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Full Length Research Paper

Antiparasitic activity of the microalgae *Cladophora crispata* against the Protoscolices of hydatid cysts compared with albendazole drug

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Antiparasitic activity of the microalgae *Cladophora crispata* isolated from Garmat-Ali River in southern Iraq was studied. Water samples were collected from river in the northern of Basrah, and cultured in chu-10 medium. Supernatants, alkaloidic and ethylacecate extracts from biomass were extracted and screened against the hydatidosis compared with albendazole drug. The present study reveal that 2- (N, N-dimethyl hydrazine) cyclohexanecarbointrile and pyridine 2,3,4,5 - tetrahyro compounds have activity against the protoscolices of hydatid cysts accordingly to the activity of albendazole based on the mean of weight of mice, mean of hydatid cysts number, mean of diameters and weights of hydatid cysts. The current study concludes that the alkaloidal and ethylacetate extracts of *Cladophora crispata* have a positive effect on the protoscolices of hydatid cyst in comparison with albendazole activity, related to reduction in the number and weight of hydatid cyst as well as the obstruction of germinated layer which is responsible for proliferation of protoscolices.

Key words: Algae, *Cladophora crispata*, bioactive chemical compounds, antiparasitic activity, hydatid cysts.

INTRODUCTION

Microalgae are a diverse group of photosynthetic microorganisms found in the soil, fresh water and marine environments (Metting and Pyne, 1986). They are able to produce a range of biochemical active compounds such as antibacterial, antifungal, antiviral, enzyme inhibitors, immunostimulants, cytotoxic, atiplasmodial activities (Ghasemi et al., 2004) and antitrypanosomal activities (Lorena et al., 2009). Most of the isolated chemical substances belong to groups of alkaloids, peptides, tannins, saponins, triterpenes and phenols (Molera and Semesi, 1996) as well as proteins (Athbi, 2011).

Diseases such as hydatid disease, hydatidosis, cystic echinococcosis, unilocularhydatid disease and echinococcosis, are referred to as the infections which are caused by cestodes of the genus *Echinococcus* and mainly by *E. granulosus* (Dar et al., 1977; Akhan et

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al., 2002; Georgopoulos et al., 2007). Hydatid cysts remains a significant public health problem in the endemic areas such as Turkey, the Middle East, South America, New Zealand, Mediterranean region, Africa, China, northern Kenya, Australia, and other sheep-raising areas (Morar and Feldman, 2005; McMnus et al., 2003). As an endemic disease, it causes social and economic losses for countries. WHO reports that approximately 100 000 people in the world are infected with these diseases every year which is more common in rural population of underdeveloped countries because of their close association with domestic and wild animals (Parija and Sheeladevi, 1999). Until recently, surgery was the only option for treatment of echinococcal cysts, however, chemotherapy with benzimidazole compounds and, cyst puncture, percutaneous aspiration, injection of chemicals, and reaspiration (PAIR) are increasingly used to as an alternative to replace a surgical method (Morar and Feldman, 2005).

The undesirable side effects associated with this classical drug, as well as the development of resistance, has encouraged researchers to use alternative synthetic or natural compounds for treatment of hydatid disease. In this regard, studies have focused on the bioactivity of natural substances derived from algae, mainly due to their accessibility and use in traditional medicine (Smit, 2004; Kladietal*,* 2008; Lorena et al., 2009); although many researches have been focused on the extracts from algae as a source of antiparasitic compounds. Early studies on bioactive chemical compounds isolated from chlorophyta have led to the discovery of several compounds, including the diterpenoids, udoteal and halimedatrial isolated from *U. flabellum* and *Halimeda sp*. respectively, and the sesquiterpenoids hipocephanal isolated from *Rhipocephalus phoenix* that inhibited cell division in sea urchin eggs (Fenical and Paul, 1984). Anuomrthine, Pronueiferine, Glaucine, Nuciferine, Yeserpin, Evodianin, Caulerpine, Leptoclinidamin-A, and Halimedin are alkaloidal chemical compounds isolated from the species of chlorophyta and act as antioxidant, antiviral, antibacterial, antifungal, and anticancer (Calixto et al., 2000; Radwan et al., 2007; Carrol et al., 2007; Everton et al., 2009). Al-Nasiri (2010) isolated an alkaloid chemical compound from *C. crispata* which was similar in structure to calothrixin and had *in vitro* antibacterial activity.

Recent studies have shown promising antimalarial activity by the alga *Laurencia sp.* (Topcu et al., 2003) and trypanocidal and leishmanicidal activity by *Fucuse vanescens, pelvetiaba bingtonil*, *Ulva lactuca* and *Sargassum natans* (Naraetal, 2000; Orhan et al., 2006). A study of Leon - Deniz et al. (2009) revealed that the activity of the organic extracts of green algae *U. conglutinata* and *U. flabellum* cause a total inhibition of the trypanosomes parasite at 24 h; 48 h and even seven days after growth. The present study was designed to examine the *in vivo* activity of bioactive chemical compounds (alkaloids and ethylaacetale) extracted from *Cladophora crispata* (Chlorophyta) against the proto-scolices of hydatid cyst of *E. granulosus* compared with a commercial drug albendazole.

MATERIALS AND METHODS

Isolation of microalga

C. crispata was isolated from Garmat - Ali River in Basra city, southern Iraq, from January to April 2012. Primary culturing was done in Chu - 10 medium. After incubation, pure cultures of the living specimens were prepared by sub culturing with agar plate method in Chu -10 medium (Stein,1975). Preserved specimens were prepared and the living specimens were inoculated in 100 ml conical flasks. Constant illumination was used at 60 µE m^{-2} Sec^{-1} intensity using white fluorescent lamps and incubated at 25 ± 2°C. Algal cultures were identified based on thier morphological characteristic following the taxonomy schemes of Prescott (1975) and Sant 'Anna (2004).

Prepration of algal extracts

Preparation of extracts was made according to Reichelt and Borowitazka (1984) using ethylacetate extract. 1 g of *C. crispata* biomass were extracted by soxhlet continuously with 250 ml of ethylacetate as solvent for 24 h. The alkaloidal extract was prepared using 0.5 g of dried culture extracted with acidic ethanol (ethanol absolute with 2% acetic acid) for 24 h in a continuous extraction by soxhlet apparatus. The extracts were filtered, and ethanol was evaporated on a rotary evaporator under vacuum at a temperature of 45°C to a small volume (about a quarter), then a small amount of NH_3 (25%) was added to make pH of 9. Subsequently, 100 ml of chloroform was added and slowly shacked for 10 min. until the alkaloidal compounds were separated from water and enter to the chloroform phase. This process was repeated three times, then total chloroform phase was evaporated, yielding a total alkaloid extract and dried under a reduced pressure and stored in -20°C for further studies.

Identification of biochemical active compounds

Ultra-violet (UV) spectrum (LKB-Sweden UV), Infra-red spectrum (IR) (Pye- Unicam Sp3- 3005 UK), and gas Chromatography Mass (GC-mass) (Agilent Technologies GC-mass 7890 AGC System) methods were applied for the identification and determination of the molecular weights and chemical structure of the isolated biochemical active compounds.

Parasite materials

E. granulosus hydatid cysts containing protoscolices were removed under aseptic conditions from livers and lungs of naturally infected sheep (The outer surfaces of the cysts were sterilized with70% ethanol before being dissected). Protoscolices were extracted according to Smyth (1980) (Figure 1).

Estimation of viable protoscolics

200 µL of hydatid fluid and 200 µL of 0.1% eosin staining solution

Figure 1. Hydatid sand containing the daughter cyst, brood capsule and protoscolex aspired from the hydatid cyst.

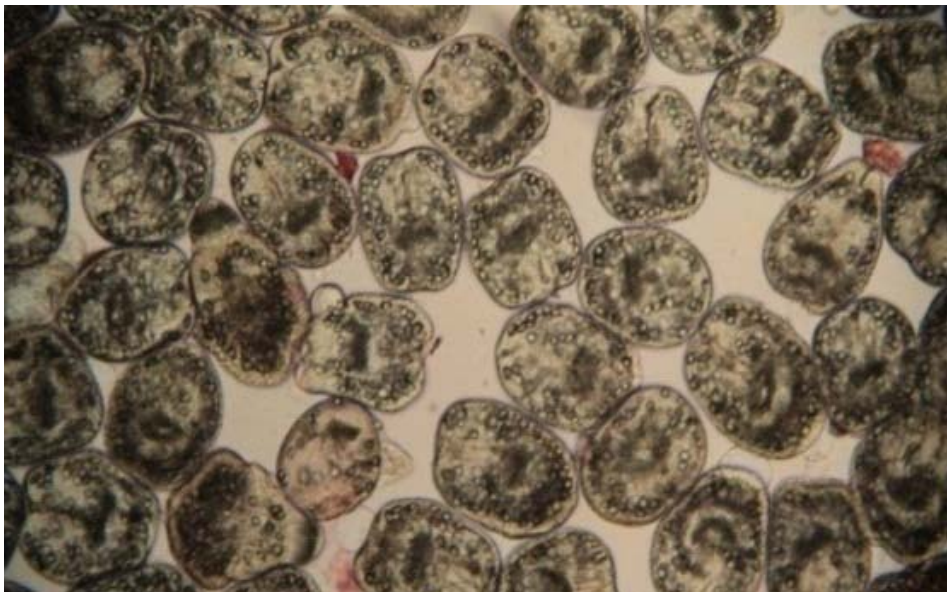

Figure 2. Viable protoscolices.

were combined in a microtube. After 20 min of incubation, the viability of protoscolices was assessed by microscopic observation. Stained protoscolices were considered as nonviable (Figure 3) and the protoscolices which have been stained with eosin were considered as viable (Figure 2) according to conventional (Taran et al., 2009).

The counting of viable protoscolices

Protoscolices were counted according to the method of Rounno et al. (1974) cited by Al- Humairy (2010). After estimating the viability of protoscolices, 10 µL of the hydatid fluid was taken by a micropipette, the count was done under dissecting microscope, and

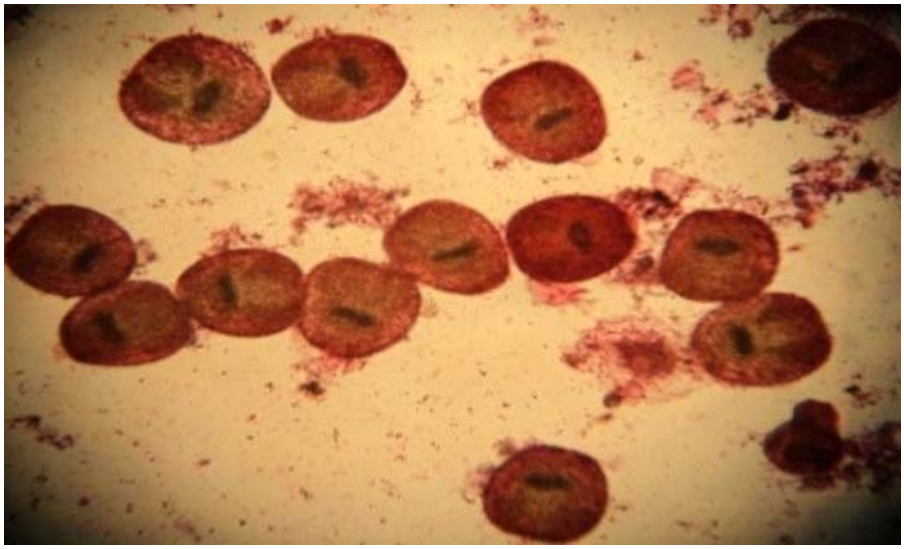

Figure 3. Non viable protoscolices.

Table 1. Experimental albino mice model.

Treatment	Number	Mice group
Alkaloids of *C. crispata*	24	T1 group
Ethylacelate extract of *C. crispata*	24	T2 group
Treated with Albendazole	8	Albendazole group
Infected without treatment	8	Positive control group
Without infection and treatment	8	Negative control group
- - - -	73	Total

repeated three times. The viable protoscolices were counted in 1 ml based on the formula:

Viability in 1 ml = number of protoscolices in 10 µL × 100

Injection of mice with protoscolices

Male Mus musculus mice Balb/C strain was injected with 0.2 ml 480/ L (2400/5 ml rate of viability) of protoscolices intraperitoneally (I.P) using 1 ml volume syringes. The region of injection was sterilized with 70% of ethyl acohol.

Determination of lethal dose (LD50)

Males of the *M. musculus* Balb/C strain were dosed orally to determine the LD_{50} using a stomach tube with bioactive chemical compounds extracted from *C. crispata* (ethylacetate and alkaloid extracts). The animals were monitored for 72 h and weakness, unstable walking, loss of balance and death was checked during this period. Injection started with low dose then continued to high dosages based on Litchfield and Wilcoxon (1949) equation:

$$LD_{50} = \text{highest dosage} - \frac{\sum ab}{n}$$

Where, LD_{50} is the lethal dose 50, highest dosage is the dose with 100% mortality of mice, a is the value of difference between the previous and next dose, b is the summation of dead animal for each dose (previous dose + next dose / 2) and n is the number of animals used for each dose .

Experimental design

Male mice of *M. musculus* Balb/c albino strain aged 6 to 8 weeks were used in this study. They were injected interperitoneal (IP) with viable protoscolices of the *E. granulosis* and left for six months before treatment.

A positive control group was infected without treatment and the negative control group was left without infection and treatment. The *in vivo* study included two parts (Table 1).

Treatment

The treatment included two groups of 24 infected male albino mice which were treated with bioactive chemical compounds. Eight infected mice were treated with albendazole and 8 more left as a positive control. Eight intact male mices were used as a negative control. More details as follows:

Treated 1 (T1) group

In this group, 24 infected male mice were dosed orally daily for one month with concentrations (280, 300, 330 µg/ml) of the alkaloidal bioactive compound extracted from *C. crispata* with 8 mices for each concentration.

Treated 2 (T2) group

This group consists of 24 infected male mice which were treated orally daily for one month with (100, 110, 120 µg/ml) concentration of ethyl acetate extracted from *C. crispata* with four pairs for each concentration.

Albendazole group

In this group, 8 of infected male mice were daily treated orally with 500 µg/ml of albendazole for 30 days.

The weight of mices and their organs were checked before and after treatment. Diameters of cysts were measured by the ruler and the number of hydatid cysts was counted based on the following formula to calculate the effective dose:

$$\text{Effective dose group} = \frac{\text{Number of cysts in positive control group - Number of cysts in treated}}{\text{Number of cysts in positive control group}}$$

Statistical analysis

The statistical analysis was conducted in SPSS 9.0; T-test at 0.05 level was used to analyze variation of the mean of viable protoscolices treated with bioactive chemical compounds and albendazole *in vitro* and *in vivo* experiments.

RESULTS

GC- mass spectrum

Alkaloid extract

In the present study, two peaks were detected by the GC-Mass analysis as a results of alkaloidal component analysis (Figure 4): Pyridine, 2, 3, 4, 5 - tetrahydro with R.T. 26.187 min and molecular weight 83 killo Dalton (kd), and the other compound was N-methyl- propylamine with R.T 26.289 min and amolecular weight of 73 kd (Figure 5).

Ethylacetate extract of *C. crispata*

The GC-mass spectrum of ethylacetate extract revealed the presence of 17 peaks as shown in Figure 6. The results indicate that the compounds were arranged as 2-(N, N- dimethylhydrazino) cyclohexanecarbonitrile which consist of 8.77% (R.T. 20.59 min) of the total extract followed by Triazolo [1, 5-a] pyrimidine carboxylic acid (7.25%) (Figure 7).

Experimental infection with hydatid cysts

The examination of experimentally infected males Balb/C mice with protoscolices, 1,2,3,4, and 6 months-post infection revealed the presence of hydatid cysts in liver, spleen, mesenteries, kidneys and lungs (Plates 1 and 2).

In vivo effects of the extracts on the weight of infected and treated mice:

Weight of the negative control group, positive group, and the treated groups of mice were checked. Results show that the weight of the positive control group weighted about 40.2 g, while the weight of the negative control group was 32.64 g and the weight of treated group with albendazole was 31.4 g. The T1 group showed 34 g of weight compared with T2 (33.8g) groups. Table 2 shows the weight of organs for each group.

In vivo activity of extracts on the number of hydatid cysts compared with albendazole

The number of hydatid cysts was decreased after the treatment with extracts of C*. crispata.* The number of hydatid cysts in spleen, lung and kidney was decreased to zero; this result is similar to those of other groups of mice treated with albendazole. Mesenteries and liver had a higher number of hydatid cysts than other organs for the positive control group (6 and 7.1 hydatid cysts) and they decreased after treatment with bioactive chemical compounds from *C. crispata* and albendazole (Table 3 and Plate 3A, B and C).

The results of the *in vivo* activity of the extract on the number of hydatid cysts of infected mice showed significant differences between groups treated with extracts and the positive control group compared with the group treated with albendazole. In T2 group, the percent number of hydatid cysts recorded 59.86% while the T1 group it was 48.6%, since the number of hydatid cysts reduced from 15.2 in the positive control group to 6.1 and 7.8 respectively in T2 and T1 groups. These results almost corresponded with the effective dose of albendazole (61.18%) compared with the T2 group and are slightly different from T1 group (Table 3).

In vivo activity of extracts on dimiter and weight of hydatid cysts compared with albendazole

The mean of hydatid cysts diameters was 8.2 mm for

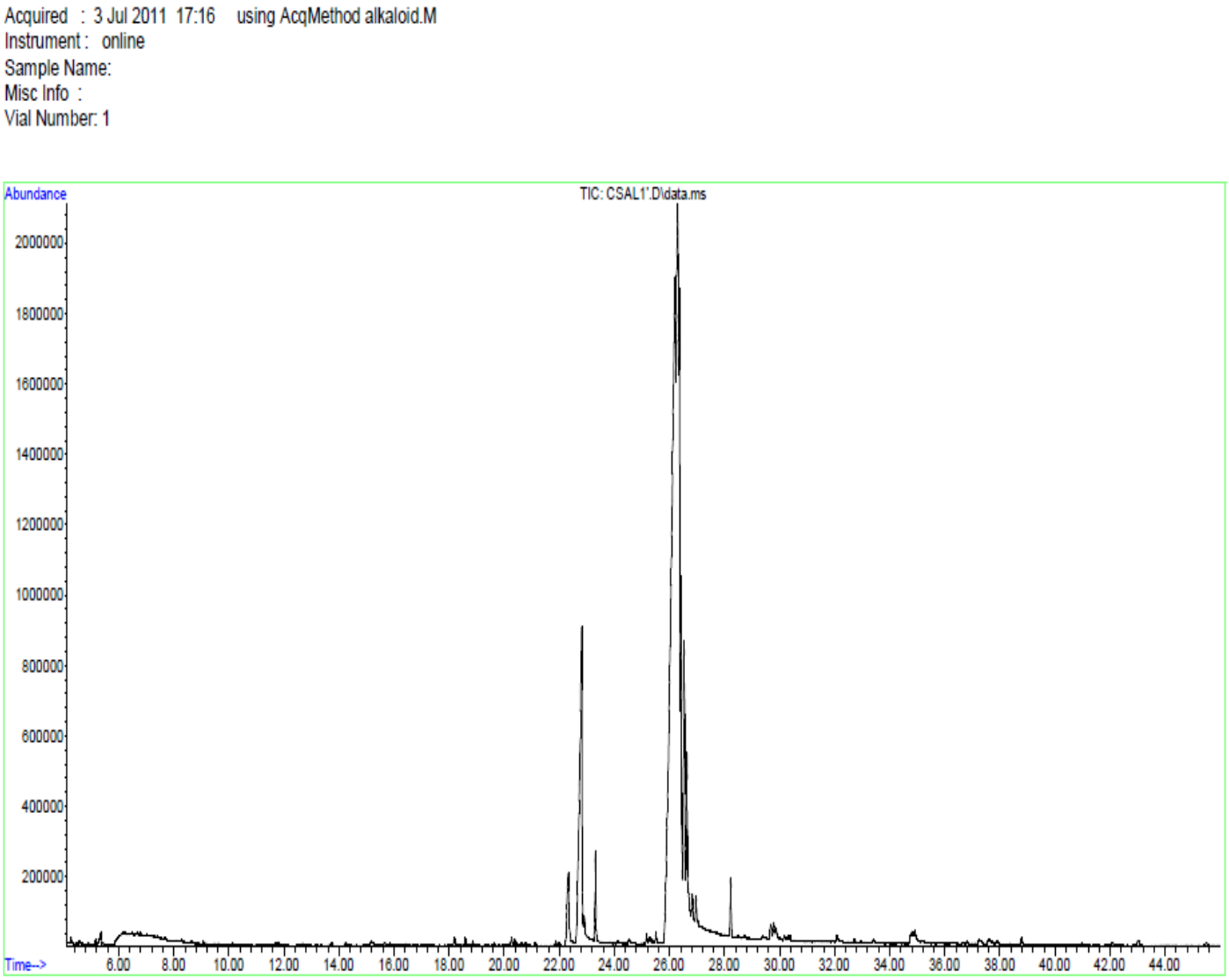

Figure 4. GC-Mass spectrum of alkaloid components of *C. crispata.*

the positive control group and was reduced to 4.2 mm in the group treated with albendazole, 4.6 mm in the T2 group, and 6.1 mm for the T1 group (Table 4, Plates 4A, B, C and 5). The weight of hydatid cysts was also studied for each group treated with the bioactive chemical compounds extracted from *C. crispata* as well as the group treated with albendazole. These results show a decrease in weight of hydatid cysts of the T2 group (0.55 g) and (0.63 g) in the T1 group compared with the group treated with albendazole recording 0.53 g where positive control group was 1.22 g in mean.

DISCUSSION

The current study demonstrated that the weights of infected experimental mice examined at 6th months - post infection, were increased compared with the negative control group. The weights of liver and spleen were also increased. On the other hand, there were slight increases in the weight of kidneys and lungs (Table 2). These results agree with those of other studies (Al-Nasiri, 2006; Al-Mobarek, 2006; Barzanjietal, 2009 and Al-Humairy, 2010). There was a significant difference of weights and diameters among the hydatid cysts of organs (livers, spleens, kidneys, lungs and mesenteries). Hepato-spleenomegaly caused by the parasite lead to an increase in weights of the experimental animals and their organs. However, many studies have explained the reasons of the increase in the weight of the organs infected with the hydatid cyst. Lightowlers et al. (2003)

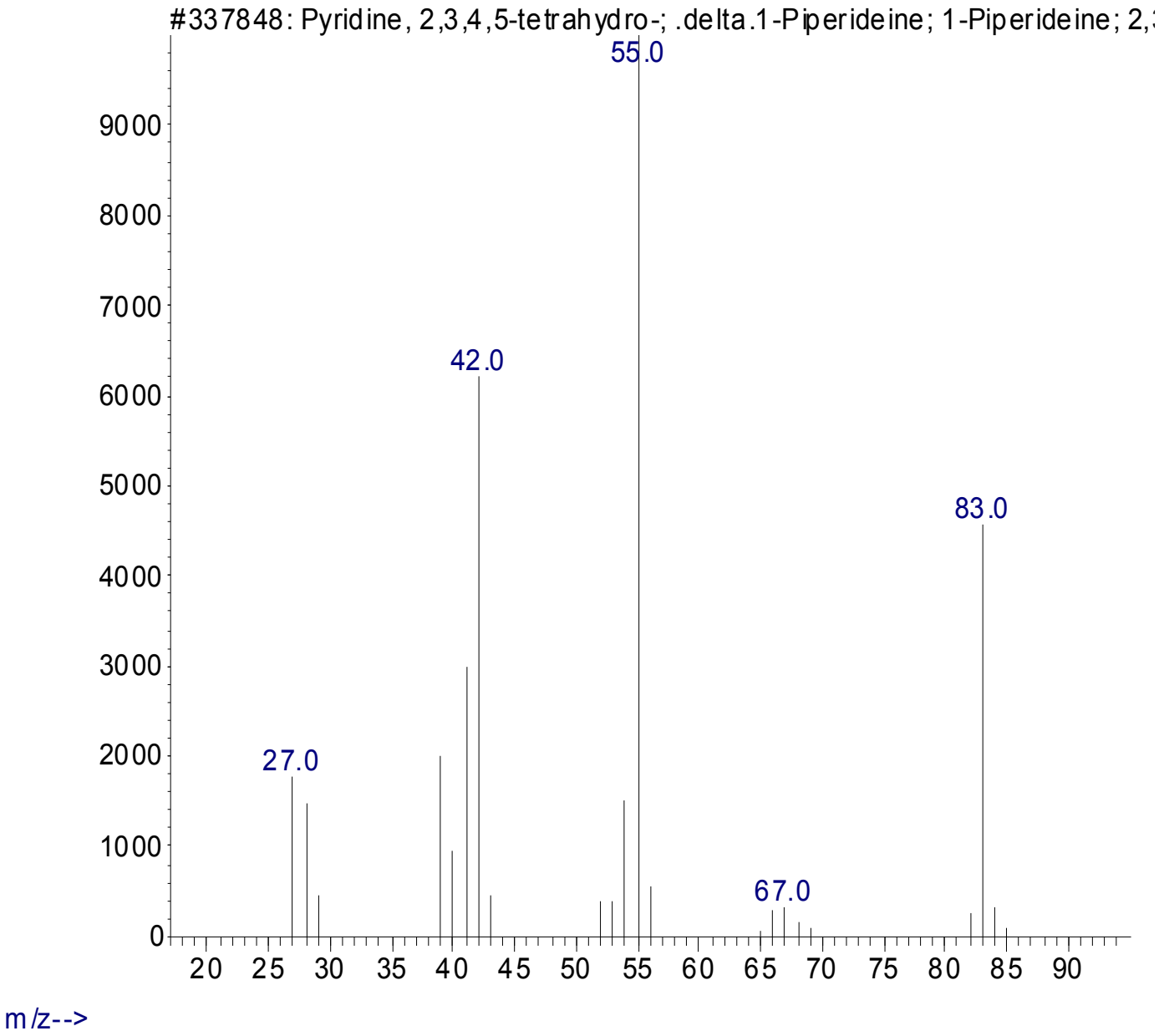

Figure 5. Mass spectrum of pyridine, 2,3,4,5 - tetrahydro.

concluded that the increase in liver and lungs weight was due to the formation of granuloma the increase in the immune cell and its migration to the target organ, so the ability of spleen to produce lymphocyte to secrete specific antibody had led to the increase of the spleen weights. The weights and diameters of hydatid cysts reported in the present study were affected by parasite infection because the weight decreased and approached the values of the negative control group especially in T2 group. The decrease in weight can be explained in relation to the decrease of number of hydatid cysts and calcification with the sloughing of the germinal layer and the disintegration of laminated layer (Maizeles and Yazdanbakhsh, 2003). However the complex layer of cyst

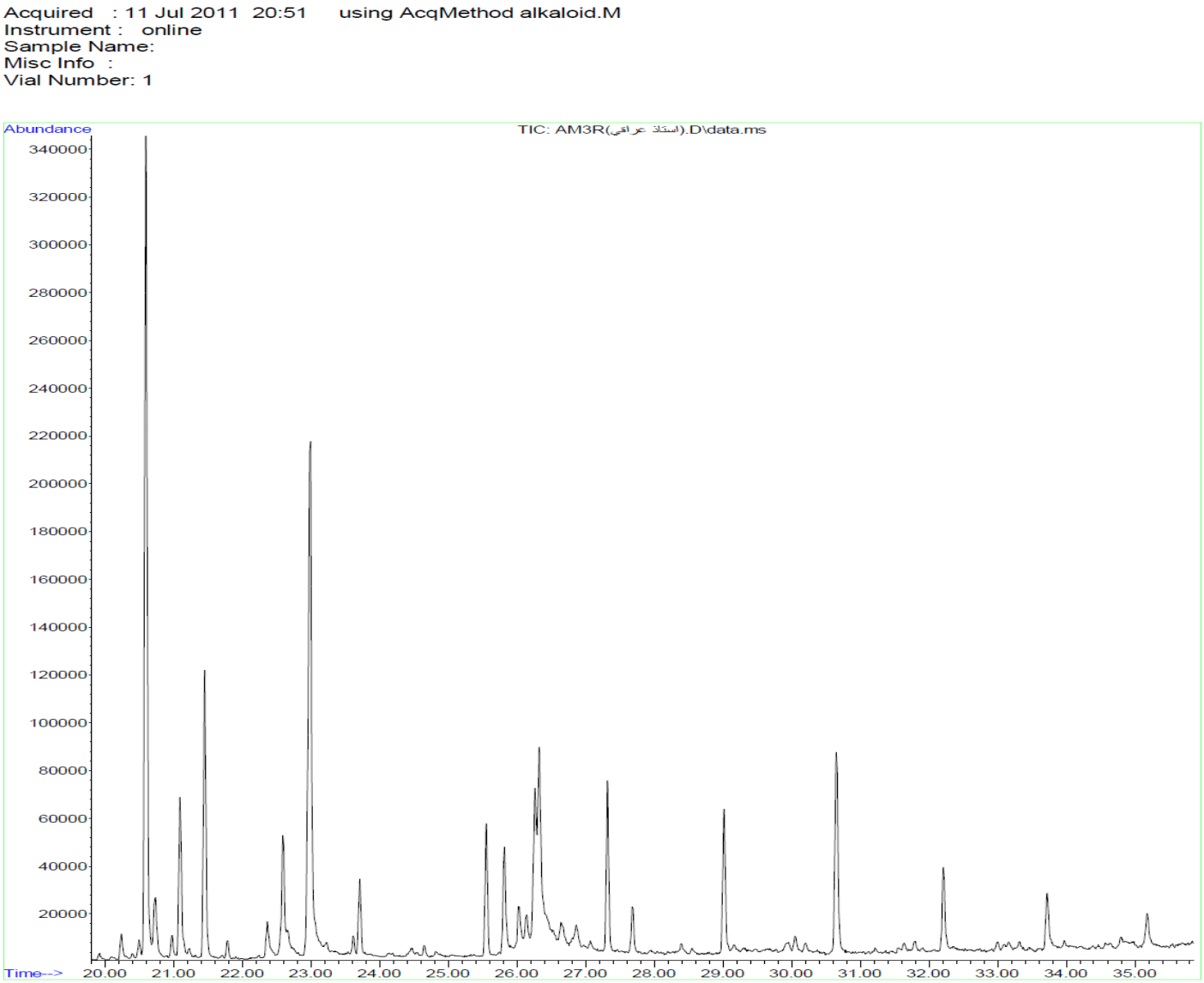

Figure 6. GC spectrum of ethylacetate extract of *C. crispata*.

has an important role in the transformation of nutritional material from serum to cyst. The knowledge of the parasite nutrition behavior can help for a drug treatment of the inoperative cyst via the selection of the effective drug and adhere them to biological material that promote distribution of drug to the cyst (Rahda et al., 2008). Compared to the bioactive chemical compounds, albendazole decreases the weights of infected testes animals more than those of the negative control group.

It is difficult to speculate the mechanism by which these bioactive compounds act as parasitic agents. In this regard, Sepulveda-Boza and Cassels (1996) suggested that many bioactive chemical compounds exhibited their parasitecidal activity by virtue of their interference with the redox balance of the parasites, acting either on the respiratory chain or the cellular defenses against oxidative stress.

It is also known that some bioactive compounds act by binding with the DNA of the parasite. For example, dihydroorotate dehy-drogenase (DHOD), the fourth enzyme in the *de novo* pyrimidine biosynthetic pathway, is essential to parasites, including the electron acceptor capacity and cellular localization (Nara et al., 2000). In this way, it has been recently demonstrated that the methanol extracts of brown algae *Ishige okamurae*, *F. evanescens* and *P. bingtonil* contain potent noncompetitive inhibitors against *Trypanosoma cruzi* DHOD (Takeaki et al., 2003; Nara et al., 2000).

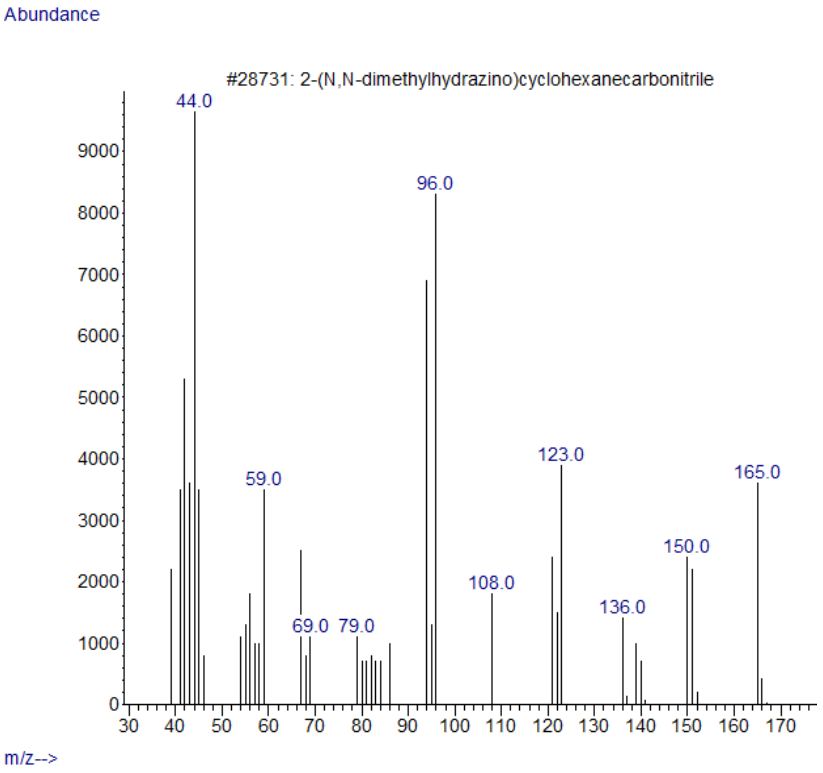

Figure 7. Mass spectrum of 2-(N,N-didimethylhydrazino) cyclohexanecarbonitrile.

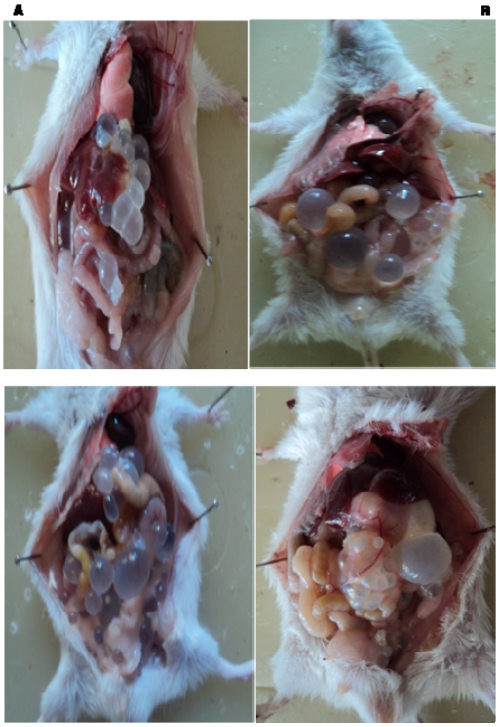

Plate 1. Pictures of experimentally infected mice. (A) mice with 4 months. (B, C, D) mice with 6 months after infection.

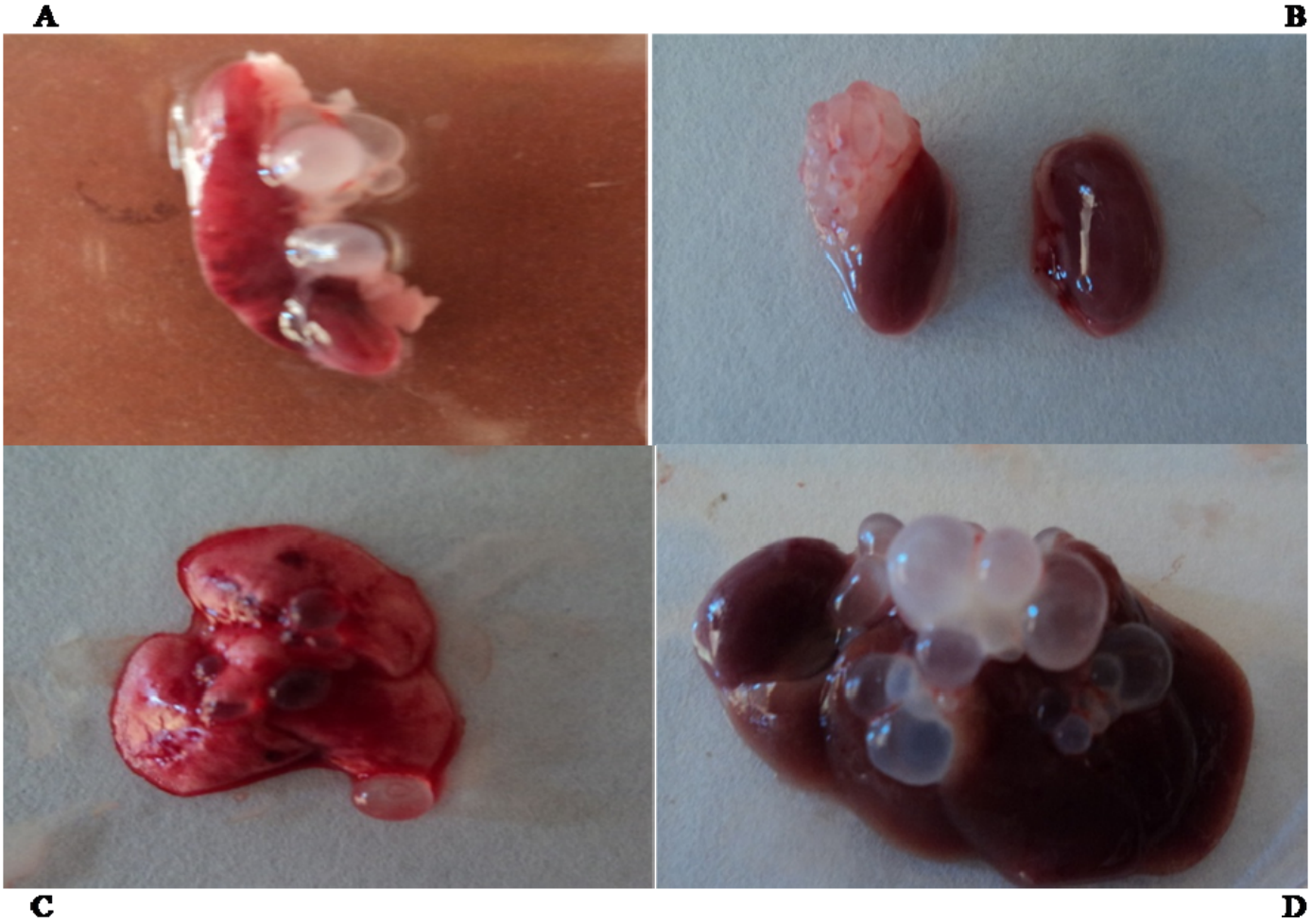

Plate 2. Pictures of infected mice organs at 6-months-post infection. A. Spleen. B. Kidney. C. Lung. D. Liver.

Table 2. Mean of weight of mice and organs treated with extracts compared with albendazole.

Group	Dose	Mean of weight/g				
		Liver	Mice	Spleen	Kidney	Lung
T1 group (alkaloid of C. crispata)	280	34.8±1.527*	4.3± 0.1152	0.45 ±0.057	0.22 ±0.100	0.26 ±0.07
	300	34.2 ±1.527	4.3± 0.100	0.45 ±0.035	0.22 ±0.100	0.26 ±0.021
	330	34.0± 1.509	4.3±0.100	0.45 ±0.051	0.23 ±0.015	0.24 ±0.007
T2 group Ethylacetate of C. crispata	100	33.8 ±0.453	3.7± 0.112	0.40± 0.034	0.23± 0.030	0.25± 0.005
	110	33.8 ±1.915	3.2 ±0.091	0.37 ±0.032	0.22 ±0.024	0.25±0.004
	120	33.8 ±0.627	3.2 ±0.092	0.37 ±0.42	0. 22 ±0.0 20	0.24 ±0.008
Albendazole	500	31.4± 1.152	3.11 ±0.404	0.30 ±0.062	0. 22 ±0.005	0.23 ±0.007
Negative control		32.64 ±1.352	2.93 ±0.264	0.36 ±0.045	0.23 ±1.566	0.24 ±0.010
Positive control		40.24 ±0.956	6.7 ±0.529	0.58 ±0.450	0.27±0.251	0.28 ±0.100
LSD		1.017	0.42	0.47	0.6	0.01

Significant differences, $P \leq 0.05$, n=8 Standard deviations*.

Table 3. Mean of hydatid cysts number in treated mice and the effective dose.

Group	Dose µg/ml	Mean of hydatid cyst number in treated mice						Effective dose (%)
		Liver	kidney	Spleen	Lung	Mesenteric	Total	
T1 group	280	4.5 ± 1.000*	0 ± 0.000	0 ± 0.000	0 ± 0.000	4 ± 0.577	8.5	44.07
(alkaloid of C. crispata	300	4.5 ± 1.000	0 ± 0.000	0 ± 0.000	0 ± 0.000	4 ± 0.577	8.5	44.07
	330	4.1 ± 1.000	0 ± 0.000	0 ± 0.000	0 ± 0.000	3.8 ± 0.577	7.8	48.68
T2 group	100	4.1 ± 1.527	0 ± 0.000	0 ± 0.000	0 ± 0.000	3.12 ± 1.000	7.22	52.63
Ethylacetate of C. crispata)	110	4.1 ± 0.577	0 ± 0.000	0 ± 0.000	0 ± 0.000	2.8 ± 1.000	6.9	54.6
	120	3.6 ± 1.000	0 ± 0.000	0 ± 0.000	0 ± 0.000	2.5 ± 0.577	6.1	59.86
Albendazole	500	3.3 ± 1.000	0 ± 0.000	0.01 ± 0.000	0 ± 0.000	2.6 ± 1.000	5.91	61.18
Negative control		0 ± 0.000	0 ± 0.000	0 ± 0.000	0 ± 0.000	0 ± 0.000	0	0
positive control		6 ± 2.000	0.7 ± 0.000	1.1 ± 1.000	0.3 ± 0.000	0.7 ± 0.003	15.2	0
L.S.D.		0.89	0.39	0.95		1.63		

Significant differences, P ≤ 0.05, n=8, Standard deviations*.

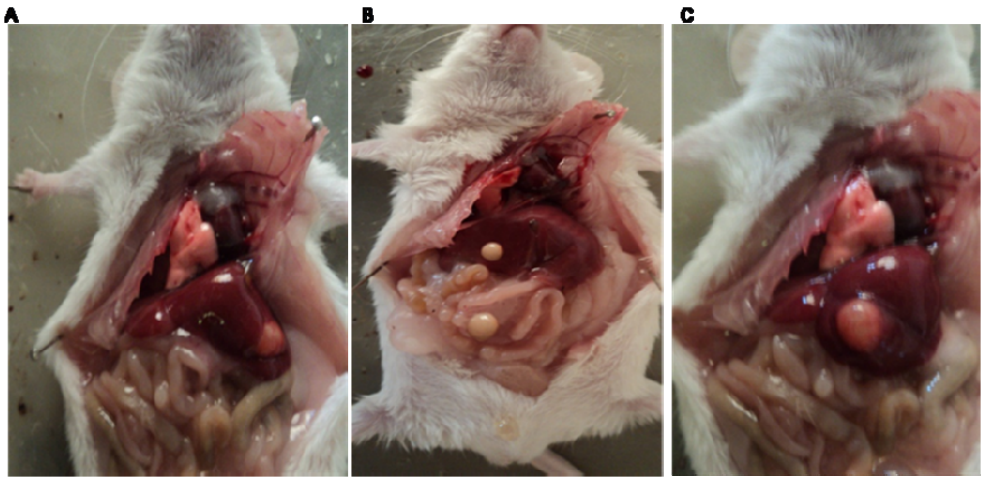

Plate 3. Pictures of treated mice. (A) T2 treated group. (B) Treated with albendazole. (C) T1 treated group in which the numbers, diameters, cysts fluids of hydatid cysts were reduced.

Table 4. Mean of diameter and weight of hydatid cysts of treated mice with bioactive chemical compound.

Group	Dose µg/ml	Mean of diameter/mm	Mean of weight/g
	280	7.4 ± 0.534*	0.75 ±0.055
T1 group (alkaloid of C. crispata	300	7.0 ± 0.64	0.66 ± 0.61
	330	6.1 ± 0.64	0.63 ± 0.069
	100	5.32 ± 0.517	0.67 ± 0.034
T2 group (Ethylacetate of C. crispata)	110	5.1 ± 1.06	0.61 ± 0.045
	120	4.6 ± 0.925	0.55 ± 0.016
Albendazole	500	4.2 ± 0.744	0.53 ± 0.034
Positive control		8.2 ± 1.724	1.22 ± 1.02
L.S.D.		0.908	0.55

Significant differences, P ≤ 0.05, n=8, *rd Standard deviation

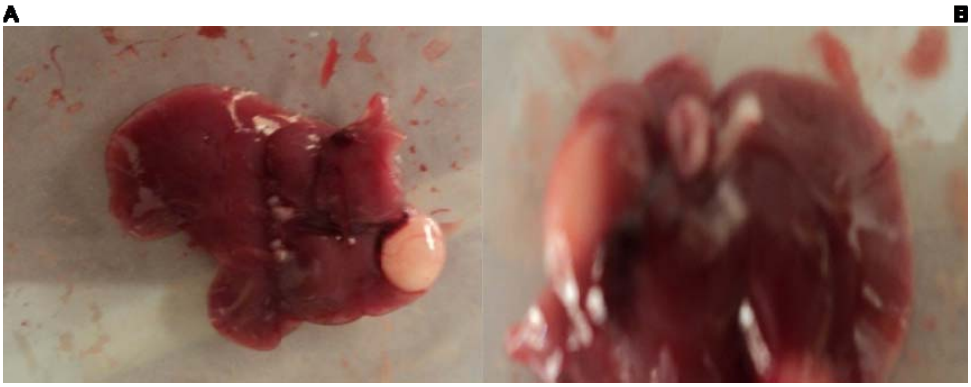

Plate 4. Pictures of treated organs of infected mice with hydatid cysts. (A) Liver from mice treated with 500 µg/ml of albendazole. (B) Liver from mice treated with 120 µg/ml of T4 group.

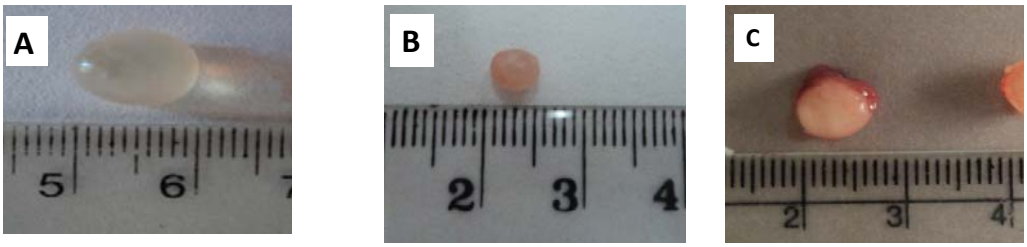

Plate 5. Pictures of hydatid cysts. (A) Positive control. (B) Hydatid cyst treated with albendazole. (C) Hydatid cyst of T2 group.

Conclusions

Overall results of the present study concluded that ethyl acetate extract is more active than alkaloid extract. The bioactive chemical compounds and albendazol decreased the weight of infected animals, and further the weight of organs (liver, spleen, kidney, lung, mesentery), and weight, number and diameter of hydatid cysts more than that of the negative control groups.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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