Effects of pomegranate (*Punica granatum* L.*)* barks of root and stem (alcoholic extract) on the viability and fatty acids content of *Echinococcus granulosus* protoscolices *in vitro* study

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Abstract

The present study was aimed to determine the scolicidal effect of stem and root alcoholic extract of *Punica granatum* L. (Pomegranate) on the viability and fatty acids content of *Ecinococcus granulosus* protoscolices. The study elucidate the scolocidal effect of both stem and root extracts. Its seemed that the scolocidal effect of root extract was higher than that of stem extract ie. LC50 and LC90 of stem extract were 5 and 9 mg/ml respectively while LC50 and LC90 of root extract were 3 and 8 mg/ml respectively. GLC analysis of *E. granulosus* protoscolices was stated presence of nine fatty acids esters, which were Lauric (C12:0); Myrisitic (C14:0); Palmatic (C16:0); Stearic (C18:0); Archidic (C20:0); Behenic (C22:0), which were saturated fatty acids, and Oleic (C18:1); Linoleic (C18:2); Linolenic (C18:3) which were unsaturated fatty acids. Treating *E.granulosus* protoscolices with LC50 of pomegranate stem bark was showing slight and swing effect on fatty acid content, while treated the protoscolices with LC50 of root bark were resulted in obvious increase in short length fatty acids like Lauric, Myrisitic and Palmatic in compare with control group. In contrast the long chain fatty acids concentration like Oleic, Linoleic and Linolenic were decrease.

Keywords: Hydatids, Pomegranate bark stem, Pomegranate bark root, Gas liquid chromatography, Lipids, *in vitro*

تأثير قلف جذر وساق الرمان (المستخلص الكحولي) على حيوية ومحتوى الاحماض الدهنية لرؤيسات المشوكة الحبيبة في دراسة خارج الجسم الحي

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الخلاصة

هدفت الدراسة الحالية لبيان فعالية المستخلص الكحولي المحمض لكل من ساق وجذر الرمان كمضادات للرؤيسات الاولية للمشوكة الحبيبية، وكذلك لتحديد تأثي هذين المستخلصين على محتوى الرؤيسات الاولية من الاحماض الدهنية. بينت الدراسة وجود تأثير مضاد للرؤيسات الاولية لكل من مستخلص الساق والجذر، وعلى ما يبدو أن تأثير الجذر أقوى من تأثير الساق، حيث بلغ تركيز مستخلص ساق الرمان القاتل ٥٠% و٩٠% من الرؤيسات الاولية ٥ و ٩ ملغم/مل على التوالي، في حين بلغ تركيز مستخلص جذر الرمان القاتل لـ٥٠% و٩٠ % من الرؤيسات الاولية ٣ و ٨ ملغم/مل. وأظهر تحليل الاحماض الدهنية بأستخدام تقنية كروماتوكرافيا الغاز السائل للرؤيسات الاولية للمشوكة الحبيبية وجود تسعة أنواع من الأحماض الدهنية وهي حامض الوريك (C12:0)، المايرستيك (C14:0)، البالمتيك (C16:0)، السيتياريك (C18:0)، الاركيديك (C20:0) و البيهنيك (C22:0) وهي أحماض دهنية مشبعة وحامض الاوليك (C18:1)، اللينوليك (C18:2) و اللينولينك (C18:3) وهي أحماض غير مشبعة. كان لمعاملة الرؤيسات الاولية للمشوكة الحبيبية بالتركيز القاتل LC50 من مستخلص ساق الرمان تأثيرا خفيفا ومتذبذبا على محتوى وتركيز الاحماض الدهنية في حين أدى معاملة الرؤيسات الاولية بالتركيز القاتل

لـ LC50 من مستخلص جذر الرمان الى ازدياد واضح في تركيز الاحماض الدهنية قصيرة السلسلة مثل حامض اللوريك، المايرستيك والبالمتيك مقارنة بمجموعة السيطرة، وعلى عكس ذلك انخفض تركيز الاحماض الدهنية طويلة السلسلة كالاوليك والينولك واللينولينيك.

Introduction

Hydatidosis is a highly pathogenic infection with an almost global incidence caused by the larval stage (unilocular hydatid cyst) of the cestode *Echinococcus granulosus*. In endemic areas it causes serious health problems for humans, livestock and wildlife animals, representing in many countries (1,2).

Man and other intermediate hosts can get infection with hydatidosis then he ingest the infective eggs those shed in the outer environment by the definitive host; the dogs and other canidian animals (3). Infectious diseases are still one of the leading causes of death all over the world. Although conventional drugs are providing an effective treatments for the infections; antibiotic resistance continues to grow among key microbial pathogens. Therefore, finding new antimicrobial agents is still an important. Medicinal plants have always been a good source to find new aim for many researchers specially agents those are derived from natural products. Recently, a wide range of plants have been screened for their antimicrobial properties (4-6).

Punica granatum commonly called pomegranate, recently described as nature's power fruit, is a plant used in folkloric medicine for the treatment of various diseases (7,8).

P.granatum L. (Punicaceae) a seeded or granular apple, is a delicious fruit consumed worldwide. The fruit grows in all worm country originally a native of persia, Afghanistan, China and India,then extended to Iraq and around countries (9).

Besides pomegranate peel extract with an abundance of flavonoids and tannins has been shown to have a high antioxidant activity and anti- inflammatory properties (10,11) furthermore, recent studies were demonstrated its anti-cancer activity in several types of human cancers because of this plant is containing polyphenolic compounds (12,13).

Methanolic extract of pomegranate fruit has been shown to induce antibacterial activity against *Listeria monocytogenes*, *Staphylococcus aureus,Escherichia coli* and *Yersinia enterocolitica* (4). The same as the pathogenic yeast and *Candida albicans* (14). Despite the many studies conducted to evaluate the efficacy of *P.granatum* in treating some diseases and microbial infections, much remains unknown about its antiecchinococcal effects. However, some studies have indicated that *P. granatum* has anti-cestodial, anti-nematoidal, anti-helmintical and anti-protozoan effects like those of (15,16).

Quercetin, luteolin, and kaempferol alkaloids were isolated from pomegranate stem and root barks extracts, Hydroquinone pyridinium alkaloid was isolated from the leaves of pomegranate (17). Various chemical constituents such as flavone glycosides (apigenin and luteolin) and tannins (punicafolin and punicalin) were separated from Pomegranate (18).

Stem and root barks were used traditionally for treating tape worms infections, (19) Tanret (1878) was isolate pelletierine from pomegranate fruits (*Punica granatum L.*). Other three allied alkaloids (methylpelletierine, pseudopelletierine, and isopelletierine) were separate from root and stem barks of *p. granatum* (20).

Lipids are vital biomolecules which considers a preserved source of energy when carbohydrates and glycogen are consume. Furthermore, lipids are essential components, those participate in the construction of membranes, nuclei, and mitochondria in cell (21). The conformation of lipids depends on the organic nature of fatty acids. In addition to their importance in the preservation of cellular membranes in all zoo systems, they play a key role in growth, reproduction and embryonic development (22). The ability of fatty acids ω-3 and ω-6 to decrease heart attack, apoplexy and carcinoma are proved, also they have a regulatory role through immune response and hypersensitivity reactions (23). The biochemical studies about lipids in the parasitic helminthes were help in developing correlation between different helminthes, and their adaptation modes in the host body (24). Lipids play an essential role in adaptation and completion of the life cycle during tapeworms and parasitic stage (25).

Fatty acids work as energy and as mediators that have ability to affect lipid metabolism and inflammatory reactions by the regulation of gene expression and cellular signaling pathways. Fatty acids ω-3, for instance, are anti-inflammatory, partially acting to inactivate the production of pro-inflammatory cytokine via effecting to nuclear factor-kappa B transcriptional network (26,27). Additionally, the ability of fatty acids in the protection of organs or tissues from infection due to a high concentration of fatty acids is detrimental to pathogens (28).

Therefore the present study was aimed to evaluate the efficacy of *P. granatum* root and stem barks alcoholic extract as natural antiechinococcal agents.

Materials and methods

Sampling

E. granulosus from cysts containing protoscoleces of sheep origin were obtained by aspiration of the content of cysts present in livers of naturally infected sheep. Cysts were collected during the routine work of local abattoirs in

Mosul/ Ninavah. Samples were suspended by addition of Potassium –phosphate buffer solution pbs (7.4 pH) after washed in several changes of sterile pbs solution (29), then refrigerated at 4°C and treated with alcohol extract acidified by H_2SO_4 (1N) for show the effect.

Preparation of root and stem barks alcoholic extracts

P.granatum barks of stem and root were collected from pomegranate trees in Mosul city at the period between Oct.-Nov. 2016, then dried and grinded to a powder.

Alcoholic barks extract were prepared as following: 100gm of each bark powder were dissolved in 1L acidified alcohol (1N H_2SO_4).

The stem and root barks were milled in to coarse powder, then extracted with ethanolic alcohol 80% that contains diluted H_2SO_4 (1N); pigments and unwanted materials were removed by shaking with chloroform, then the alcoholic extract dried to a powder to prepare stock solution for each bark were prepared at a concentration of 1% (1 gm/100 ml pbs pH 7.4) ie10mg/ml (30).

Estimation of lethal concentration 50 (LC50) for root and stem alcoholic extract to *E.granulosus* protoscolices

Gradient concentration of each extract (root and stem) were prepared as following 1,2,3,4,5,6,7,8,9 and 10 mg/ml pbs. In addition to control group (contain pbs pH 7.4 only).

Each concentration were put (2 ml) in Siliconized test tube, then (0.2 ml) of protoscolices suspension (about 1500 psc) were added to each tube. Tubes were refrigerated at 4ºC, for 24hrs. The viability of protoscolices were determined depending on reaction with vital stain (Eosin 0.1%) and flame cells activity (31).

Three replicates for each treatment were applied, then LC50 for root and stem bark alcoholic extract were determined according to protoscolices viability (32).

Preparation of protoscolices extract

Protoscolices suspension of (1500 psc/ml) were treated with LC50 for root and stem bark extracts, then the treated protoscolices were washed with distilled water several times, then centrifuged, then protoscolices sediment were collected, then disruped using ultrasonic disintegration (MSE), a12000 vibration/second for 30 second in ice bath four cycles of sonication were applied with the suspensions. Ultracentrifugation at 15000g/30 minutes was done using (MSE) super speed ultracentrifuge. The suspension for each control group and treatments were lyophilized for the subsequent study.

Fatty acids extraction

Protoscolices extract was prepared and lyophilized according to Pappas and Narcisi method (33), using Christ-BETA-Lyophilizer /Germany. Then fatty acids were extracted depending on Al-Kaisy *etal.* modified method (34). 200mg of dried extract were dissolved in (10 ml) of 7.5N NaOH (which prepared in 60% methanol). The mixture then heated to 105 ºC for 90 minutes. After cooling; 12 ml of distilled water added the mixture then acidified using 20% sulphoric acid to adjustified pH to 2. Fatty acids mixture then withdrawn with separating funnel after mixing with diethyl ether. Rotary vacuum evaporator was used to extract fatty acid mixure.

GLC analysis fatty acids

Gas liquid chromatography GLC technique (Instrument: PA. CKARD, model 4.38, USA) was used to analyze fatty acids mixture. 1ml of methyl ester was prepared immediately ($25cm^3$ methanol +$0.1cm^3$ Acetyl chloride), then added to the crude fatty acids which were prepared before (34). Analysis of the protoscolices fatty acids and authentic fatty acids were performed in Ibn Sena Company Labs/ University of Baghdad utilized the following conditions:

Column type: SE/30, length: 3 m, diamer: 1/8"; carrier gas and flow: Heat at 30ml/minute; Column temperature: 100-300 ºC at 10º C /minute; Detecter: FID; Detecter temperature 325 ºC; injector temperature: 230 ºC; fatty acids esters were detected then, using photoelectric cell, type Schimazu-UV spectrophotometer at wavelength 230nm.

Results

The effect of pomegranate extracts on the viability of *E.granulosus* protoscolices, Its seemed that the viability of treated protoscolices were decreased with the root and stem barks concentration increase. So that treating protoscolices with 8 mg/ml of stem bark was reducing protoscolices survival to 9%, while 4% of protoscolices were survive after treating with 10 mg/ml of the stem extract. The same as for root bark, which reduce survival of protoscolices to 40% when treated with 4mg/ml and to 0% when treated with 9 mg/ml (Table 1).

It is worthy to say that pomegranate root extract had more increase effect on the viability of *E. granulosus* protoscolices than that of stem bark. So that LC50 and LC90 of root bark were 3 and 8 mg/ml respectively, whereas LC50 and LC90 of stem bark were 5 and 9 mg/ml respectively (Figure 1). Effect of root and stem barks of pomegranate on the fatty acids content of *E. granulosus* protoscolices. In the present work, according to GLC analysis and compared with retention time of authentic fatty acids (Table 2) there were nine fatty acids esters those found in the *E.granulosus* protoscolices. Six of them were saturated which were: Lauric acid (C12:0); Myrisitic acid (C14:0); Palmatic acid (C16:0); Stearic (C18:0); Archidic acid (C20:0) and Behenic acid (C22:0). The other three

were unsaturated fatty acids which were Oleic acid (C18:1); Linolic acid (C18:2) and Linolenic acid (C18:3) (Figure 2) .

Treating protoscolices of *E.granulosus* with LC50 of root bark showed obvious change in fatty acids concentration when compared with control group (Figure 3 and Table 3). While protoscolices were treated with LC50 stem bark was showing slight and swing on the fatty acid content when compared with control group (Figure 4 and Table 3). Its seemed that long chain fatty acids concentration were decrease which include Oleic acid, Linolic acid and Linolenic acid. While short chain fatty acid were decreased which include Lauric acid, Myrisitic acid and Palmatic acid.

Table 1: Show the effect of root and stem barks extract in the viability % of *E.granulosus* protoscolices in different concentrations, after 24hrs at 4°C

Barks	Concentrations mg/ml										
	Control	1	2	3	4	5	6	7	8	9	10
Stem	99%	98%	81%	77%	63%	51%	43%	39%	28%	13%	4%
Root	99%	97%	68%	52%	40%	32%	27%	21%	9%	0	0

* three replicates were used for each treatment,
-Initial number of protoscolices used in each treatment were≈ 1250 protoscolices /20µl.

Table 2: Retention time for the authentic fatty acids esters those found in *E.granulosus* Protoscolices using GLC technique (Instrument: PA. CKARD, USA).

Fatty acids esters	Lauric C12:0	Myristic C14:0	Palmatic C16:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2	Linolenic C18:3	Arachidic C20:0	Behenic C22:0
Retention time (minutes)	10.38	12.73	14.60	15.45	16.00	16.51	17.36	19.41	20.62

Table 3: Types and concentration% of fatty acid esters those found in *E.granulosus* protoscolices after exposure to LC50 of root and stem bark extract of pomegranate compared with control group

Fatty acids esters	Lauric C12:0	Myristic C14:0	Palmatic C16:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2	Linolenic C18:3	Arachidic C20:0	Behenic C22:0
Control	2.17	5.64	12.36	19.61	33.47	14.55	6.72	3.92	1.56
Stem	0	12.93	16.12	15.66	29.41	15.30	7.30	2.16	1.12
Root	12.53	24.70	13.64	19.79	16.87	5.80	3.79	2.37	0.51

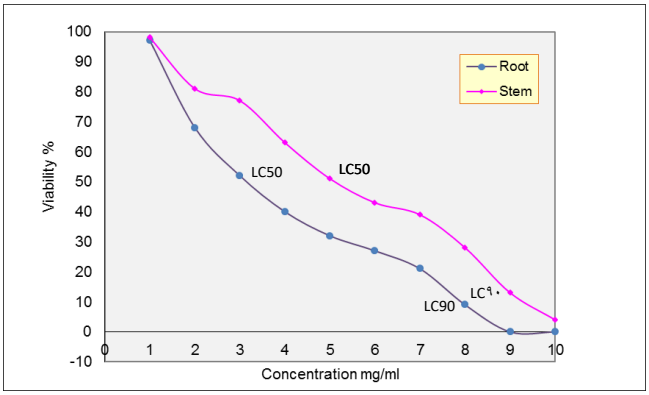

Figure 1: Lethal concentration 50 (LC50) and (LC90) Effect of root and stem bark of pomgranate on the *E.granulosus* protoscolices.

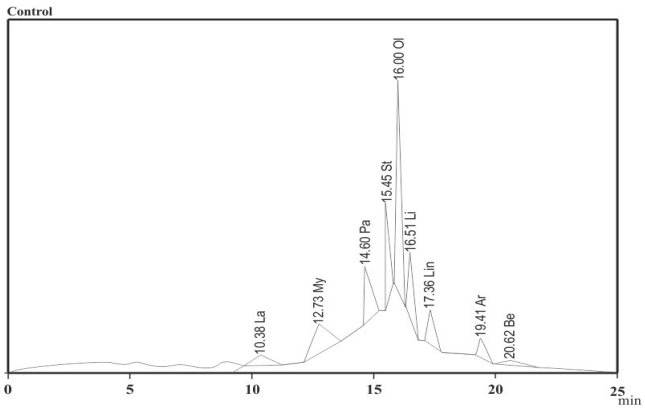

Figure 2: Chromatogram of fatty acids esters, which were separated from the extract of *E.granulosus* protoscolices.

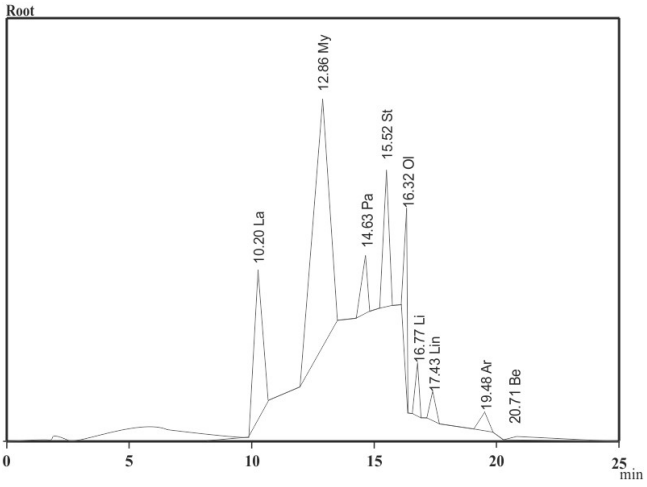

Figure 3: Chromatogram of fatty acids esters, which were separated from the extract of *E.granulosus* protoscolices after exposure to (LC50) of pomegranate root extract.

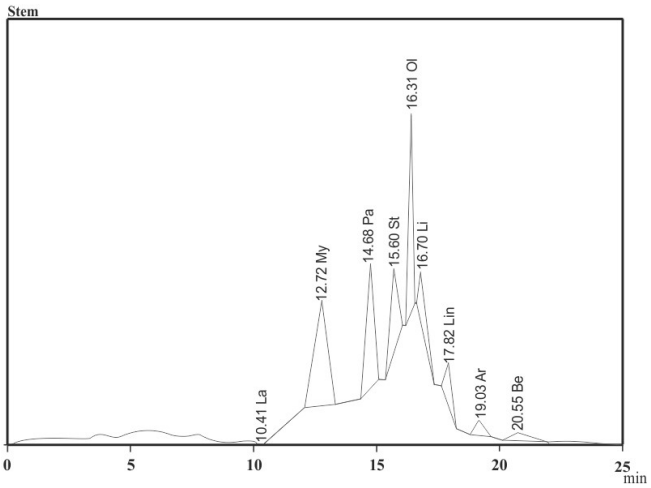

Figure 4: Chromatogram of fatty acids esters, which were separated from the extract of *E.granulosus* protoscolices after exposure to (LC50) of pomegranate stem extract.

Discussion

In this study, treatments compared control yielded to increase concentration % of short chain fatty acids in specially on protoscolices extract treated with root bark significant but stem not significant at morality level 0.05 by using chi square test, In the other side there is decrease in concentration of fatty acids long chain in both treatments (stem, root) barks. The reduction was observed great relatively in protoscolices treated with LC50 from root bark,which show that treatments obstructed The elongation process in fatty acids,whereas hydatids were anaerobic parasite larvaes not depended on *de novo* pathways to synthase fatty acids but depended on salvage pathway by taking short chain fatty acids from host and change its structure on suitable of parasite metabolism, This is consistent with what Zayed and Hanan (35) found two concentration of *Callistemon lanceolatus* and *Ambrosia martima* were used (LC10 and LC25) for one week, snail *Biomphalaria alexandrina* treated with these plants were then collected and identification of fatty acids composition in snail tissue was carried out using GLC. The obtained results declared that treating snail with the plant powder was resulted in reducing concentration of both short and long fatty acids chain, and then disturbance of snail and the endo parasite physiological adaptation and thus abolishing the parasite. Furthermore, Sadeghian *et al.* (36) explained that pomegranate fruit skin has emerged as medicinal plant with potential antimicrobial activity gram positive *Staphylococcus aureus* and negative gram *Pseudomonas aeroginosa* bacteria. As well as against pathogenic yeast, *Candida albicans* on inhibition zone.and this plant used to evaluate the effect of its peel on suckling mice infected with experimental *Cryptosporidium parvum* (37).

From the results of the present study it could be suggested that the pomegranate root bark extract can be considered an effective anti scolocidal agents.

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