Antiviral Activities and Phytochemical Constituents of Egyptian Marine Seaweeds (*CystoseiraMyrica*(S.G. Gmelin) C. Agardh and *Ulva Lactuca* Linnaeus) Aqueous Extract

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ABSTRACT

Background: some natural and synthetic compounds can prevent, suppress, or reverse the progression of virus infection. Natural products have proven to be the most effective in terms of their ability to act as an antiviral. In the present study, the antiviral potentiality of the bioactive compounds derived from aqueous extract of two Egyptian marine seaweed species (*Cystoseiramyrica* and *Ulva lactuca*)were assessed on different viruses. **Materials and methods:**these two species were collected from Hurghada at the Red Sea and Al-Agami area in Alexandria Mediterranean Sea, Egypt.The assay of cytotoxicity and antiviral activity by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenlytetrezolium bromide] and by neutralization methods were conducted. **Results:**these two species have a bioactive compound affected hepatitis A virus ($HAV\text{-}H_{10}$), Coxsackie B_4 virus,herpes simplex virus types-1 (HSV-1) and type2 (HSV-2), grow and cytopathic effect (CPE) in *Vero* cells.

Keywords: Antivirus, Marine seaweed, *Cystoseiramyrica*, *Ulva lactuca*, Hurghada, Red Sea, Al-Agami, Alexandria, Egypt.

INTRODUCTION

For thousands of years,algae resources have been sought ability to prevent disease and prolong life was a belief.Seaweeds are potentially excellent sources of bioactive metabolites that could represent useful leads in the development of new functional ingredients in cosmetic andpharmaceutical industries. Their derived chemical compounds have a broad spectrum of biological activities.Already, they are used as herbal medicine, fertilizer, fungicides, herbicides and direct sources in human nutrition too[1,2].

Traditional and modern medicines have relatively exhausted most of their resources in land plants. However, the marine environment by its biological and chemical diversitycan be a source of new types of agents against cancer and infectious diseases[3,4]. During the last decades, numerous novelcompounds have been isolated from marine organisms and many of these substances havebeen demonstrated to possess interesting biological activities[5,6].

In particular, antiviral effects of sulfated polysaccharides and terpenes from marine seaweeds against a variety of enveloped viruses, such as Herpes Simplex Virus type 1 (HSV-1) and 2 (HSV-2), Human Immunodeficiency Virus (HIV), human cytomegalovirus, dengue viruses, respiratory syncytial and influenza viruses have been reported[7].

The antimicrobial potential of macroalgae from Mediterraneancoasts remains partially unexplored. Many chemically unique compounds ofmarine algae with antimicrobial activity have been isolated and a number of them are underinvestigation and/or are being developed as new pharmaceuticals such as brominated phenols,sterols, terpenoids, polysaccharides, peptides, proteins, acrylic acid, terpenes, chlorophyllides,phenols and heterocyclic carbons etc.[8,9]. The present investigation was per-formed with the following objectives: To evaluate the antiviral activity of the two seaweeds as well as to reveal the chemical constituents in the two seaweeds using GC-MS, IR and NMR**)** analysis.

MATERIALS AND METHODS

Collection and identification of seaweeds:

The studied algal species were collected from the coastal areas of Hurghada Red Sea and Al-Agami Alexandria Mediterranean Sea Egypt. Algal samples were cleaned of epiphytes, and necrotic parts were removed. Then, cleaned samples were rinsed with sterile water to remove any associated

Received: 20/08/2016
Accepted: 28/08/2016
DOI : 10.12816/0029034

debris. The cleaned fresh materials were shade air-dried and ground into fine powder, as described by **Nasr and Aleem**[10,11]. The samples were identified as *Cystoseira myrica* (S.G. Gmelin) C. Agardh and *Ulva lactuca* Linnaeus.

Preparation of seaweed extracts:

Sixty grams of powdered samples were extracted with 100ml of water. The samples were kept in the dark for 24h at 45°Cwith intermittent shaking. After incubation, the solutionwas filtered through filter paper, and the filtrate was collected (crude extracts). The aqueous extract was evaporated by rotary evaporator and the residues were completely dried to constant weight by placing it in a porcelain dishes inside desiccators with calcium carbonate. Then the residue was stored at -12°C until further uses. Five grams of the residue was dissolved in 100ml of sterile distilled water to make 5% seaweed concentrations.Extracts were filtered with 0.2mm filter to be free from bacteria and other micro-organisms under aseptic conditions to be tested later as antiviral.

Determination of extract cytotoxicity:

For cytotoxicity assay, the two aqueous crude extract was prepared as described by **Van den Berghe*et al*.**[12].

MTT Assay Protocol Procedure:

For antiviral assay, herpes simplex virus types-1 (HSV-1) and type2 (HSV-2), hepatitis A virus (HAV-H_{10}), and Coxsackie B4 virus were used. The viruses were obtained from Microbiology Center in Medicine Faculty in Azhar University (Girls Branch). Test the effect of two seaweeds crude extract on these viruses to determine antiviral activity *invitro* using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenlytetrezolium bromide] method as reported by **Van Meerloo*et al*.**[13] and by cytopathic effect (CPE) reduction assay according to the method described by **Serkedjieva and Ivancheva**[14].

Separation and purification of the active compounds of the tested algae extracts:

For identifying the highly active compounds extracted from algae, purification techniques were carried out. The active crude extracts were subjected to fractionation as follows:

Column chromatography:

The seaweed crude extracts were fractionated to explore the active compounds. The Glass column of 50 cm length and 2.5 cm diameter were packed by Sephadex (G l00), following the wet method. All fractions were tested with the most sensitive viral cell line to determinate the most active fraction for each extract and the purity of each fraction was investigated by TLC. Then, the fractionation was performed using HPLC system (Pump model MINI Puls3, detector UV/VIS-151 FC 203B, Gilson) at Regional Center for Mycology and Biotechnology Al-Azhar University

Bioassay of the active compounds:

After active compounds of algae fractions were separated by TLC technique, bioassay experiment process was carried out.

Identification of the most active compounds:

Gas chromatography - Mass spectrum analysis:

The mass spectrum of the active fractions was analyzed using the direct inlet unit in the Shimadzu QP-5050 GC-MS at the Regional Center for Mycology and Biotechnology, Al- Azhar University to ensure the purity of the active compound and give the mass fragmentation of its constituents.

Infra-Red (IR) spectrum:

The Infra-Red absorption spectrums of the purified fractions were estimated using BRUKER, Vector 22, Germany at the Micro-Analytical Center, Faculty of Science, CairoUniversity.

Nuclear Magnetic Resonance (NMR) spectra:

The active fractions of interest were subjected to the proton proton (1H) NMR analysis and were recorded in deutrated dimethyl sulphoxide (DMSO-d6) on a Varian Mercury-VX-300 NMR spectrometer at the Micro-Analytical Center, Faculty of Science, Cairo University.

RESULTS

Cystoseiramyrica

The silica gel plate using the solvent system Butanol: Acetic acid: Water 3:3:1 v/v and few drops of ammonia as well as column chromatography revealed the presence of 13 fractions (Table 1).

From Table (1) it was clear that fraction No. (7) exhibited the highest antiviral activity. The suggested chemical structure configuration of the most active antiviral constituents of the *Cystoseiramyrica* aqueous extracts. The proposed configuration satisfies and complies with the analytical identification

in characteristics shown by the IR; 1H-NMR; and by GC/MS can be deduced that the compound has the following characteristics.

1- IR spectra:

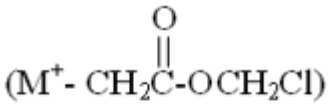
The absorption band which is a broad band appeared at 3320 cm^{-1} indicating the presence of OH group and appearance of stretching band at 1643 $cm^{-1}$for carbonyl group of ester for acetate moiety. The bands at 1335 cm^{-1} for CH of CH_3 and the bands at 759 cm^{-1} depicts the presence of C-Cl. Finally, the band at 1014 cm^{-1} for O-CH_2 (Figure 1).

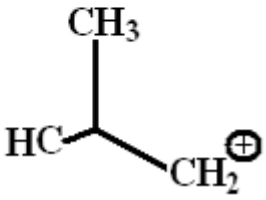
2- NMR spectra:

1HNMR (dimethyl sulphoxide (DMSO-d6) on a Varian Mercury-VX-300 NMR) δ at 3.3ppm (S,1H, 4OH), 2.4-2.5 (S, 2H, OCH_2), 1.7 (m, CH and CH2) and 1.2 (S, 3H, and CH_3) Figure (2).

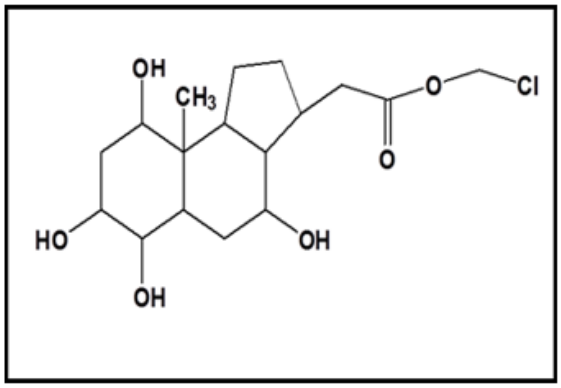
3- Mass Spectra:

Ms m/z (%): 362.5 (M^+), 327 (M^+-Cl), 254 and base Peak at 43.09 for (CH_3-$CH=CH_2^+$)figure (3).





This compound contains naphthalene skeleton which has high intensity of four adjusted hydroxyl group at 4, 6,7,9 carbons and free methyl group–CH_3 at C_9 on it. Also, it contains halogen atom (Cl), acetate group and cyclo pentane. The molecular weight is 362.5, and its molecular formula is $C_{17}H_{27}O_6Cl$. Finally, the nomenculature of the compound is identical to (rank) of GC mass figures, Chloromethyl 2-(dodecahydro-4,6,7,9-tetrahydroxy-9a-methyl-1H-cyclopenta[α]naphthalene-3-yl) acetate figure (7).



2- Ulva lactuca

The silica gel plate using the solvent system Butanol: Acetic acid: Water 3:3:1 v/v and few drops of ammonia as well as column chromatography revealed the presence of 15 fractions (Table and Plate).

From Table (2) it was clear that fraction No.(10) exhibited the highest antiviral activity. The suggested chemical structure configuration of the most active antiviral constituents of the *Ulva lactuca* aqueous extracts. The proposed configuration satisfies and complies with the analytical identification in characteristics shown by the IR; ^{1}H-NMR; and by GC/MS can be deduced that the compound has the following characteristics.

1- IR spectra:

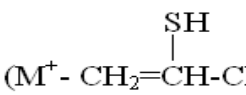
A broad band for OH group and SH group appears at 3410 cm^{-1} and stretching band at 1635 cm^{-1} for olefinic (C=C) and bands at 2623 and 2673cm^{-1} for CH_2. Appearance of band at 601 cm^{-1} for C-Cl and absorption band at 493 cm^{-1} for C-S (Figure 4).

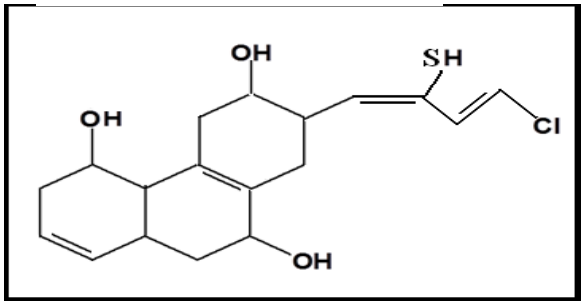
2- NMR spectra:

^{1}HNMR (dimethyl sulphoxide (DMSO-d6) on a Varian Mercury-VX-300 NMR) δ at 4.0 ppm (S,1H, SH), 3.3 ppm (S, 3H, OH), 2.1-2.6 (m, 8H, CH_2 = CH_2 and CH=CH) and 1.4-1.5 (m, CH_2) Figure (5).

3- Mass Spectra:

Ms m/z (%): 359.5 (M^+), 324 (M^+-Cl), 237 and finally, base Peak at 44.03for(CH_3-CH_2-CH_3^+) Figure(6).





This ccompound contains phenanthrene skeleton which has high intensity of three adjusted hydroxyl group at 3,5,10 carbons on it. Also, it contains halogen atom (Cl), mercapto group (–SH). The molecular weight is 359.5, and its molecular formula is $C_{18}H_{23}O_3SCl$. Finally, the nomenculature of the compound is identical to (rank) of GC mass figures, 2-((1E,3E)-4-Chloro-2-mercaptobuta-1,3-dienyl)-1,2,3,4,4b,5,6,8a,9,10-decahydrophenanthrene3,5,10-triol.

DISCUSSION

Interest in employing antiviral compounds from naturalsources like plants or algae has been enhanced byresearchers and the consumers' preference for naturalmedicines and concerns about the toxic effects ofsynthetic antiviral materials. Marine organisms are a rich source of structurally novel and biologically active metabolites. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry. To date, many chemically unique compounds of marine origin with various biological activities have been isolated and some of them are under investigation and are being used to develop new pharmaceuticals[15,16]. In most studies done in this area, theviruses tested belonged to the herpesviridae family,especially herpes simplex viruses. In the present studywe chose HSV-1 for our research because of its ability toperform different clinical complications and its increasingprevalence in communities. Since, HSV-1 isa good example of enveloped viruses and HAV as an undeveloped. Therefore, thediscovery of natural antiviral compounds should beinteresting. In some studies in this direction, some brownalgae were tested. However, until now there has been noother study on the antiviral effect of *C. myrica* and new compound from *U.lactuta*. This wasvaluable for us especially because we had easy accessto this alga on the Egypt coast.

In another step of our research, we tested thesterilized and filtered extract for their probable postattachment inhibition effects on HSV-1 replication[17]. Therefore,the water extracts of *C. myrica*and *U.lactuta*also exhibit hepatitis A virus (HAV-H_{10}), Coxsackie B_4 virus herpes simplex virus types-1 (HSV-1) and type-2 (HSV-2)to infect the *Vero*cells. Also, in another studythe antiviral effect of diterpens from *Dictyotapfaffi* aBrazilian brown alga, on HSV-1 was evaluated and theresults indicated that the diterpenes affected an earlystep of the replicative cycle[18]. The present results suggested that *C. myrica* water extract might be acandidate for natural antiherpetic compound development.

Kim *et al*.[19] found that the ethyl-ether extract of algae in vitro exhibited a broad-spectrum of antibacterial activity, but not antifungal activity[19], whereas**Lee *et al*.** and **Deveau*et al*.** demonstrated that *U. lactuca*methanolic extracts inhibit a variety of clinically relevant human pathogenic bacteria and fungi strains and showed maximum inhibitory from methanol extract than acetone, chloroform, hexane and ethyl acetate solvents[20,21,22,23,24]. On the contrary, **Sarith*aet al*.** showed that the acetone extract was the best in inhibiting pathogenic microorganisms[4]. On the other hand, **Mendes *et al*.** investigated *Ulva fasciata* were collected from Rasa beach and Forno beach, Rio de Janeiro, Brazil for having antiviral activity on the replication of human metapneumovirus (HMPV)[25].

So, in this study the seaweed extracts of *Cystoseiramyrica* and *Ulva lactuca* possessed noticeable activity against different virus. after separation, purification, and identification we get the new bioactive compounds from seaweed extracts calledChloromethyl 2-(dodecahydro-4,6,7,9-tetrahydroxy-9a-methyl-1H- cyclopenta [α]naphtalen-3-yl)acetate from *Cystoseiramyrica* aqueous extracts and 2-((1E,3E)-4-Chloro-2-meracaptobuta-1,3-dienyl)-1,2,3,4,4b,5,6,8a,9,10-decahydrophenanthrene3,5,10-triol from *Ulva lactuca*.It is evident from the present study that the aqueous extracts of *Cystoseiramyrica* and *Ulvalactuca* could be utilized as a good source of antimicrobial agent in pharmaceutical industry.Further in *vivo*investigations of thepurifiedcompound from waterextract are suggested forfuture studies.

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Table (1): The antiviral activity of isolated fractions from *Cystoseiramyrica* against the tested viruses.

Fractions	HAV-H_{10}	$CoxB_4$	HSV-1	HSV-2
1	+	-	+	-
2	-	-	-	-
3	-	-	-	-
4	+	-	-	+
5	+	-	++	-
6	++	++	+++	++
7	++++	+++	++++	++++
8	+++	++	++	++
9	+	-	+	-
10	++	++	++	+
11	+	+	+	+
12	-	-	-	-
13	-	-	-	-

Table (2): The antiviral activity of isolated fractions from *Ulva lactuca* against the tested viruses.

Fractions	HAV-H_{10}	$CoxB_4$	HSV-1	HSV-2
1	-	-	-	-
2	-	-	-	-
3	-	+	+	-
4	++	+	++	+
5	+	+	+	+
6	+	+	+	-
7	-	-	-	-
8	+	-	++	-
9	++	+	++	++
10	+++	++++	++++	+++
11	++	++	+++	++
12	++	-	+	+
13	+	-	+	+
14	-	-	-	-
15	-	-	-	-

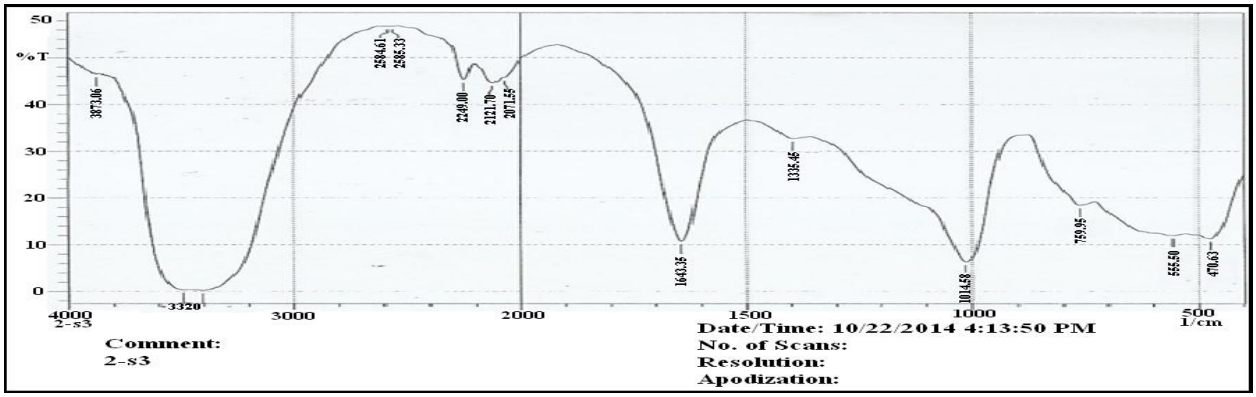

Figure (1): The infrared spectrums (IR) of the pure active compound obtained from *Cystoseiramyrica*

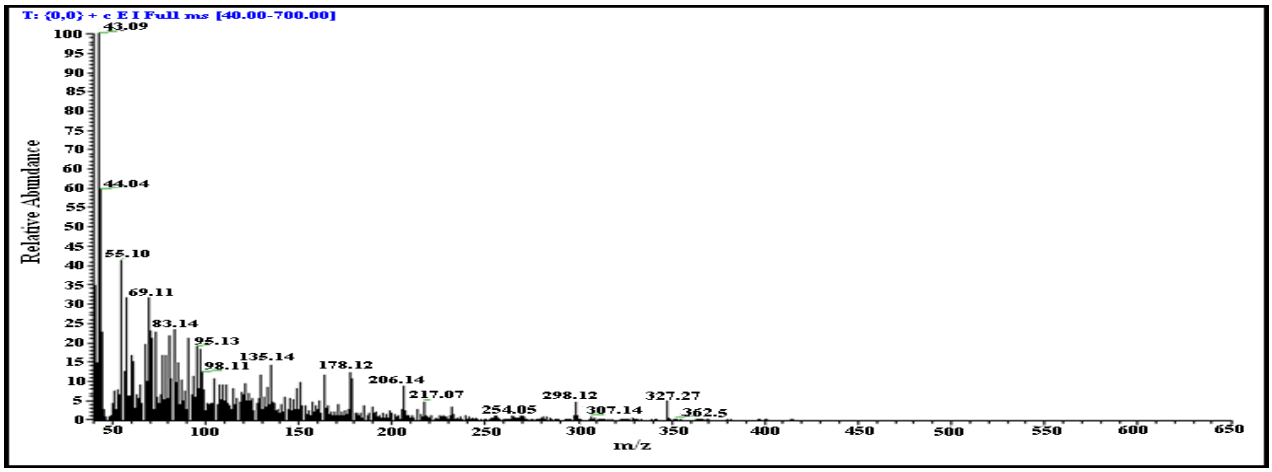

Figure (2): The ^{1}H-NMR spectrum of the pure active compound obtained from*Cystoseiramyrica*

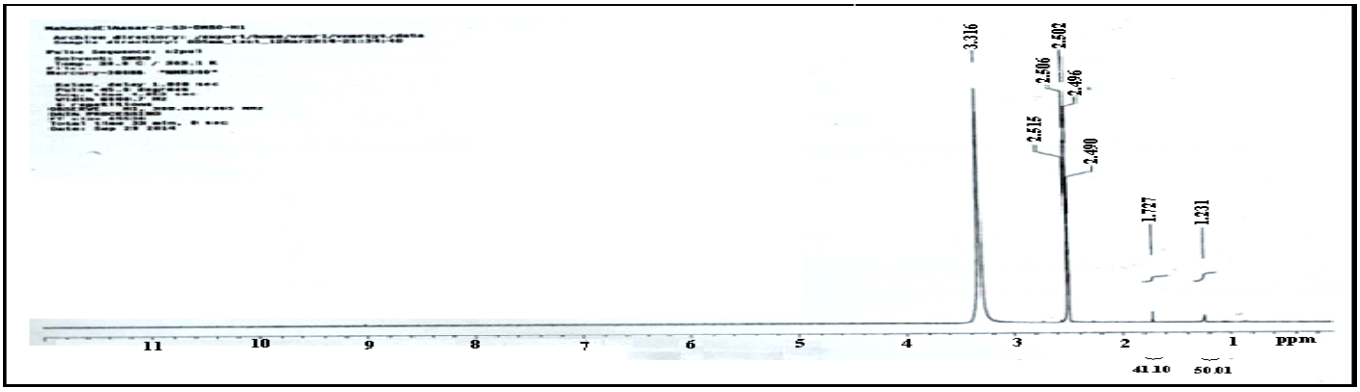

Figure (3): The mass spectrum of the pure active compound obtained from*Cystoseiramyrica*

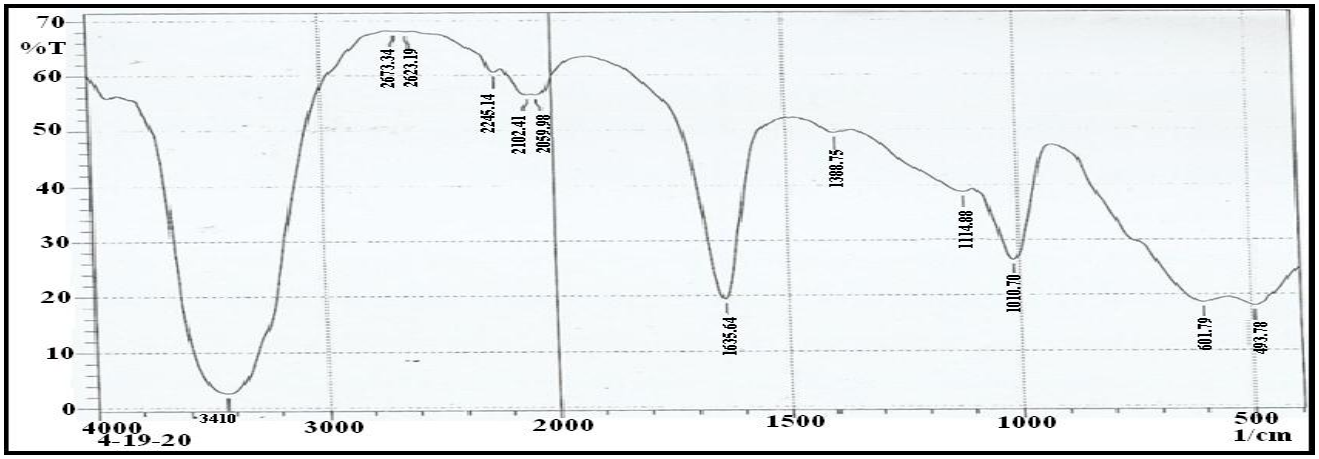

Figure (4): The infrared spectrums (IR) of the pure active compound obtained from *Ulva lactuca*

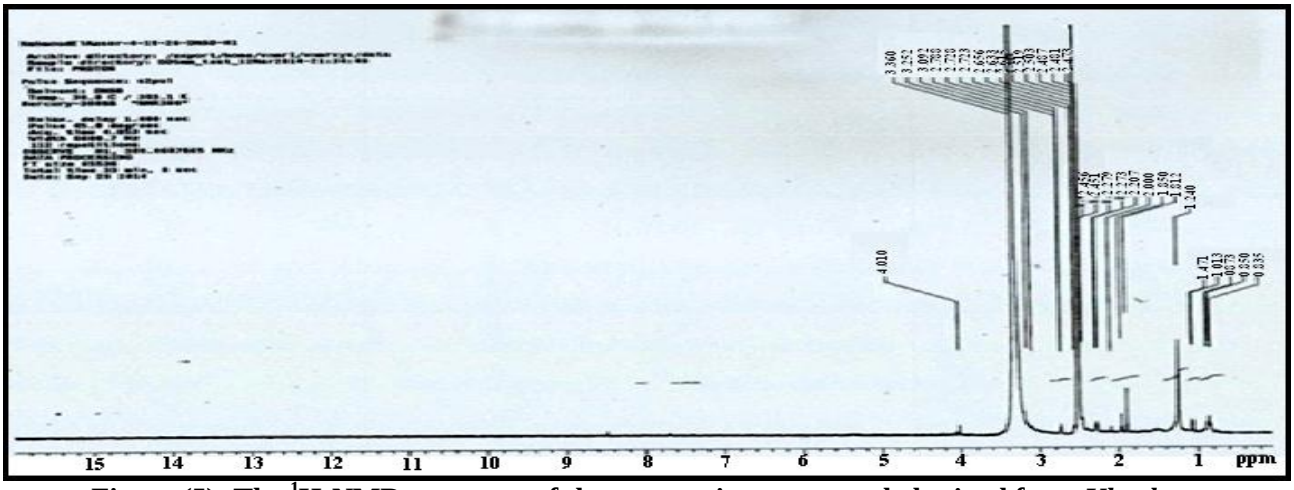

Figure (5): The ^{1}H-NMR spectrum of the pure active compound obtained from *Ulva lactuca*

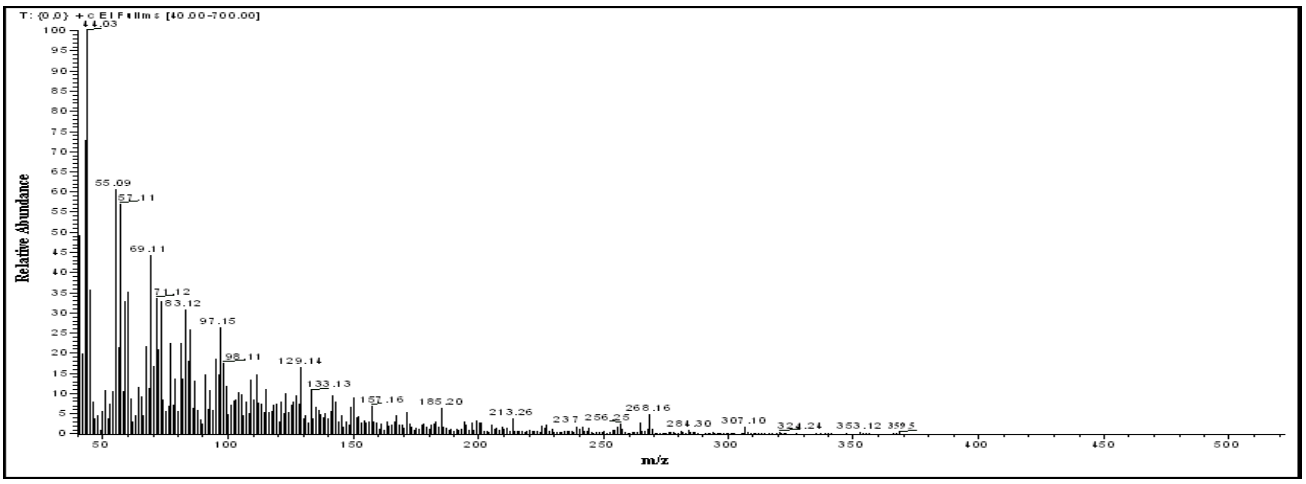

Figure (6): The mass spectrum of the pure active compound obtained from *Ulva lactuca*