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RESEARCH ARTICLE

Antiprotoscolices effects of ethanolic extract of *Zingiber officinale* against *Echinococcus granulosus invitro* and *invivo*

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Abstract

Ginger (*Zingeber officinale*) is one of the world's best known spices, and it has also been universally used throughout history for its health benefits. The purpose of this study to evaluates the effect of ethanolic extract of *Z. officinale* (EZO) as antiprotoscolices *in vitro* and *in vivo*. Protoscolices of *Echinococcus granulosus* were collected from sheep livers containing hydatid cyst and were exposed to three different concentrations of EZO extract (150,100,50)mg /ml for 0, 30,60,90, 120 min . The rate of alive of protoscolices was 97% in control group . Protoscolices were exposed to ethanolic extract of *Z. officinale* at concentration of 50 mg /ml was 60%, 46.2% ,15.37% ,7.7% and 0% after 0, 30, 60 ,90 and 120 min respectively. Viability rate of this extract at concentration 100 mg/ml was 51.33%, 37.53%,20% and 0% after 0,30,60 and 90 min respectively. Zero percent viability rate was observed at concentration of 150 mg/mL after 60 min of exposure.

To determine the *in vivo* efficacy, mice were inoculated intraperitoneally with viable protoscolices and then treated with EZO in the next day. The result showed in treated group significant reduction in weight of liver and spleen . Histological sections of the liver from mice treated with EZO at concentration 50 mg /ml showed proliferation of kupffer cells as a defensive cell with few mononuclear cells in sinusoids and absence of hydatid cyst comparing with control group revealed Hydatid cyst wall and necrosis of hepatocytes. These results showed that EZO have protoscolicidal effects *in vitro* and *in vivo*.

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Introduction

Hydatid disease, also called hydatidosis or echinococcosis, is a cyst-forming disease resulting from an infection with the metacestode, or larval form, of parasitic dog tapeworms from the genus *Echinococcus* .The vast majority of human diseases are from *Echinococcus granulosus* and *Echinococcus multioccularis* which cause cystic echinococcosis and alveolar echinococcosis . Humans may become infected though the ingestion of food and water contaminated with infective eggs released in the feces of dogs harboring the adult tape worm. (Satoskar *et al.* ,2009). Currently the basic approaches for treatment of hydatid disease are surgery and chemotherapy. However, operative leakage may lead to dissemination of viable protoscolices to adjacent tissues and thus to intrapritoneal hydatid disease(Zhenguo *et al*.,2005).

Ginger (*Zingiber officinale*) is one of the world's best known spices, and it has also been universally used throughout history for its health benefits.The main constituents of ginger include volatile oil, phenolic derivatives

(zingerone) and oleoresin (gingerols and shogaols) are main antioxidant compound in ginger (Kikuzaki and Nakatani, 1993). Gingerol is of the major pungent compounds in ginger and can be altered to shogaols, zingerone, and paradol (Govindarajan and. Connell,1983) whichtakes part in several activities such as hepatoprotective (Ezeonu *et al*.,2011) antiparasitic (Forouzan *et al.*,2012) , antiflarial (Mostafa *et al*.,2011) ,antimicrobial (Karuppiah and Rajaram , 2012) .Echinococcosis is most commonly treated with albendazole. However, albendazole is poorly absorbed in the intestinal tract and thus its hepatic concentration is low, a fact that reduces its efficacy since the liver is one of the organs most affected by echinococcosis. Patients with echinococcosis taking albendazole may have serious adverse reactions such as encephalitis syndrome, influenza- like syndrome, allergic purpura, drug rash, etc., and the treatment is not cost effective. Moreover, Echinococcus granulosus protoscolices have developed resistance to albendazole (Urrea-Paris *et al*.,2000)

MATERIALS AND METHODS

Preparation of alcohol plant extract

A100 g based on dry weight powdered rhizomes of *Zingiber officinale* added to adequate amount Ethanol 70% (500 ml) and putting in electrical shaker for two hours and the products were squeezed through gauze cloth to remove the practice and . The suspension was filtered through a Whatman paper No.1 and the crude ethanol extracts were drying at 37˚C by oven .The powder extract was stored at 4˚C until use.

Protoscolex collection :

Echinococcus granulosus protoscolices were aseptically removed from liver hydatid cysts obtained from sheep at the municipal abattoir in Baghdad,Iraq. Cysts were washed several times in sterile Phosphate Buffer Saline, pH 7.2. Cyst surfaces were sterilized by 70% alcohol and their fertility was determined by the presence of free protoscolices in cystic fluid by microscopic examination of a wet mount drop. Hydatid fluid with protoscolices was collected as previously described by (Smyth ,1967)

Hydatid fluid containing protoscolices was evacuated completely into tubes and it was left to precipitate for an hour to obtain hydatid sand at room temperature. Protoscolices were maintained in a sterile preservative solution made of a mixture of Krebs-Ringer solution and hydatid cyst fluid (4:1) (Health *et al*.,1975).

The protoscolices were washed 3 times in PBS (pH 7.2) by centrifugation for 15 min with speed 3000 rcf .The concentration of protoscolices was confirmed as the number of protoscolices per ml of the hydatid fluid in PBS containing 3×10^3 protoscolices in 1 ml with more than 90% viability was used in further use.

The viability of protoscolices was determined prior to the experiments by staining 0.1% aqueous eosin stain, as a vital staining,and was checked microscopically after adding 10 µl of eosin solution to 10 µl of protoscolices for 5 min. Unstained protoscolices were considered as viable while stained protoscolices were considered as non-viable (Smyth and Barrett,1980) .Non-treated protoscolices (with plant extracts) were considered as the control group. The percentage of viable protoscolices (viability rate) was determined by counting a minimum of 100 protoscolices (as a ratio of number of viable protoscolices to total protoscolices).

***In vitro* study:**

Stock solutions of alcoholic extracts of *Z. officinale* were prepared by dissolving 1.5 g of the dried extract in 10 ml distilled water (150 mg/ml) concentrations of 150, 100, and 50 mg/ml of stock solutions for were used.

The *in vitro* study was carried out in sets of 12 tubes. A 1.0 ml solution of viable protoscolices (approximately 3000 viable protoscolices) was placed in each tube, and 1.0 ml of plant extract for each concentration was added to each tube, mixed gently and incubated at 28 C. The control group were contain solution of protosclices without extract plant . Percentages of viable protoscolices or survival were determined for each experiment after 5 min and in serial period of time (every 30 min) until all of the protoscolices were dead. The data were derived from the mean value of 3 replications.

***In vivo* study:**

This study males' Swiss albino mice weighing 30 g with age 6-8 weeks were divided into three groups injected two groups into intra peritoneal (approximately 3000 viable protoscolices) The next day were injected one group with alcoholic extracts of *Zingiber officinale* concentrations50 mg / ml, while positive control not injected with extract either negative control injected with saline solution only, the amount of dose used for all groups 0.2 ml. The eliminate the liver and spleen from each mouse and weighted. An organ hypertrophy indicator for these organs was calculated as follow :

$$\text{Organ Hypertrophy Indicator} = \frac{\text{Organ weight}}{\text{Animal weight- Hydatid cyst weight}} \times 1000$$ (Kroes and Tanner ,1987)

Histological procedure :

Animals were killed and small piece of liver and spleen tissues were taken and were fixed in 10% neutral formalin, alcohol-dehydrated, paraffin-embedded and the section to mean thickness of 4 µm. The histological examination was evaluated by assessing the morphological changes with hematoxylin and Eosin (H&E) stains (Bancroft and Stevens,1982).

Statistical Analysis

The Statistical Analysis System- SAS (2010) was used to effect of difference factors in study parameters. The Chi-square-χ^2 test at the comparative between percentages in this study

Results:

Effect of *Zingiber officinale* extract in protoscolieces viability *in vitro.*

The results showed a significant effect in P<0.05 table (1) when treated protoscolices of *E. granulosus* with alcoholic extract of *Z. officinale*. The protoscolices treated with concentrations of 150 ,100, 50 mg/ml started to show a decrease in viability at Zero time to 45.53% , 51.33% ,60 % respectively . All treated protoscolices with 150,100 ,50 mg \ ml perished completely at 60, 90,120 min respectively.

Table1. Effect of *Zingiber officinale* extract in protoscolieces viability *in vitro*

Concentration	Rate of alive protoscolieces (No. alive/No. tested)				
	0 min.	30 min.	60 min.	90 min.	120min
150mg/ ml	45.53±1.63	23.67± 1.59	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
100mg/ml	51.33±1.25	37.53± 1.84	20.00± 0.85	0.0 ± 0.0	0.0 ± 0.0
50mg/ml	60.0±1.15	46.2± 1.36	15.37± 1.05	7.70± 0.73	0.0 ± 0.0
LSD Value	6.723*	7.452*	7.643*	6.53*	0.00NS

Positive control = 97%

* (P<0.05)

Effect of *Zingiber officinale* extract *in vivo* and Histological Changes

Dissecting of mice in positive control group revealed the presence of secondary hydatid cysts in liver, peritoneal membrane, diaphragm, and spleen. In the liver (which was especially infected) , cysts either partially embedded in the parenchyma or attached to the surface (Figure 1) . Protoscolices viability test from these cysts revealed 100% viability . Cysts in treated mice with 50mg/ml concentration were not found in the visceral organs especially the liver ,as well as Dissecting of mice in negative control group showed no hydatid cysts (Figure 2). Lower liver hypertrophy indicator in treated mice group (49) and negative control group (41) than positive group(90.0). As well as lower spleen hypertrophy indicator in treated mice (6.1) and negative control group (4.0) than positive group (15.2) This results was highly significant in P<0.05(Table 2) .

Histological section of liver from infected control mice showed Hydatid cyst wall and necrosis of hepatocytes (Figure 3) , and showed proliferation of kapffer cell and congestion of central vein blood ,infiltration of inflammation cells with portal area. Some hepatocytes degeneration with fatty vacuoles (Figure 4). Sections of spleen from these mice revealed fibrosis ,amyloidosis and distended white pulp (Figure 5).

Figure (6) showed section of liver from non – infected mouse group with normal size of hepatocytes , sinusoids and kupffer cell. While figure (7) showed section of spleen from non – infected mouse group normal size of white pulp compared with red pulp .

Histological sections of the liver from treated mice with extract of *Z. officinale* at concentration 50 mg /ml shows proliferation of kupffer cells with few mononuclear cells in sinusoids (Figure 8).

Section of spleen show shows proliferation of lymphocytes in the periarteriolar sheath (Figure 9).

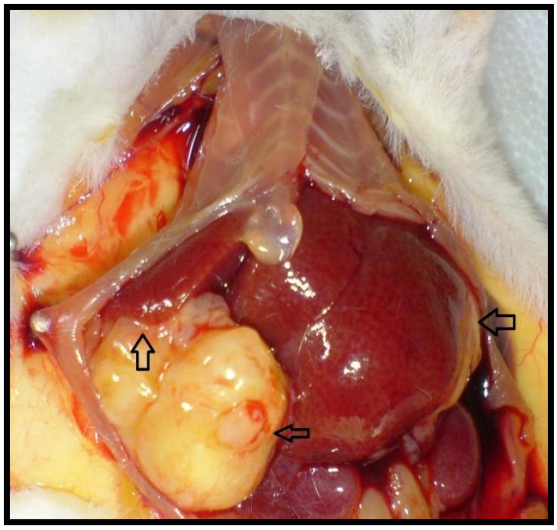
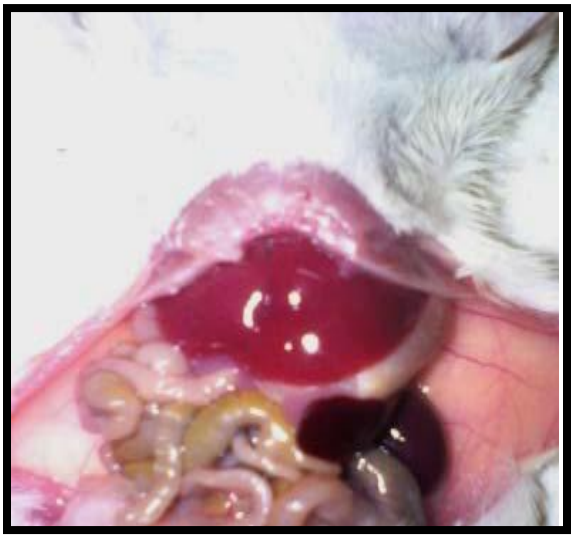


Figure (1) Secondary hydatid cysts in Intern al organs of mice in control groups.



Figure(2) Mice non- infected group with Hydatid cysts

Table 2. Effect of *Zingiber officinale* extract in liver & spleen

Groups	Average liver wt(g)	Liver hypertrophy indicator	Average spleen wt(g)	Spleen hypertrophy indicator
Infected control	2.5± 0.02	90.0 ± 2.54	0.40 ± 1.18	15.2 ± 0.81
Non-infected control	0.98 ± 0.00	41 ± 0.97	0.11± 0.02	4.0 ± 0.30
Treated with 50mg	1.2 ± 0.01	49 ± 1.0	0.15 ± 0.1	6.1 ± 0.63
LSD Value	0.745 *	14.535 *	1.274 *	4.133 *

*** (P<0.05)**

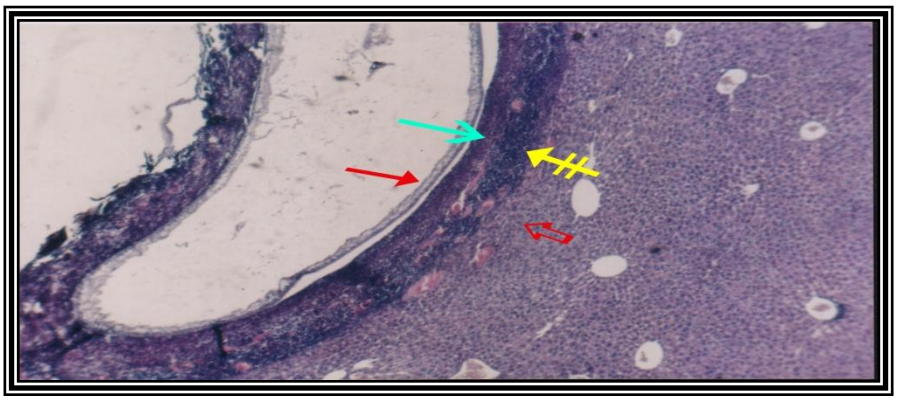


Figure (3) Histological section of liver from mouse control group showing Hydatid cyst wall germinated layer ( ) , laminated layer (←) , connective layer ( ) and degeneration of hepatocytes(⇦) (H&E 10X)

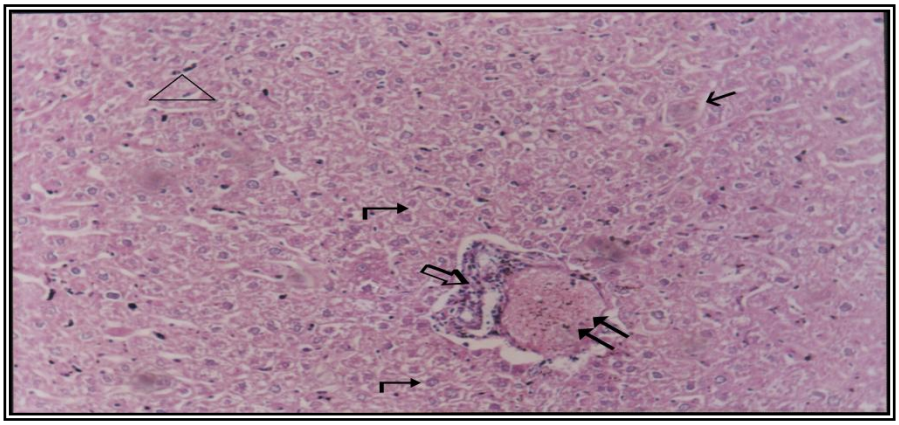
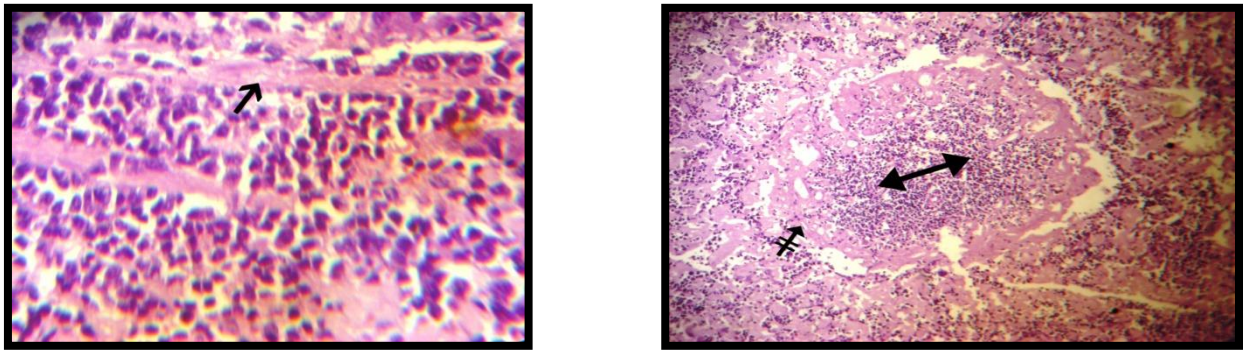


Figure (4) Histological section of liver from mouse control group showing proliferation of kapffer cell (△) , congestion of central vein blood (⇇),infiltration of inflammation cells with portal area(⇨),degeneration of hepatocytes with fatty vacuoles(↱) (H&E 10X)



B **A**

Figure (5) Histological section of spleen from mouse control group showing:
(A)- Amyloidosis(↨)and distended white pulp(↔ .) (H&E 10X).
(B)- Fibrosis(➔) (H&E 40X) .

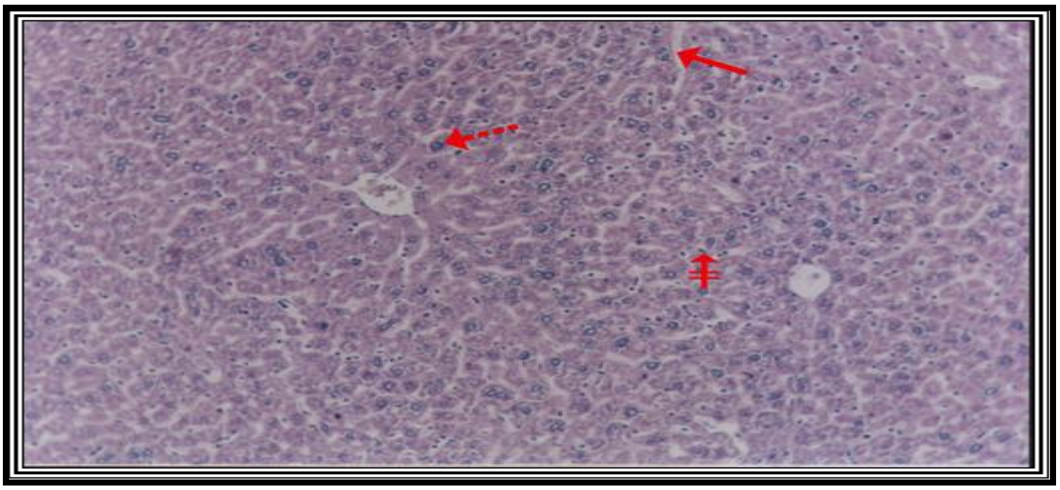


Figure (6) Histological section of liver from non – infected mouse group showing with normal of hepatocytes (↼) , sinusoids (↙) and kupffer cell (↨) (H&E 10X)

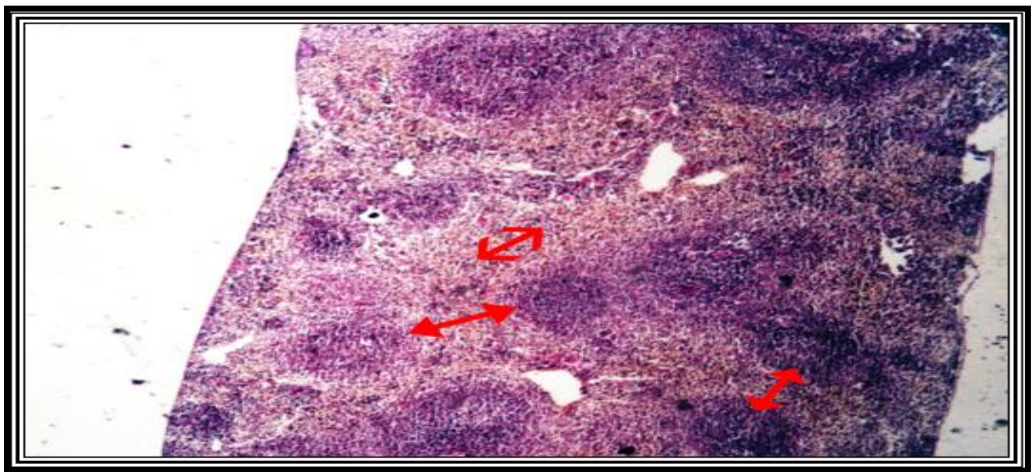


Figure (7) Histological section of spleen from non – infected mouse group showing normal size of white pulp (↔) compared with red pulp(⇔) (H&E 10X)

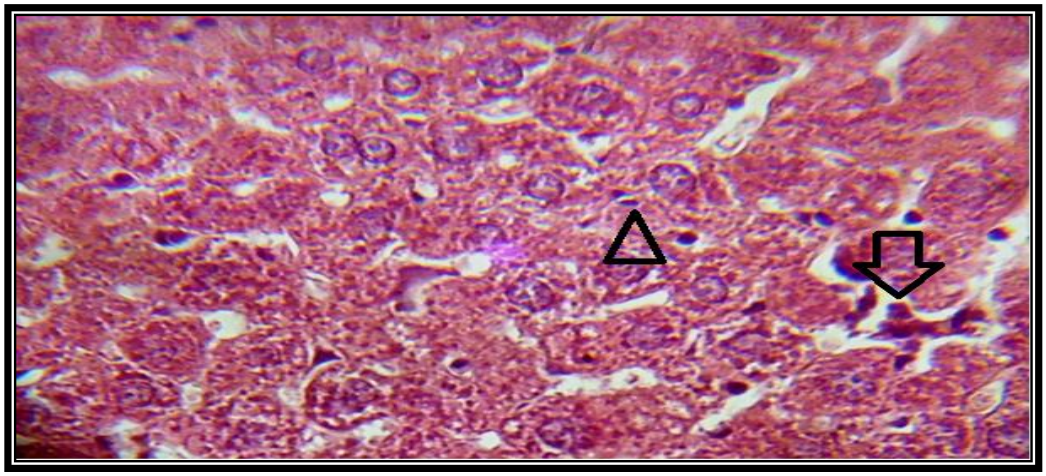


Fig (8) Histological section of liver from mouse treated with 50mg/ml showing proliferation of kupffer cells (△) with few mononuclear cells in sinusoids(⇨) (H&E stain 40X)

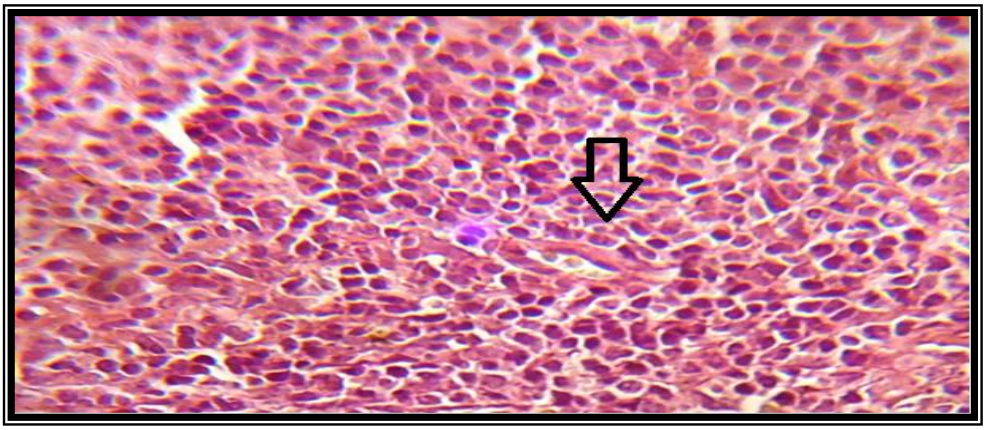


Fig (9) Histological section of spleen from mouse treated with 50mg/ml showing proliferation of lymphocytes in the periarteriolar sheath(⇨) (H&E stain 40X**)**

Discussion :

Effect of *Zingiber officinale* extract in protoscolieces viability *in vitro*.

Although surgery is the most effective choice of treatment, a number of drugs are being used and various degrees of success have been claimed. However, the metabolites ofcertain drugs including benzimidazole, mebendazole, albendazole, and albendazole sulfoxide are potentially toxic in some subjects (Davis *et al.*, 1989; Whittaker and Faustman ,1991; Pawlowski, 1997).This results came in harmony with previous reports were using medicinal plants to kills protoscolices of *E.granulosus* , such as: Zibaei *et al.* (2012) Investigated the *in vitro* scolicidal effect of aqueous extract of olive leaf (*Olea europaea)* tested, 0.1% and 0.01% concentrations had strong scolicidal effects in 120 min. and *S. khuzestanica* 0.1% had very strong scolicidal effects in 30, 60, and 120 min of exposure times and the mortality rate decreased with the lower concentration.

Moazeni and Roozitalab (2011) investigated the *in vitro* scolicidal effect of methanolic extract of *Zataria multiflora*. *Z. multiflora* extract at a concentration of 10 mg/mL killed 68.9%, 93.7%, and 100% of protoccoleces after 1, 2, and 3 min respectively. They reported 100% scolicidal effect of this extract at a concentration of 25 mg/mL after 1 min of application.

Showed Gholami *et al.*(2013) the efficiency of that methanolic extract of *S. ebulus* fruit *in vitro* at the concentration of 50 and 100 mg / ml killed 88.3% , 98.6% of the protosclices respectively after 60 min.

Barzinji *et al*. (2009) showed Concentrations of 5000 and 1000 µg/mL, for *D. socotrana* and *J. unicostata*, respectively, exhibited the highest protoscolicidal activity, significantly reducing and/or stopping protoscolex

Z. officinale contains about 1-2% of volatile oil and 5-8% of resinous matter, starch and mucilage. The volatile oil contains monoterpenes, sesquiterpenes and sesquiterpene alcohol zingiberol, gingerol and shagoals. Most of the pharmacologically active constituents reside in the volatile oils(Lakshmi and Sudhakar,2010)

In this study we investigated the effectiveness of alcoholic extract of *Z. officinale* on the protoscolices of hydatid cyst compared to Formalin, hydrogen peroxide, cetrimide, pure alcohol, hypertonic saline and silver nitrate have been used as effective scolicidal agents, but may cause unacceptable side effects, limiting their use. (Kadir *et al*.,2004)

Effect of *Zingiber officinale* extract *in vivo* and Histological Changes

New effective alternative treatment is extremely important in today's climate, where species are becoming resistant and there is resurgence in the use of natural alternative therapies, instead of synthetic pharmaceuticals that often have severe side effects (Harris *et al*.,2000).

Barzinji *et al*., 2009 Oral and intraperitoneal administration of the extracts in white mice invoked noticeable inhibitory effects on the *in vivo* development of secondary hydatid cysts.

Ma *et al*. (2007) showed that the combined treatment with alkaloids of *Sophora moorcroftiana* + albendazole against echinococcosis in mice had a marked effect as indicated by reduced hydatid cyst weight and by structural cyst changes such as the loss or destruction of microtriches, the appearance of numerous lipid droplets together with accumulations of glycogen and lysosomes, loss of organelles, breaking and pyknosis of nuclei in the germinal layer.

Shuhua *et al.* (2002) indicated that efficiency of Albendazole emulsified with 30% soybean oil in reducing the size of hydatid cysts caused by *E. granulosus* protoscolices.

In control mice ,liver and spleen hypertrophy as result to increased granulomatous lesion in tissue them The local inflammatory response around *E. multilocularis* metacestodes develops by recruitment of effector cells, e.g. neutrophils, monocytes and lymphocytes, to the site of infection (Gottstein & Hemphill 1997).

Histopathological examination of the cyst revealed typical double layered wall and in some cases with clear fluid inside. This result came harmony with what found Hashemitabar *et al*.(2006) Some samples revealed a precystic structure, which consisted of connective tissue and scattered hyaline cells showing a necrotic basophilic structure infiltration of mononuclear and eosinophil that resembled a cubicula membrane. Zakim and Boyer (1996) showed that atrophy, degenerated in hepatocytes surrounding with hydatid cyst and increased in kupfer cells that act as defenses cells to clearing and elimination bacteria ,yeast and parasite from human body.

MacSween and Whaley(1992) indicated that occurrence splenomegaly when infected with hydatid cyst .

Ali-Khan(1983)showed that amyloidosis accompaniment to alveolar hydatid cyst.

The spleen in amyloidosis is enlarged, firm and of rubbery, the deposition is in arteriolar walls and extends into surrounding lymph follicles and amyloidosis are some disturbance of protein metabolism (Anderson, 1971) .

Hydatid cyst induces inflammatory response by infiltration of lymphocyte, neutrophil and macrophage to the site of injury. This inflammatory response was reduced in mice treated with *Z. officinale*. The plant contains high level of total phenolic and flavonoid, responsible for its high antioxidant activities (Ghasemzadeh *et al*.,2010)

In general, the observed efficacy could probably be due to the phytochemical constituents of this plant. The antioxidant action of *Z. officinale* has been proposed as one of the major possible mechanisms for the protective

actions of the plant against toxicity. It has been shown that gingerol is endowed with strong antioxidant action both *in vivo* and *in vitro*, in addition to strong anti-inflammatory and anti-apoptotic actions. (Kim *et al*., 2007)

Conclusion:

In this present study, the activity of *Zingiber officinale* extract was explored the possibility of more investigation *in vitro* and *in vivo* protoscolicidal effect . The results suggested that *Zingiber officinale* could be used for further investigation into potential discovery of new natural bioactive compounds and it was isolated of active protoscolicidal constituents are in progress and may lead to the discovery of compounds with improved therapeutic value.

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