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ANTIPROTOSCOLICES ACTIVITY OF NONADECOIC ACID ; PHTHALIC ACID, DIFLOROPHENYL UNDECYL STER AND 1,2- BENZENDICARBOXYLIC ACID , BIS (2-ETHYLHEXYL) ESTER EXTRACTED FROM CLADOPHORA CRISPATA AND HAPALOSIHON AUREUS COMPARED WITH ALBENDAZOLE

Amal ,KH. KHalaf* ; Sabeeh ,H.Al-Mayah **
and Ahmed , M. Athbi**

ABSTRACT:

The present study has aimed the hydatid disease , which is endemic in Iraq especially in Basrah city . Fatty acids compound (Nonadecoic acid ; Phthalic acid, diflorophenyl undecyl ster and 1,2- Benzendicarboxylic acid , bis (2-ethylhexyl) ester) extracted from *Cladophora crispata* and *Hapalosihon* have used and compared with albendazole . The results of the present study have found that compounds have activity against the protoscolices of hydatid cyst similar to the activity of albendazole and in low concentration .

INTRODUCTION :

There are more than 50 different organisms that may be transmitted from dog to man . *Echinoccocus* is one of these organisms , the causative agent of Echinoccocosis and is a major world zoonosis affecting humans as well as domestic animals (1). The word echinococcus originates from greek meaning “hedgehog berry” a term descriptive of gross pathology of lesion. Hydatid is also a Greek word meaning “a drop of water”. This disease process probably was known to Hippocrates who described “liver….Filled with water” (2). Until recent decades, 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 (1). The undesirable side effects associated with this classical drug, as well as the development of resistance, are encouraging research into alternative synthetic or natural compounds effective for the treatment of hydatid disease . 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). In this regard , the present study has tried to test the effects of bioactive chemical compounds extracted from *Cladophora crispata* on viability of protoscolices of

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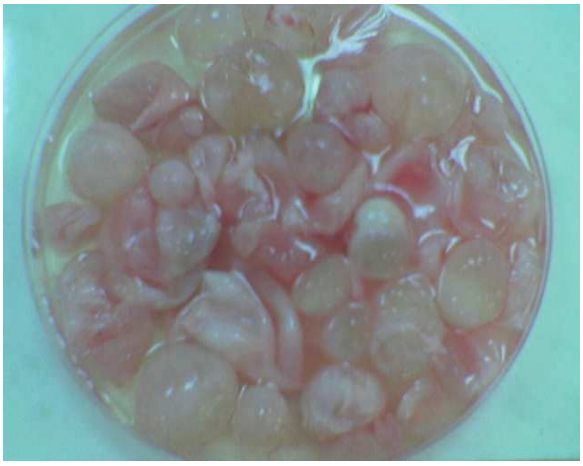
hydatid cyst *in vitro* compared with albendazole .

MATERIALS & METHODS

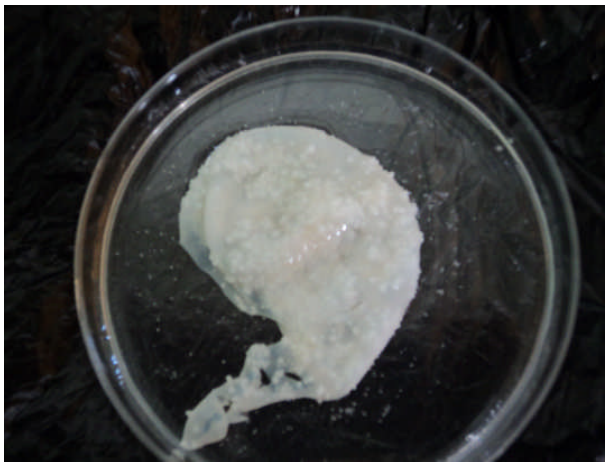
-Parasite materials:

Fresh hydatid cysts were obtained by surgery from livers and lungs of human infected with hydatid disaese 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 protoscoleces were extracted.

E. granulosus hydatid cysts containing protoscoleces were removed under aseptic conditions from liver and lungs of human. The outer surfaces of the cysts were sterilized with 70% ethanol before being dissected. Protoscoleces were extracted according to (7)method.



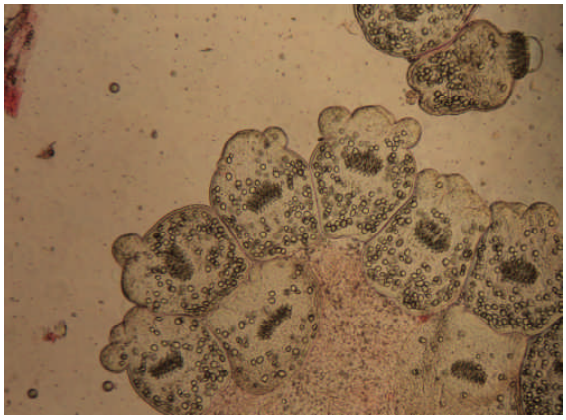
Infected Lung of human containing hydatid cysts



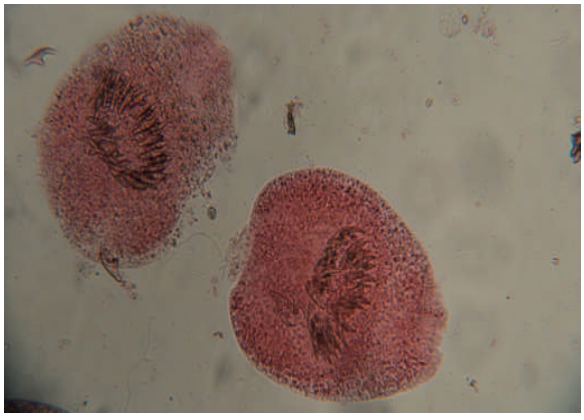
Germinal layer containing protoscoleces removed from hyhatid cyst

- Estimation of protoscolices viability :

There after 200µl of hydatid fluid and 200µl of 0.1% eosin staining solution were combined in a microtube. After 20 min incubation, the viability of protoscoleces were assessed by microscopic observation. Stained protoscoleces were considered as nonviable and the protoacoleces,which had not stained with eosin, were considered as viable according to conventional.



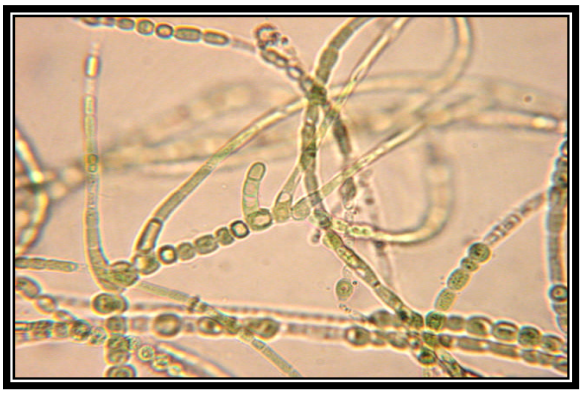
Viable protoscolices



Non viable protoscolices

- Chlorophyta and Cyanophyta species :

Chlorophyta and Cyanophyta were collected from different locations in Basrah city. Cyanobacteria and green algae were collected by 500 ml plastic jars, transferred to the laboratory to declare each chlorophyta and cyanophyta were found. Chlorophyta (*Cladophora crispata*) were collected as biomass from Garmat Ali. In Basrah city , washed with a much amount of distilled water to remove all extraneous matters, and left to dry with room temperature and then more dried was in 60°C for24 hours, grounded and kept in plastic sack until use. Cyanophyta (*Hapalosiphon aureus*) were cultured by using Chu – 10 medium , briefly , jars of 5 liters were filled with 3 liters of liquid medium , inoculated with desired cyanobacteria, and then transferred to growth chamber at 30-35 ^{0}C. Cyanobacteria was harvested at the end of logarithmic phase by using GFA pre weighed filter paper and centrifuge methods . Freeze – dried weighted again to reach a fixed weight of dried cyanobacteria .



Hapalosiphon aureus

Cladophora crispata

- Preparation of extracts :

Preparation of the extracts was done according to (8) with some modification as follows:

- Preparation of Fatty acid:

Cyanobacteria and green algae were continuously extracted by soxhlet using 250 ml of hexane for 24 hours, and then the extracts concentrated at room temperature.

- Preparation of methanol extract :

The methanol extracts to be prepared; dry mass in ratio (1: 15 g/ml) was extracted using magnetic starrier through 24 hours. The precipitates were removed by filtration and left to dry until use, and then the filtrates were concentrated at room temperature.

Design of experiment :

The effect of bioactive chemical compounds were studied *in vitro* compared with albendazole after determination of viability of protoscolices , lethal concentrations were chose from LD_{50}, *In vitro* study was designed based on (8,9) methods as following :

1. Three concentration from each bioactive chemical compounds extracted as describe previously (methanolic , and hexane extract) and three concentration of albendazole , each of them were added alone to test

tube containing 4 ml of Kreb ́ s ringer maintain medium .

2. The suspension of protoscolices were shaking and added to test tubes containing bioactive chemical compound in volume of 1 ml for each tube , approximately 2000-2500 of protoscolex based on the viability counting .
3. control group was prepared with each experiment and include a test tube containing hydatid cyst fluid (Kreb ́ s ringer mention medium + hydatid sand , 4:1) with the same viability .
4. The viability of protoscolices was observed from the fist hour continuously for seven days and repeatedly three times for each concentration to calculate the mean of viable protoscolex .

-Methanol extract :

Methanolic extract from *Cladophora crispata* in (230 , 240 , 250 µg \ml) concentrations was used to kill the protoscolices maintained in Krebs ringer medium and the viability was calculated based on (8) .

- Hexane extracts :

Two extracts of hexane were used *in vitro* against the protoscolices of hydatid cyst . (150 , 160 , 170 µg \ml) concentrations of hexane compound extracted from *Cladophora crispata* and (125,135,145, µg\ml) concentrations from *Hapalosiphon aureus* .

- albendazole :

Three concentrations (250 , 500 , and 1000 µg \ ml) of albendazole drug were chose *in vitro* against the protoscolices of hydatid cyst for comparison with bioactive chemical compound extracted from *Cladophora crispata* and *Hapalosiphon aureus* .

- GC-Mass spectra analysis:

GC-Mass spectra of fraction was done in Bruker company , Iran and
Al- Elbait university in Jordin.

- **Statistical analysis :**

Statistical analysis was done using analysis of variance (ANOVA) and L.S.D. test at 0.05 was used to analyze differences in the mean of viability of protoscolices treated with bioactive chemical compounds and albendazole . (SPSS, 10)

RESULTS :

Testing of methanol extract of Cladophora crispata:

Methanol extract of *Cladophora crispata* recorded high activity at 250 µg\ml after 5 – days post treatment , while 230 µg\ml and 240 µg\ml had activity after 6 days – post treatment since the protoscolices still viable after 5 days – post treatment recording 6.2 and 2.3 mean of viability **. Table(1)**

Table (1):Viability of protoscolices treated with methanol extract of *Cladophora crispata*

Concentration\ time of treatment	Mean of viability								
	1 h	4 h	1 day	2 days	3 days	4 days	5 days	6 days	7days
230 µg\ml	79.33	58.33	42	31.66	20	13.66	6.66	0	0
240	76.33	55	38.66	26.33	15.66	9.33	3.33	0	0
250	69.33	54.33	37.66	25.33	13	8	0	0	0
Control	95.66	92.66	91	88.33	82.66	75.66	71.33	64.66	60.33
L.S.D.	0.854								
Significant differences , P ≤ 0.05									

3.2.5. Testing of hexane extract of *Cladophora crispata:*

170 µg\ml of hexane extracted from *Cladophora crispata* revealed an activity against the protoscolices of hydatid cyst after 5 days – post treatment while 160 µg\ml record 5.3 mean of viability after 5 days – post treatment and the activity of 150 µg\ml was observed after 7 days – post treatment. Table(2)

Table(2) :Viability of protoscolices treated with hexane extract of *Cladophora crispata*

Concentration\ time of treatment	Mean of viability								
	1 h	4 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days
150 µg\ml	80.3	65.3	54.6	40.3	23.66	11.33	6.66	2.66	0
160	78	63.3	53	38	22	10.33	4.66	0	0
170	76.6	61.6	51.6	35.6	19.66	8.66	0	0	0
Control	95.6	92.3	88	84.3	80	76.6	69.33	64	59.3
L.S.D.	0.765								
Significant differences , P ≤ 0.05									

Testing of hexane extract of *Hapalosiphon aureus* :

Three concentrations (125, 135, 145, µg\ml) of hexane extract of *Hapalosiphon aureus* show activity after 6 days – post treatment and this explain the hexane extract has high activity than alkaloid extract of the same cyanophyte and when compared with hexane extract of *Cladophora crispata* is less . **Table(3)**

Table (3) : Viability of protoscolices treated with hexane extract of *Hapalosiphon aureus*

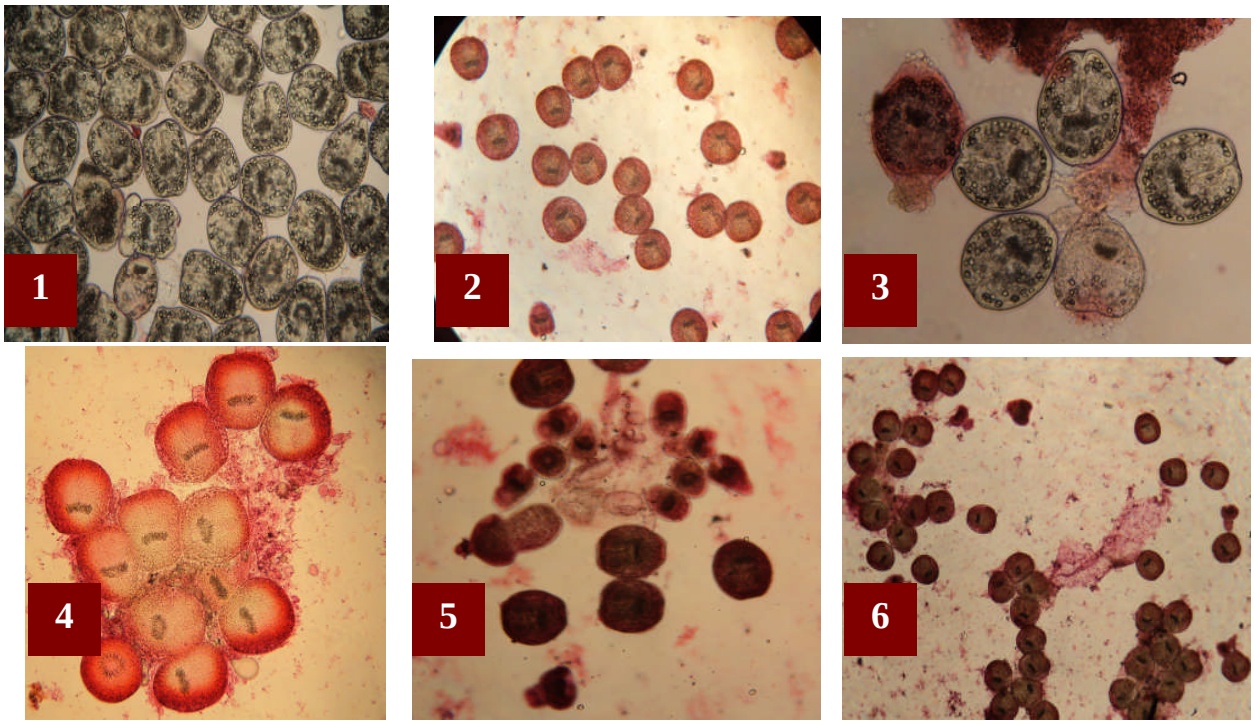
Concentration \time of treatment	Mean of viability								
	1 h	4 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days
125 µg\ml	82.33	68.66	55	44.33	32.66	23.66	13.66	6.66	0
135	80.66	66	52.66	43	30	20.66	11	4.66	0
145	77.33	60.66	51.66	40.66	28.66	18.33	9.66	0	0
Control	95	91.66	88.33	85.33	80.66	75	70.66	65.66	59.66
L.S.D.	0.666								
Significant differences , P ≤ 0.05									

Testing of albendazole activity :

High activity of albendazole has revealed at 1000 µg\ml after 3 days – post treatment while 500 µg\ml concentration of albendazole has recorded 7.6 mean of viability after 4 days – post treatment and high activity after 5 days – post treatment . Table(4)

Table (4) : Mean of viability of protoscolices treated with albendazole

Concentration\ time of treatment	Mean of viability								
	1 h	4 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days
250 µg\ml	85.6	71	54.6	46.3	32.6	20	9.3	0	0
500	79	63.66	49.6	33.6	19.6	7.6	0	0	0
1000	74	47	30	12.33	0	0	0	0	0
Control	96.3	92.3	90.6	86	81	74.3	70	66.3	59.6
L.S.D.	0.88								
Significant differences, P ≤ 0.05									



Pictures of treated protoscolices where :

1, control group (viable protoscolices).

2, protoscolices treated with Phthalic acid,3,5- diflurophenyl, undecyl ester after six days post treatment

3, protoscolices treated with Nonandioic acid , dimethyl ester after one days post treatment

4, protoscolices treated with 1,2-Benzendicarboxylic acid , bis(2-ethylhexyl) ester after seven days post treatment

5, protoscolices treated with albendazole after six days post treatment

6, protoscolices treated with Nonandioic acid , dimethyl ester after six days post treatment

GC – Mass analysis of Extracts :

The methanolic , and hexane extracts of *Cladophora crispata* and alkaloid , hexane of *Hapalosiphon aureus* were subjected to GC – Mass spectroscopy analysis as follow :

- Methanol extract of *Cladophora crispata* :

GC – Mass spectrum of methanol extract has recorded 22 peak , Phthalic acid, 3,5-diflurophenyl , undecyl ester consist 22.57% (R.T. 22.647) of total extract followed by ethyl linoleolate (13.13% and 25.946 min of R.T.) , other compounds have tabled below :

Peak	R.T.	% of total	Compound
1	22.647	22.57	- Phthalic acid,3,5- diflurophenyl, undecyl ester
2	25.946	13.13	- Ethyl linoleolate
3	20.264	3.26	- Phytol
4	22.33	2.73	- 2.6,6- Trimethyl- bicycle [3,1,1] hept-3-ylamine
5	32.411	1.65	- Diterpine

R.T: retention time , M.W. molecular weight

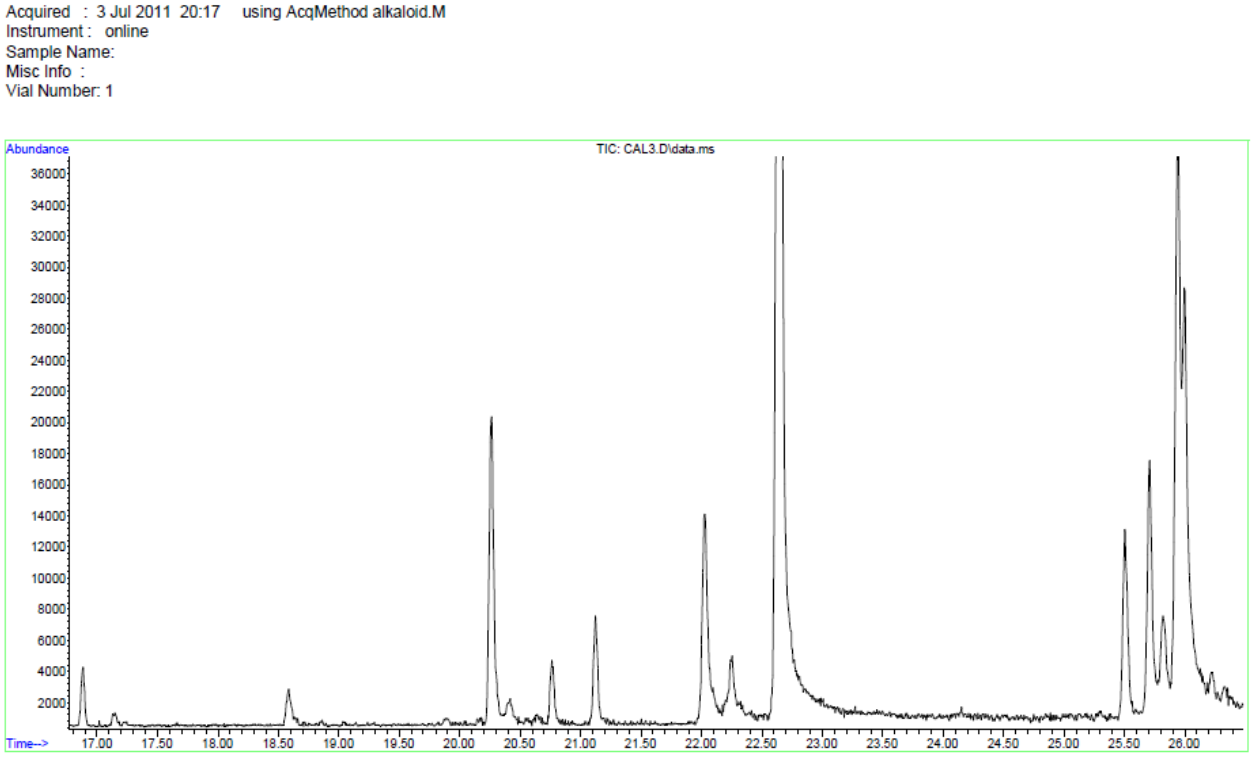


Figure (1) : GC-Mass spectrum of methanol extract of *Cladophora crispata*

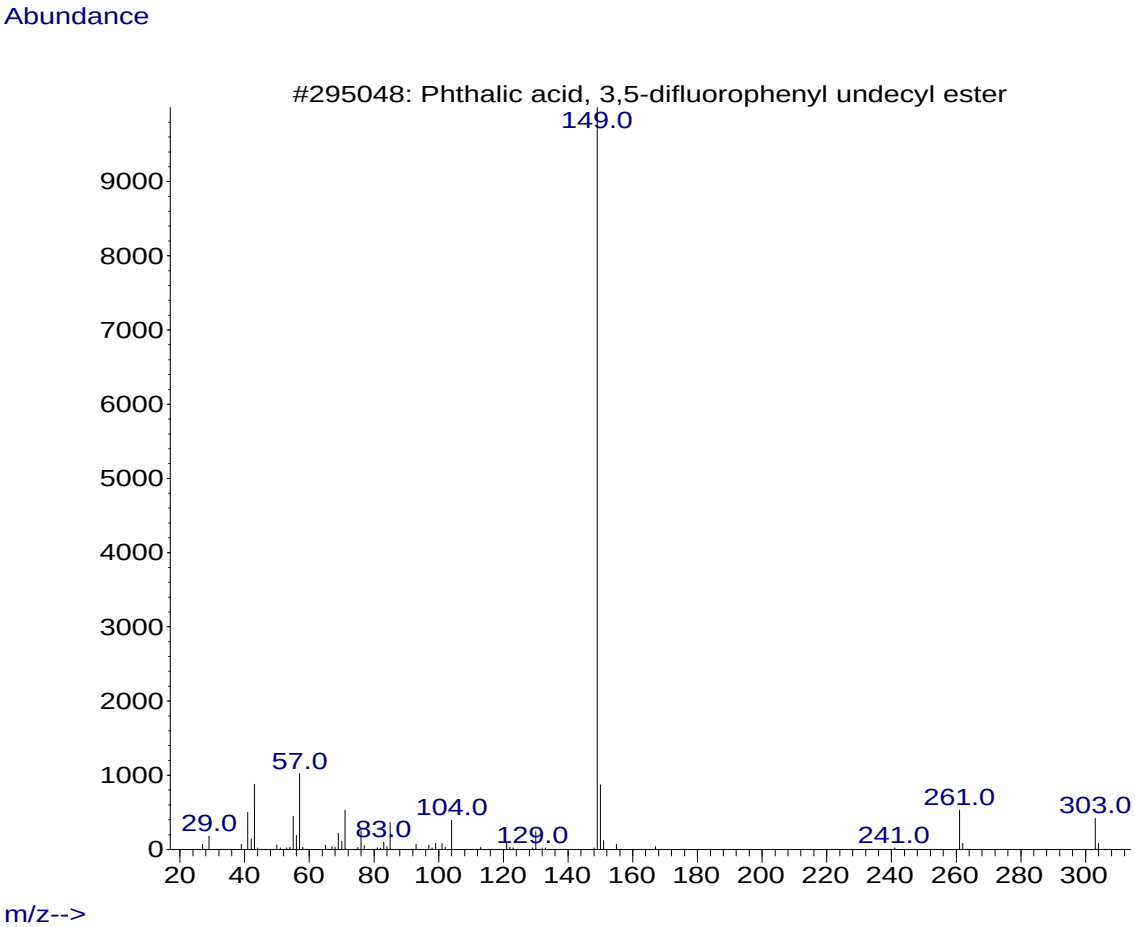


Figure (2) : Mass spectrum of Phthalic acid ,3,5-diflurophenyl undecyl ester compound

Hexane extract of *Cladophora crispata* :

GC – Mass spectrum (Fig,4) of hexane extract of *Cladophora crispata* revealed that are 27 peak in different size and six of them arranged based on the percentage of the total extract and the results of spectrum showed Nonandioic acid, dimethyl ester (C11H20O4) consist 12.28 % of total hexane extract followed by hexadecanoic acid (12.03 %) as the following :

Peak	R.T.	% of total	Compound	M.W.
1	25.951	12.28	-Nonandioic acid , dimethyl ester	216
2	22.673	12.03	-Hexadecanoic acid	256
3	18.628	4.63	- 3,7,11,15,tetramethyl- 2- hexadecan-1-ol	296
4	30.362	3.42	- Isopropyl palmitate	298
5	27.02	3.27	- Eicosanoic acid	312
6	34.905	2.26	- 9 – octadecanoic acid	282

R.T.: retention time , M.W. : molecular weight

Figure (3) Chemical structure of Nanondiocoic acid , dimethyl ester

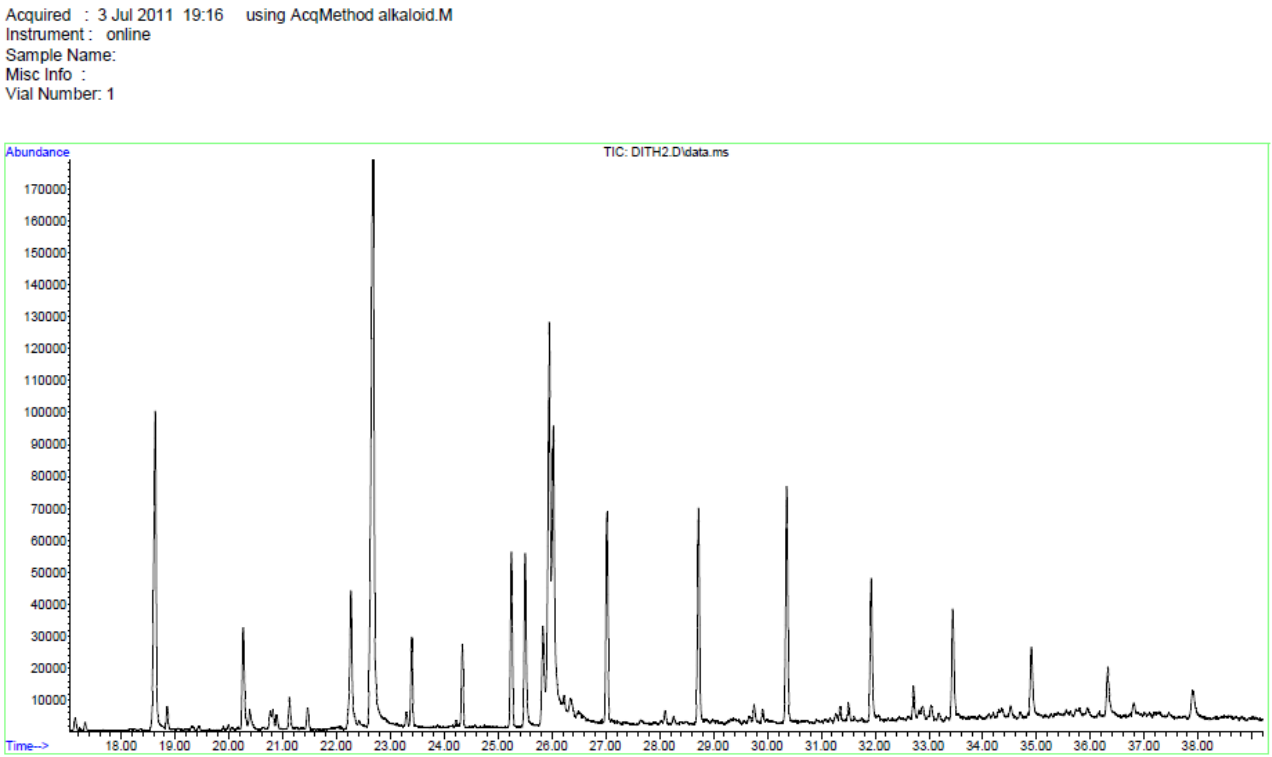


Figure (4) : GC – Mass spectrum of hexane extract of *Cladophora crispata*

- Hexane extract of *Hapalosiphon aureus* :

Analysis of GC – Mass spectrum of hexane extract of *Hapalosiphon aureus* in the present study showed 14 peak (fig,5) and five of them consist high percentage of the total hexane extract , 1,2-Benzendicarboxylic acid , bis(2-ethylhexyl) ester consist 30.52 % (26.548 of R.T.) of total extract followed by 4- Acetylbutric acid which consist 14.74 % (23.240 of R.T.) and other have described as follow :

Peak	R.T.	%of total	Compound
1	26.548	30.52	- 1,2- Benzendicarboxylic acid , bis (2-ethylhexyl) ester
2	23.240	14.74	- 4- Acetylbutyric acid
3	27.141	2.68	- Dibutyl phthalate
4	30.660	1.51	- 2L, 4L- Dihydroxyeicosane
5	24.664	1.35	- Tetradecanoic acid

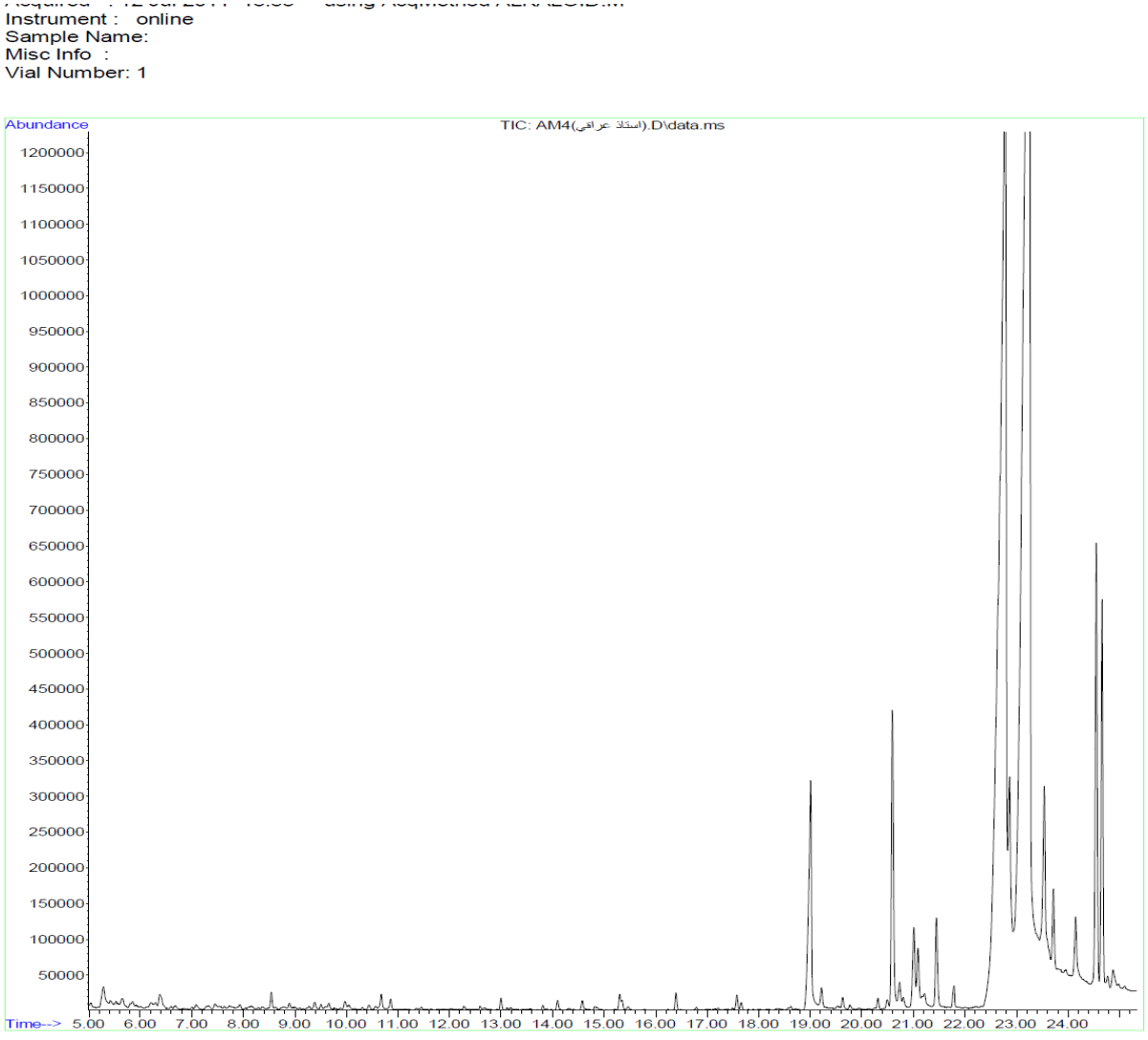


Figure (5) : GC-Mass spectrum of hexane extract of *Hapalosiphon aureus*

Discussion :

Emergence of hydatid disease in the world implies serious loses. Usage of commercial antibiotics for hydatid disease treatment produces undesirable side effects (10) . Natural products have been the source of therapeutics since the advent of traditional medicine and healing, and remain a dominant source to date. The World Health Organization (WHO) approximates that 80% of the world's inhabitants depend mainly on traditional medicine for their primary health care. Chlorophyta and Cyanophyta are rich source of structurally novel biologically active metabolites. Cell extracts and active constituents of various algae may be potential bioactive compounds of interest in the pharmaceutical industry (11 ,12) .

Three concentrations of methanol extract of *Cladophora crispata* were used in the present study and only one concentration has *in vitro* activity against the protoscolices of hydatid cyst after 5 days – post treatment but the other two concentrations showed activity after 6 days – post treatment , this means the time has played an important role in treatment since decreased concentration leads to increase the time of treatment . Comparison between *in vitro* activities of methanol extract of *Cladophora crispata* and albendazole revealed that each of them record activity after 5 days – post treatment but methanol extract in one concentration. The activity of methanol extract returned to the presence of Phthalic acid,3,5-diflurophenyl, undecyl ester compound as GC- Mass spectrum showed in figure (1) .

Testing *in vitro* and *in vivo* activities of two species hexane extracts of taxa studied, showed *Cladophora crispata* , and *Hapalosiphon aureus* were positive. Hexane extract of *Cladophora crispata* has showed *in vitro* activity after 5 days – post treatment similar to of ethyl acetate extract and slightly different from hexane extract of *Hapalosiphon aureus* where recorded activity after 6 days – post treatment . The analysis of GC-Mass spectra of hexane extracts of each *Cladophora crispata* and *Hapalosiphon aureus* has detected that Nonandecoic acid, dimethyl ester consisted the high percentage of the total hexane extract of *Cladophora crispata* and 1,2-Benzendicarboxylic acid , bis (2-ethylhexyl) ester consisted high percentage of the total hexane extract of *Hapalosiphon aureus* . The activity of hexane extracts were tested previously as antibacterial and antifungal by (13) , whereas the activity of hexane and other extracts of *Cladophora crispata* and *Hapalosiphon aureus* have tested as antiprotoscolex for the first time

Like other parasitic tape worm , this organism cannot be synthesise most of the lipids it requires , so these molecules must be aquired from the environment or from the host (14) . A 15 kDa protein identified as a marker of the asexual reproductive phase was shown to be involve in acquisition , storage and transport of lipid . This protein, termed EgFABp1 (*E. granulosus* fatty acid binding protein) , belong to a family of proteins known to bind fatty acids , although their precise role is not known and the full picture of function is still unclear (15) . However , their importance for fatty acids uptake and transport has been demonstrated and they have proposed to be involved in transport of fatty acids to specific metabolic pathways , modulation of gene expression , cell growth and differentiation , regulation of enzymes activities , promotion of cellular uptake and utilization of fatty acids , and regulation of signal transduction (16,17,18) and it is possible that fatty acids

will bind to the carrier proteins and penetrate the membrane of the organism without being transported into the cell. This could cause high local concentrations of fatty acids within the membrane resulting in disruption of its structure. The concentration of fatty acid at which disruption occurs would depend on the nature of the acid, the structure of the membrane and the ability of the transport system to recognize various fatty acids (16,18) . Further, Fatty acids may inhibit the uptake of oxygen leading to reduce the building of ATP and then death of organism, or fatty acids may acts as uncoupling agents that change the permeability of membranes to protons leading to inhibition in ATP synthesis (19).

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الفعالية الضد الرؤيسات الاولية للمركبات الدهنية (Nonadecoic acid و Phthalic acid, diflorophenyl undecyl ster و (Benzendicarboxylic acid , bis (2-ethylhexyl) ester المعزولة من الطحلب الاخضر *Cladophora crispata* والطحلب الاخضر المزرق *Hapalosiphon aureus* مقارنة بالالبندازول

*** امل خضير خلف و أ. د. صبيح هليل المياح و أ.م.د. احمد محسن عذبي**

المستخلص :

استهدفت الدراسة الحالية مرض الاكياس العدرية ، وهو من الامراض المتوطنة في العراق وخاصة في محافظة البصرة اذ استخدمت المركبات الدهنية (Nonadecoic acid و Phthalic acid, diflorophenyl undecyl ster و (Benzendicarboxylic acid , bis (2-ethylhexyl) ester المعزولة من الطحلب الاخضر *Cladophora crispata* والطحلب الاخضر المزرق *Hapalosiphon aureus* مقارنة بالعقار التقليدي الالبندازول المستخدم في علاج داء الاكياس العدرية. وجدت الدراسة الحالية بان هذه المركبات تمتلك فعالية ضد الرؤيسات الاولية المكونة للسائل العدري ومقاربة الى فعالية الالبندازول وبتراكيز اقل .

*** فرع الاحياء المجهرية ،كلية الطب ،جامعة ذي قار**

**** قسم علوم الحياة ، كلية التربية ، جامعة البصرة**