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USE OF *CITRULLUS COLOCYNTHIS* FRUITS AND *QUERCUS* SPP. BARK EXTRACTS AS SCOLICIDAL AGENTS FOR PROTOSCOLECES OF *ECHINOCOCCUS GRANULOSUS IN VITRO*

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Abstract

The present study was achieved for the period from October 2018 to March 2019 in the Department of Biology-Faculty of Education for Girls-University of Kufa, which aims to study the scolicidal effects of methanolic extracts of *Citrullus colocynthis* fruits and *Quercus* spp. bark in the viability of the protoscoleces of the *Echinococcus granulosus in vitro*. The results showed that the methanolic extracts of the *C. colocynthis* fruits and the *Quercus* spp. bark were effective in the destruction of the protoscoleces, and that the percentage of mortality increased with increasing concentration and exposure time of the extracts. At 4 mg / ml, the mortality rate was 35.6% and 58.3%, respectively, after 30 minutes of exposure to the extracts, while the mortality rate was 37.7% and 70.2%, respectively, after 120 minutes of exposure to the extracts and at 16 mg / ml, the mortality rate was 83.3% and 76.9%, respectively, after 30 minutes of exposure to the extracts, while the mortality rate was 100% after 120 minutes of exposure to the extracts. The results indicated that the methanolic extract for *C. colocynthis* fruits was more effective than the methanolic extract for *Quercus* spp. bark in the mortality of protoscoleces, where the lethal concentration for 50% (LC50) of the protoscoleces was 6.31 mg / ml and 6.6 mg / ml, respectively, after 30 minutes of exposure to the extracts, while the LC50 was 3.16 mg / ml and 3.4 mg / ml respectively, after 120 minutes of exposure to the extracts. The results also showed that the methanolic extract for *C. colocynthis* fruits led to the destruction of the protoscoleces in less time than the methanolic extract for *Quercus* spp. bark when exposed to the concentration of 4 mg/ml, where the lethal time for 50% (LT50) of the protoscoleces was 47.32 minutes and 50.12 minutes, respectively while exposed to the concentration of 16 mg / ml, where the LT50 of 15.85 minutes and 20.89 minutes, respectively. The statistical analysis showed the existence of a positive correlation between the time and concentration and the rate of mortality of protoscoleces at the level of probability 0.05.The conclusion from the present study that the fruits of the *C. colocynthis* was more effective from *Quercus* spp. bark in the destruction of the protoscoleces of the *E.granulosus* and they are natural materials that can be used as lethal substances for the protoscoleces before surgical operations to remove the hydatid cysts.

Keywords **:** Protoscoleces; *Echinococcus granulosus*; *Citrullus colocynthis*; *Quercus* spp.; Methanolic extract; *In vitro*

Introduction

Hydatid cystic disease (HCD) is one of the most serious epidemiological health problems in most parts of the world (Naguleswaran *et al*., 2006). It is known as *Echinococcosis, Cystic echinococcosis, Hydatidosis, Uniocular hydatidosis* (Roberts & Janovy, 2000) is a common disease between humans and animals (Zoonotic disease). The countries of the Middle East, North Africa, Sudan, the Caspian Basin and some of South America are hyperendemic diseases (Wen *et al*., 1993). The disease is caused in human and other intermediate hosts (sheep, cows, buffalo, camels, horses and other animals) for the larval stage of the tapeworm parasites of *Echinococcus*, which includes several species, most important of which are *E. granulosus* and *E. multilocularis*. This stage can attack any organ of the intermediate host body (Kismet *et al*., 2008). The disease spreads in rural areas where farm animals and carnivores are abundant, helping to complete the life cycle of this parasite, which needs the intermediate hosts and the final hosts (dogs, wolves, hyenas, leopards, and other wild animals) (Marquardt *et al*., 2000). The spread of this disease can be attributed to two reasons: The first is the inability to detect the infection in the early stages because it shows no symptoms of the disease only after increasing the size of the cysts, which leads to pressure on the adjacent tissues, the second reason is the loss of therapeutic. This disease is similar to the spread of cancerous tumors in the metastasis stage (Eckert & Deplazes, 2004). This disease in Iraq is still an endemic disease affecting social and economic as well as the effects on the health of human being, which led many researchers to investigate the means of treatment, although surgical intervention is one of the most important methods of treatment despite the serious problems that a patient experiences during surgery , which are sometimes difficult to perform and sometimes impossible (Mentes *et al*., 2000; Elissondo *et al*., 2002), that the patient is not surgically qualified, because of age or anesthesia or the occurrence of the cysts in places difficult for the surgeon to deal with it as in the cysts of the brain or heart or spine and hence the importance of using materials or extracts of a different chemical nature may help in the treatment of patients. Surgery is accompanied by a secondary infection, which results from the spills of protoscoleces into the peritoneal cavity. Many scolicidal chemical agents have been used to discourage cyst contents, but most are associated with adverse side effects. Some studies have suggested that traditional plants have an effective effect in preventing post-surgical secondary infection (Rostami *et al*., 2016). According to the World Health Organization survey, about 70-80% of the world's population relies on non-conventional medicines, especially from herbal sources, for primary health care. This is especially the case in developing countries where the cost of consulting a physician and the price of medication exceed the possibility of most people (Al-Snafi, 2016). *Citrullus colocynthis* is known for its many names, bitter apples and bitter cucumber, a small plant belonging to the Cucurbitaceae family, it is home in the Mediterranean basin and Asia especially Turkey and the desert region of India and Pakistan. The *C. colocynthis* contains carbohydrates, proteins, amino acids, tannins, saponins, phenolics, flavanoids, flavone glucosides, terpenoids,

alkaloids, anthranol and steroids (Al-Snafi, 2016). The fruit extracts of *C. colocynthis* showed an antimicrobial effect such as *Pseudomonas*, *Staphylococcus* and *Candida* (Marzouk *et al*., 2009) and antifungal drugs such as *Aspergillus flavus* (Amrouche *et al*., 2011) and the effect of hypoglycemia on patients with type 2 diabetes (Huseini *et al*. 2009) and rats (Dallak, 2011). In addition, *C. colocynthis* extract was found to be anti-*leishmania major* (Baloch *et al.,* 2013) and against molluscs *Biomphalaria arebica* (Zaid *et al*., 2013) and anthelmintic, *Haemonchus contortus* (Ullah *et al*., 2013). The oak is a plant belonging to the Quercus genus and belongs to the family of Fagaceae, growing in Minor Asia, Iran and Greece and is well known for its medicinal properties around the world (Dar *et al*., 1976; Panahi *et al*., 2012). Furthermore, *Q. infectoria* was used as a natural remedy for various diseases and is abundant in the Zagros Mountains of western Iran (Dar *et al*., 1976). The oak plant and its derivatives have been used in many studies as antimicrobial (Wan *et al*., 2014), antifungal (Baharuddin *et al*., 2015), antivirals (Hussein *et al*., 2000) and antiparasites (Kheirandish *et al*., 2016). The presence of polyphenolic compounds in the crude extract of the oak plant makes it anti-oxidant and anti-inflammatory, anti-microbial and anti-cancer. These compounds include gallic acid, tannins and some other flavonoid compounds such as quercetin (Hashim *et al*., 2013). Due to the lack of studies on the effect of *C. colocynthis* fruits and *Quercus* spp. bark in the viability of the protoscoleces of the *E. granulosus*, so this study, which aims to evaluate the effectiveness of methanolic extract of the *C. colocynthis* fruits and *Quercus* spp. bark in the viability of the protoscoleces of the *E. granulosus in vitro*.

Materials and Methods

The present study was achieved for the period from 1/10/2018 to 1/3/2019 in the Department of Biology – Faculty of Education for Girls - University of Kufa, where the fruits of the *C. colocynthis* and the bark of the *Quercus* spp. had been obtained from the local markets of Al-Najaf province, and was grinded by an electric grinder for the purpose of obtaining powder. The process of extracting the fruits of the *C. colocynthis* was done according to the Baloch *et al*.(2013) and the bark of the *Quercus* spp was extracted depending on the Malekifard and Keramati (2018) . (50) g of each powder were combined with (200) ml of methyl alcohol 97% (volume / volume) in a glass flask with a capacity of 500 ml and leave the beakers at laboratory temperature and in a dark place for 72 hours . The mixture was stirred by shaker to ensure that the substance is exposed to alcohol. The samples were filtered by filtration papers and the extract was then dried by rotary evaporator at a temperature of 40 °C. The dry extract was then stored in a sterile container and at a temperature of 4 ° C until use .

The samples of the infected sheep 's livers were collected from the Najaf province massacre and placed in clean containers. They were transferred to the laboratory in the Department of Biology-Faculty of Education for Girls - University of Kufa. The preparation and counting procedures were carried out in no more than two hours. The protoscoleces was isolated and examined the viability depending on the Landa-Laracia *et al*. (1997). About 75% of the hydatid cyst fluid was pulled and placed in a sterile glass container and then removed the germinated layer and washed with sterile saline solution 0.9% for three times . Use the centrifuge at 3000 rpm for three minutes to precipitate the protoscoleces in the hydatid fluid and the wash solution of the germinated layer, where the leachate was neglected and the precipitation containing the protoscoleces was suspended by 0.9% saline solution. The protoscoleces were calculated using the fixed size transfer method by micro pipette. The total number of protoscoleces was calculated in the volume of 10 µl of the suspension of the protoscoleces using a compound microscope under 20x. The number rate was calculated for three replicates in the calculation of the total number of protoscoleces and examined the viability of the protoscoleces using 0.01% eosin dye, where they mixed equal amounts of the suspension of the protoscoleces with the eosin dye, and examined by compound microscope under 20x. A manual meter was used as 200 primaries were counted from live and dead protoscoleces where the living protoscoleces appear in a greenish color, while the dead protoscoleces appear red. Prepare a stock solution for the extracts by dissolving 2 g of each dry extract in 100 ml of the saline solution 0.9%, then, concentrations of 4, 8 and 16 mg / ml were prepared for each extract and kept at 4 °C until use in bioassay experiments *in vitro*. The suspension is well shaked in order to regularize the distribution of protoscolices in suspension. The total number of protoscolices was calculated in 1 milliliters of suspension using the fixed-size transfer method. Twenty-two test tubes with a tight lid were used with three tubes per concentration and control, and then transfer (1) milliliters of protoscolices suspension containing approximately 2000±20 protoscolex. The viability of protoscolices were calculated using 0.01% eosin dye at intervals of 30, 60 and 120 minutes after treatment. (200) of living and dead protoscolices were counted, where the living protoscolices appear in the a greenish color, while the dead protoscolices appear red (Landa-Laracia *et al*., 1997).

Statistical Analysis

The method of least squares for value deviation was applied to calculate the lethal concentration for 50 (LC_{50}) and lethal time for 50 (LT_{50}) of the protoscoleces and the regression coefficient (r) was calculated (Finney, 1977).

Results and Discussion

The results of the current study indicated in Table (1) and (2) that the methanolic extracts of the fruits of the *C. colocynthis* and the bark of the *Quercus* spp. were very effective in the destruction of protoscoleces, and that the percentage of mortality increases with increasing concentration and exposure time of the extracts. At 4 mg / ml, the mortality rate was 35.6% and 58.3%, respectively, after 30 minutes of exposure to the extracts, while the mortality rate was 37.7% and 70.2%, respectively, after 120 minutes of exposure to the extracts and at 16 mg / ml, the mortality rate was 83.3% and 76.9%, respectively, after 30 minutes of exposure to the extracts, while the mortality rate was 100% after 120 minutes of exposure to the extracts.

The results also showed, that the methanolic extract of *C. colocynthis* fruits was more effective than the methanolic extract of *Quercus spp.* bark in the destruction of the protoscoleces, where the LC_{50} of the protoscoleces was 6.31 mg/ml and 6.6 mg/ml, respectively, after 30 minutes of exposure to the extracts, while the LC_{50} of the protoscoleces was 3.16 mg/ml and 3.4 mg/ml, respectively, after 120 minutes of exposure to the extracts (Table 3). The results also showed that the methanolic extract of the *C. colocynthis* fruits resulted in the destruction of the protoscoleces in less

time than the methanolic extract of *Quercus* spp. bark, where the LT_{50} of the protoscoleces was 47.32 and 50.12 minutes, respectively, at a concentration of 4 mg/ml, while at the concentration 16 mg/ml, the LT_{50} of the protoscoleces was 15.85 minutes and 20.89 minutes respectively (Table 4).

Table 1: Percentage rate of protoscoleces treated with the methanolic extract of the *C. colocynthis* fruits.

Time (minute)	% Mortality			
	Control	4 (mg / ml)	8 (mg / ml)	16(mg / ml)
30	0	35.6	52.1	83.3
60	0	65.3	60.9	90.5
120	0	37.7	98.9	100

Table 2 : Percentage rate of protoscoleces treated with the methanolic extract of the *Quercus spp.* bark.

Time (minute)	% Mortality			
	Control	4(mg / ml)	8(mg / ml)	16(mg / ml)
30	0	58.3	62.2	76.9
60	0	60.0	69.3	88.3
120	0	70.2	89.7	100

Table 3: LC_{50} of the protoscoleces for *E. granulosus* parasite

Extracts	LC_{50} (mg / ml)		
	30 minute	60 minute	120 minute
C. colocynthis fruits	6.31 6.31 (r =0. 941)*	3.98 (r =0. 971)*	3.16 (r =0. 977)*
Quercus spp. bark	6.6 (r =0. 901)*	5.8 (r =0. 933)*	3.4 (r =0. 978)*

* Correlation coefficient, Significant levels at 0.05

Table 4 : LT_{50} of the protoscoleces for *E. granulosus* parasite.

Extracts	LT_{50} (minute)		
	4(mg/ml)	8(mg/ml)	16(mg/ml)
C. colocynthis fruits	6.31 47.32 (r =0. 893)*	31.62 (r =0. 991)*	15.85 (r =0. 997)*
Quercus spp. bark	50.12 (r =0. 983)*	39.81 (r =0. 930)*	20.89 (r =0. 976)*

* Correlation coefficient, Significant levels at 0.05

Due to the increasing development of drug resistance and the undesirable effects of existing antimicrobials, resulting in the search for new antimicrobial compounds was the focus of a number of studies (Paller *et al*., 2004). Interest in natural products has increased during in recent years, medicinal plants have been identified as sources of biologically active compounds, were isolated and analyzed to determine mechanisms and target locations (Ishida *et al*., 2006). Baloch *et al*. (2013) mentioned that the methanolic extract of *C. colocynthis* fruit is highly effective in the complete inhibition of the growth of the proastigotes stage of the *Leishmania major* at a concentration of 500 µg/ml. Ullah *et al*. (2013) studied the effect of methanolic extract of *C. colocynthis* fruit at 100 mg/ml against *Haemonchus contortus in vitro*. All worms were found to be dead after 4 hours of exposure to the extract. Swarnakar and Kumawat (2014) studied the effect of the alcohol extract of the *C. colocynthis* fruit in the in the adult worms of *Orthocoelium scoliocoelium in vitro*, and found that the extract with a concentration of 40 mg / ml was effective in loss of movement and paralysis after 5 hours of exposure to the extract. The results of the present study are consistent with the findings of Malekifard and Keramati (2018), who studied the effect of different concentrations (10, 25, 50) mg/ml of methanolic extract of *Quercus* spp. at different intervals (10, 20, 30, 60) minutes on the protoscoleces of *E. granulosus in vitro*, and found that the methanolic extract of the *Quercus* spp. at a concentration of 50 mg/ml had killed all the protoscoleces (100%) after 20 minutes of exposure to the extract and concluded that the toxicity of the extract for the protoscoleces is due to its containment of the flavonoid compounds. Studies on the effects of the *Quercus* spp. extract indicate that it is composed of various components such as polyphenolic compounds and flavonoid compounds, which target several points in microbial cells (Mahmoudvand *et al*., 2016). Kheirandish *et al*. (2016) found that the high amounts of phenolic compounds found in *Quercus* spp. extracts may be responsible for anti-leishmanial activity. The conclusion from the present study that the fruits of the *C. colocynthis* was more effective from *Quercus* spp. bark in the destruction of the protoscoleces of the *E. granulosus* and they are natural materials that can be used as lethal substances for the protoscoleces before surgical operations to remove the hydatid cysts.

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