Assessment of the effect of *Allium sativum* on serum nitric oxide level and hepatic histopathology in experimental cystic echinococcosis in mice

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Abstract The current study was carried out to evaluate the prophylactic and therapeutic effects of *Allium sativum* on experimental cystic echinococcosis by measuring the serum nitric oxide level and studying hepatic histopathological changes. The experimental animals were divided into five groups, ten mice in each, group (I): prophylactic; group (II): therapeutic; group (III): prophylactic and therapeutic; group (IV): infected nontreated; group (V): non infected non treated. The results showed that serum nitric oxide was significantly increased as a result of infection in all infected groups compared to group V. Statistical significant difference was noted in serum nitrate level in group I at 1st and 8th week post infection compared to the same time interval in group IV. In group II, statistical significance was noticed only at the 1st week post infection. Statistical significant difference was noted in serum nitrate level in group III at 1st, 4th, 6th and 8th week post infection compared to same time interval in group IV. Hydatid cysts developed in livers of mice of group IV as early as 4 weeks of infection while no cysts were found in groups I, II and III. Histopathologically there were moderate pathological changes in group I and group II as hepatocytes showed moderate steatosis, moderate venous congestion and inflammatory cellular infiltrate with foci of degeneration and necrosis. While livers of mice of group III showed mild steatosis, mild venous congestion, mild inflammatory cellular infiltrate, no necrosis and no biliary hyperplasia. Accordingly, that garlic

(*Allium sativum*) may be a promising phototherapeutic agent for cystic echinococcosis

Keywords Cystic echinococcosis · *Allium sativum* · Garlic · Nitric oxide · Histopathology

Introduction

Cystic echinococcosis (hydatid disease) is caused by infection with the larval stage of dog tapeworm *Echinococcus granulosus*. It is a major zoonotic disease of worldwide distribution and especially prevalent in sheep-raising countries (McManus et al. 2003). In man, the majority of hydatid cysts occur in the liver, causing symptoms that may include chronic abdominal discomfort, occasionally with a palpable or visible abdominal mass, hepatomegaly, biliary cirrhosis, portal hypertension and ascites. If a cyst becomes infected with bacteria, it resembles an abscess (Becker et al. 1997; Garcia 2007).

A number of options are available for treatment of cystic echinococcosis, including surgery, puncture-aspiration-injection-reaspiration (PAIR), and chemotherapy. Surgery is generally considered the treatment of choice for a complete cure, although this approach is limited to unilocular cysts in operable body sites. Cases where multiple cysts are present in several different sites, or in patients with a high surgical risk, PAIR and chemotherapy are considered appropriate options. Because there is always the risk of cyst fluid spillage during surgery, pre- and/or post-operative use of chemotherapy may be appropriated. During the last few years, experience with the benzimidazole derivatives mebendazole and albendazole suggests that under certain circumstances, chemotherapy may replace surgery. Although relapses after chemotherapy have been reported, patients are usually

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sensitive to retreatment (Pawlowski et al. 2001). However, resistant cases that do not respond to medication were reported (Ulger et al. 2013). Therefore assessment and finding of some new agents especially from herbal origin with a desired scolical effect attracts great attention for treatment and pre-surgical use to prevent the hydatid cyst recurrence (Eskandarian 2012).

Garlic (*Allium sativum*) is one of the most extensively researched medical plants. The antimicrobial activity of garlic depends on allicin produced by enzymatic activity of allinase (a cysteine sulfoxide lyase) on alliin after crushing or cutting garlic clove (Ross et al. 2001). Allicin and other thiosulfonates are believed to be responsible for the range of therapeutic effects reported for garlic. Garlic extract has been reported to inhibit growth of various gram-positive and gram-negative bacteria (Fani et al. 2007). Allicin caused inhibition of growth of *Entamoeba histolytica* (Mirelman et al. 1987). Its effect as anti-giardial and antihelminthic agent in human patients has been proven by Soffar and Mokhtar (1991). Diallyl trisulphide (organosulphur compound of garlic) inhibited the growth of *E. histolytica*, *Giardia lamblia* and *Trypanosomes* in vitro (Lun et al. 1994). There was an evident that feeding *Biomphalaria alexandrina* snail on garlic powder exerted some biological and biochemical changes (Mantawy and Mahmoud 2002). Garlic chloroformic extract is safe and potent protoscolicid and might be used in hydatid cyst treatment and pre-surgery to prevent secondary cyst recurrence (Eskandarian 2012).

Nitric oxide (NO) is an important vascular and neuronal messenger molecule which has emerged as an important mediator of a variety of biological functions. It is a soluble free radical, generated from arginine by at least three isoforms of NO synthase, one of which is the inducible NO synthase (iNOS) (Moncada and Higgs 1993). One of the most prominent functions of NO is its participation in antimicrobial defense (Bogdan et al. 1997). Many cell types, e.g. macrophages, can express high levels of iNOS when activated by immunological stimuli, e.g. interferon gamma (IFN- γ) or tumour necrosis factor (TNF), thus leading to the killing of parasites (Liew et al. 1997).

In *Echinococcus granulosus* infection, an aggressive cellular immune response against the protoscoleces is observed involving activated macrophages, neutrophils, eosinophils and lymphocytes and approximately 10 % of the protoscoleces will survive to develop into hydatid cysts as this cellular attack is responsible for initial parasite death (Rogan and Craig 1997). One probable killing mechanism is nitric oxide as this initial attack is under Th1 control, there was increase in IFN- γ levels during initial phase of murine infection peaking after 1 month, and reduced levels of interleukin (IL)-4 and (IL)-10. The second phase of experimental infection occurs once the hydatid cyst is fully formed and is characterized by a more Th2 type response

involving elevated IL-4 and IL-10 and a decrease in IFN- γ and tumor necrosis factor- α (TNF- α) (Rogan 1998). However, it is possible that fully formed hydatid cysts may also be susceptible to macrophage attack if activated by IFN- γ . In human elevated levels of NO and IFN- γ have been observed in sera from some patients with *E. granulosus* infection (Touil-Boukoffa et al. 1998). *In vitro*, murine hydatid cysts of *E. granulosus* were damaged and killed when incubated in presence of NO produced by IFN- γ activated peritoneal macrophages (Steers et al. 2001).

The aim of the present study is to evaluate of the prophylactic and therapeutic effects of *Allium sativum* on experimental cystic echinococcosis by measuring the serum nitric oxide level and studying hepatic histopathological changes.

Materials and methods

Preparation of aqueous garlic extract

Peeled garlic (30 g) was crushed in 60 ml distilled water in a mortar. The crushed material was carefully decanted by pressing and the fluid was collected. One milliliter of aqueous extract contained 500 mg of garlic materials (Sener et al. 2003). Raw garlic juice was placed in 1.5 ml tubes and stored in a -20°C freezer. Working solution (50 mg/kg body weight) was made from the stock solution by dilution with distilled water. The dose selected for the present work is equivalent to the daily amount of garlic recommended for an average human to maintain good health (~ 4 gm) (Riad et al. 2009). Garlic was intragastrically administered, as crude juice, in a dose of 50 mg/kg body weight/mouse.

Mice

Adult female Swiss albino mice six to eight weeks old weighing 25–30 gm inbred at *Schistosoma* Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Giza, Egypt was used in throughout this study. Animals were housed in Ain-Shams University, Experimental Animal Unit. The mice were maintained under standard laboratory care (25°C , with a relative humidity of 40–60 %, normal diet of commercial pellets and potable water ad libitum). All the animal experiments were performed according to the rules and regulation of the Animal Ethics rules, Ain-Shams University, Cairo, Egypt.

Experimental design

The animals were randomly divided into five groups with ten mice in each, group (I): mice were treated with garlic

day after day for 2 weeks before infection (prophylactic group); group (II): treated with garlic day after day starting 3rd day after infection till the end of the 8th week post-infection (therapeutic group); group (III): mice were treated with garlic day after day starting from 2 weeks before infection till the end of the 8th week post-infection (prophylactic and therapeutic group) (Riad et al. 2009); group (IV): infected non-treated group; group (V): non-infected non-treated group. Blood samples were collected one week post infection then on 3rd, 4th, 6th and 8th week post-infection. Serum separation was performed by centrifugation at 1,000 g for 15 min, under cooling for the subsequent analysis of nitric oxide production.

Parasite and infection

Hydatid cysts from camel livers or lungs were obtained from Egyptian slaughter house. *E. granulosus* protoscoleces were obtained by aseptic puncture of the cysts according to Baz et al. (1995). Parasites were washed several times with phosphate buffered saline (PBS) pH 7.2–7.4 (Sigma Chemical Company, St. Louis, MO, USA) containing gentamycin (40 µg/ml) (GIBCO-BRL Life Technology, NY). Parasite vitality was determined by eosin exclusion (Robinson and Arme 1985). Mice were inoculated intra-peritoneally with 200 µl of a suspension containing 2000 live protoscoleces in PBS (Araj et al. 1977; Dematteis et al. 1999).

Nitric oxide production determination

Nitrate accumulation in sera was performed according to Miranda et al. (2000) as an indication of NO production. This was based on the reduction of nitrates by vanadium (III) chloride (VCl₃) combined with detection by acidic Griess reaction. Before NO estimation, sera were deproteinized to reduce turbidity. Two hundred µl of absolute ethanol was added to 100 µl sample followed by centrifugation at 3,000 rpm for 5 min. Saturated solution of 200 mg VCl₃ (Sigma-Aldrich) was prepared in 25 ml 1 M HCl (ADWIC, Egypt). Excess solid was removed by filtration, and the blue solution was stored in the dark at 4 °C. Griess reagents was composed of 1:1 of mixture of .1 % *N*-(1-naphthyl) ethylenediamine (BDH) in deionized H₂O and 2 % sulfanilamide (Sigma-Aldrich) in 5 % HCl and premixed immediately prior to application. Each solution was filtered to remove trace particulates. Experiments were performed at room temperature by adding 100 µl VCl₃ to 100 µl samples followed by rapid addition of 100 µl Griess reagents (1:1:1). After incubation for 30–45 min the absorbance at 540 nm was measured using a spectrophotometer (Helios α theromospectonic). Nitrate standard solution was serially diluted (10 dilutions starting from

0.5 mM). The absorbance was referred to a nitrate standard curve to determine the nitrate concentration in samples.

Histopathology

Mice were sacrificed on 4th, 6th and 8th week post infection. Liver samples fixed in 10 % formalin and paraffin blocks, (4 mm thick) were prepared and stained with Hematoxylin and Eosin stains and then were studied histopathologically to evaluate structural alterations of the hepatic parenchymal cells (Beiting et al. 2004; Al-Kuraishi 2009).

Statistical analysis

Continuous variables were expressed as mean and Standard Deviation. Student *t* test was used to compare a continuous variable between two study groups. ANOVA Test was used to assess the statistical significance of the difference between more than two study group mean with Post Hoc Test for comparisons of all possible pairs of group means. Paired *t* test was used to assess the statistical significance of the difference between variables measured twice for the same study group. A significance level of $p < 0.05$ was used in all tests. All statistical procedures were carried out using SPSS version 15 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Serum nitric oxide levels

Serum nitrate levels were significantly increased ($p < 0.05$) in mice infected with *E. granulosus* in all groups of the study compared to its level in normal non-infected mice.

When serum nitrate levels in mice of groups I, II and III were compared with the levels in infected mice that did not received any dose of *A. sativum* (group IV), at each time interval (Table 2), statistical significant difference was noted in group I at 1st week ($p < 0.05$) and 8th weeks ($p < 0.001$) post infection compared to the same time interval in group IV. While in group II, statistical significance was noticed only in serum nitrate level at 1st week ($p < 0.05$) post infection.

Statistical significant difference was noted in group III at 1st, 4th and 6th weeks ($p < 0.05$) and 8th weeks ($p < 0.001$) post infection compared to the same time interval in group IV.

Histopathology results

Examination of livers of mice of different group of the study revealed focal or spottynecrosis with infiltration

of inflammatory cells (lymphocytes), hydropic degeneration, with fatty changes (steatosis). Also there was congestion and dilatation or widening of the sinusoids (figures 1–6). The severity of these findings varied from one group to another according to table (2).

Discussion

Evaluation of the prophylactic and/or therapeutic effect of *A. sativum* on cystic echinococcosis was the aim of this study. Experimental hydatidosis was induced by intra-peritoneal inoculation of protoscoleces into mice. Serum nitric oxide levels and pathological changes in liver parenchyma were assessed to reflect the effect of oral administration of *A. sativum* on cystic echinococcosis.

To study the immunological relationship between the intermediate host and the parasite, mice were the experimental model in hydatidosis. In this model, the host reacts to the protoscoleces producing innate local and adaptive systemic responses (Severi et al. 1997).

Nitric oxide is a soluble free radical, synthesized by a family of nitric oxide synthase isoforms using L-arginine as the substrate. It is an important vascular and neuronal messenger molecule which has emerged as an important mediator of a variety of biological functions (Moncada and Higgs 1993). One of the most prominent functions of nitric oxide is its participation in antimicrobial defense (Bogdan et al. 1997). Nitric oxide has been the focus of many investigations attempting to define its role in the immune response. Under different conditions, nitric oxide can have cytoprotective or cytotoxic effects depending not only on the initial insult but also on the source, rate of production, and concentration of nitric oxide in the biological milieu (Brunet et al. 1999). Hence, it is the balance between nitric oxide and other inflammatory cytokines and mediators (such as TNF- α , IL-1 β , IFN- γ) that is crucial in determining its effect on the host (Liew et al. 1997). Several studies have suggested that nitric oxide plays a unique role in the liver, and its involvement in a variety of hepatic processes is supported by the finding that the majority of cells found in the liver, such as hepatocytes, Kupffer cells, fat-storing cells, and endothelial cells, are able to produce nitric oxide under adequate stimulation (Brunet et al. 1999).

Garlic (*A. sativum*) has been reported as the source of some antihelminthic substances and it is a well-known and important dietary and medicinal plant with several useful properties. For instance, its antioxidant, antitumor, antiviral, antifungal, antibacterial, antiprotozoal, antihelminthic and antiprotoscolex effects have been reported (Eskandarian 2012). In vitro, *A. sativum* extracts had a high protoscolecidal activity (Sadjjadi et al. 2008; Moazeni and Nazer 2010).

Both water and alcoholic extracts of garlic activated nitric oxide synthase activity in isolated platelets in vitro as well as in placental villous tissue. Activation of calcium-dependent nitric oxide synthase and the subsequent production of nitric oxide is probably the most novel mechanism yet claimed by which garlic can exert its therapeutic properties (Das et al. 1995). To study the role of garlic in prevention of cardiovascular disease, the effect of garlic extract on nitric oxide production was estimated by measuring the nitric oxide metabolites nitrite and nitrate in the plasma of mice. Garlic extract increased nitric oxide production by 30–40 %. Garlic extract increased nitric oxide production by activating constitutive nitric oxide synthases, but not inducible nitric oxide synthases (Mori-hara et al. 2002).

In the present study, serum nitric oxide level in group IV was nearly equal to that in normal mice (group V) at first week of infection then significantly increased as the infection progressed to reach higher levels at 4th, 6th and 8th weeks post infection. This coincides with results of Dematteis et al. (1999) who reported that Th2-type response was stimulated by live protoscoleces at the beginning of infection as only IL-4, IL-5 and IL-10 were secreted by splenocytes from infected mice sacrificed one week postinfection. A shift to a Th1-type response was observed 4 weeks post infection with a significant increase of IFN- γ secretion. The very early Th2-polarization induced by live protoscoleces was lost after four weeks of infection that is at the time where cysts are expected to develop (Araj et al. 1977).

Allium sativum has significant enhancing effects on the immune system (Schäfer and Kaschula 2014). It has an ability to increase nitric oxide synthase activity intracellularly which explains why many of the therapeutic actions of garlic parallel the physiological effects of nitric oxide (Mori-hara et al. 2002). Therefore, in the present study, the statistically significant higher levels of nitric oxide in group III, at 1st, 4th, 6th and 8th weeks post infection compared to group IV (Table 1) indicate the prophylactic and therapeutic effect of *A. sativum* in murine cystic hydatidosis. This protective effect of *A. sativum* could be mediated by nitric oxide production as, in vitro, murine hydatid cysts of *E. granulosus* were damaged and killed when incubated in presence of nitric oxide produced by IFN- γ activated peritoneal macrophages (Steers et al. 2001).

Although nitric oxide levels significantly increased in group I at week 1 then at week 8 post infection and in group II at week 1 post infection hence this effect was not able to protect mice's livers from necrosis and other pathological changes even in absence of hydatid cyst (Table 2).

Livers of 7–8 weeks old female mice experimentally infected with cystic echinococcosis showed histopathological changes in the form of inflammatory cell

Table 1 Comparison of the serum nitric oxide levels between each of groups I, II, III, and IV

Time of sampling	Groups				<i>p</i>
	(I) Prophylactic		(IV) Infected non-treated		
	Mean (μmol/L)	±SD	Mean (μmol/L)	±SD	
1st week	128.00	13.53	96.53	2.83	0.017*
3rd week	191.80	58.30	206.67	26.72	0.709
4th week	230.00	87.67	251.93	26.83	0.700
6th week	320.00	124.89	262.97	54.21	0.508
8th week	384.00	9.17	280.13	20.10	0.001*
Time of sampling	(II) Therapeutic		(IV) Infected non-treated		<i>p</i>
	Mean (μmol/L)	±SD	Mean (μmol/L)	±SD	
	Mean (μmol/L)	±SD	Mean (μmol/L)	±SD	
1st week	160.00	16.00	96.53	2.83	0.002*
3rd week	203.00	50.51	206.67	26.72	0.917
4th week	213.75	87.63	251.93	26.83	0.510
6th week	223.90	109.15	262.97	54.21	0.608
8th week	388.83	178.75	280.13	20.10	0.354
Time of sampling	(III) Prophylactic and Therapeutic		(IV) Infected non-treated		<i>p</i>
	Mean (μmol/L)	±SD	Mean (μmol/L)	±SD	
	Mean (μmol/L)	±SD	Mean (μmol/L)	±SD	
1st week	160.27	27.70	96.53	2.83	0.017*
3rd week	260.23	39.86	206.67	26.72	0.125
4th week	384.00	9.17	251.93	26.83	0.037*
6th week	517.93	82.19	262.97	54.21	0.011*
8th week	963.17	153.85	280.13	20.10	0.002*

p *p* value* Statistical significant difference (student *t* test)**Table 2** Pathological findings of different studied groups

Groups	Time of sampling	Pathological findings						
		Inflammation	Necrosis	Degeneration	Fatty changes	Congestion	Biliary hyperplasia	Hydatid cyst
(I)	4th week	+1	+1	+2	+2	+1	+1	–
	6th week	+2	+2	+2	+2	+2	0	–
	8th week	+2	+2	+2	+2	+2	0	–
(II)	4th week	+1	+1	+1	+2	+2	0	–
	6th week	+2	+2	+2	+2	+1	0	–
	8th week	+2	+2	+2	+2	+2	+1	–
(III)	4th week	+1	0	+1	+1	+1	0	–
	6th week	+1	0	+1	+1	+1	0	–
	8th week	+1	0	+1	+1	+1	0	–
(IV)	4th week	+2	+3	+3	+2	+3	+2	Present + dense inflam. daughter cyst
	6th week	+2	+3	+3	+2	+3	+2	
	8th week	+2	+3	+3	+2	+3	+2	

(0): No pathological findings

(+1): Mild pathological findings

(+2): Moderate pathological findings

(+3): Severe pathological findings

Fig. 1 Liver biopsy of group IV showing 4 wks of infection shows a cyst containing a small daughter cyst. The cyst is surrounded by a dense chronic and acute inflammatory cellular infiltrate

Fig. 3 Liver biopsy of group II showing moderate degree of congestion and inflammatory cellular infiltrate

Fig. 2 Hepatocytes showing moderate steatosis in group II

Fig. 4 Liver biopsy of group I showing foci of degeneration and necrosis

infiltration, increase of lymphoid tissues, mild necrosis, sinusoid dilation, increase in biliary ducts (hyperplasia) and severe hydropic degeneration, with multiple cysts appeared as early as one month postinfection (Al-Kuraishi 2009). This coincides with the results of this study as intense inflammatory response with severe necrosis, degeneration, fatty changes, congestion and biliary hyperplasia were noticed in livers of mice of group IV (Table 2). Moreover, cysts (Fig. 1) were noticed in livers of mice of this group started to appear as early as 4 weeks of infection in some mice.

When the histopathological changes in the liver tissues of mice in groups I, II and III were studied in the present work, moderate pathological changes were noticed in group I and group II as hepatocytes showed moderate steatosis (Fig. 2) moderate degree of congestion and inflammatory cellular infiltrate (Fig. 3) with foci of

Fig. 5 Liver biopsy of group III showing mild venous congestion and mild inflammation of hepatic parenchyma

Fig. 6 Hepatocytes showing mild steatosis in group III

degeneration and necrosis (Fig. 4) While livers of mice of group III showed mild venous congestion and mild inflammation of hepatic parenchyma (Fig. 5) mild steatosis (Fig. 6), no necrosis and no biliary hyperplasia. What is notable is the absence of hydatid cyst from all groups that received *A. sativum* as prophylaxis or treatment (groups I, II and III). This emphasizes the effect of increased levels of nitric oxide that induced by administration of *A. sativum* for both prophylaxis and treatment.

An overall look on the results of this work, it is clear that when *A. sativum* was administered before induction of murine cystic echinococcosis only (group I) or after induction of the disease only (group II), hydatid cyst were absent with moderate pathological changes in spite of non statistically significant increase in the level of serum nitric oxide at the same time intervals in both groups. While when *A. sativum* was given to mice both before and after induction of the disease (group III), mild pathological changes and absence of cysts were noticed which correlate to the statistically significant increase in the level of serum nitric oxide.

In conclusion, this study demonstrated that garlic (*A. sativum*) is efficient as prophylactic and therapeutic agent in cystic echinococcosis with the added advantage of being a natural product.

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