

Antiprotoscolices effect of methanolic extract of *Zingiber officinale*, *Artemisia aucheri* and *Eucalyptus globulus* against *Echinococcus granulosus* in vitro

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ABSTRACT

First choice in the treatment of hydatid disease is still surgery. Leakage the live protoscolices during surgery and the risk of secondary cysts have been a permanent fair for surgeons. Various methods and materials have been used to kill protoscolices as well as drug therapy that most of them had serious side effects. The main objective of this study was to evaluate the in vitro scolicidal effect of methanolic extract of eucalyptus, artemisia and ginger. Protoscolices were collected aseptically from the hydatid cysts of the sheep; washed three times in sterile PBS and stored at 4 °C until use. Live protoscolices were exposed for 5, 10, 25, 40 and 60 min for three concentrations of the each extract (25, 50, 100 mg/ml). The viability of the protoscolices were confirmed by 0.1% eosin staining method. The methanolic extract of ginger at concentration of 100 mg/ml, and eucalyptus at concentrations of 50, 100 mg/ml killed 100% of protoscolices after 40 min respectively. Scolicidal effect of all concentrations of the methanolic extract of ginger and eucalyptus was significant comparing to the control groups at all exposure times ($P < 0.001$). No significant relationship was observed between the artemisia extract at different concentrations and exposure times comparing to the control groups ($p = 0.99$). The results of this study showed that the eucalyptus and ginger extract have high scolicidal activity and can be used as natural scolicidal agents.

Keywords: *Protoscolex, Hydatid cyst, Ginger, Eucalyptus, Artemisia, In vitro*

Introduction

Hydatidosis is a common zoonotic disease caused by the larval stages of *Echinococcus granulosus* [1]. In the life cycle of this parasite, domestic and wild carnivores, mainly dogs are the definitive hosts, and herbivores and occasionally humans are the intermediate hosts of this parasite [2]. While infection of carnivores with adult stage of *E. granulosus* cannot cause important disease unless in heavy infection, the establishment of larvae (cyst) in various organs of intermediate host (especially liver, lungs, and sometimes the brain, heart and spinal cord), can cause severe disease and it can be even fatal [3]. This disease is more common in rural areas where there is a close relationship between dog and livestock and human [4]. This disease, according to the significant

mortality rate caused great economic losses, can be considered as a public and the socio-economic health concern in both the public health and livestock industry [2]. Surgery is still the main method of the treatment of cystic echinococcosis [5]. Although at surgical treatment, if surgeons ignore the necessary measures, will be a risk of leakage of protoscolices and it will seeded in the internal organs and tissues during surgery that is one of the main causes of recurrence and the formation of secondary hydatid cysts [6]. To avoid the leakage of cyst contents, it is necessary to use the effective scolicidal agents in order to reduce the secondary infection rate [7]. Since anti-parasitic properties of some of herbal extracts have been well proven as well as the chemical scolicidals that have toxic and side effects; therefore,

this study conducted to evaluate the efficacy of in vitro protoscolicidal effect of artemisia, ginger and eucalyptus extracts on the protoscolices of hydatid cyst at different concentrations and exposure times.

MATERIALS AND METHODS

In this experimental study, hydatid cyst infected livers of sheep obtained from the Hamadan slaughterhouse carried to the research laboratory of Parasitology & Mycology, Faculty of Medicine, Hamadan University of Medical Sciences. In aseptic conditions, all the contents of the cyst were aspirated by a disposable syringe and transferred into the falcon tubes and left in stationary state for 30 min, then, supernatants were discarded. The cysts fluid which have the highest rate of viable protoscolices was used for experiment. After washing with sterile normal saline during three times, the percentage of early live protoscolices was determined by observing their flame cells movement (vibrations of a flame cell and contraction the body) and also using vital staining of eosin 0.1% and observing with a light microscope. The live protoscolices did not take the dye and stained protoscolices were considered dead. Finally, the live protoscolices were transferred into dark container at 4 °c temperature until use.

Plant materials, ginger rhizomes, eucalyptus leaves, and artemisia fruits, were purchased from the herbal market in Hamadan province, Iran, and was identified and authenticated at College of Pharmacy, Hamadan University of Medical Sciences. After washing with tap water and drying, each plant material was grinded to yield a fine powder. The powdered material of each plant (200 g) was extracted by maceration in methanol (3* 1 L) at room temperature for three times, each time for three days. Then, each methanolic extraction was evaporated under reduced pressure until completely dried. The dried extractions were kept in refrigerator until use. In this study, three concentrations (25, 50 and 100 mg/ml) and five different exposure times (5, 10, 25, 40 and 60 min) were examined for each plant extract. In order to prepare the methanolic extract, amount 200 gr of the eucalyptus leaves, artemisia fruits and ginger root each from with pure methanol were mixed at a ratio 3:1 slowly for one hour with shaker utterly blend. Obtained mixture was placed at room temperature for 24 hours. The obtained solution was smooth and it moved to Rotary devices for removing the solvent, then were placed in containers cristalizor at room temperature until completely dried and the materials were kept at refrigerator temperature. The scolical tests were carried out based on Moazeni and Nazer [8]. For preparation of extract, the concentrations 0.25, 0.5 and 1 g of dried extract were dissolved in 10 mL of normal saline, respectively. Then, 2 ml of each the concentration of extract was placed in a test tube and added a drop of solution suspension of parasite contains protoscolices (at least 1000 larvae) and mixed gently. The tube was incubated at 37°C and was examined at 5, 10, 25, 40 and 60 min. After finishing the exposure time for each tube, upper

part of the solution was removed carefully by pipette and settled protoscolices two milliliters of 0.1% eosin stain was added and mixed gently. Upper part of the solution was discarded after 15 minutes. Then, to determine the protoscolices viability, they were examined under a light microscope with *10 objective and percent of dead larvae was determined. Also, in order to evaluate the possible factors affecting on the live protoscolices including the passage time, at the same times protoscolices were placed with the proximity of normal saline in the another test tube and the results were recorded. The experiments was repeated three times (triplicate). Analysis of the data was performed by SPSS software, using the chi-square for comparing between groups ($P < 0.05$).

RESULTS

The mortality rate of protoscolices at concentrations and various exposure times of methanolic extract of these plants have been shown in Tables 1-3.

According to the results of this study, the mortality rates of exposed protoscolices by ginger extract at concentrations of 25, 50 and 100 mg/ml was 80.61%, 85.21%, 100% after 60 min exposure respectively, while the death protoscolices in the control group was 13%. Scolicidal effect of methanol extract of eucalyptus in concentrations of 25, 50 and 100 mg/ml after 40 minutes of exposure, was 84.78%, 100% and 100% respectively, while mortality rate was 15% in the control group. Artemisia extract had very low killing activity on the live protoscolices. So that after 60 min exposure at concentrations of 25, 50 and 100 mg/ml killed 5.19%, 11.29% and 17.33% of protoscolices, while mortality rate in the control group was 4%.

DISCUSSION

The present study showed that methanolic extract of ginger and eucalyptus had high scolical activity, while the methanolic extract of artemisia had low effect on the protoscolices of hydatid cysts. Until recent years, surgery was the treatment of choice for cystic echinococcosis. Different scolical materials such as formalin, hydrogen peroxide, cetrimide, ethanol, hypertonic saline and silver nitrate have used as scolical in hydatid cyst surgery, that most of these agents have serious side effects [9]. *Z. officinale* contains 1-2% of volatile oil and 5-8% of resinous matter, starch and mucilage. The volatile oil containing monoterpenes, sesquiterpenes and sesquiterpene alcoholzingiberol, gingerol and shagoals. These fractions are the most pharmacological active constituents in the volatile oils [10]. In this study, the obtained results of protoscolicidal effect of ginger extract showed that the methanolic extract of ginger with concentration of 100 mg/ml, killed all protoscolices in 40 min of exposure that was in accordance with Moazeni et al (2011) [8], which reported that at 50 mg/ml concentration after 40 min 100% of protoscolices were destroyed. Another study showed also, the killing effect of this herbal extract on the microorganisms.

Table 1: Scolicial effect of ginger extract at concentration and various exposure times

Con (mg/ml)	Exposure time(min)														
	5			10			25			40			60		
	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)
25	426.51±74.77	54.69±14.33	12.37	543.17±36.18	128.37±9.39	23.60	660.18±87.76	273.73±38.43	41.36	699.94±36.96	423.44±17.91	60.62	653.05±56.70	529.32±57.44	80.61
50	479.98±40.36	119.42±6.97	25.25	557.12±46.95	205.22±17.4	36.83	573.58±101.60	289±15±53.82	50.10	520.54±28.83	314.19±7.85	60.70	660.31±35.84	561.93±26.16	85.21
100	422.88±84.00	189.62±31.20	45.58	534.79±179.79	374.13±136 ±52	66.83	529.39±116.46	464.31±95.07	89.79	466.52±101.54	466.52±101.54	100	-	-	-
Control (n)	500	20	4	500	36	7.2	500	50	10	500	59	11.8	500	65	13
P<0.001															

Con: concentration, PSC: protoscolices, SD: Standard deviation

Table 2: Scolicial effect of eucalyptus extract at concentration and various exposure times

CON (MG/ML)	EXPOSURE TIME(MIN)														
	5			10			25			40			60		
	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)
25	502.10±71.44	75.22±15.41	14.32	574.14±97.37	173.13±31.52	29.97	434.86±74.36	217.98±32.75	50.74	652.71±59.94	503.28±69.26	76	590.54±57.89	501.48±51.60	84.778
50	545.92±70.74	183.59±18.09	33.98	596.99±14.02	304.17±12.52	50.96	535.90±73.19	373.00±51.06	69.51	411.81±81.17	411.81±81.17	100	-	-	-
100	472.42±77.57	185.11±36.68	37.92	529.10±31.07	307.19±7.80	58.33	509.19±80.49	455.36±70.02	89.70	426.74±78.15	426.74±78.15	100	-	-	-
CONTROL(N)	300	20	6.66	500	50	10	500	65	13	400	60	15	500	75	15
P<0.001															

Con: concentration, PSC: protoscolices, SD: Standard deviation

Table 3. Scolicial effect of artemisia extract at concentration and various exposure times

Con (mg/ml)	Exposure time(min)														
	5			10			25			40			60		
	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)	PSC (Mean±SD)	Dead PSC (Mean±SD)	Mortality (%)
25	365.36±48.86	12.56±1.64	3.43	367.77±42.22	13.55±1.64	3.67	371.35±2.63	15.68±0.47	4.21	351.42±48.44	16.29±2.24	4.63	368.81±31.39	19.10±1.38	5.19
50	341.31±47.77	21.38±2.95	6.26	380.71±49.58	27.27±3.79	7.13	347.39±53.32	31.08±4.98	8.92	372.13±45.06	35.32±4.55	9.46	321.45±21.99	35.84±2.53	11.14
100	370.78±43.50	41.98±4.92	11.32	410.62±36.89	52.135±4.96	12.67	454.16±62.15	65.38±8.93	14.40	516.29±111.47	84.05±16.97	16.45	435.22±96.64	76.34±17.32	17.42
Control(n)	300	9	3	300	9	3	306	11	3.59	300	12	4	300	12	4
P=0.99															

Con: concentration, PSC: protoscolices, SD: Standard deviation

In Noor Nihad study, ethanolic extract of ginger at 50, 100 and 150 mg/ml after 120, 90 and 60 min respectively; 100% of protoscolices killed [11]. Merawin et al. in 2010 showed strong microfilaricidal activity of aqueous extract of ginger against *Dirofilaria immitis* in vitro [12]. Other studies showed antidiabetic [13] and antioxidant effect of aqueous extract of ginger [14]. Eucalyptus belongs to the family Myrtaceae and the leaf extract has anti-cancer effects, anti-inflammatory, analgesic, anti-oxidant, anti-malarial, anti-fungal and antiviral effects. Eucalyptus globulus, the most represented species of eucalyptus genus in the international pharmacopeia, has many effects such as antiseptic, astringent, disinfectant, deodorant, expectorant, febrifuge, inhalant, and insect repellent, vermifuge. The eucalyptus leaf extract has eucalyptol (1,8-cineol), citronellol, citronellal, citronellyl acetate, p-cymene, eucamalol, limonene, linalool, β -pinene, γ -terpinene, α -terpinol, alloocimene, aromadendrene. Eucalyptol as the main component of eucalyptus leaf has antiparasitic and antifungal effects. In this research, eucalyptus showed profound protoscolocidal effects, which can be due to the presence of eucalyptol. Eucalyptus in concentrations of 50 and 100 mg/ml after 40 min of exposure killed all protoscolices. The results of Safarnejad Tameshkel showed that, the fatality effect of methanolic extract of eucalyptus on *Giardia lamblia* cyst after 60 min exposure with concentration of 200 mg/ml was 63.3% [15]. In Abdollahzade study, the result of in vivo and in vitro evaluation showed that acetonetic and ethanolic extract of eucalyptus comparing to aqueous extract has the most effective antimicrobial activity on *Brucella* and they can be useful in treatment of human and animal Brucellosis [16]. The genus *artemisia* that is belonging to *Astraceae* family [17], anti-tumoral activity in some species of this family has been attributed to the presence of flavonoids, sesquiterpene lactones, lignans, acetylenes, triterpenes or glycolipids [18]. *Artemisia* spp was used to treatment of helminth infections and *Enterobius*, *Ascaris* in the first century [19]. Various species of the genus *Artemisia* have been used for treatment of malaria, hepatitis, hypertension, inflammation and infections caused by fungi, bacteria and viruses [20]. *Artemisia* extract at none of surveyed concentrations (25, 50 and 100 mg/ml) hadn't effect on protoscolices that might be due to the resistance of protoscolices against extract and also used concentrations and times, because two variables of concentration and time have the major role in protoscolices mortality. Based on Zhuliang study and et al. the *Artemisia lancea* extract at concentration of 10 mg, causing restrain of larvae growth about 99% against *Haemoncus contorus* [21]. The results of Barati et al. showed that *artemisia* extract in concentrations of 31.25, 62.5, 125, 250 and 500 mg/ml against promastigotes of *Leishmania major* is ineffective and only at concentration of 5000 mg/ml is in accordance with drug control [22]. No surveys about the effect of eucalyptus and *artemisia* extract on protoscolices of hydatid cysts are reported previously.

CONCLUSION

This study showed that the methanolic extract of ginger and eucalyptus may be considered as a natural and effective scolicidal agent. Therefore, we can recommend it as the complementary treatment and in surgery of cyst for patients with hydatid cyst after investigation on the animal models and clinical trials in human populations.

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