Antiparasite a ctivity of the microalgae Cyanobacteria *Hapalosiphon aureus* against the protoscolices of hydatid cyst , compared with albendazole drug .

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Abstract

Antiparasite a ctivity of the microalgae *Hapalosiphon aureus* from Basrah river in southern Iraq was studied. Water samples were collected from Shatt-Alarab river in the southern of Basrah , the algae cultured in BG-11 medium. Supernatants , alkaloidic and hexane extracts from biomass are isolated and screened against the Hydatidosis and compered with albendazole drug. The present study has resulted that 2- Methyl - 1-pyrroine and Ethylhexyl phthalate compounds have activity against the protoscolices of hydatid cyst similar to the activity of albendazole from the mean of weight of mice , mean of hydatid cyst number , mean of dimeter and weight of hydatid cyst.

.**Key words :** Algae , bioactive chemical compounds , antiparasite , isolation and identification .

فعالية الطحلب الأخضر المزرق (السيانوبكتريا) *Hapalosiphon aureus* ضد مرض الاكياس العدرية مقارنة بدواء الالبندازول

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المستخلص :

اختبرت فعالية الطحلب الاخضر – المزرق *Hopalosiphon aures* المعزول من انهار البصرة جنوب العراق . جمعيت عينات المياه الحاوية على الطحلب من مياه شط العرب . زرع في الوسط الزرعي BG-11 بعد عزله وتنقيته . اختبرت فعالية المستخلص القلويدي والزيتي للطحلب ضد الطفيلي المسبب لداء العدريات .استنتجت الدراسة الحالية ان المركب القلويدي 2-Methyl 3- pyrroine و المركب الزيتي Ethylhexyl phthalate امتلكا فعالية ضد مرض الاكياس العدرية مشابهة لفعالية دواء الالبندازول وقد لوحظ انخفاض في وزن الفئران المختبرية المعاملة بالمستخلصات الطحلبية وفي عدد الاكياس بالاضافة الى معدل اقطار واوزان الاكياس العدرية .

Introduction :

Microalgae are adiverse group of photosynthetic microorganisms found in the soil and fresh water environments (Metting and pyne , 1986) . They are able to produce a range of biochemical active compounds as antibacterial , antifungal , antivirale , enzyme inhibiting , immunostimulant , cytotoxic , ntiplasmodial activities (G hasemi *etal* ., 2oo4). and Antitrypanosomal (Lorena *etal* ., 2009) . Most of the isolated substances belong to groups of alkaloids , peptides , Tannins , Saponins , Triterpenes and phonols (Molera and semesi ,1996 as wellas carbohychates (Athbi 2010) . Hydatid disease, hydatidosis, cystic echinococcosis , Unilocular hydatid disease and *Echinococcus granulosus* Echinococcosis, all describe infections which are caused by cestodes of genus Echinococcus usually *Echinococcus granulosus* (Dar *etal.* , 1977 ; Akhan *etal* . , 2002 ; Georgopoulos *etal* . , 2007).

Hydatid cyst remains a significant public health problem in 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 , 2003 ; McMnus *etal.*, 2003) . As an endemic disease, it causes social and economic losses for countries. WHO reports state that approximately 100,000 people in the world are infected with this disease every year (Roming,2003) which is 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, more recently, cyst puncture, and percutaneous aspiration, injection of chemicals, and reaspiration (PAIR) are increasingly seen to supplement or even replace surgery as the preferred treatment (Morar and Feldman , 2003)

The screening of microalgae and macroalgae for antibiotics and pharmacologically active compounds received ever increasing interest. A range of pharmacological activities have also been observed with extracts of algae and cyanobacteria as antibacterial , antifungal ,anticancer ,and anti-parasitic compounds (3,4,5,6). They are able to produce a wide range of active substances with antibacterial, antifungal, antiviral, antiplasmodia, enzyme inhibiting,

imunostimulatant and cytotoxic activities (Ghasemi *et al.*, 2004). As well as antiprotoscolices (Khalaf, 2011). Part *et al.* (1944) were the first to isolate an antibacterial substance from *Chlorella which is* mixture of fatty acids, named Chloralin exhibited negative bacteria .Although very little research has focused the extracts of algae as asource of anti-parasitic compounds, recent studies have shown promising antimalarial activity in alga *Laurencia sp* . (Topcu *etal* ., 2003) in addition to their trypanocidal and leishmanicidal activity in *Fucus evanescens , pelvetia babingtonil* , *Ulva lactuca* and *Sargassum natans* (Nara *etal* ., 2000 ; and Orhan etal ., 2006) . The present study were design to examine the *in vivo* activity of bioactive chemical compounds (alkaloids and ethylaacetale) extracted from Cyanobacteria *(Hapalosiphon aureus)* against the protoscolices of hydatid cyst of *Echinococcus granulosus* and compared with albendazole drug.

Materials and methods

Isolation of microalgae

The microalga *Hapalosiphon aureus* is isolated from Shatt-Alarab River in Basra city, southern Iraq from January to April 2012. Primary culturing is done in BG-11 medium. After colonization, pure culture of the living specimens are prepared by using subculturing with agar plate method in Chu – 10 medium (Stein, 1975). Preserved specimens are prepared and the living specimens are incubated in 100 ml – conical flasks. Constant illumination is used at 60 $\mu E\ m^{-2}\ Sec^{-1}$ intensity with white fluorescent lamps. Temperature is 25 ±2°C. The resulted culture is identified based on morphology following taxonomy schemes of Prescott (1975) and Sant 'Anna (2004).

Preparation extracts :

Preparation of extracts is done according to (Reichelt and Borowitazka, 1984) . In N-hexane extract 1 gm of *Hapalosiphon aureus* biomass are extracted by soxhlet continuously with 100 ml of ethyl acetate as solvent for 24 hour and then the extracts are concentrated at room temperature.The alkaloidal extract preparation for take 0.5 gm of dried culture extracted with acidic ethanol (ethanol absolute with 2% acetic acid) for 24 hour in a continuous extraction (soxhlet) apparatus . The extraction are filtered , and ethanol is evaporated on a rotary evaporator under vacuum at a temperature of 45C to a small volume (about a quarter) . Then a small amount of

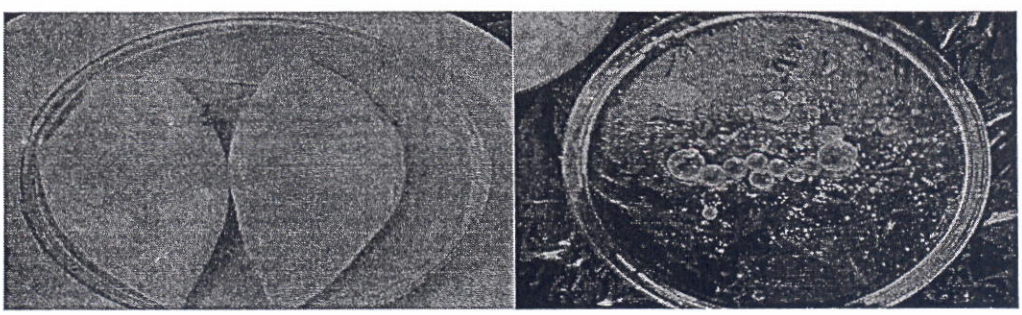
NH_3 (25%) is added to make pH of 9 . Subsequently , 100 ml of chloroform was added and slowly shacked for 10 mintes until alkaloid is separated for water and enter to the chloroform phase . This is repeated from three times and then total chloroform phase was evaporated , yielding a total alkaloid extract are dried under reduced pressure and stored in -20 C for further studes .

Identification of the Biochemical Active Compounds

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

Parasite materials :

Fresh hydatid cysts were obtained by surgery from human infected with hydatid disease from Al- Sadir Teaching Hospital in Basrah city . They were wrapped carefully in clean plastic bags, placed in an ice box, and transported to the Department of Biology, College of Education , Basrah University, where protoscolices were extracted (Smyth , 1964). . *E. granulosus* hydatid cysts containing protoscolices were removed under aseptic conditions from liver and lungs of naturally infected sheep and human. The outer surfaces of the cysts were sterilized with 70% ethanol before being dissected. Protoscolices were extracted according to (Smyth , 1964) (fig. 1).



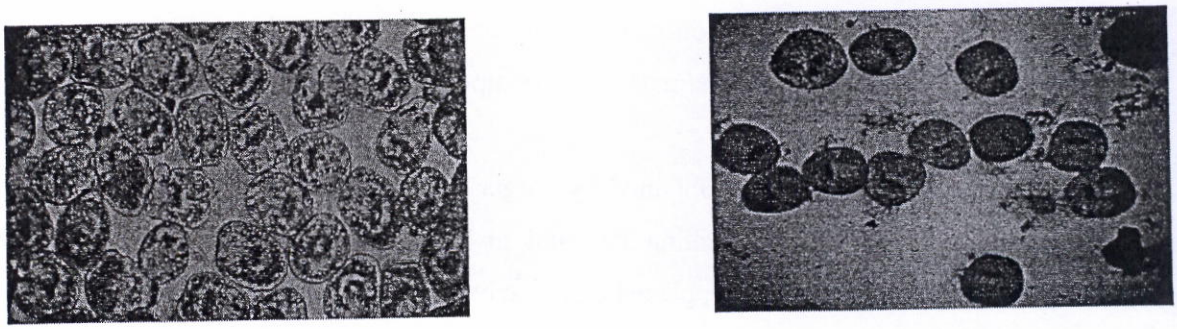
Hydatid cyst removed from human liver

Hydatid sand containing the daughter cyst , brood capsule and protoscolex after aspired from the hydatid cyst

Figure 1 . Hydatid cyst of *Echinococus granulosus*

Estimation of protoscolices viability :

200µl of hydatid fluid and 200µl of 0.1% eosin staining solution were combined in a microtube. After 20 minites incubation, the viability of protoscoleces are assessed by microscopic observation. Stained protoscoleces were considered as nonviable and the protoacoleces,which have not been stained with eosin, were considered as viable according to conventional.(Taran *et al* ., 2009) (fig. 2).



Viable protoscolices Non viable protoscolices

Figure 2 . Viable and non viable protoscolices

The counting of viable protoscolices

Protoscolices were counted according to the method 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 (type Wild No.3) and repeated three times. The viable protoscolices were counted in 1ml based on the formula Viability in 1 ml = number of protoscolices in (10 µl) × 100 .

Injection of mice with protoscolices

A number of male *Mus musculus* mice Balb\C strain were injected with 0.2 ml 480/ (2400/5ml rate of viability) of protoscolices intraperitoneally (I.P.) by 1ml syringe syringe volume). The region of injection was sterilized with 70 % of ethyl alcohol . Al- Humairy (2010). .

ermination of Lethal Dose (LD_{50})

Males of the *Mus musculus* Balb \ C strain were dosed orally to determine LD_{50} by using a stomach tube with bioactive chemical compounds extracted from *H. aureus* (hexane and alkaloid extracts). The animals were monitored for 72 hours and weakness , unstable walking , loss of balance and death were observed during this period . Ingestion started with low dose then continued to high dosages based on (Litchfield and Wilcoxon , 1949) equation.

Experimental design :

Male mice aged 6 – 8 weeks of *M. musculus* Balb\c albino strain used in this study; they were injected inter peritoneal (I.P.) with viable protoscolices of the hydatid cyst and left for six months before treatment. In addition, a positive control group was infected without treatment and the negative control group was left without both of infection and treatment. The *in vivo* study included two parts (Table-1) .

A-Treatment :

It included two groups of (48) infected male albino mice which were treated with bioactive chemical compounds and albendazole together with a positive control (8 infected male mice) and a negative control (8 intact male mice) which are described as follows :

Treated 1 (T1) group :

In this group , 24 infected male mice were dosed orally daily for one month with concentrations (230 , 240 , 250µg / ml) of the alkaloid bioactive compound extracted from *H. aureus* with four pairs of mice for each concentration.

Treated 2 (T2) group :

This group consistes of 24 infected male mice which were treated orally for one month with (125 , 135, 145 µg \ ml) concentration of hexane extracted from *H. aureus* four pairs for each concentration.

Albendazole group :

atistical analysis:

The statistical analysis was conducted by using T- test. The L.S.D. test at 0.05 level was used to analyze differences in the mean of viability of protoscolices treated with bioactive chemical compounds and albendazole in the *in vivo* and studies (SPSS, 1998) .

Results

Classification of alga

Hapalosiphon aureus

It is found in fresh water. True branched filamentous species in two levels from a parent filament. The individual cell of this cyanobacterium has typically a thick, gelatinous cell wall and it is 6-9 µm in diameter and 21 – 24 µm in length (Fig. 3) . They lack flagella, but hormogonia is intercalated as a motile filament and they may move about by gliding along the surface (Anagnostidis and Komarek, 1990) . The growth curve of *H. aureus* was measured by using the spectrophotometer at 650 nm and the lag phase of this algae continued for 2 days, 8 – 10 days in the exponential phase, and 10 – 24 days in the stationary phase. It had a growth constant (K) of 0.44, whereas the generation time (G) was 0.68.

Division: Cyanobacteria

Class : Cyanophyceae

Order :Nostocales

Family : Stigonemataceae

Genus : *Hapalosiphon*

In this group , four pairs of infected male mice were orally and daily treated with 500 µg /ml of albendazole for one month.

Positive control group :

It included four pairs of infected male mice which were left without treatment as a positive control group.

Negative control group :

In this group, four pairs of male mice were left intact without infection and treatment and considered as a negative control group.

The weight of mice and their organs were checked before and after treatment , so the 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 :

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

Mice groups	Number	Treatment	Doses µg\ml
T1 group	24	Alkaloid extract of *H. aureus*	250 230 240
T2 group	24	Hexane extract of *H. aureus*	125 135 145
Albendazole group	8	Treated with Albendazole	500
Positive control group	8	Infected without treatment	
Negative control group	8	Without infection and treatment	
Total	73	- - - -	

Table 1.Experimental albino mice model

Species : *Hapalosiphon aureus* West and G.S. West

Figure 3 . Alga of *Hapalosiphon aureus*

The GC- Mass spectrum of the alkaloid extract of *H. aureus* has recorded three peaks starting with 2- Methyl-1- pyroline which consist 39.40 % (R.T. 4.231min) of the total extract followed by 18- Nonadecen-1- amine (28.61%, 1.443 min of R.T.) as in table 2. Fig.4.

Peak	R.T.	% of total	Compound	M.W.
1	1.443	28.61	- 18- Nonadecen-1- amine	283
2	2.032	27.41	- Acetaamide	59
3	4.231	39.40	- 2- Methyl-1- pyrroline	83.13

Table 2 . Alkaloid Bioactive Chemical Compounds Extracted from *H. aureus*

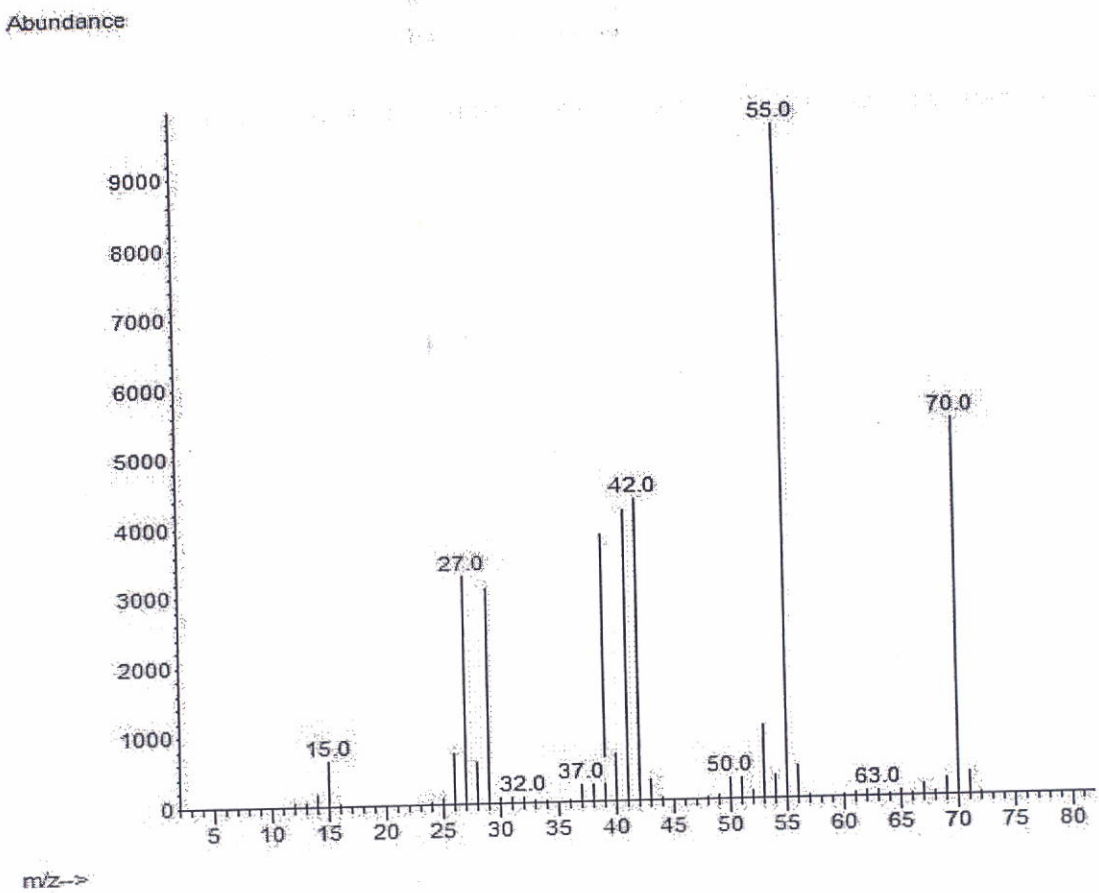


Figure 4 . Mass spectrum of -2- methyl-1- pyrroline

Hexane extract of *H. aureus* :

The analysis of GC – Mass spectrum of the hexane extract of *H. aureus* in the present study shown 14 peaks. Ethylhexyl phthalate consist 30.52 % (26.548 min of R.T.) of the total extract followed by dihydroxyl tecosane 14.74 % (23.240 min of R.T.) table 3. Fig 5.

Peak	R.T.	%of total	Compound	M.W.
1	23.240	14.74	- Di hydroxyl tecosane	214.15
2	24.664	1.35	- Tetradecanoic acid	228
3	26.548	30.52	- Ethylhexyl phthalate	280

Table 3 . Bioactive chemical compounds of hexane extract of *H. aureus*

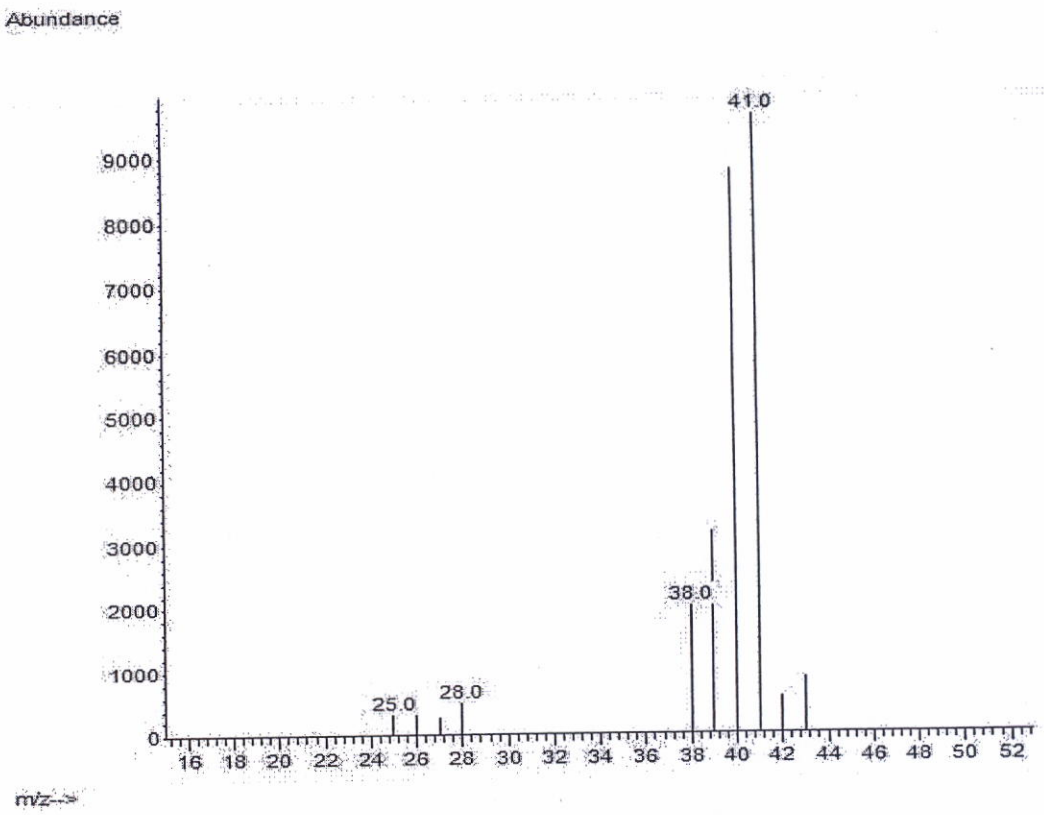
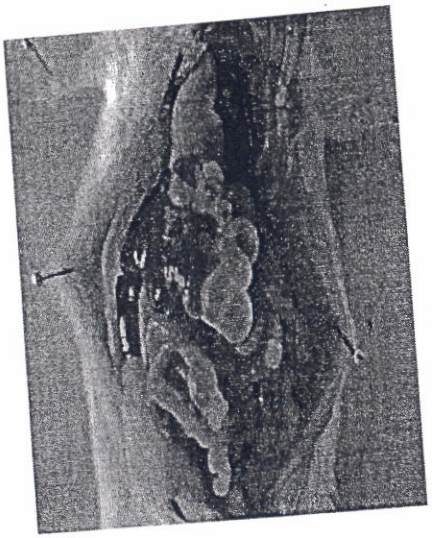


Figure 5 . Mass spectrum of Ethylhexylphthalate

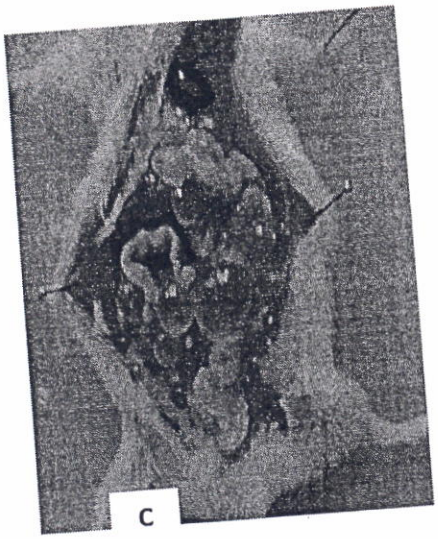
...ital infection with hydatid cysts

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



A

B



C

D

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

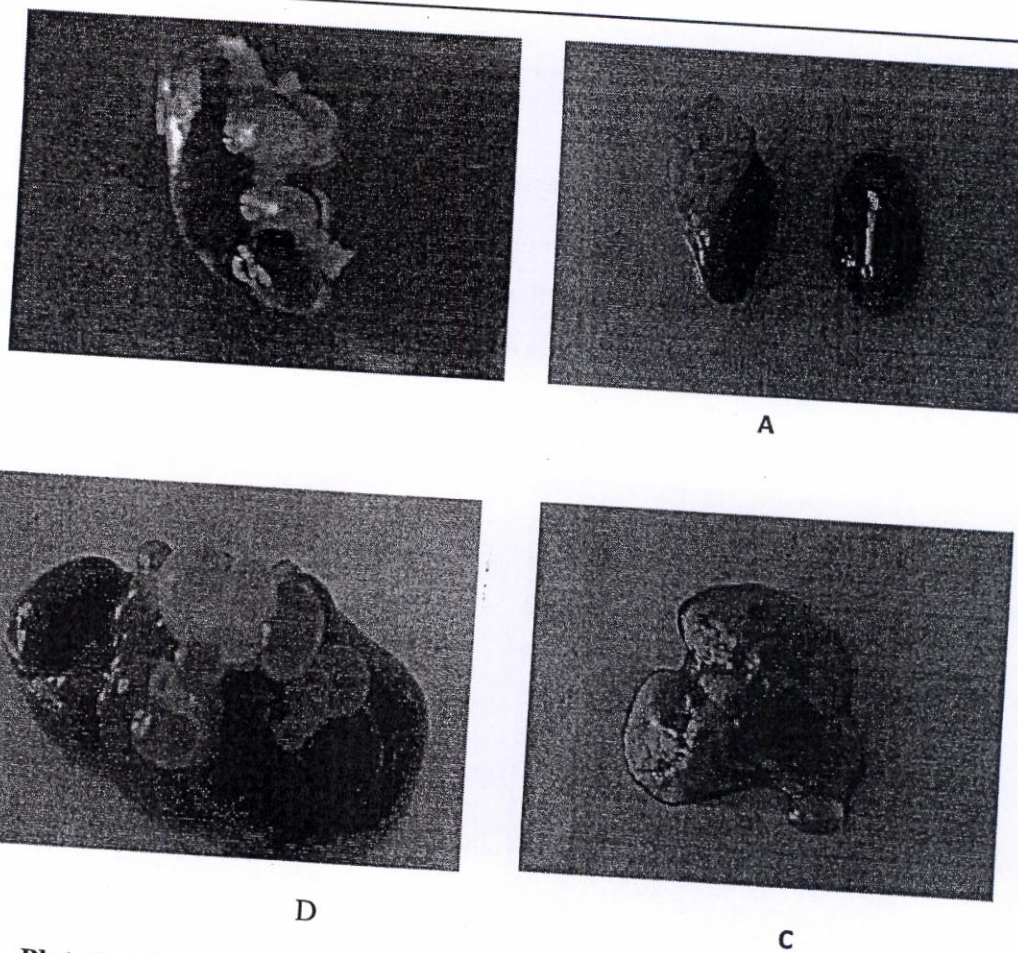


Plate 2 . Pictures of infected mice organs at 6 months – post infection :

A. spleen B . kidney C. lung and D. liver

***In vivo* activity of extracts on the weight of infected and treated mice**

The weight of the negative control group, the positive control group , and the treated groups of mice were checked and the results shown that the weight of the positive control group increase about (40.2 gm) while the weight of the negative control group was 32.64 g and the weight of treated group with albendazole is 31.4g (Plate 2 ; pictures, A,B,C). The T1 group shown 35 g of weight compared with T2 (34g) groups. This means that there are clear significant differences between two groups that responding to the drug. Table (4) shown the weight of organs for each group .

Groups	Doses µg\ml	Mean of weight\ gm				
		Mice	Liver	Spleen	Kidney	Lung
T1 group Alkaloidal extract	230	35.3	4.83	0.50	0.22	0.26
	240	35.3	4.7	0.50	0.22	0.26
	250	35.0	4.5	0.46	0.22	0.26
T2 group Hexane extract	125	34.5	4.1	0.48	0.24	0.25
	135	34.2	3.8	0.46	0.23	0.25
	145	34.0	3.8	0.46	0. 23	0.25
Albendazole	500	31.4	3.11	0.30	0. 22	0.23
Negative control		32.64	2.93	0.36	0.23	0.24
Positive control		40.24	6.7	0.58	0.27	0.28
L.S.D.		1.017	0.42	0.47	0.6	0.01

Significant differences , $P \leq 0.05$, n=8

Table 4 . Mean of weight of mice and organs treated with extracts compared with albendazole

***In vivo* activity of extracts on the number of hydatid cysts in organ of infected mice compared with albendazole**

The number of hydatid cysts decreased after the treatment with extracts of *H.aureus* since the number of hydatid cysts in spleen , lung and kidney decreased to zero (The result is similar to other groups treated with albendazole . Mesenteries and liver have a high number of hydatid cysts than other organs in the positive control group which were 6 and 7.1 hydatid cysts and they decreased after treatment with bioactive chemical compounds extracted from *H.aureus* and albendazole since the

number of hydatid cysts of the liver was 4.5 in the T2 group compared with 3.3 in the group treated with albendazole while the number of mesenteric hydatid cysts decreased to 3 in the T2 group compared with a number of 2.6 of hydatid cysts in the group treated with albendazole (Table 5) .

Groups	Dose µg\ml	Mean of hydatid cyst number in treated mice						Effective dose (%)
		Liver	kidney	spleen	lung	mesenteric	total	
T1 group Alkaloid extract	230	5.8	0	0.1	0	4.5	10.4	31.57
	240	5.6	0	0.03	0	4.5	10.13	33.35
	250	5	0	0	0	4.2	9.2	39.47
T2 group Hexane. Extract	125	4.2	0	0	0	4	8.2	46.05
	135	4.5	0	0	0	3.7	8.2	46.05
	145	4.5	0	0	0	3	7.5	50.6
Albendazole	500	3.3	0	0.01	0	2.6	5.91	61.18
Negative control		0	0	0	0	0	0	-
positive control		6	0.7	1.1	0.3	7.1	15.2	-
L.S.D.		0.89	-	0.39	-	0.95	-	1.63

Significant differences , $P \leq 0.05$, n=8

Table 5 . Mean of hydatid cysts number in treated mice and the effective dose

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

The mean of hydatid cysts diameters was 8.2 mm in the positive control group and was reduced to 4.2mm in the group treated with albendazole , 5.8 mm in the T2 group, and 6.5 mm in the T1 group. The weight of hydatid cysts was also studied for

treated with the bioactive chemical compounds extracted from *H. aureus* as the group treated with albendazole. These results shown a decrease in the weight of hydatid cyst in the T2 group (0.58gm) and (0.68 gm) in the T1 group compared with that group treated with albendazole recording 0.53gm where positive control group was 1.22gm in mean(Table 6).

Groups	Dose µg\ml	Mean of diameter\mm	Mean of weight \gm
T1 group Alkaloidal extract	230	7.2	0.81
	240	6.5	0.7
	250	6.5	0.68
T2 group Hexane extract	125	6.32	0.72
	135	6.2	0.66
	145	5.8	0.58
Albendazole	500	4.2	0.53
Positive control		8.2	1.22
L.S.D.		0.908	0.55

Significant differences , $P \leq 0.05$, n=8

Table 6 . Mean of diameter and weight of hydatid cysts of treated mice with bioactive chemical compound

DISCUSSION:

The er nce of hydatid disease in th ld implies serious losses. The usage of commercial antibiotics for hydatid disease treatment produces undesirable side effects (Kern , 2003) . Natural products are the source of therapies since the advent of traditional medicine and healing remain a dominant source till now. The World Health Organization (WHO) estimates that 80% of the world's inhabitants depend mainly on traditional medicine for their primary health care. Algae such as

cyanophyta and chlorophyta are rich sources of bioactive chemical compounds as secondary metabolites (Rodrigues *etal.*,2004 and Tuney *etal.*, 2006) .

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 *Udotea flabellum* and *Halimeda sp.*, respectively, and the sesquiterpenoid rhipocephanal 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 *etal.*,2000; Radwan *etal.*,2007; Carrol *etal.*, 2007; and Everton *etal.*, 2009). Al – Nasir (2010) isolated an alkaloid chemical compound from *H. aureus* which was similar in structure to calothrixin and had *in vitro* antibacterial activity .

The weights of infected experimental animals were examined at 6^{th} month – post infection in the present study and results have shown an increase in the weight of experimentally infected animals compare with to the negative control group . The weight of liver and spleen also increased. On the other hand, there were slight increases in the weight of kidneys and lungs (Table,4). These results agree with other studies (Al – Nasiri , 2006 ; Al- Mobarek, 2006 ; Barzanji *etal.* , 2009 and Al-Humairy , 2010) . The presence of a large number of hydatid cysts in different weights and diameters (liver , spleen , kidneys , lungs and mesenteries) and hepatospleenomegaly caused by the parasite leads to the increase in weight 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 *etal.* (2003) concluded that the increase in liver and lungs weight was due to the formation of granuloma and the increase in the immune cell and its migration to the target organ , so the ability of spleen to produce lymphocyte and the division of spleen cells to secrete specific antibody had led to the increase in the weight of the spleen.

Bioactive chemical compounds have revealed an activity on the weight of the infected animals and their organs. Further more the weights and diameters of hydatid cysts were also affected because the weight has decreased and approached that of the negative control group especially in theT2 group treated. The decrease in weight may be explained in relation to the decrease in the number of hydatid cysts and hydatid cysts calcification with the sloughing of the germinal layer and the disintegration of laminated layer (Maizeles and Yazdanbakhsh, 2003) since the complex layer of cyst has an important role in the transformation of nutritional material from serum to cyst. The knowledge of the parasite nutrition behavior can help us to drug treatment in the inoperative cyst via the selection of the effective drug and the adherence of them to biological material that promote distribution of drug to the cyst (Rahdar *etal.*, 2008). Compare with the bioactive chemical compounds , albendazole decreased the weight of infected experimental animals more than those of the negative control group since albendazole affected the weight of intact experimental animals in the study conducted by Ahmed (2009).

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