Protoscolicidal effects of ferulic acid on viability of protoscolices of hydatid cyst: *In vitro* study

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الخلاصة

حامض الفيروليك هو احد مشتقات المركبات الفينولية والذي يحتوي على قدرة كبيرة لتثبيط النمو المايكروبي, ان الدراسة الحالية تهدف للتحقق من تاثير هذا الحامض على حيوية الرؤوس الاولية لدودة المشوكات الحبيبية, استخدم في هذه الدراسة 126 عينه من الرؤوس الاولية استخلصت من اكباد الاغنام المصابة باالاكياس المائية, وقد عرضت هذه الرؤوس الى ثلاث تراكيز مختلفة من حامض الفيروليك وهي 10 و 20 و 30 ملغ\ مل, بالاضافة الى تركيز 50% من محلول الكلوكوز المركز الذي استخدم كمحلول سيطرة, تم حساب التاثير القاتل للمحاليل بعد مرور 1 و 3 و 7 دقيقة وقد تم قياس نسبة القتل بالاعتماد على اختبار نفاذ صبغة الايوسين الذي يعتمد على تغير لون الاخضر للرؤوس الحية الى احمر بعد قتلها, وقد اثبتت الدراسة ان التركيز 30 ملغ\ مل هو التركيز الامثل حيث كانت نسبة القتل في هذا التركيز 100% بعد مرور 7 دقيقة من العلاج , في حين كانت نسبة القتل لمحلول الكلوكوز المركز 94% لنفس الفترة الزمنية.

Abstract

Ferulic acid is one of phenolic compounds derivatives that reported to be a successful antimicrobial agent. This study aimed to investigate the effect of Ferulic acid solution on the viability of *Echinococcus granulosus* protoscolises. To evaluate the effects of various concentrations of Ferulic acid, 126 samples of live protoscolices of hydatid cyst were exposed to 10, 20 and 30 mg/ml concentration of Ferulic acid and 50% hypertonic glucose solutions as a positive control and 30% dimethylsulfoxide (DMSO) as a control and solvent. The protoscolicidal effect evaluated after 1, 3 and 7 minute, the protoscolicidal effects were determined by 0.1% eosin (eosin exclusion test), in this test protoscolices viability determined depending on the change in protoscolices green color to red after dye.

A 30 mg/ml of Ferulic acid revealed perfect protoscolicidal effect (100% fatality rate) in 7 minute post treatment wile 50% hypertonic glucose made a significant decrease in protoscolices viability (94% fatality rate) in the same duration of exposure.

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The results showed that Ferulic acid solution is highly effective in killing protoscolices of *Echinococcus granulosus* in vitro.

Introduction

Hydatidosis is a zoonotic disease that infects both humans and domestic animals. This disease is widespread in developed countries, especially in rural communities and the infection rate in females (62.7%) which is higher than in males (37.2%). The treatment of this disease depends on surgical removal of the cyst or its contents. The disease has a worldwide distribution and is endemic in several countries including China, Kenya, Turkey and Iran (1)(2). In Iraq, hydatid cyst is hyper endemic and considered as one of the major helminthic diseases, the first reported case was in woman eye in 1925 (11). National reports have been published on the prevalence of the parasite in Iraq, in Al- Diwania the incidence rate reach 21%, and in Baghdad 521 hospital cases have been recorded According to the annual reports of Ministry of Health (7). Humans are infected by ingesting of parasite eggs leading to cyst formation mainly in the liver and lung (3). The best treatment is considered surgery. During the surgery and before evacuation of the cyst, protoscolicidal agents are used to be injected into the cyst in order to prevent secondary cyst formations (2). Currently most common protoscolicidal agents used are hypertonic saline, silver nitrate, cetrimide and formalin; each has a variety of dangerous complications such as biliary tract fibrosis and liver necrosis (4)(5)(6)(7). In this regard, World Health Organization (WHO) purposed an urgent need to find new protoscolicidal agents which are more effective and with less complications (8). Ferulic acid is one of phenolic compounds derivatives; Ferulic acid was reported to be a successful antimicrobial agent such as bactericidal activity against *Streptococcus beta-haemolyticus* (9). However, its protoscolicidal efficacy and the best concentration were not tested yet. Thus, this study was undertaken to evaluate the protoscolicidal effects of various concentrations of Ferulic acid.

Materials and methods

Collection of samples: All the samples used in this study were obtained from the livers of infected sheep of Najaf and Diwaniya slaughter houses. The livers with intact cysts were transferred in cool boxes (ice-containing) with sterile isotonic saline solution to the research laboratory in the Department of Microbiology and Parasitology, College of Medicine / Al Qadisiya University. The cysts were immediately processed. Abnormal, ruptured and small sized cyst (≤3 cm in diameter) were excluded.

Isolation of protoscolices: The cyst surface was washed for several times with sterile physiological (normal) saline then with 70% ethanol to decrease contamination with host tissue. According to McManus and Symth (1978), the cyst contents {fluid and PSCs} were aspirated via aseptic technique into sterile flask. Aseptic dissections of the cysts were performed and all the remaining fluid and PSCs were aspirated and added to the flask content. The flask content was left to stand for 10 minutes. 1ml of the precipitate was poured into a test tube; 10 ml normal saline was added and centrifuged at 3000 rpm for 5 minutes. The germinal layer was pealed off by a forceps and washed for several times with isotonic saline solution to free the remaining attached PSCs. The suspension was centrifuged at 3000 rpm for 5 minutes, and the pellet of PSCs was collected.

Estimation of viability and counting of protoscolices: The viability of the freshly collected PSCs was determined by eosin exclusion test; According to Smyth & Davis (1974), the number of PSCs per ml has been calculated by using the method of constant volume transfer. This procedure depends on transferring a constant volume of isotonic saline solution containing PSCs to the slide, mixing it with the same volume of eosin stain and examining the mixture under 40x objectives to record the average of 3 readings:

$$\text{Viability} = \frac{\text{No. of viable PSCs}}{\text{Total No. of PSCs}}$$

When the percentages of viable PSCs were more than 90% they are considered to be appropriate for the study. 0.01ml of pooled PSCs was transferred over a slide and mixed with equal volume of 0.1% eosin and

the viability was evaluated by 40x power microscopy after one minute. Dead PSCs absorb eosin and colored red while the living one remains green. Also the viability of PSCs is determined according to the biological feature represented by muscular movement of PSCs, and flame cell activity as observed by direct microscopy examination at x 100.

Preparation of tested agents:

1- **Hypertonic glucose (control):** A 100 ml vial of hypertonic glucose solution (50%) was purchased from a local pharmacy (Ajanta Company, India). The required volume was applied to the tested slide accordingly.
2- **Dimethyl sulfoxide (control & solvent):** A 1000 ml bottle of DMSO solution (100%) was purchased from Sigma Aldrich Company, Batch no: 28216. A 30 % solution of DMSO was prepared according to volume/ volume equation (N1 x V1= N2 x V2) (10). The DMSO (30%) was used as a solvent to Ferulic acid.
3- **Ferulic acid (tested agent):** A 5 g crude powder of Ferulic acid was purchased from Sigma Aldrich Company (Batch no. 76755369),

Ferulic acid powder was dissolved in 30% DMSO in order to prepare three different concentrations (10 mg/ml, 20 mg/ml and 30 mg/ml).

Evaluation of Protoscolicidal effect of studied chemicals: In order to asses the protosolicidal effect, 0.01ml of pooled PSCs was transferred over a slide and mixed by 0.01 ml of 0.1% eosin and the viability of PSCs was evaluated by 40x power microscopy after one minute. Then 0.01 ml of Ferulic acid solution was added to the slide and mixed by wooden stick. The protoscolicidal effect was tested in three different concentrations (10 mg/ml, 20 mg/ml and 30 mg/ml), and three different durations 1, 3 and 7 minute of exposure. In addition, the protoscolicidal effect of the control agents; hypertonic glucose solution (50%) and DMSO (30%) were tested by using same exposure durations.

Statistical analysis:

Statistical analyses were done by using SPSS version 13 computer software (Statistical Package for Social Sciences). The outcome variable for the present study was calculated as follows:

Count of dead protoscolices = Count of total alife protoscolices (at baseline) – Count of alife protoscolices after treatment

Fatality rate = [Count of dead protoscolices / Count of total viable protoscolices] x 100 (At baseline)

Treatment effect was assessed by paired t-test. The R^2 called the determination coefficient is used to measure the amount of variation in the dependent variable. P value less than the 0.05 level of significance was considered statistically significant.

Result and discussion

Table 1: Effects of Ferulic acid on the viability of protoscolices (PSCs) stratified by duration of treatment and concentration of solution.

Ferulic acid	Baseline count of viable PSCs pretreatment	Count of viable PSCs after treatment	Count of dead PSCs (attributed to treatment)	Fatality rate of PSCs (%)	P (Paired t-test)
After 1 minute treatment					
10mg/ml concentration					**<0.001**
Mean	325.7	291.3	34.3	10.5	
SE	4.98	4.91	0.67	0.24	
20mg/ml concentration					**<0.001**
Mean	181.7	119	62.7	34.5	
SE	2.19	1.73	0.88	0.38	
30mg/ml concentration					**<0.001**
Mean	190	70	120	63.2	
SE	3.21	2.65	1.15	0.84	
After 3 minutes treatment					
10mg/ml concentration					**0.001**
Mean	290	249.3	40.7	14	
SE	7.23	5.78	1.45	0.15	
20mg/ml concentration					**<0.001**
Mean	181.7	97.7	84	46.3	
SE	4.33	3.93	1.15	1.01	
30mg/ml concentration					**0.001**
Mean	177	34	143	80.7	
SE	3.79	1.53	5.29	1.28	
7 minutes post treatment					
10mg/ml concentration					**0.001**
Mean	288.3	227	61.3	21.3	
SE	4.1	2.08	2.33	0.52	
20mg/ml concentration					**<0.001**
Mean	187	49	138	73.9	
SE	7.09	5.2	2.89	1.9	
30mg/ml concentration					**0.001**
Mean	184.3	0	184.3	100	
SE	4.7	0	4.7	0	

Table 2: Effects of hypertonic glucose (50%) on the viability of PSCs stratified by duration of treatment.

Hypertonic glucose (50%)	Baseline count of viable PSCs pretreatment	Count of viable PSCs after treatment	Count of dead PSCs (attributed to treatment)	Fatality rate of PSCs (%)	P (Paired t-test)
After 1 minute treatment					0.002
Mean	290.7	236.7	54	18.6	
SE	3.18	4.7	2.31	0.88	
After 3 minutes treatment					0.001
Mean	305	199.7	105.3	34.5	
SE	3.79	6.17	3.76	1.43	
7 minutes post treatment					<0.001
Mean	288.7	16.3	272.3	94.3	
SE	3.53	1.76	2.4	0.58	

Table 3: Effects of DMSO (30%) on the viability of PSCs stratified by duration of treatment.

DMSO (30%)	Baseline count of viable PSCs pretreatment	Count of viable PSCs after treatment	Count of dead PSCs (attributed to treatment)	Fatality rate of PSCs (%)
After 1 minute treatment				
Mean	157.7	157.7	0	0
SE	4.7	4.7	0	0
After 3 minutes treatment				
Mean	159.7	159.7	0	0
SE	9.21	9.21	0	0
7 minutes post treatment				
Mean	165.7	165.7	0	0
SE	6.74	6.74	0	0

Table 4: The mean fatality rate of PSCs (%) after Ferulic acid treatment stratified by treatment duration and concentration of tested solution.

	Concentration of tested solution (mg/ml)			Linear trend (correlation) with changing conc of treatment
Ferulic acid	10	20	30	
After 1 minute	(n=3)	(n=3)	(n=3)	r=0.95 P<0.001
Mean	11	35	63	
SE	0.2	0.4	0.8	
After 3 minutes	(n=3)	(n=3)	(n=3)	r=0.95 P<0.001
Mean	14	46	81	
SE	0.2	1	1.3	
7 minutes post treatment	(n=3)	(n=3)	(n=3)	r=0.97 P<0.001
Mean	21	74	100	
SE	0.5	1.9	0	
Linear trend (correlation) with changing duration of treatment	r=0.95 P<0.001	r=0.95 P<0.001	r=0.97 P<0.001	
	y=8.7+1.79Dur P<0.001 R2=0.988	y=27.3+6.62Dur P<0.001 R2=0.987	y=59.5+5.95Dur P<0.001 R2=0.967	

Table 5: The mean fatality rate of PSCs (%) after hypertonic glucose treatment by treatment duration.

Hypertonic glucose	Concentration of tested solution 50%
After 1 minute	(n=3)
Mean	18.6
SE	0.9
After 3 minutes	(n=3)
Mean	34.5
SE	1.4
7 minutes post treatment	(n=3)
Mean	94.3
SE	0.6
Linear trend (correlation) with changing duration of treatment	r=0.95 P<0.001

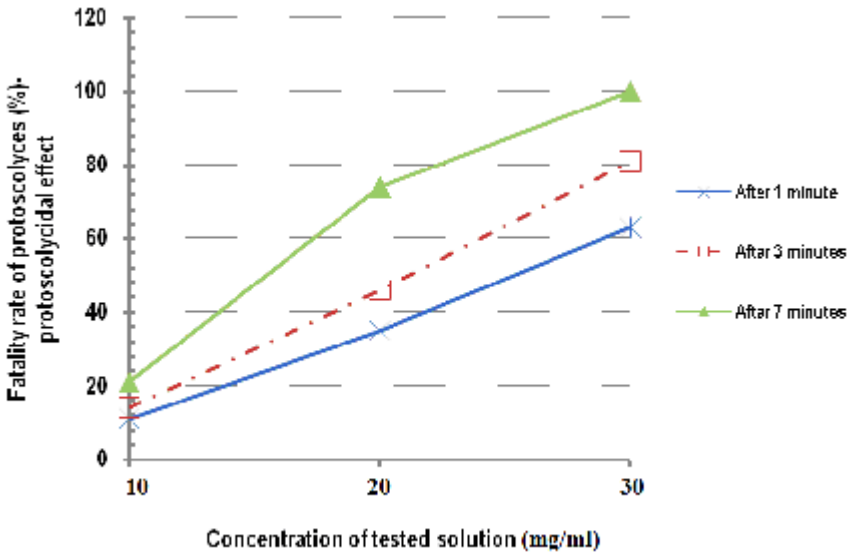

Figure 1: Line graph showing the effect of increasing Ferulic acid solution concentration after 3 different treatment durations.

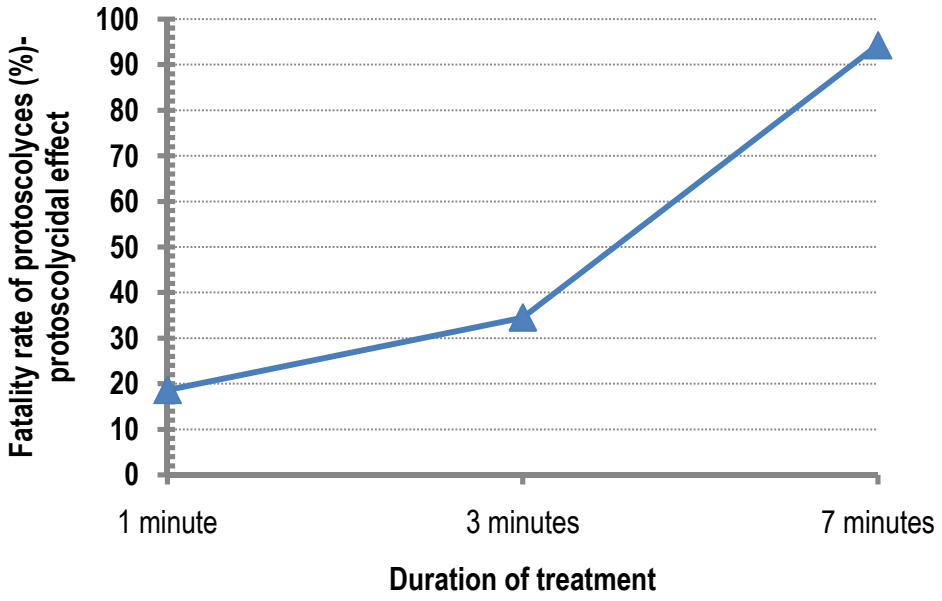

Figure 2: Line graph showing the effect of increasing the duration of exposure to 50% hypertonic glucose solution.

The protoscolicidal effect of ferulic acid solution was tested in relation to possible linear effects: concentration of tested solution and duration of contact with solution. The effect of increasing concentration from 10mg/ml to 30mg/ml at each of 3 different durations of treatment was assessed and then the effect of increasing duration of treatment from 1 to 7 minutes at each of the 3 different concentrations was also substantiated.

As shown in table (4) and figure (1), at the minimal concentration (10mg/ml) and shortest duration of treatment (1 minute) FA was associated with 11% protoscolicidal effect. Increasing the concentration of FA had a significant effect after 1 minute of treatment that reach 35% of fatality rate, since an increase of 10mg/ml in concentration is associated with huge mean increase in fatality rate of 63%, although fatality rate showed a statistically significant strong positive linear trend with concentration at the 3 minutes duration. At the 7 minutes duration fatality rate showed a strong positive linear correlation with concentration that reach 100% fatality rates.

At the 10mg/ml concentration an increase in duration had a weak significant linear trend with fatality rate that reach 14%, duration increasing had strong effect at 20mg/ml concentration in 46% fatality rate, in 30mg/ml concentration increase duration had a strong linear trend with 810mg/ml fatality rate. An increase of 4 minute in duration (after 7 minutes) was associated with a small mean increase in fatality rate of 21% in 10mg/ml concentration. The 20mg/ml concentration had a very strong linear trend with fatality rate 74%, in 30mg/ml concentration the effect of changing duration was significantly the strongest and biggest fatality rate that kill all the PSCs (100%). The duration had a statistically significant strong positive linear correlation with fatality rate. An increase in duration of 1 minute is associated with a statistically significant mean increase in fatality rate of 6.62 and 5.95 at 20mg/ml and 30mg/ml concentration respectively.

These findings are in consistency with the study of Aziz, which reported that ferulic acid able to inhibit growth of *Seed-borne* fungi (11). Another study by Franke et al., reported that ferulic acid inhibits the respiratory syncytial virus reproduction (12). The possible explanation of the antimicrobial effect of ferulic acid is the ability to interrupt the activity of the enzyme that responsible for DNA replication such as topoisomerase (11).

Recommendations

Further studies are recommended

1. To examine the effects of the studied agent on the integrity of hydatid cyst membrane.
2. To clarify the pharmacological/toxicological profile of the agent.

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