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Effect of Saussurea costus extracts in the viability of Echinococcus granulosus protoscoleces of sheep origin In vitro

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Abstract

Cystic echinococcosis is one of the most prevalence and dangerous zoonotic parasitic disease in the world. Iraq is one of the most countries that affected by this disease. Surgery is usually the most effective therapy and can be used with injection of drugs in hydatid cysts before surgery to kill protoscoleces to avoid anaphylactic shock as a result of spilling of hydatid cyst fluid into peritoneal cavity, as well as this reduce the chance of secondary hydatid cysts production. Therefore, the current study aimed to evaluate the effectiveness of *Saussurea costus* extracts on protoscoleces viability of *Echinococcus granulosus* , where the protoscoleces of *E. granulosus* exposed to four different concentrations of *Saussurea costus* extracts at four different exposure times including; 15, 30, 45 and 60 min *in vitro*, The results of ethanolic extract showed highest efficacy at concentration 20,30mg/ml in 45,60min and 40mg/ml at all times. The highest scolicidal effect of petroleum ether extract was reported at 10 and 15mg/ml during 60min, while 20mg/ml reported similar effect at 45 and 60min. The data of cool aqueous extract showed reduction of protoscoleces viability to 0% at 200 and 250mg/ml in 45 and 60min, respectively, while 300mg/ml showed same reduction of viability at all experiment time periods. The data of hot aqueous extract showed 100% kill rate by using 350mg/ml at 60min and 400mg/ml at 45 and 60, as well as 450mg/ml at all experiment period times. Comparing to the control group. Generally, *Saussurea costus* extracts have concentration and exposure time-dependent effect on protoscoleces viability.

Keyword: *Echinococcus granulosus, Saussurea costus* , protoscoleces

تأثير مستخلصات القسط الهندي في حيوية الرؤيسات الأولية للمشوكة الحبيبية من اصل اغنام خارج

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الخلاصة

يعد داء المشوكات الكيسي من اكثر الامراض الطفيلية المشتركة بين الانسان والحيوان انتشاراً وخطورة في جميع انحاء العالم. ويعتبر العراق من الدول المتأثرة بهذا المرض .عادة ما تكون الجراحة هي العلاج الأكثر فعالية للمرض ويمكن استخدامها مع حقن الادوية في الاكياس العدرية قبل اجراء الجراحة لقتل الرؤيسات الأولية لتجنب حدوث الصدمة (التأقية) نتيجة أنتشار سائل الكيس العدري في التجويف البريتوني، بالإضافة الى ان هذا يقلل من فرصة تكون أكياس عدرية ثانوية. لذلك، هدفت الدراسة الحالية تقييم فعالية مستخلصات نبات *Saussurea costus* المعروف بالقسط او القسط الهندي على حيوية الرؤيسات الأولية للمشوكة الحبيبية. حيث عرضت الرؤيسات الأولية لطفيل المشوكة الحبيبية الى أربعة تراكيز مختلفة لمستخلصات نبات القسط الهندي خلال فترات تعرض وهي 15،30،45 و60 دقيقة خارج الجسم الحي. أظهرت نتائج المستخلص الايثانولي اعلى فاعلية له عند التركيز 20 و 30ملغم/مل في 45و60 دقيقة ،40ملغم/مل في جميع الأوقات. وكان اعلى تأثير قاتل لمستخلص بتروليوم أيثر عند التركيز 15و10ملغم/مل بعد 60 دقيقة و20ملغرام/مل بعد45 و 60 دقيقة وقد حققت نتائج المستخلص المائي البارد انخفاض نسبة الحيوية الى 0% عند تراكيز 200 و 250ملغم/مل في 45،60 دقيقة و300ملغم/مل في الأوقات15، 30، 45 و60دقيقة اما نتائج المستخلص المائي الحار فقد سجلت نسبة قتل 100% عند تراكيز 350ملغم/مل في 60دقيقة و400ملغم/مل في 45و 60دقيقة و450ملغم/مل في جميع الأوقات المستخدمة بالمقارنة مع مجموعة السيطرة. على العموم، فقد تناسب تأثير مستخلصات القسط الهندي تناسباً طردياً مع زيادة التركيز وزيادة مدة التعريض.

الكلمات المفتاحية: المشوكة الحبيبية، القسط الهندي، الرؤيسات الأولية

Introduction

Echinococcosis or Hydatidosis is a zoonotic disease, caused by the metacestode or larval stage of a dog tapeworm *Echinococcus granulosus* [1,2]. *E. granulosus* spread in central Asia, China, North and East Africa, Australia and South America [3] . Life cycle of *E. granulosus* in definitive host, represented by the canids which harbors the adult worms in their intestines, and intermediate host includes livestock, as well as human which act as an accidental host. The intermediated host can be infected by orally ingestion of parasite eggs which develop into fluid-filled cysts in their liver, lungs, and other organs [4]. The disease causes expanded mortality and morbidity in human and livestock [5]. Echinococcosis disease is asymptomatic for several years, and the clinical symptoms can appear due to increase of cyst size which cause pressure on nearby tissues **[6]**.

There are several methods of treatment echinococcosis including surgery, percutaneous technique, chemotherapy [7]. Surgery is the most effective therapy of the disease but with quite difficult for some cases because the cyst diffusion in to many organs or formed in risky location [8]. Chemotherapy by singing benzimidazole derivatives are necessary in cases of possible recurrence, injury to several organs in the body and addition to cases of the advanced stages [9]. However, it has side effects such as nausea, vomiting, diarrhea, abdominal pain, headache, it may cause gastro-intestinal and liver functions disturbances, hematuria and leukopenia [10]. In order to reduce the risk of hydatid fluid spillage of the cysts during surgery and to prevent reoccurrence of echinococcosis, which may occur in approximately 10% of postoperative cases[11]. Therefore, the use of more effective and less harmful agents to treat this disease is important [12], such as *pistacia vera*, *zataria multiflora* and other plants [13,14].

Saussurea costus belongs to family of Asteraceae is one of the main species of the genus Saussurea [15] which mostly grow in the humid regions at altitude of 2600-4000m in Western Himalayan region of India and Pakistan, and it is also been started to cultivate in new areas for commercial purposes in 1920 [16]. The roots of *S. costus* are used for the treatment many diseases including chronic gastritis, stomach ulcer, asthma, bronchitis and rheumatoid [17], oil extracts from the roots of S. costus has been used in the treatment of leprosy [18]. Chemical studies of the S. costus plant have shown it contains many phytochemical compounds with biological activity, including alkaloids, flavonoids, sesquiterpene terpenes, anthraquiones and tannins [19,20].

This study aims to determinate the effect of *S .costus* extracts on viability of *E. granulosus* protoscoleces *In vitro*.

Materials and methods

Samples collection and protoscoleces isolation:

The infected livers of sheep (figure1) were collected from butcher shops in Mosul city and protoscoleces of E. granulosus were isolated according to Smyth [21] by sterilizing the surface of hydatid cyst with 70% ethanol following by aspiration of hydatid cyst fluid using G21 needle. Protoscoleces were washed three times with phosphate buffered saline (PBS) using centrifuge at 3000rpm for 10minutes, with the addition of 1gm of streptomycin and 20000 UI of penicillin before starting a second wash.

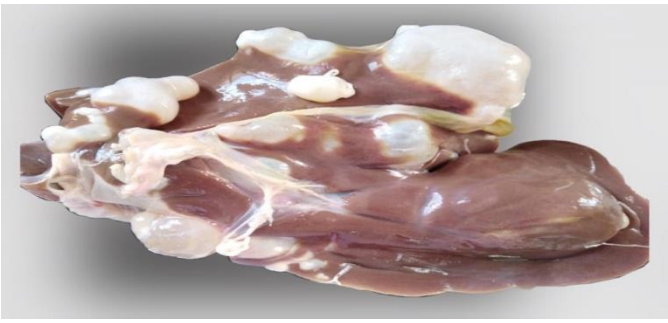

Figure1 : Hydatid cysts in the liver of sheep

Estimation of protoscoleces viability:

The assessment of the viability of protoscoleces was estimated by taking 20 ul of the hanging of protoscoleces was mixed with 20 ul of 0.1% eosin stain on the glass slide. Protoscoleces that appear by bright green in color considered alive (figure 2) while protoscoleces red in color dead because stain entry inside it (figure 3) [22].The viability of the protoscoleces in this study was 100%.

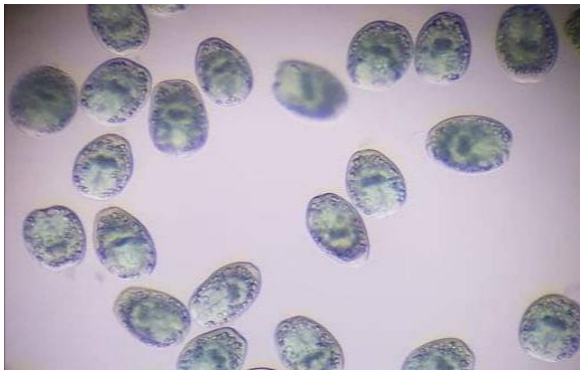

Figure 2 : Live protoscoleces before treated with extracts after dye it with 0.1% eosin

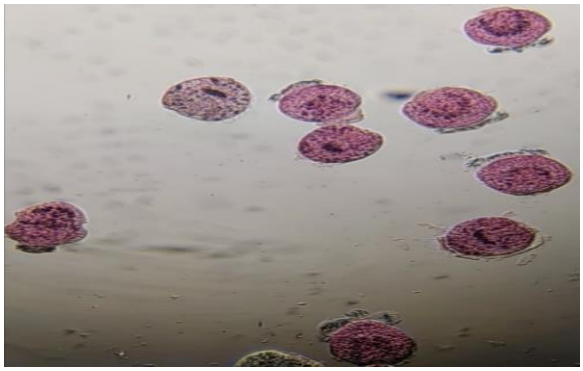

Figure 3 : Dead protoscoleces after treated with extract and dye it with 0.1% eosin

Collection and preparation of the *S. costus* extracts

S. costus plant was bought from perfumery shops in Mosul city. 25 gm from a root of plant powder were added to 500ml of petroleum ether for 72 hours on magnetic stirrer, then solution were filtered by Whatman No.1 filter paper, 500ml Ethanol 70% is added to the precipitant for 72 hours on magnetic stirrer [23], after filtering the solution, distilled water was added to precipitant on magnetic stirrer at medium speed and temperature 60C° to obtain a warm water extract, The solvent was removed by using rotary evaporator at 40C° [24] . Cool aqueous extract was prepared according to Rios et al. method [25], where 40g of root plant powder was added to 400 ml of distilled water following by mixing process using blender and then magnetic stirrer. The samples underwent to soak and mix for 24 hours at 4C°.The samples were filtered using Whatman filter paper No.1 following by store the crude extracts at 4C°, until been used.

Effect of *S. costus* extracts on protoscoleces viability *In vitro*

To evaluate the effect extracts on viability of the protoscoleces, experiments were designed that included four different concentrations at four different times, in addition to the control group. In each test tube 1ml of extract dissolved in PBS was added, with the addition of 2000 protoscoleces in the same tube and placed in a water bath at 37C◦ according to the specified times washed with PBS solution to get rid of the effect of extracts and examined protoscoleces under a light microscope to count the living and dead ones.

Statistical analysis

Data analysis was carried out using the SPSS statistical analysis system, the means and standard error were used, in addition to using Duncon's test to measure a significant difference between the means of experimental study at the level of significance $p \leq 0.05$.

Results and Discussion

The results of this study showed the effect of petroleum ether extract *S. costus* on protoscoleces viability *in vitro* using four concentrations 5,10,15 and 20 mg/ml at four different times 15,30,45 and

60minutes. The data of petroleum ether extract indicated 0% of viability rate at 10mg/ml and 15mg/ml during 60min, while 20mg/ml indicated 0% of viability at 45 and 60min. The highest percentage of viability (47.785%) reported at 5mg/ml during 15min (see table-1).

Table 1. The effect of petroleum ether extract of S. costus on viability percentage of protoscoleces.

Con.	Control	Viability of protoscoleces (%) at different time			
		15min.	30min.	45min.	60min.
5mg	100	47.785±1.278 f	25.850±2.626 e	19.440±0.329 d	2.500±1.443 a
10mg		42.565±1.405 e	18.220±0.408 d	13.330±0.408 c	0 a
15mg		17.140±0.790 d	9.830±1.056 b	1.495±0.863 a	0 a
20mg		10.800±0.408 bc	1.515±0.874 a	0 a	0 a

Similar letters in the table above indicate there are no significant differences between values at $p \leq 0.05$, while different letters indicate there are significant differences between values at $p \leq 0.05$.

The current study results showed the effect of Ethanolic extract *S. costus* on protoscoleces viability *In vitro* using four concentrations 10,20,30 and 40 mg/ml at four different times 15,30,45 and 60minutes. Lowest percentage of viability rate reported at 20mg/ml and 30mg/ml during 45 and 60min, while 40mg/ml indicated lowest viability percentage (0%) at all experimental time periods. The highest viability percentage (100%) was reported at 10mg/ml during 15min. (see table-2).

Table 2 . The effect of ethanolic extract of S. costus on viability percentage of protoscoleces.

Con.	Control	Viability of protoscoleces (%) at different times			
		15min	30min	45min	60min
10mg	100	100.000±0.000g	69.7900±3.181f	28.570±0.408e	23.640±0.871d
20mg		27.470±1.749e	18.570±0.825c	0a	0a
30mg		7.566±1.261b	1.110±0.702a	0a	0a
40mg		0a	0a	0a	0a

Similar letters in the table above indicate there are no significant differences between values at $p \leq 0.05$, while different letters indicate there are significant differences between values at $p \leq 0.05$.

The current data showed the effect of hot aquatic extract of *S. costus* on protoscoleces viability in *vitro* using four concentrations 300,350,400 and450mg/ml at four different times 15,30,45 and 60min Lowest percentage of vitality rate (0%) Showed at 350mg/ml and 400mg/ml during 60 and 45min, respectively, while 450mg/ml indicated lowest viability percentage (0%) at all experimental time periods. The highest viability percentage (52.165%) was reported at 300mg/ml during 15min. (see table-3).

Table 3 . The effect of hot aquatic extract of *S. costus* on viability percentage of protoscoleces.

Con. mg/ml	Viability of protoscoleces (%) at different times				
	Control	15min	30min	45min	60min
300mg	100	52.165±3.559 f	39.950±0.871 e	24.162±3.314 c	2.940±1.697 a
350mg		30.337±1.998 d	22.525±0.574 c	1.095±0.632 a	0 a
400mg		24.215±1.425 c	11.425±3.299 b	0 a	0 a
450mg		0 a	0 a	0 a	0 a

Similar letters in the table above indicate there are no significant differences between values at $p \leq 0.05$, while different letters indicate there are significant differences between values at $p \leq 0.05$.

The data of cool aqueous extract of *S. costus* on protoscoleces viability *in vitro* using four concentrations 150,200,250 and 300,mg/ml at four different times 15,30,45 and 60minutes. Indicated lowest viability percentage (0%) at 200mg/ml during 45 and 60min, while 250mg/ml showed 0% of viability at 30, 45 and 60min, in addition to 300mg/ml showed 0% of viability during all experimental time periods (see table-4).

Table 4 . The effect of cool aquatic extract of *S. costus* on viability percentage of protoscoleces.

Con. mg/ml	Viability of protoscoleces (%) at different times				
	Control	15min	30min	45min	60min
150mg	100	48.680±1.166 d	31.700±2.136 c	9.530±0.779 b	2.470±0.867 a
200mg		31.245±1.244 c	2.732±1.034 a	0 a	0 a
250mg		2.652±1.885 a	0 a	0 a	0 a
300mg		0 a	0 a	0 a	0 a

Similar letters in the table above indicate there are no significant differences between values at $p \leq 0.05$, while different letters indicate there are significant differences between values at $p \leq 0.05$.

The viability rate of protoscoleces for petroleum ether extract decreased to zero at concentration 10 and 15mg/ml after 60min and 20mg/ml after exposure time of 45and60min, results are converged with a study of El-Bahy et al. [28] that achieved the maximum mortality rate among the protoscoleces 100% when used *Nigella sativa* oil at 100mg/ml concentration after 120min with the superiority of a current study in time and focus. The current study also outperformed in terms of achieving a high killing rate compared to the results of Hesari et al. [29] study , where they reported highest mortality rate 4% when used petroleum ether extract of *Cucurbita moshata* at concentration 10 mg/ml in 60min.

The present study showed that *S.costus* extracts inhibitive efficiency against protoscoleces of *E. granulosus* to different concentration at times different . The current data of ethanolic extract indicated 100% kill rate at 20 and 30 mg/ml during 45 and 60min, while the same kill rate was reported at 40mg/ml during all experiment time periods. This data is in an agreement with the data that obtained by previous study [26], where they reported 100% kill rate by using 50mg/ml of ethanolic extract of Salvadora persica at 20min.These results were superior in terms of concentration, time and killing rate on results Al-Aloosi et al. [27] when used alcoholic extract of *viscum album* showed high killing (80.7%) at 1500mg/ml in60 min.

The concentration 450mg/ml of hot aqueous extract showed highest kill rate (100%) at all experiment time periods. These results are in an agreement with previous data [30], where he reported reduction of protoscoleces viability from 87% to 0% by fourth day of experiment by using hot aqueous extract of ziziphus spina.

Regarding to the current data of cool aqueous extract, 100% kill rate was reported at 250mg/ml at 15, 30 and 45 min, while 300mg/ml reported same kill rate during all experiment time periods. These results are agreed with the data obtained previously [31], where cool aqueous extract of Citrus aurantifolia reported 100% kill rate at 15,20,50 and 100mg/ml after 24, 48 and 96 hours.

The scolicidal effect of these extracts may be attributed to the medicinal properties of the roots *S. costus* plant Including antimicrobial, anti- inflammatory, anticancer, analgesic and heptoprotective properties in humans[18]. In addition to the death of protoscoleces determined by entering eosin dye inside parasite which caused some structural changes including distribution of hooklets in protoscoleces cytoplasm and membranous swellings of protoscoleces plasma membrane in some cases.

Conclusion

It is concluded that all extracts of *S. costus* have scolicidal effect on *E. granulosus* protoscoleces which represented by concentration and exposure time-dependent effects.

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