IN VITRO ACTIVITY OF ALKALOIDS EXTRACTED FROM CHLOROPHYTA AND CYANOPHYTA AGAINST THE HYDATID DISEASE COMPARED WITH ALBENDAZOLE

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

ABSTRACT:

The activity of alkaloids compound extracted from Chlorophyta and Cyanophyta were test against pathogenic bacteria and fungi without parasites ,thus, the present study has aimed to test the activity of alkaloids as antiparasite for the first time in Iraq and against Hydatidosis among the world .The present study has resulted that 2-(N,N-dimethylhydrazino)cyclohexanecarbonitrile compound has activity against the protoscolices at five days –post treatment in vitro and in low concentration from that used with albendazole.

INTRODUCTION :

Hydatid disease, hydatidosis, cystic echinococcosis , Unilocular hydatid disease , *Echinococcus granulosus* Echinococcosis , and Al - akyas al-mai'yah' ; 'al atash' (in Arabic) all terms describing infections which are caused by cestodes of genus Echinococcus usually *Echinococcus granulosus* (1 ; 2 ; 3). The dogs are the definitive host and the adult worms are found in their small intestine. Humans get infected either by contact with the definitive host or by consuming vegetables and water contaminated with the hydatid ova (4). 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 (5 ;6) . As an endemic disease, it causes social and economic losses for countries. WHO reports stated that approximately 100,000 people in the world are infected with this disease every year (7) which is common in rural population of underdeveloped countries because of their close association with domestic and wild animals (8) . 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 (9). 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 . In this

* **Microbiology department, college of medicine , university of Thi-qar.**
** **Biology department , college of education , university of Basrah.**

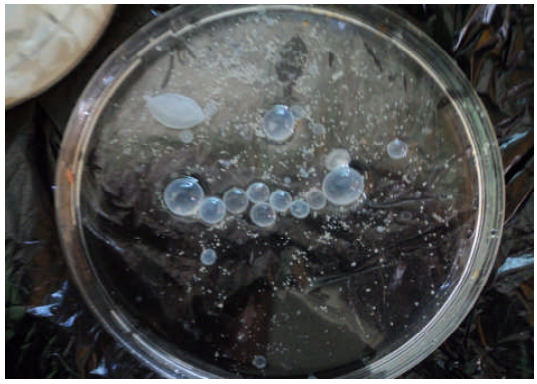
regard, most studies have been focused on activity of natural substances derived from chlorophyta and cyanophyta , mainly due to their accessibility and use in traditional medicine . A range of pharmacological activities have also been observed with extracts of chlorophyta and cyanophyta as antibacterial , antifungal ,anticancer ,and anti-parasitic compounds (10; 11; 12; 13; 14; 15). The present study were design to examine the *in vitro* activity of bioactive chemical compounds (alkaloids) extracted from chlorophyta (*Cladophora crispata*) and cyanophyta (*Hapalosiphon aureus*) against the protoscolices of hydatid cyst of *Echinococcus granulosus* and compared with albendazole .

MATERIALS & METHODS

-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. *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 (16) method.

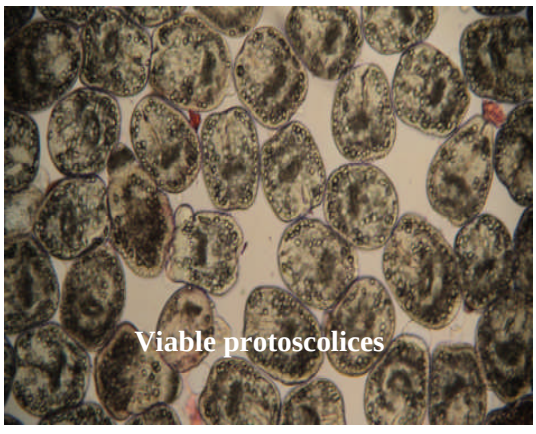
Hydatid cyst removed from human liver

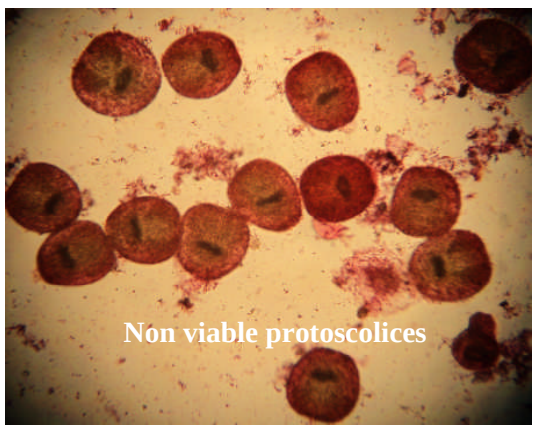


Hydatid sand containing the daughter cyst , brood capsule and protoscolex after aspired from the hydatid cyst

- 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.





- 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 .

-Preparation of extracts :

Preparation of the extracts was done according to (17) with some modification as follows:

- Preparation of ethyl acetate extract :

10 gm of *Cladophora crispata* biomass were extracted by soxhlet continuously with 250 ml of ethyl acetate as solvent for 24 hour .

- Preparation of alkaloids:

The dry mass of samples (*Cladophora crispata* and were grinded and then were extracted with acidic ethanol (ethanol absolute with 2% acetic acid) for 24 hour in a continuous extraction (soxhlet) apparatus . the extraction were filtered , and ethanol was evaporated on a rotary evaporator under vacuum at a temperature of 45°C to a small volume (about 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 minutes until alkaloid separated for water and enter to the chloroform phase . this was repeated for three times and then total chloroform phase was evaporated , yielding a total alkaloid extract.

- 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 (18;19) 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 .

- alkaloids :

Three concentration of alkaloids bioactive chemical compound were studied on the

viability of protoscolex based on LD_{50} determination , which were (100 , 110 , 120 µg \ ml) of alkaloid bioactive chemical compound extracted from *Cladophora crispata* and (150 , 180 , 200 µg \ml) of alkaloid bioactive compound extracted from *Hapalosiphon aureus* .

- Ethyl acetate extract :

(280 , 300 , 330 µg \ml) concentrations of ethyl acetate extracted from the green algae *Cladophora crispata* and the experiment was done as describe previously .

-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 compounds 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 alkaloid extract of *Cladophora crispata* :

The results of *in vitro* activity of bioactive alkaloid compound extracted from the green algae *Cladophora crispata* revealed an activity of extract at 120 µg\ml in 6day – post treatment , while the two other concentration show an activity after 7 days – post treatment compared with control group see table(1).

Testing of alkaloid extract of *Hapalosiphon aureus* :

The activity of bioactive alkaloid compound extracted from *Hapalosiphon aureus* was less when compared alkaloid of *Cladophora crispata* where the activity of 200 µg\ml was observe after 7 days – post treatment and the mean of protoscolices viability at 150 µg\ml was 6.0 after 7 days – post treatment .see table(2)

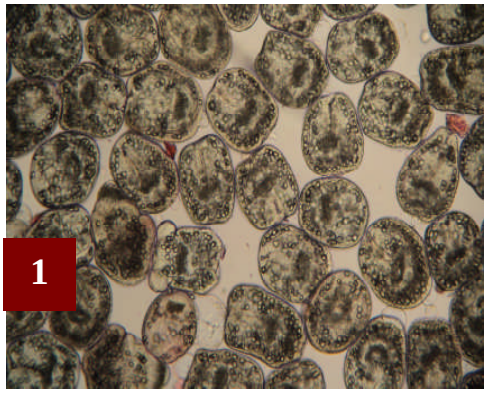
Testing of ethyl acetate extract of *Cladophora crispata* :

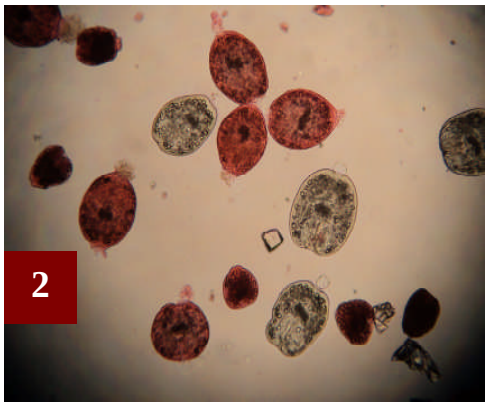
Two concentration of ethyl acetate extract show high activity after 5 days – post treatment that were 300 and 330 µg\ml while 280 µg\ml show 4.5 mean of viability after 5 days – post treatment . Compared with control group and other bioactive chemical compound extracted from *Cladophora crispata* , ethyl acetate extract was more active as antiprotoscolices agent. see table(3)

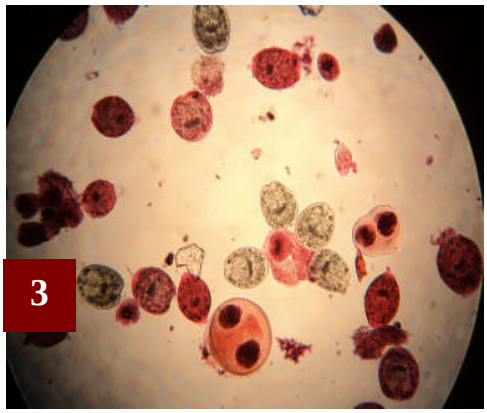
Testing of albendazole activity :

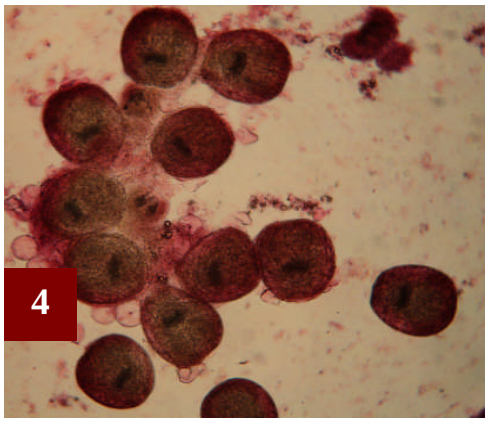
High activity of albendazole has revealed at 1000 µg\ml after 3 days – post treatment while 500 µg\ml concentration of albendazole has recorded 7.6 mean of viability after 4 days – post treatment and high activity after 5 days – post treatment .

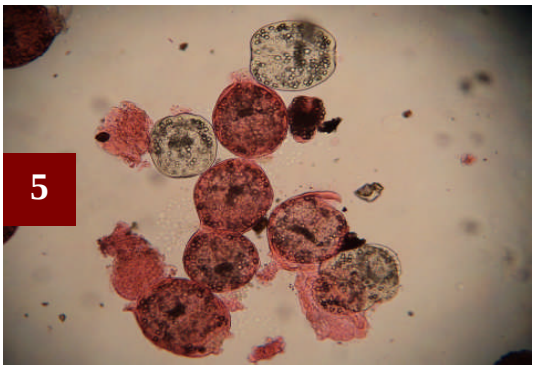
Comparison of *in vitro* activity between albendazole and bioactive chemical compound extracted from *Cladophora crispata* and *Hapalosiphon aureus* has explain that ethyl acetate extract of *Cladophora crispata* has similar activity to albendazole at 300 and 330 µg\ml of concentration after 5 days – post treatment , . see table(4)











Pictures of *in vitro* treated protoscolices where :

1, control viable protoscolices ;

2, protoscolices treated with Pyridine,2,3,5 – tetrahydro of *Cladophora crispata* after thee days – post treatment.

3, protoscolices treated with 2-(N,N-dimethylhydrazino)cyclohexanecarbonitrile of *Cladophora crispata* after four days – post treatment.

4, protoscolices treated with albendazole .

5, protoscolices treated with Benzeldehyde ,2-nitro, diaminomethylledenhydrazone of *Hapalosiphon aureus* after six days – post treatment.

GC – Mass analysis of Extracts :

The methanolic , alkaloid , ethyl acetate and hexane extracts of *Cladophora*

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

3.5.1. Alkaloid of *Cladophora crispata* :

21 number of peak were detected by GC – Mass analysis as results of alkaloid extract in the present study , Pyridine , 2, 3, 5 – tetrahydro consist 43.72 % (R.T.32.570 min) of total extract followed by 1-[2-Adamantylidene] semicarbazide (9.38% , 22.626 of R.T.) as explained in table (5)

-Ethyl acetate extract of *Cladophora crispata* :

GC – mass spectrum of ethyl acetate extract revealed the presence of 67 peak as showed in figures (3) , the results of compounds were arranged in table based on their percents in extract starting with 2–(N,N-dimethylhydrazino)cyclohexanecarbonenitrile which consist 8.37% (R.T. 20.601) of total extract followed by methyl ester of decyclotrenudine (7.28%) . Other compounds have explained in the table(6) :

Alkaloid extract of *Hapalosiphon aureus* :

GC- Mass spectrum (Fig,5) of alkaloid extract of *Hapalosiphon aureus* has recorded seven peaks started with Benzeldehyde ,2-nitro, diaminomethylledenhydrazone which consist 39.40 % (R.T. 4.231) of total extract followed by 18- Nonadecen-1-amine (28.61%, 1.443 of R.T.) see table (7)

Discussion :

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 . In this regard, most studies have been focused on activity of natural substances derived from Chlorophyta and Cyanophyta, mainly due to their accessibility and use in traditional medicine . Chlorophyta and Cyanophyta – like plants – produce a variety of remarkable compounds collectively referred to as secondary metabolites. They are synthesized by the organism in cultures at the end of the primary growth phase and into the stationary phase. The relevant substances are diverse in their chemical structure and physiological function (20;21) .

In vitro activity of bioactive chemical compounds extract from *Cladophora crispata* and *Hapalosiphon aureus* recorded that ethyl acetate extract had high activity among the six extracts used in the present study followed by methanol and hexane extracts , whereas alkaloids extracts of the *Cladophora crispata* and *Hapalosiphon aureus* has low activity compared with the other extracts .Indicators used in the present study and description by each of (22) and (23) explained that the ethyl acetate and methanol extract may contain anthocyanin, terpenoids, saponins, tannins, xanthoxyllines, totarol, quassionoids, lactones, flavones, phenones, and polyphynols. Therefore, ethyl acetate and methanol was used as a solvent for the extraction of biologically active compounds from Cyanophyta , Chlorophyta ,and plants in many studies such as (22 ; 23 ; 24). Ethyl acetate was widely used as solvent to extract the bioactive chemical compounds from chlorophyta , cyanophyta and plants, thus ethyl acetate is used to extract the bioactive chemical compounds from the chlorophyte *Cladophora crispata* in the present study . Ethyl acetate has showed high *in vitro* and *in vivo* activity against the

protoscolices of hydatid cyst because it show activity after 5 days post-treatment.

GC-Mass spectrum of ethyl acetate extract analysis revealed that the activity of its returned to the presence of the alkaloid named 2–(N,N-dimethylhydrazino)cyclohexanecarbonenitrile which consist 8.37% of total extract , alkaloids were extracted previously (24) from chlorophyta such as *Cladophora crispata* and explained that the activity of alkaloid returned to the presence of amine and amid groups in its structure . The biochemical medicinal activity of alkaloids results from inhibition of enzymes action by interaction with thymol group (Sh) of enzymes and linking with DNA & RND , then destruction of these nucleic acids and finally inhibition the biosynthesis of cell proteins , metabolism of each of carbohydrates , and lipids .

Comparison between *in vitro* activities of ethyl acetate extract of *Cladophora crispata* and albendazole revealed that each of them record activity after 5 days – post treatment . The activity of ethyl acetate extract returned to the presence of 2–(N,N-dimethylhydrazino) cyclohexanecarbonenitrile compound as GC- Mass spectrum showed in figure (4) . Among the two extract used against the hydatid cyst in the present study , the activity of alkaloids extract of each *Cladophora crispata* and *Hapalosiphon aureus* is less because it was a time consumption where the extracts recorded positive results after 6 days – post treatment in the case of *Cladophora crispata* and 7 days – post treatment in the case of *Hapalosiphon aureus*.

In the present study , GC- Mass spectra of the two alkaloids extracts has detected each of Pyridine,2,3,5 – tetrahydro from *Cladophora crispata* and Benzeldehyde ,2-nitro, diaminomethylledenhydrazone from *Hapalosiphon aureus* . Pyridine,2,3,5 – tetrahydro has activity different from the activity of 2-nitro, diaminomethylledenhydrazone , since Pyridine,2,3,5 – tetrahydro record activity after 6 days – post treatment while the activity of Benzeldehyde ,2-nitro, diaminomethylledenhydrazone because its activity has explained after 7 days –post treatment and only two concentration revealed activity against the protoscolices of hydatid cyst . Comparison with alkaloids extracts of each *Cladophora crispata* and *Hapalosiphon aureus* , ethyl acetate extract has considered a suitable for extract of alkaloid in the present study than the other method used in extraction of alkaloids alone as pure compounds since ethyl acetate extract contains not only alkaloids but also lipids , glycosides , esters , terpens , phenols , and others based on GC –Mass spectra analysis , further , the bioactive chemical compounds acts in a synergistic action (19). Therefore, alkaloid compound 2–(N,N-dimethylhydrazino) cyclohexanecarbonenitrile extracted by ethyl acetate solvent has showed high activity than other for *in vitro* and *in vivo* studies . Alkaloids extracts of all algal species has low antiprotoscolices activity. These results could be related to solubility of the bioactive compounds in ethyl acetate, methanol and even hexane rather than acidic ethanol (25). The second explanation may be due to lysis of the algal cells of *Cladophora crispata* and *Hapalosiphon aureus* by that organic solvents, which lead to release of membrane-bound vesicles that contain the active metabolites. Such condition might have occurred for all solvents used in the present study, with the exception of acidic ethanol (25) and this result has disagreed with the study of (24). It is difficult to speculate the mechanism by which these

bioactive compounds act as parasitecidal agents. In this regard (26) suggest that many bioactive chemical compounds exhibit their parasitecidal activity by virtue of their interference with the redox balance of the parasites, acting either on the respiratory chain or on 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 dehydrogenase (DHOD), the fourth enzyme in the *de novo* pyrimidine biosynthetic pathway, is essential to parasite, including the electron acceptor capacity and cellular localization (21). In this way, it has been recently demonstrated that the methanol extracts of brown algae *Ishige okamurae* Yendo, *Fucus evanescens*, and *Pelvetia babingtonii* contain potent noncompetitive inhibitors against *T. cruzi* DHOD (21) .

Table(1) : Viability of protoscolices treated with alkaloid 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
100 µg\ml	87	80	66	49.33	39.67	19	10.67	5	0
110	84.67	73.33	62.33	46.33	32	15	8.67	2.67	0
120	82	71.67	61.3	44.33	30	13.33	8.67	0	0
Control	95.33	92.33	87.33	82.67	79.67	73.67	68.33	60.67	58.33
L.S.D.	4.903								
Significant differences , P ≤ 0.05									

Table (2) : Viability of protoscolices treated with alkaloid 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
150 µg\ml	88.6	75.6	65	55	45.6	29.66	20	12.66	6.33
180	87.3	73.3	64	49.33	35.6	19.33	12.66	5.33	0
200	85.3	72	61.6	46.6	31	17	11.33	4.33	0
Control	92.3	90	87.3	83.6	76.33	72.66	65.66	60	58.33
L.S.D.	0.984								
Significant differences , P ≤ 0.05									

Table (3) : Viability of protoscolices treated with ethyl acetate extract of *Cladophora crispata*

Concentration \time of treatment	Mean of viability								
	1 h	4 h	1 day	2 days	3 days	4 days	5days	6 days	7days
280 µg\ml	80.66	61.66	44.66	28.66	17.33	11.66	4.66	0	0
300	75.33	58	33.66	21.66	12.66	6	0	0	0
330	69.66	56.66	32.33	18.66	10	4.66	0	0	0
Control	96	93.333	91.33	83.66	80.66	75.66	73.33	65.66	58.66
L.S.D.	0.716								
Significant differences, P ≤ 0.05									

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									

Table (5)

Peak	R.T.	% of total	Compound	M.W.
1	32.570	43.72	- Pyridine,2,3,5 – tetrahydro	
2	22.626	9.38	- 1-[2- Adamantylidene] semicarbazide	
3	39.682	5.51	- Amyl nitrite	
4	20.264	5.17	- Acetaamid	
5	23.298	2.54	- N-Methyl- propylamine	

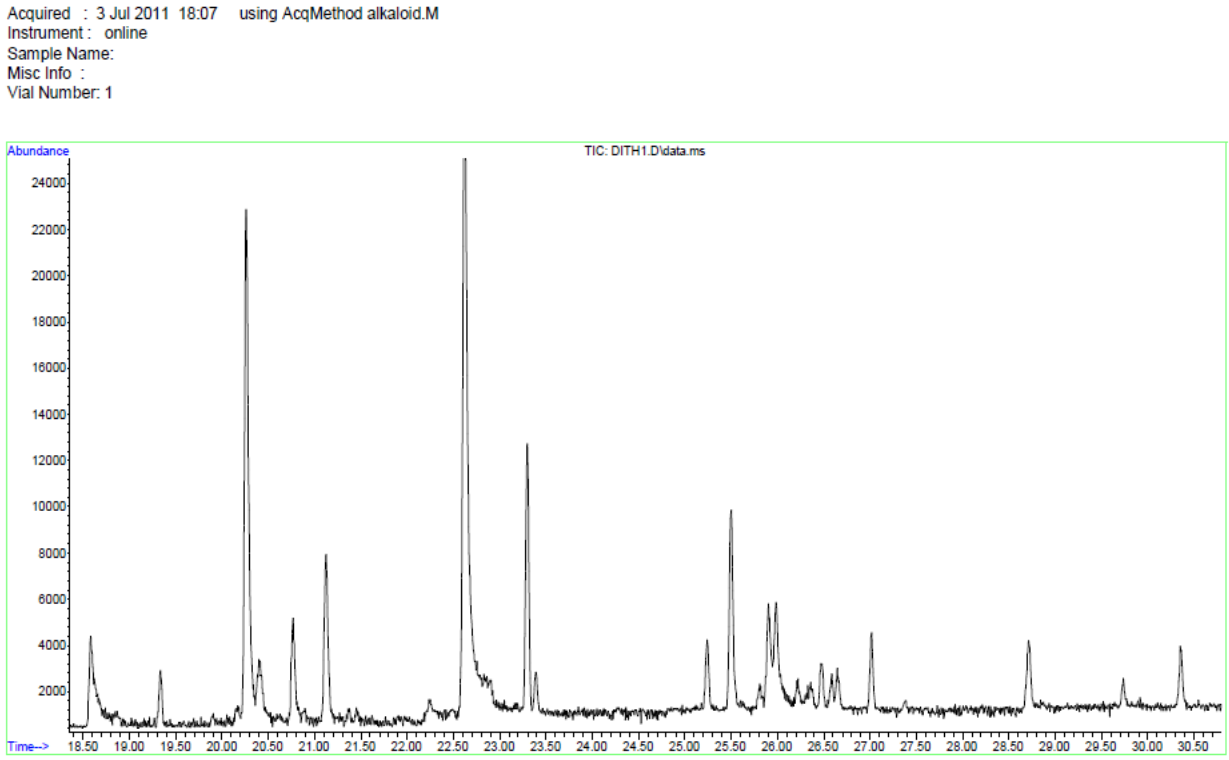
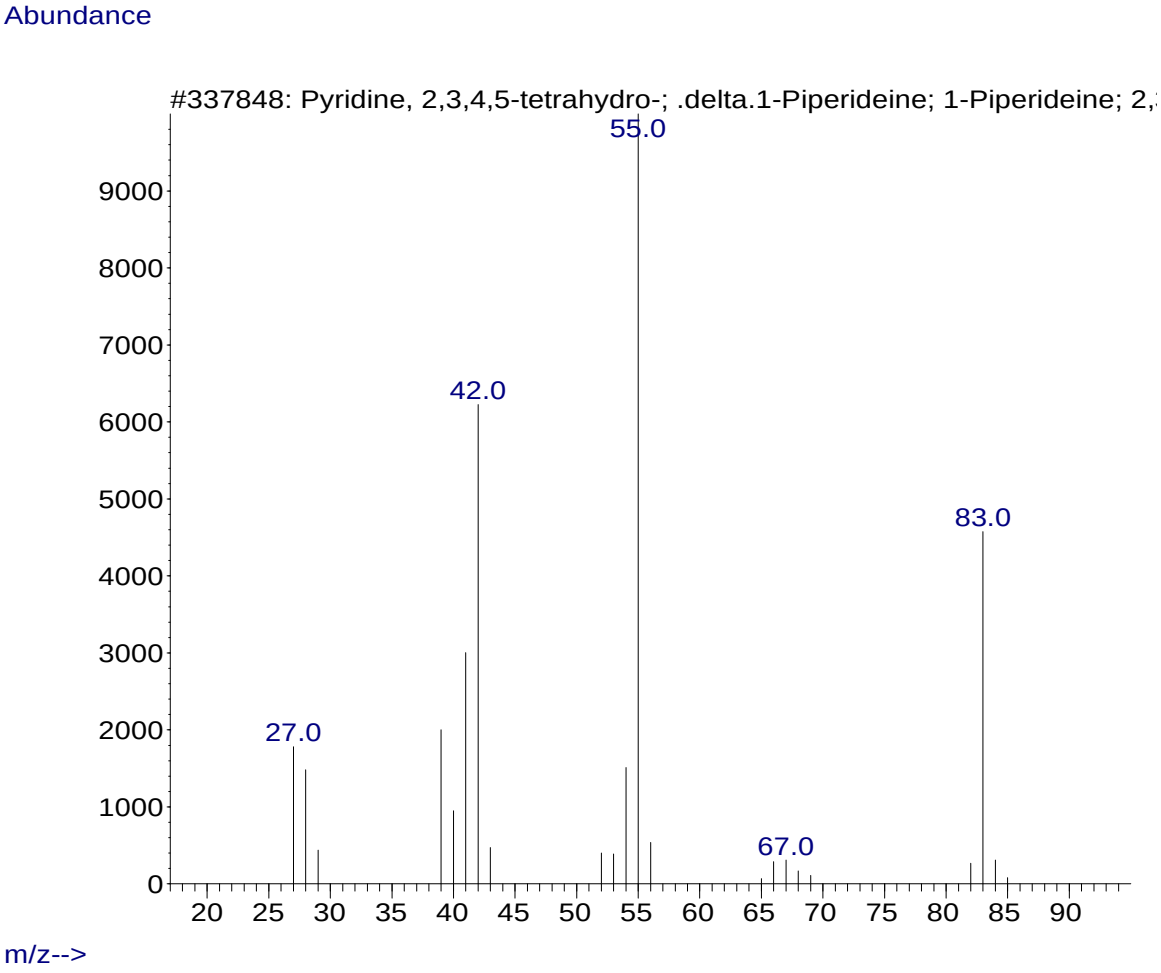


Figure (1) : GC-Mass spectrum of Alkaloid extract of *Cladophora crispata*



Figure(2): Mass spectrum of Pyridine , 2, 3, 5 – tetrahydro

Table(6)

Peak	R.T.	% of total	Compound	
1	20.601	8.37	-2-(N,N-dimethylhydrazino)cyclohexanecarbonitrile	
2	22.991	7.28	- Methyl ester of decyclotrenudine	
3	26.653	6.40	- Isoquinoline, decahydro	
4	21.452	2.87	- Phthalic acid, cyclohexylmethyl isohexyl ester	
5	27.323	2.03	- Methyl 20 hydroxyeicosan- 5(Z),8(Z),11(Z),14(Z)-tetranoate	

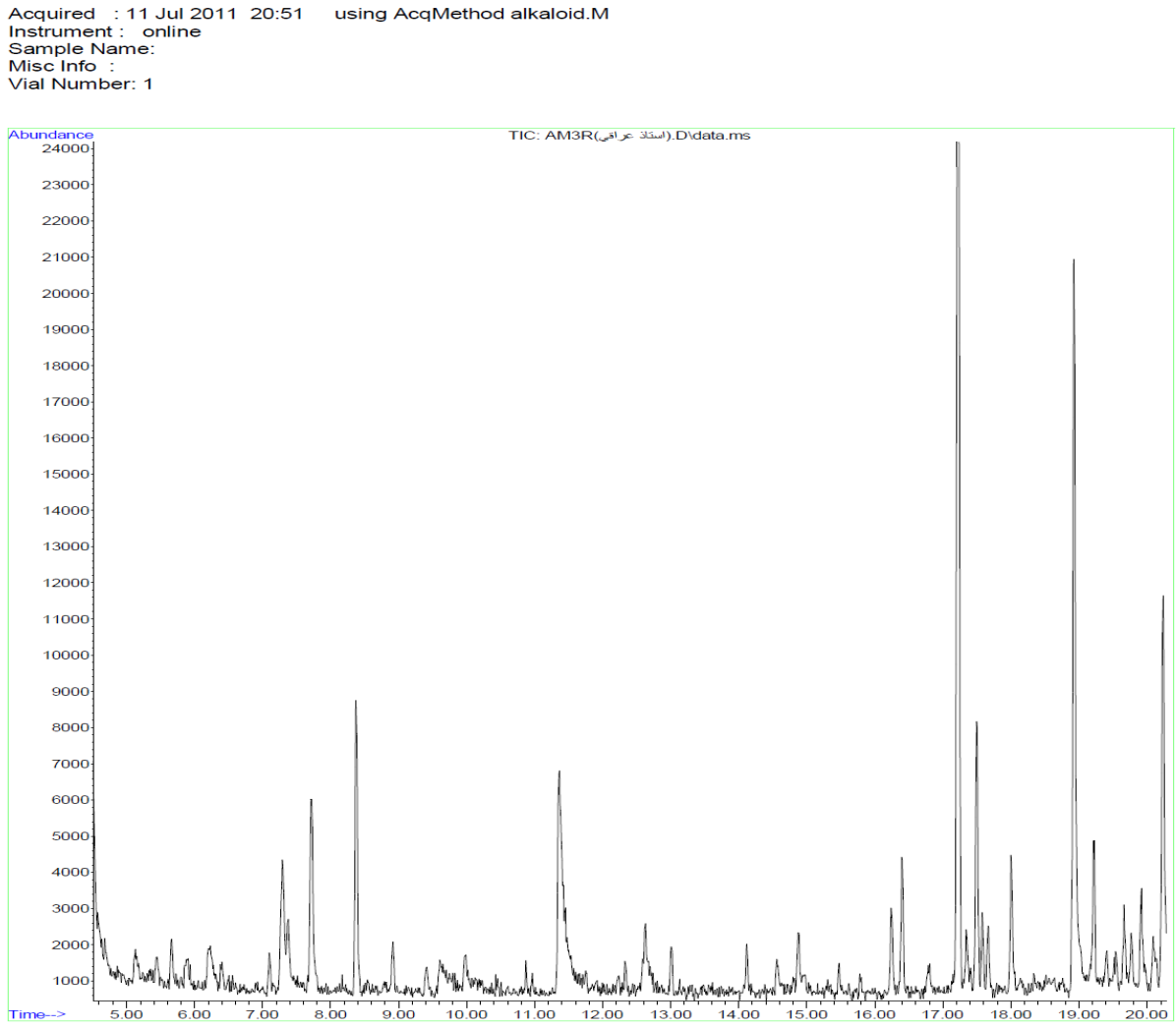
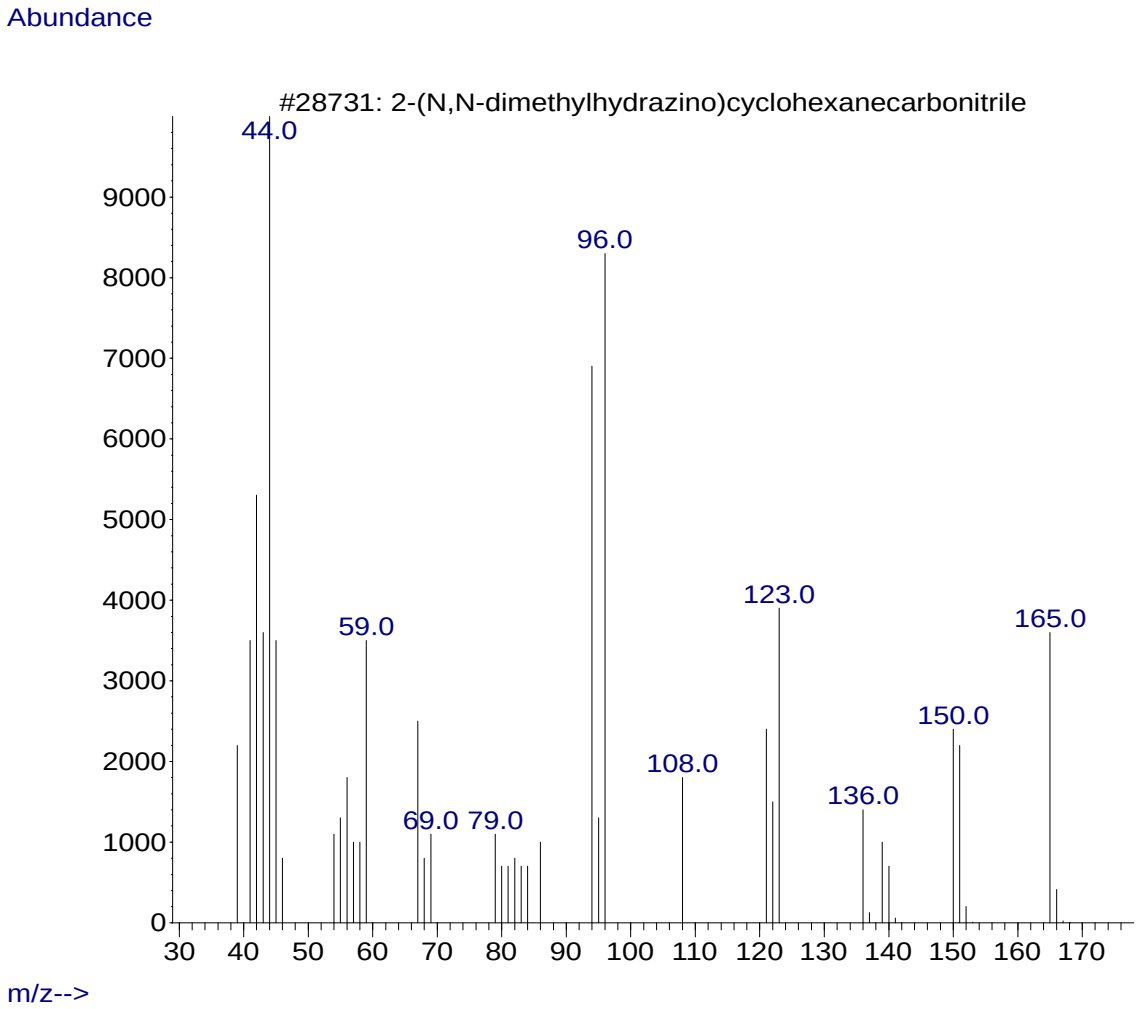


Figure (3): GC- Mass spectrum of ethyl acetate extract of *Cladophora crispata*



Figure(4): Mass spectrum of 2-(N,N- didimethylhydrazino) cyhexanecarbonitrile

Table(7)

Peak	R.T.	% of total	Compound	
1	4.231	39.40	- Benzeldehyde ,2-nitro, diaminomethylledenhydrazone	
2	1.443	28.61	- 18- Nonadecen-1- amine	
3	2.032	27.41	- 1,5- cyclooctadiene (E,Z) -	
4	20.818	5.40	- (E)-3- pentenamide	
5	19.210	1.33	- 2- Methyl-1- pyrroline	

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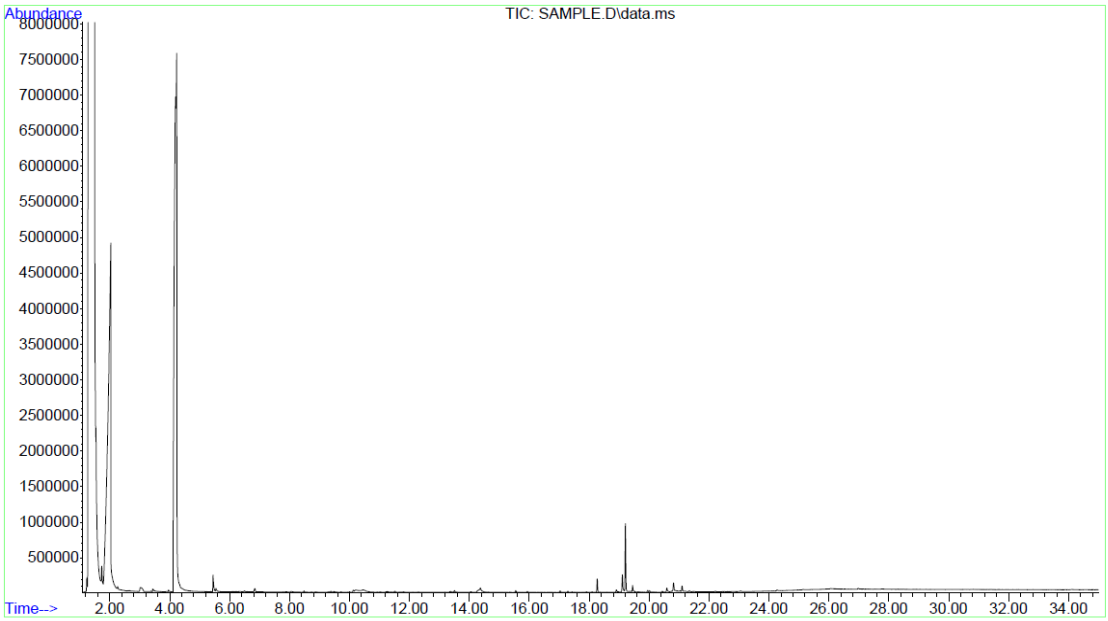

Figure (5): GC – Mass spectrum of alkaloid extract of *Hapalosiphon aureus*

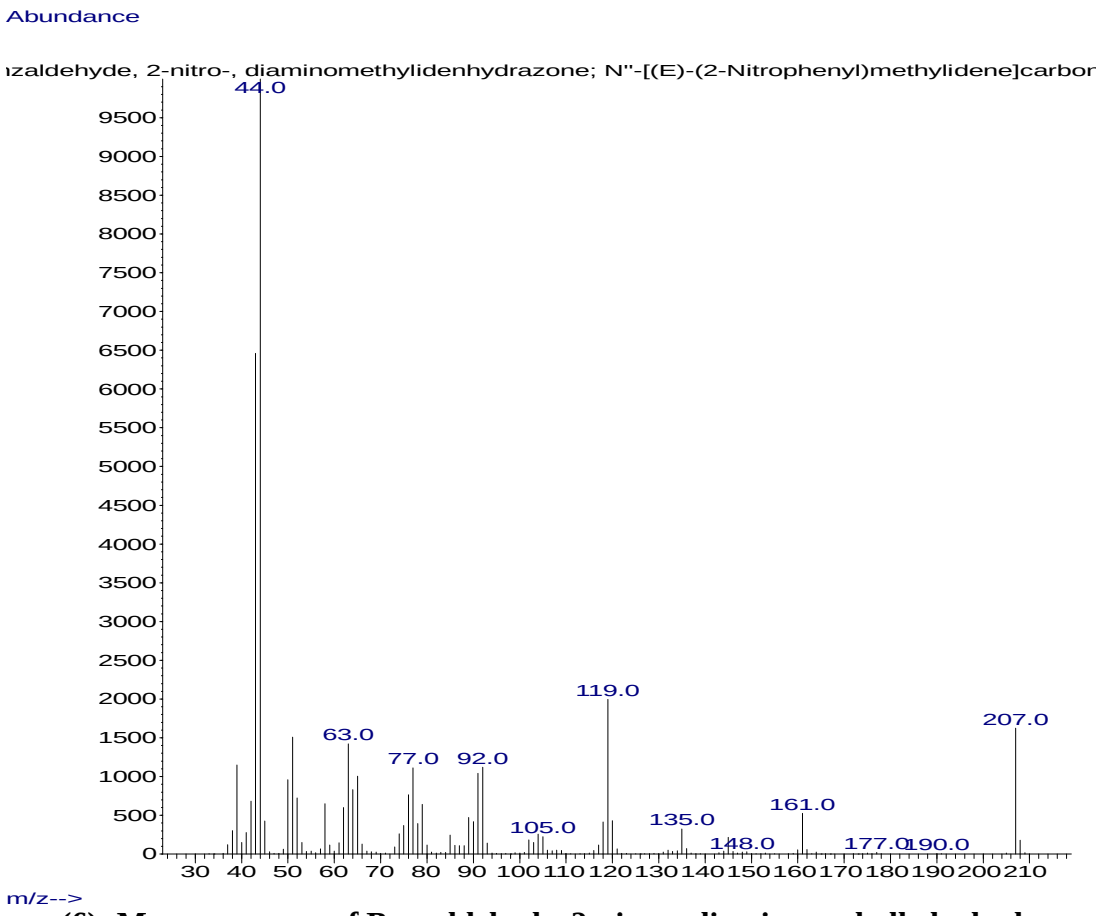

Figure (6) :Mass spectrum of Benzeldehyde, 2-nitro-, diaminomethylledenhydrazone

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فعالية المركبات القلويدية المعزولة من الطحالب الخضر والطحالب الخضر المزرقة ضد مرض الاكياس العدرية مقارنة بالالبندازول داخل المختبر

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

المستخلص :

اختبرت فعالية المركبات القلويدية الطبية المعزولة من الطحالب الخضر والطحالب الخضر المزرقة ضد البكتريا والفطريات الممرضة ولم يتم التطرق الى فعاليتها ضد الطفيليات ، لذلك استهدفت الدراسة الحالية الى اختبار فعالية المركبات القلويدية لهذه الطحالب لأول مرة في العراق ضد الطفيليات وعالميا ضد الطفيلي المسبب لداء العدريات . استنتجت هذه الدراسة امتلاك المركب 2-(N,N-dimethylhydrazino)cyclohexanecarbonitrile فعالية في قتل الرؤيسات الاولية للكيس العدري عند اليوم الخامس من معاملة الرؤيسات الاولية داخل المختبر وبتراكيز اقل مما استخدم من تراكيز من الالبندازول .

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* فرع الاحياء المجهرية ، كلية االطب ، جامعة ذي قار و ** قسم علوم الحياة ، كلية التربية ، جامعة البصرة