than it is of ethanolic extract. We recommend the use of methanolic extract of Iraqi honey bee wax as fatal agent to protoscolices before a surgical procedure to remove the hydatid cysts .

Index Terms- Iraqi honey bee wax ; Protoscolices ; Echinococcusgranulosus ; In vitro

I. INTRODUCTION

The hydatid cystic disease (H.C.D.) is one of the serious epidemic health problems in most parts of the world [1]. And it is known by many names, such asechinococcosis , cystic echinococcosis cysts , hydatidosis and unilocularhydatidosis[2].It is a common disease among humans and animals (Zoonotic diseases), and the Middle East and North Africa, Sudan and the Caspian Sea basin and some countries of the South American countries arehyperendemic for this disease[3]. Disease, in humans and other intermediate hosts, arises by the larval stage of the parasitic tapeworms belonging to the genus *Echinococcus*, which includes many species. The most medically important species is the *E. granulosus* and *E. multilocularis*.This stage can attack any organ of the body of intermediate host[4]. Spreading the disease in the rural regions where farm animals breeding and carnivores helps to complete the parasite life cycle asintermediate host (sheep, cows, buffaloes, camels, horses and other animals) and the final host (dogs, wolves, hyenas, leopards and other carnivore animals) [5].The spread of the disease can be attributed to two reasons: the first is the inability to detect the infection in the early stages because it does not show satisfactory symptoms only after increasing the size of the cyst, leading to pressure on adjacent tissues, and the second reason is the loss of therapeutic means, and the disease resembles the tumors in the metastasis stage[6].This disease is still endemic in Iraq and socially and economically influential as well as its consequences for the health standpoint to humans, prompting many researchers to investigate the means of treatment, though surgery is one of the most important methods of treatment despite the serious problems exposed during the patient surgery, which are difficult and sometimes impossible to take place at other times[7,8].The fact that the patient is not eligible is because of age or surgically anesthesia or the occurrence of the cysts in places difficult for the surgeon to deal with it,such as the cysts in the brain or in the heart or inspine;and hence the importance of the use of materials or extracts with a different chemical nature may help in the treatment of patients.Of the drugs used in the treatment by many researchers and veterinarians are:Albendazole (ABZ) , Mebendazole (MBZ) , Praziquantel , Epsiprantel , Isoprinosine , Cyclosporine A,Ivermectin and Fenbendazole and other chemicals that have a partial impact in the treatment of patients and for long-term [9,10].Natural products were used for thousands of years to treat multiple diseases, despite the fact that several of them havebeen replaced by traditional medicine, and there is currently a wide interest and great use of natural products by people[11]. In recent years, multi-drug resistance evolved by microorganisms, causing human disease as a result of the indiscriminate use of antimicrobial commercial drugs commonly used in the treatment of infectious diseases,and this situation has necessitated the search for new antimicrobial compounds[12].The justifications and reasons that led to the search for new anti-microbial materials to these compounds shown few side effects compared to the severe side effects of some drugs currently used[13].So there is an increasing need for new substances with anti-microbial activity. That natural products have opened new therapeutic methods, it contributed to the understanding of many biochemical pathways and their value as a valuable tool in biochemistry and molecular and cell biology

[14]. Honeybee products possess great importance for being anti-bacterial , anti-fungal , anti-viral and anti-parasitic. The most important of these products include natural bee honey,propolis,honey bee pollen and honey bee wax[15]. Beewax is from honey bee products which is secreted by the wax glands of honey bee workers.It is a mixture of esters , fatty acids , alcohols and hydrocarbons as well as unsaturated aromatic materials and dyes[16].Because of the medical importance of honey beewax and the antimicrobial effectiveness ,few studies on this material proposed research which aim to assess the efficiency ofethanolic and methanolicextracts of honey bee wax in the viability of theprotoscolices of the *E. granulosus*parasite*In vitro*.

II. Materials & Methods

The current study was conducted for the period from 1/9/2015 until 1/3/2016 at the Department of Biology - College of Education Girls- University of Kufa, where the bee wax was collected from beekeeping Apiaries, located in groves in Kufa - Najaf province - Iraq and bee wax cut into small pieces.In this study, two types of solvents, namely 99.9% methanol and 96% ethyl alcohol, as the blending 70 ml of each of the solvents with 30 grams of beeswax in flasks 250 ml were used.So, the total volume of 100 ml each were covered and the flasks were left in the laboratory in a dark place for a period of 48 hours with stir the mixture by using a Shaker for exposing the material to alcohol[16]. Extracts werefiltrated by filtration papers (Whatman no.1) for three consecutive times, and then they were dried by rotary evaporator at 40 ° C [17].Dry extracts were preserved in dark containers at 4° C until use[18]. The liver samples of sheep infected with hydatid cysts were collected from the massacre of Najaf province and placed in clean containers and transported to the laboratory of postgraduatestudies in the Department of Biology- College of Education for Girls- University of Kufa. Preparation and counting process of protoscolices was conducted in a period not exceeding two hours, where the sterility of the outside of the cyst by 70% ethyl alcohol and withdrawal of about 75% of the hydatid fluid and placed in a sterile glass container and then removed the germinated layer and placed in a glass dish and washedwith 0.9% sterile normal saline three times.The centrifuge in speed of 3000 rpm for a period of three minutes were used to precipitate the protoscolices in hydatid fluid and wash-germinated layer solution and then neglected the filtrate and suspended the sediment, which contains the protoscolicesby 0.9% sterile normal saline. The preliminary count ofprotoscolices was done by using the transfer method of fixed size by micropipette , where counting the total number of protoscolices in 10 µl of the prtoscolices suspension was achievedbyusing compound microscope under 20X and the number rate for three replicates in counting the total number of protoscolices was adopted.Theviability of theprotoscolices were examined by using 0.01% eosin dye, where equal amounts of protoscolices suspension were mixed with eosin dye, and examined with a microscope under 20X. The manual counter was used for counting 200live and dead protoscolices, where the live protoscolices werecolored withgreenish color, while the dead protoscolices were colored with red[19]. Stock solutions were attended for each extract by dissolving 1 gram of dry extract in 100 ml of 0.9% saline solution and then attended the concentrations of 2,4 and 8 mg / ml for each extract and preserved at 4° C until use in the viability assay . The protoscolices suspension were shakedwell for a regular distribution of protoscolicesin the suspensionand the total number of protoscoliceswas counted in 1 ml of suspension by using the transfer method of fixed size above, and was attended 21 test tubes with a tight lid for extracts and control (three tubes for each treatment), and then transfer 1 ml of protoscolices suspensionwhich contains nearly 2000 ± 20protoscolices .Thetubes containing protoscolices were treated with 1ml from the concentrations of 2,4, and 8 mg / ml for each extract, and the controltubeswere treated with 0.9%. saline solution. The viability of the protoscolices were calculated by using 0.01% eosin dye and at intervals of 15,30, 45 and 60 minutes after treatment,having been counted 200 live and dead protoscolices, where the live protoscolices were colored with greenish color, while the dead protoscolices were colored with brown and thenthe percentage of mortality was calculated[19].

III. Statistical Analysis

The results were analyzed statistically by using the table of analysis of variance(ANOVA table), and the standard deviation (S.D) was calculated and less significant difference (L.S.D) was used in the diagnosis of statistical differences between treatments[20].The method of least squares for value deviation was applied to calculate the lethal concentration for 50(LC50) and lethal time for 50 (LT50)of the protoscolicesand the regression coefficient (r)was calculated for them[21].

IV. Results & Discussion

The results of the current study shown in table (1) and (2), indicated that theethanolicand methanolic extractsof the Iraqi honey bee wax have great efficiency in the destruction of the protoscolices, and that the mortality percentage increases steadily with increasing the concentration and duration of exposure to both extracts.There was a significant correlation coefficient at 0.05 between the concentration and the mortality and between the time and the mortality of the protoscolices as in the table (3) and (4)**.**The mortality percentage, when the treatment with 2 mg / ml of the ethanolic and methanolic extractsofhoney bee wax,was 16.7±1.43% and20.2±2.26 % , respectively, after 15 minutes of exposure to extracts, while the concentration8 mg / ml giving mortality percentage96.5±2.91 % and 98.7±1.09% respectively after 60 minutes of exposure to extracts. Statistical analysis showed that there are significant differences between the mortality percentage for the destruction of protoscolicesin treatments and control at the level of probability of 0.05. As the results showed, themethanolic extractof honey beewax was more effective than theethanolic extract in the destruction of the protoscolices, where the LC50 of the protoscolices was 3.5 mg / ml and 4.9 mg / ml, respectively, after 15 minutes of exposure to the extracts, while the LC50 of the protoscolices was 1.7 mg / ml and 2 mg / ml, respectively, after 60 minutes of exposure to the extracts (table 3). The results showed that the LT50 of the protoscolices was 39.8 minutes when exposed to the

concentration of 2 mg / ml of the methanolic and ethanolic extracts, while themethanolic extractled to decimation of protoscolices in less time than theethanolic extracts, when exposed to the concentration of 8 mg / ml, which was the LT50 11.2 minutes and 20.9 minutes, respectively (table 4).

Table (1):The effect of ethanolic extract of honey bee wax in the protoscolices of *Echinococcusgranulosus* parasite.

Concentration (mg / ml)	Mortality percentage of the protoscolices in the time periods (minute) ±SD				L.S.D at the level 0.05
	15	30	45	60	
Control	0	0	0	0	
2	16.7±1.43	25.6±3.42	43.4±0.82	59.2±2.51	4.6
4	30.8±2.57	41.7±1.62	58.9±3.31	80.3±2.57	5.1
8	46.3±1.59	60.7±2.22	72.6±4.11	96.5±2.91	7.2
L.S.D at the level 0.05	6.1	5.5	6.4	5.8	

Table (2):The effect of methanolic extract of honey bee wax in the protoscolices of *Echinococcusgranulosus* parasite.

Concentration (mg / ml)	Mortality percentage of the protoscolices in the time periods (minute) ±SD				L.S.D at the level 0.05
	15	30	45	60	
Control	0	0	0	0	
2	20.2±2.26	33.3±3.34	48.2±0.79	68.6±3.11	4.8
4	45.2± 0.78	51.6±2.66	67.6±2.02	88.7±3.45	6.2
8	68.5±1.59	79.3±2.54	87.8±1.44	98.7±1.09	7.9
L.S.D at the level 0.05	5.3	6.1	5.7	6.8	

Table (3): LC50 of the protoscolices of the *Echinococcusgranulosus*parasite.

Time (minute)	LC50 of the protoscolices (mg / ml) when exposed to extracts			
	Ethanolic	Correlation coefficient (r *)	Methanolic	Correlation coefficient (r *)
15	4.9	0.996	3.5	0.975
30	3.8	0.998	2.9	0.992
45	2.8	0.988	2.3	0.985
60	2.0	0.989	1.7	0.979

***Significant levels at 0.05**

Table (4): LT50 of the protoscolices of the Echinococcusgranulosus parasite.

Concentration (mg/ml)	LT50 of the protoscolices (minutes) when exposed to extracts			
	Ethanolic	Correlation coefficient (r *)	Methanolic	Correlation coefficient (r *)
2	39.8	0.994	39.8	0.926
4	30.2	0.977	22.4	0.989
8	20.9	0.933	11.2	0.850

***Significant levels at 0.05**

The review of literature does not exhibit any study on the effect of honey bee wax extracts in the parasites, but most studies have focused on the effectiveness of anti-microbial to bacterial and fungal. It has been documented that honey bee wax was recommended as antimicrobial treatments in the European and Asian regions over centuries [22]. [23] studied the effect of certain plant-based compounds present in beewax, andthey found that these compounds have antimicrobial effectiveness.Also,[24] summarized the uses of beewax at the present time, including cosmetics and antibacterial properties, where he found that it is effective especially against some bacterial species such as *Bacillus subtilis,Proteus vulgaris*, *Salmonella gallinarum* and *Bacillus alvei*, and it is partially effective against some types of bacteria

such as *Salmonella pullorum*,*Salmonella dublin*,*Escherichia coli* and *Bacillus larvae*.He also found that beewax is effective against some types of pathogenic bacteria, especially *Bacillus alvei*,*Proteus vulgaris*,*Salmonella gallinarum* and *Bacillus subtilis*.Moreover[25] studied the impact of mixing of natural honey , beewax and olive oil on the growth of the yeast *Candida albicans* and the bacteria*Staphylococcus aureus* and found that it does not have growth in the bacteria*S. aureus* and the yeast *C. albicans* on the medium containing the natural honey, while light to moderate growth over the medium containing a beewax.A study conducted by [16]showed that the bees wax samples were effective against the gram negative and gram positive bacteria, and showed aclearly inhibitory effect against one kind of yeast that is *Candida albicans*. In a study by [18], it was found that the bee wax extracts were effective against some types of pathogenic bacteria such as *Listeria monocytogenes* ,*Psedomonasaeruginasa*,*Staphylococcus aureus*,*Salmonella enteric* and *Escherichia coli* and against some types of microscopic fungus such as *Aspergillusfumigatus* ,*Aspergillusflavus* and *Aspergillusniger*, and detrimental for some of the different strains of yeasts such as *Candida krusei*,*Candida albicans*,*Candida glabrata*,*Candida parapsilosis*, *Candida tropicalis*,*Geotrichumcandidum* and *Rhodotorulamucilaginosa*.The results of the current study indicate that the methanolic extract of the Iraqi honey bee wax is effective in the destruction of the protoscolicesof the *Echinococcusgranulosus* parasite for a period of time less than that required for ethanolic extract used for the same purpose . We recommend the use of methanolic extract of Iraqi honey beewax as fatal agent to protoscolices before any surgical procedure to remove the hydatid

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