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RESEARCH ARTICLE

EFFECT OF ALCOHOLIC EXTRACT OF CLOVE (SYZYGIUM AROMATICUM) ON VIABILITY OF PROTOSCOLICES OF SHEEP HYDATID CYSTS IN VITRO

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Abstract

Clove (Syzygium aromaticum) is one of the most valuable spices that has been used for centuries as food preservative and for many medicinal purposes such as antispasmodic, carminative, antioxidant and antimicrobial. This study was aimed to evaluate the effects of alcoholic extract of clove, on the viability of Echinococcus granulosus protoscolices in vitro. Different concentrations of the clove extract were assessed (1, 50 and 100 mg /ml). The results of the current study showed significant complete inhibition (100%) of protoscolices viability in vitro treated with100 mg/ml of alcoholic extract of S. aromaticum after 3 hours of exposure. Also, 85% inhibition with concentration of 50 mg/ml after 3 hours, progressing to complete inhibition of protoscolices viability after 4 hours. With concentration of 1 mg /ml, 70 % inhibition was observed after 3 hours, progressing to 100% inhibition after 5 hours of exposure to S. aromaticum alcoholic extract. There were significant correlations between efficacy and concentration and between efficacy and exposure time.

Keywords: Echinococcus granulosus, Protoscolices, Clove (Syzygium aromaticum), Extract, Hydatid cysts.

Introduction

Cystic echinococcosis also known as hydatid cyst disease is one of the most important zoonotic parasitic disease affects both humans and herbivorous animals [1-3]. Cystic echinococcosis has a worldwide distribution and caused by larva of *Echinococcus granulosus* (*E. granulosis*), the dog tapeworm which belongs to the Platyhelminthes phylum. Dogs are the definitive hosts and contain the adult worm in the intestines**.** Humans and animals such as sheep, camels, goats, pigs, horses, donkeys, monkeys and other animals are serve as intermediate hosts**.** In the typical life cycle, adult worms in the dog's intestine liberate thousands of eggs, which are ingested by sheep or humans accidently through food contaminated with dog feces containing the eggs [3]. The hydatid cysts form primarily in intermediate host's internal organs such as the liver, lungs, and rarely spleen, kidneys, bone, brain, and other organs [3].

It has been suggested that surgical removal in combination with chemotherapy such as albendazole is still the most important treatment method; however, this treatment method may complicated by hydatid fluid leakage into the abdominal cavity during surgery and protoscolices may cause secondary hydatid cysts and anaphylactic shock**.** In addition, 20% hypertonic sodium chloride, 0.5% silver nitrate, formalin, and cetrimide have been used as protoscolicidal agents [4-6], however these protoscolicidal agents are toxic and may cause complications like liver necrosis and cholangitis. Consequently, the World Health Organization (WHO) proposed an urgent need for new protoscolicidal agents which would be more effective and with fewer complications. As a result, many studies have aimed at alternatives of this therapy through the effective, safe and economical herbal medicine for studying the effect of the different herbs extracts and its effects on the viability of the protoscolices [7, 8]. Recently, several plant extracts, such as garlic and olive leaf extracts have been evaluated as scolicidal agents instead of using chemotherapy in conjunction with surgery [7]**.** Therefore, the objective of this study was to determine the scolicidal efficacy (i.e., the mortality rate of the protoscolices) of alcoholic extract of clove *(Syzygium aromaticum*), on the protoscolices of sheep hydatid cysts *in vitro*.

Materials and Methods

Parasite material

Livers infected with hydatid cysts of *E. granulosus* were obtained from slaughtered sheep in Al-lohom area abattoirs, Aden Governorate, Yemen. Infected livers placed in boxes with ice and transported within 3 hours to the laboratory of biology at faculty of Education where hydatid cysts removed from livers.

Collection of hydatid fluid

Cysts were washed several times in sterile PBS, PH 7.2. The exposed surfaces of cysts were sterilized by 70% ethyl alcohol. Then the fluid was aspirated by using large size syringe with needle (20 ml). The aspirated hydatid fluid collected in sterile tubes (500 ml) and left to precipitate for an hour and the protoscoleces were settled down at the bottom of the tubes. The fertility of hydatid cysts determined by the presence of free protoscolices in cyst fluid by examination of wet mount preparation using the microscope. These procedures were applied according to Smyth [9].

Preparation of protoscolices

After the deposition of the protoscolices, the supernatant was removed by Pasture's pipette and the yielded protoscoleces were maintained in a sterile preservative solution made of a mixture of Krebs-Ringer solution (KRS) and hydatid cyst fluid 4:1 for all experiments. This preservative solution contains nutritional factors and a number of minerals such as glucose, magnesium, chloride, potassium, chloride, sodium chloride, sodium phosphate dibasic and sodium phosphate monobasic, that keep protoscolices alive for a long period of time. In addition, this preservative solution does not contain any antibiotic or antifungal drugs [10]. Protoscolices measuring a total of 30000/mL to 40000/mL were collected.

Viability of protoscolices

In this study, eosin stain with 0.1% concentration (1g of eosin powder in 1000 mL of distilled water) was used for the viability test of protoscolices. The viability of protoscolices was determined prior to the experiments. A 0.01 ml solution of pooled protoscolices was transferred over a slide and mixed with 0.01 ml of 0.1% aqueous eosin stain, as a vital staining, and was evaluated by low power microscopy after 5 min. Unstained protoscolices were considered as viable (alive) while stained in red colour protoscolices were considered as non-viable (dead) [11]. The samples of protoscolices were selected while the viability was 95% or more.

At the end of each exposure time, the percentage of viability protoscolices was calculated using this formula [11].

Live protoscoleces % =

$$\frac{No.\,of\ the\ live\ protoscoleces\ in\ 1ml}{No.\,of\ total\ sample\ protoscoleces\ in\ 1\ ml}\ X\ 100$$

The procedure was repeated three times in a row and the survival rate was taken.

Plant collection

The flowers buds of clove (*S. aromaticum*) were collected from the local market in Lahj governorate, Yemen. The plants was identified by experts from the Department of Biology, Faculty of Education, Aden University, Yemen. Cloves were cleaned and ground directly. Then placed in a sterile dark-colored bottle. The bottles were kept in the refrigerator until use.

Table1: The plant under study [12].

English name of the plant	Scientific name	Family	Part in use
Clove	*Syzygium aromaticum*	Myrtaceae	Flowers buds

Preparation of alcoholic extract

The plant powder (50gm) was taken in Soxhlet apparatus. Then 500 ml ethyl alcohol 70% was added. The plant materials were extracted until the color of the extract disappeared and then, it was left undisturbed. The solvent was evaporated by rotary evaporation. The mixture was finally placed in clean Petri dishes. Then, the dry mass was transferred to hot air oven and kept for 24 hours at 50 °C. It was weighed and kept in the refrigerator in sterile and dark-colored containers until use [13]. Different concentrations of 1 mg /ml, 50 mg /ml, and 100 mg/ml of clove alcoholic extract were prepared.

In vitro treatment of the protoscolices:

A total of 15 tubes were selected and divided into 3 sets each with 3 groups (clove alcoholic extract group, positive and negative control group) of 3 tubes. Each set was used for different concentration (1 mg/ml, 50 mg/ml, and 100 mg/ml).

In a sterile condition, a 0.1 mL of a fluid containing 2000–3000 protoscolices was transferred to each tube. The groups were divided as follows: (1) clove alcoholic extract, (2) sterile preservative solution as the negative control, and (3) albendazole sulfoxide (ABZSO) 800 µg/ml as the positive control. The experiments were carried out at different concentrations (1 mg/ml, 50 mg/ml, and 100 mg/ml) in each group as follow: in the first step: 2 ml of alcoholic extract of the clove at a concentration of 1 mg/ml was added to the tubes containing 2000- 3000 live protoscolices in first group of the first set. In the second step: the first group of the second set was treated with alcoholic extract of the clove at a concentration of 50 mg/ml. In the third step: treatment of the first group of the third set with clove

alcoholic extract at a concentration of 100 mg/ ml. After adding the extract at each stage, the tubes are placed in the incubator at 37°C. Then the viability of the protoscolices was assessed for each experiment at different and specific times by taking a sample with a pipette of 0.1 ml and examined for viability using 0.1% eosin. The process was repeated three times and the medium was taken.

In positive control group, live protoscolices were treated with the chemical drug albendazole sulfoxide (ABZSO) 800 µg/ml described by Yones *et al*., [14], by adding 2 ml to live protoscolices (2000-3000 protoscolices), and placed in the incubator at 37°C.Then the protoscolices were examined by adding 0.1% eosin stain at different exposure periods to compare it with the results of other experiments. In negative control group, no substance was added except for the preservative solution (negative control) and 2000-3000 live protoscolices, and it was subjected to the same procedures as in the previous groups.

Ethical considerations:

The study protocol was approved by "the Committee of Research and Postgraduate Studies, at the biology department (Animal specialty) faculty of Education, University of Aden. Permissions were obtained from the faculty of Pharmacy at Aden University where the samples examined and from the Al-lohom area abattoirs, Aden Governorate, where the samples collected.

Statistical analysis:

Statistical calculations were carried out by 2-way variance analysis (ANOVA), using the Statistical Package for Social Sciences (GenStat) software version 20.. A p- value of ≤0.05 was regarded as statistically significant.

Results:

The scolicidal efficacy (mortality rate among protoscolices of sheep hydatid cyst) was evaluated for alcoholic extract of *S. aromaticum* at different concentrations (100, 50 and 1 mg/ml) and exposure times (Table 2).

In the current study, concentration of 100 mg/ml showed the greatest potential scolicidal effect on the vitality of the protoscolices, where complete inhibition (100%) of protoscolices viability was observed after 3 hours of exposure, followed by a concentration of 50 mg/ml. where 85% inhibition was observed after 3 hours of exposure, progressing to complete inhibition of protoscolices viability after 4 hours. The lowest scolicidal effect (70 %) of *S. aromaticum* alcoholic extract on viability of protoscolices was observed with 1 mg/ml after 3 hours of exposure, progressing to 100% inhibition after 5 hours of exposure.

Table 2: Effect of alcoholic extract of *S. aromaticum* at different concentrations and different exposure times on the protoscolices of sheep hydatid cyst *in vitro***

Time (min.)	Concentration mg/ml		
	1 mg/ml	50 mg/ml	100 mg/ml
0.0	96%	96%	96%
30	84%	80%	79%
60	63%	59%	29%
120	48%	38%	10%
180	30%	15%	0.0 %
240	14%	0.0	
300	0.0		

*Least significant difference; **P value for one-way ANOVA was <0.001

In this study, there was association between scolicidal efficacy and both concentration and exposure time as seen in tables 2 and 3.

The scolicidal effects of *Syzygium aromaticum* alcoholic extract was dose-dependent and time-dependent. In table 2, the scolicidal efficacy of *S. aromaticum* alcoholic extract became higher after 1, 2. and 3 hours than after 30 min of exposure, and this difference was highly significant ($P < 0.05$). Also, the mean efficacy was proportionally increased with the extract concentrations that were applied, given that as the concentration increased, the scolicidal efficacy also increased significantly ($P < 0.05$). Therefore, we can conclude that the results of this study showed significant differences at the probability level of $p \leq 0.05$ for all concentrations and in all exposure times.

Table 3: Comparison of the effect of different concentrations of *S. aromaticum* alcoholic extract on viability of protoscoleces at different exposure times *in vitro*.**

Concentration	Time (hours)			Mean of concentration
	1	2	3	
1 mg/ml	63.33	48.33	29.67	47.11
50 mg/ml	59.33	37.67	15.33	37.44
100 mg/ml	29.33	10.00	1.67	13.67
Means of time	50.67	32.00	15.56	
LSD*	Concentrations=3.246 Time= 3.246 Concentrations× Time= 5.622			
CV%	10.0			

*Least significant difference; **P value for one-way ANOVA was <0.001

In the negative control group, the protoscolices remained viable for 10 days after being incubated in sterile preservative solution at 37˚C. Minimal differences in viability between the first and 6th day was recorded

(where 40.0% of the incubated protoscolices died), while starting from the 7th day, there was significant decrease in the viability of protoscolices till total death of all protoscolices that occurred at the end of the 10th day (Fig.1). Also, in the positive control group treated with 800 µg/ml albendazole, significant effect on the viability of protoscolices was exhibited at the third day of treatment, where 60% of the treated protoscolices died and the complete scolicidal effect was occurred at the sixth day of treatment (Fig.1).

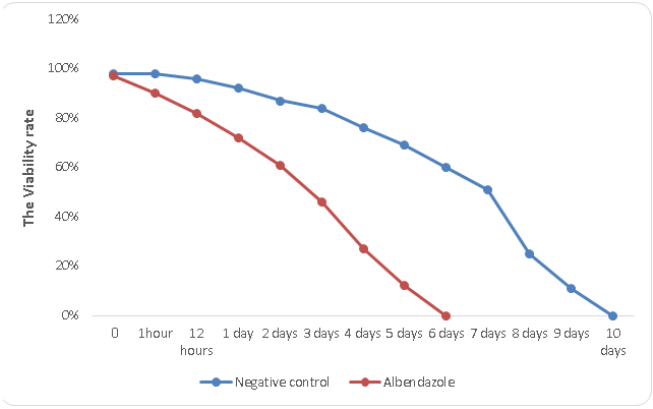

Fig. 1: Comparison between protoscolices viability rate in negative and positive control group.

The effects of 1 mg/ml alcoholic clove extract on the viability of protoscolices was compared with positive and negative control groups as seen in table 4. The results show a clear effect of 1 mg/ml alcoholic extract on killing protoscolices after 30 min and 60 min of exposure compared to the positive and negative control groups. At the concentration of 1 mg/ml, clove alcohol extract showed a greater reduction in the vitality of the protoscolices with a mean viability of 73.50%, albendazole (92.17%), and negative control group with a mean viability of 97.17%. The results showed significant difference in terms of protoscolicidal effects between the clove alcohol extract (1 mg/mL) at 30 min and 60 min of exposure when compared with control groups.

Table 4: The effects of 1 mg/ml alcoholic clove extract on the viability of protoscolices in comparison with positive and negative control groups**

Groups of Agents	Time		Mean viability
	30 min.	60 min.	
A clove (Syzgium aromaticum)	84.33	62.67	73.50
Negative control	97.67	96.67	97.17
Albendazole	94.33	90.00	92.17
Mean of time	92.11	83.11	
*LSD 5%	Extraction = 2.905, Time = 1.677 Extraction × Time = 4.109		
CV %	3.0		

*Least significant difference; **P value for one-way ANOVA was <0.001

Discussion:

There is a need for new therapeutic agents against hydatid cysts with high effectiveness and low toxicity. Thus, in recent times, the study of plants used by traditional medicine is a mean of alternative treatment, and several anti-parasitic properties of natural products have been identified [7]. Therefore, the present study was carried out to investigate the *in vitro* effects of *Syzygium aromaticum* extract of different concentrations on viability of protoscolices of sheep hydatid cyst *in vitro*.

The clove (Syzygium aromaticum L.), a member of the Myrtaceae family, is an aromatic tree, currently grown in many tropical areas. It is an evergreen tree, 10–20 m tall, with spear-shaped leaves and cluster-like, yellowish flowers. Dried flower buds are commonly used in cooking, pharmacy, perfumery and cosmetics. The main ingredient (up to 20%) is essential oil, characterised by the presence of eugenol, eugenol acetate and a- and b-caryophyllene. Eugenol is the main bioactive compound.

The clove is an important medicinal plant and has a broad spectrum of pharmacological effects such as antimicrobial, antiviral, antioxidant, and antinociceptive, and has been used in traditional applications for centuries [15-19].

In the current study, the results showed that the 100 mg/ml concentration of the *Syzygium aromaticum* alcoholic extract exhibited more potent and faster effect on protoscoleces viability of sheep hydatid cyst compared to other concentrations (1 mg/ml & 50 mg/ml). Where the complete inhibition (100%) of protoscolices viability in vitro treated with100 µg/ml alcoholic extract of *Syzygium aromaticum* was after 3 hours of exposure. Also, 85% inhibition with concentration of 50 µg/ml after 3 hours, progressing to complete inhibition of protoscolices viability after 4 hours. With concentration of 1 µg/ml, 70 % inhibition was observed after 3 hours of exposure, progressing to 100% inhibition after 5 hours of exposure to *S. aromaticum* alcoholic extract. In this study, significant correlations between efficacy and concentration and between efficacy and exposure time were found as seen in tables 1 and 2. In addition, all treatments of protoscolices with the three different concentrations of *Syzygium aromaticum* alcoholic extract revealed dose-dependent and time-dependent scolicidal effects on the protoscolices of sheep hydatid cyst.

The results of this study in accordance, with Ueda-Nakamura *et al.*, [20] who observed 100% inhibition of *Leishmania amazonensis* with the concentration of 100 µg/ml. In a study conducted by Hayam M Ezz Eldin [21], a significant inhibition of 95.1% was observed with concentration of 100 mg/ml after 24 h, which also progressed to complete inhibition (100%) after 72hours of treatment. In addition, the anthelminthic activity of the

eugenol of *Syzygium aromaticum* against the fish parasite *Gyrodactylus* sp. was evaluated. After one hour exposure to 5 and 10 mg/L eugenol, mortality of the parasites was near 80 and 90%, respectively, and differed significantly from 0 h [22]. Furthermore, a significant decrease in proliferation of *Giardia lamblia* was reported, after treatment with *S. aromaticum* at a concentration upper than 200 µg/ml [23]. Moreover, it was reported that *S. aromaticum* cause apoptosis in *L. donovani* promastigotes [24]. likewise, in sheep supplemented with ground cloves, the number of strongyloid eggs per gram of faeces decreased by 40.6% on average. This decrease in the number of excreted eggs was attributed to bioactive substances in cloves such as alkaloids, glycosides, essential oils, tannins, etc., in particular Eugenol, that constitutes 89% of the plant essential oil [25, 26], which is thought to act on the cuticle of the parasite, and tannins, which are known to have anthelminthic properties [27, 28].

However, several researchers documented the evidence of anthelminthic properties of cloves *in vitro* and *in vivo*. They indicate a similar mode of action of the clove on helminths as with some anthelminthic drugs [21, 29–32]. As, the antihelminthic effects of clove extracts could be attributed to their strong corrosive effect on the cuticle and tegument of helminths, inhibition of energy metabolism of parasites by inhibiting fumarate reductase and succinate dehydrogenase activity [31]. Moreover, clove ethanol extract affects on acetylcholinesterase activity and motility in the paramphistome Cotylophoron cotylophorum [32].

On other hand, clove essential oil is generally recognised as a safe substance when consumed in quantities lower than 1500 mg/kg [33], and the World Health Organization (WHO) established that the daily quantity acceptable of *S. aromaticum* essential oil per day in humans is about 2.5 mg/kg of weight [34].

Conclusion:

The results of the study highlighted that *S. aromaticum* extracts in particular 100 mg /ml has a potent lethal effect and safe agent for treatment of hydatid cysts. Further studies on the *Syzygium aromaticum* and its effect on the protoscolices of hydatid cyst *in vivo* are suggested to be done.

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مقالة بحثية

تأثير المستخلص الكحولي لنبات القرنفل (Syzygium aromaticum) على حيوية الرؤيسات الاولية للمشوكة الحبيبية Echinococcus granulosus خارج الجسم الحي

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المُلخّص

يعتبر نبات القرنفل (Syzygium aromaticum) واحد من التوابل القيمة والذي تم أستخدامه منذ قرون كمادة لحفظ المواد الأغذية وللعديد من الأغراض الطبية كمضاد للتشنج، طارد للغازات، مقاوم للتأكسد، ومضاد للميكروبات. هدفت هذه الدراسة لمعرفة تأثير المستخلص الكحولي لنبات القرنفل على الرؤيسات الاولية للمشوكة الحبيبية Echinococcus granulosus في الزجاج in vitro أذ استخدم المستخلص الكحولي لنبات القرنفل وبالتراكيز 1 و 50 و 100 ملغم/مليلتر. اظهرت النتائج أنخفاظا معنويا في نسبة حيوية الرؤيسات الى 0% عند الزمن 180 دقيقة عند التركيز 100ملغم/مليلتر، بينما أنخفضت نسبة الحيوية الى 85% عند استخدام التركيز 50 ملغم/مليلتر عند الزمن 3 ساعات و 0% عند الزمن 4 ساعات. بينما انخفضت نسبة الحيوية عند استخدام التركيز 1,0 ملغم/مليلتر الى 70% و100% عند الزمن 3 ساعات و 5 ساعات على التوالي. اظهرت نتائج الدراسة وجود علاقة ذات دلالة معنوية بين فعالية المستخلصات وكلا من التراكيز وفترة التعرض.

الكلمات المفتاحية: المشوكة الحبيبية، الرؤيسات الاولية، القرنفل، المستخلص، داء الاكياس العدرية.

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