J.Agric.Chem.and Biotechn., Mansoura Univ.Vol. 7 (3): 67- 74, 2016

CONTROL OF SOME MICROBIAL SKIN DISEASES BY SOME MARINE ALGAL EXTRACT

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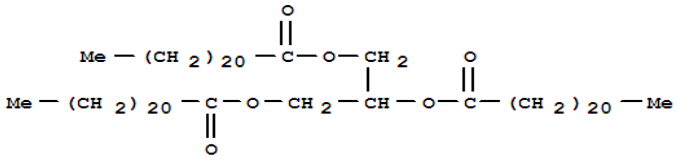
ABSTRACT

In this study, thirty two bacterial isolates were randomly isolated from skin, wound and hair of patients. Antibiotic susceptibility of the collected bacterial isolates to seventeen antibiotics was studied using the disc diffusion method. Results revealed that bacterial isolates number (9) and (17) were proved to be the most multi-drug resistant isolates recording 100% resistance. Bacterial isolate number (9) was subjected to molecular identification by using 16S rRNA gene sequence, the tested bacterial isolate revealed a similarity of 98% with *Staphylococcus aureus* subsp. *aureus* JH1.

The antibacterial activity of *Saragassum latifolium*, *Ulva lactuca and Jania rubens* were tested against the most multi-drug resistant bacterial isolate. *Saragassum Latifolium* extract appeared the highest antibacterial activity. Ultraviolet spectrum of some compounds is recorded in conjunction with other spectral data such as infrared and GC-MS analysis as an attempt to deduce the compounds which caused inhibition to the growth of bacterial isolate. The suggested chemical structure of purified antagonistic material isolated from *Saragassum latifolium.*



INTRODUCTION

The skin is the largest organ of the body and has the largest surface area when compared with other organs. Diseases involving human skin, hair and nail diseases are common and may be caused by bacteria, fungi or viruses (Hainer, 2003). Human bacterial floras are approximately ten times more than human cells in the body (Simon et al., 2005).

Antibiotics abuse spread all over the world specially in Egypt, this lead to appear multi-drug resistant bacteria, also side effects which result from using antibiotics lead to search for new substances with antimicrobial activities and high tolerability, therefore it is necessary to take measures to reduce microbial resistance and to explore alternative antimicrobial sources (Maseleno and Hasan, 2013).

Seaweeds consider a source of major metabolites that possess bioactive efforts (Colwell, 1983 ; Oh et al., 2008), such as polysaccharides, fatty acids, proteins, carotenoids, vitamins, sterols, and many other fine chemicals. These metabolites have a broad range of biological activities, such as anti-viral, anti-tumoral, anti-oxidant and antimicrobial activity against bacteria and fungi (Devi et al., 1997 ; Hellio et al., 2001a ; Hellio et al., 2001b).

The aim of the present work is the following:

- Searching for new antimicrobial algal substances alternative for the use of antibiotics.
- Identification the extracted antimicrobial compounds.

MATERIALS AND METHODS

Materials

1. Culture media for bacterial isolates

- Nutrient broth (N.B.) medium: (Marshall, 1993).
- Nutrient agar medium: (Sood, 2006).

2. The antibiotics used in sensitivity screening on the bacterial isolates from skin infections of patients.

Seventeen selected antibiotic discs (Bio analyse, Turkey) were used (amoxicillin, oxacillin, imipenem, doxycycline, nitrofurantoin, cefuroxime, cephalothin, rifampin, cefotaxime, gentamicin, levofloxacin, ampicilline, chloramphenicol, trimethoprine, erythromycin, tetracycline and vancomycine), table (1).

Table (1): Susceptibility of bacterial isolates to different antibiotics

Antibiotic Class	Antibiotics	Concentration µg/disc potency	Susceptiblity		Resistance	
			No.	%	No.	%
Ansamycins	Rifampin (RA)	5	15	46.87%	17	53.12%
Aminoglycosides	Gentamicin (CN)	15	17	53.12%	15	46.87%
Carbapenems	Imipenem (Imp)	30	24	75%	8	25%
	Cefotaxime (Ctx)	30	5	15.62%	27	84.37%
Cephalosporins	Cefuroxime (cxm)	30	9	28.12%	23	71.87%
	Cephalothin (Kf)	30	9	28.12%	23	71.87%
Folate pathway inhibitors	Trimethoprine (Stx)	30	13	40.62%	19	59.37%
Fluoroquinolones	Levofloxacin (lev)	10	21	65.62%	11	34.37%
Glycopeptide	Vancomycine (VA)	30	14	43.75%	18	56.25%
Macrolide	Erythromycin (Ery)	15	19	59.37%	13	40.62%
Nitrofuratoins	Nitrofurantoin (Nit)	12	22	68.75%	10	31.25%
	Ampicillin (p)	10	0	0	32	100
Penicillins	Amoxicillin (AMP)	10	0	0	32	100
	Oxacillin (Oxa)	17	4	12.5	28	87.5%
Polyketide	Doxycycline (Do)	15	17	53.12%	15	46.87%
	Tetracycline (Te)	30	12	37.5%	20	62.5%
Prototypical broad spectrum	Chloramphenicol (Chl)	30	13	40.62%	19	59.37%

3. Types of Algal extracts tested on bacterial isolates.

In this study, three species of seaweeds were collected from two sites Ras-Al Teen and Al-Mountazah, Alexandria. All seaweeds were identified (Aleem, 1993). The collected species from different algal phyla were *Jania rubens*, *Ulva Lactuca and Sargassum latifollium.*

Methods:

4. Collection of samples:

Thirty two bacterial isolates were collected from skin infections, wounds and burns at Tanta University Hospitals, Department of Dermatology and Burns immediately placed in nutrient broth transport medium and then transferred to laboratory of bacteriology in Botany Department, Faculty of Science, Tanta University. Bacterial isolates were grown on nutrient agar plates. Plates were incubated over night at 37ºC.

5. Molecular characterization of the best bacterial isolates by 16S rRNA they used the following protocol for extraction:

- DNA extraction by using protocol of Gene Jet genomic DNA purification Kit (Thermo): (Corkill et al., 2008).
- 16S rDNA Amplification: (Drancourt et al., 2000).
- loading of 4ul from the PCR mixture to examine the PCR product on 1% agarose gel against 1Kb plus ladder (Fermentas).

  F:- AGA GTT TGA TCC TGG CTC AG

  R:- GGT TAC CTT GTT ACG ACT T
- Purification of PCR Product: (Sambrook et al., 2001)
- Sequencing of partial 16S rRNA gene: (Mignard et al., 2006).

6. Antibiotic susceptibility of isolated bacterial:

Susceptibility of bacterial isolates to seventeen different antibiotics was studied using the disc diffusion method (Cursino et al., 2005). Bacterial isolates were subcultured on nutrient agar plates at 37ºC for 24 h. Bacterial suspension was prepared using few separate colonies for each isolate and 1-2 ml of phosphate buffer. Each suspension was diluted using phosphate buffer to obtain cell count of about 10^6 CFU/ml using standard turbidity. A volume (0.1 ml) of each of the previous suspensions was dropped on the center of two well dried plates of nutrient agar and was then spread homogeneously using sterile glass rod and left to dry at 37ºC for 15min. The antibiotic discs were then applied to the prepared plates using sterile forceps with 1cm part, and they were pressed gently in site and incubated at 37ºC for 24 hours. Diameters of inhibition zones were measured and compared to a reference table to differentiate the isolates into sensitive, intermediately resistant or resistant (Bemer and Drugeon, 2001).

Algal collection

Seaweeds were collected from two sites Ras Al-Tin and Al-Mountazah along the north coast of Egypt, Alexandria region. Tested seaweeds were collected by hand from different depth ranged from 1 feet to 1 meter. All samples were brought to laboratory in plastic bags containing sea water to prevent evaporation. The algae were cleaned from epiphytes and rock debris, then given aquick fresh water rinse to remove surface salts. Some of the collected seaweeds were preserved for identification.

Preparation of the extract.

The clean material was air dried in the shade at room temperature 25^{o}C on absorbent paper, then grounded to fine powder in an electrical coffee mill. The extraction was carried out with a solvent by soaking the material in the solvent (1:15 v/v) within aconical flask, plugged with cotton wool, then Kept on a rotary shaker at 150 rpm at room temperature for 96 hr. The extraction with acetone as a solvent was carried out on samples. The extracts were pooled and filtered using filter paper, the obtained filtrate was freed from solvent by evaporation under reduced pressure. The residues (crude extracts) obtained were suspended in the respective solvent to final concentration of 100 mg/ml and stored at -20^{o}C in airtight bottle.

Gas chromatography mass spectra (GC-MS) of the antagonistic material:

Sargassum latifollium extract content was examined by gas chromatography, Mass sepectroscopy in Claurs 580/560S. Work was done with column 30.0 m x 250µm, Rtx–5MS (crossbond 5% diphenyl 95% dimethylpolysiloxane), Perkin Elmer Company in Central lab, Tanta University, equipped with heated FID .The GC conditions were employed using Helium as carrier gas (0.8 ml/min) and the temperature program was 60°C for 0 min, followed by an increase of 10°C/min to 235°C for the remainder of the run. Detector and injection point heaters were 230 and 280°C, respectively and typically 0.1 or 1.0 ml was injected at a 20: 1 split.

UV spectra of the antagonistic material:

The UV spectra of the tested material were determined by spectrophotometer (UV 2101/pc) using cuvette containing the antagonistic material dissolved in pure acetone and the blank was prepared using pure acetone. The wavelength ranged from 200-1000 nm. The appearance of different peaks at certain wavelength may indicate the polymeric structure of the compound.

The infrared spectra (IR) of the antagonistic material:

Using Perkin Elmer 1430 infrared spectrophotometer the molecular structure of the antagonistic material was partially identified. Because the antagonistic material is liquid at room temperature so it can be examined directly as a thin film. Measurements were carried out at infrared spectra between 200-4000nm.

RESULTS

Isolation of Bacteria from Infected Human skin

A total of thirty two bacterial isolates were randomly collected from skin, wound and hair of patients attending the outpatient clinic of the dermatology of Tanta university hospital using swabs immersed in nutrient broth medium then transferred to laboratory of bacteriology in botany department, faculty of science, Tanta university, Egypt . These isolates were grown on nutrient agar and incubated at 37ºC over night.

Antibiotic susceptibility of clinical bacterial isolates:

Antibiotic susceptibility of the collected bacterial isolates to seventeen antibiotics was studied in the present study using the disc diffusion method. Results in table (1) shown that the highest resistance of bacterial isolates were recorded to ampiciline and amoxicillin was (100%), oxacillin (87.5%) and cefotaxime (84.37%). On the other hand, the lowest resistant was recorded by imipenem (25%) and Nitrofurantoin(31.25%). Cephalothin and cefuroxime have the same bacterial resistance (71.87%), While the resistance of the tested bacterial isolates to the aminoglycoside drugs was 46.87%, also the bacterial isolates showed resistance to chloramphenicol (59.37%). Finally, the tested bacterial isolates can resist some antibiotics like macrolide antibiotic erythromycin (40.62%), quinolone antibiotic levofloxacin (34.37%), glycopeptide antibiotic vancomycine (56.25%), polyketide antibiotic tetracycline 62.5%, doxycycline (46.87%) and trimethoprine (Stx) was (59.37%) in addition to ansamycins group rifampin (RA) which recorded (53.12%). The data showed in table (2) revealed that among the bacterial isolates number (9) and (17) were proved to be the most multi-drug resistant isolates recording 100% resistance. However, antibiotic showed the highest susceptibility toward bacterial isolate number (27) recording 70.58%.

Table (2): Sensitivity of the bacterial isolates to seventeen tested antibiotics.

Bacterial isolates	Antibiotic Number	Resistance %	Antibiotic Number	Susceptiblity %
1	13	76.5	4	23.5
2	10	58.82	7	41.17
3	11	64.7	6	35.3
4	10	58.82	7	41.17
5	10	58.82	7	41.17
6	11	64.7	6	35.3
7	14	82.35	3	17.64
8	10	58.82	7	41.17
9	17	100	0	0
10	6	35.3	11	64.7
11	8	47	9	53
12	10	58.82	7	41.17
13	11	64.7	6	35.3
14	6	35.3	11	64.7
15	10	58.82	7	41.17
16	11	64.7	6	35.3
17	17	100	0	0
18	6	35.3	11	64.7
19	10	58.82	7	41.17
20	9	53	8	47
21	6	35.3	11	64.7
22	8	47	9	53
23	11	64.7	6	35.3
24	11	64.7	6	35.3
25	11	64.7	6	35.3
26	10	58.82	7	41.17
27	5	29.41	12	70.58
28	10	58.82	7	41.17
29	11	64.7	6	35.3
30	10	58.82	7	41.17
31	12	70.5	5	29.5
32	12	70.5	5	29.5

Molecular identification of the selected multi-drug resistant bacterial isolate.

Among the tested bacteria isolates number (9) and (17) recorded the highest multi-drug resistance, so bacterial isolate number (9) was subjected to molecular identification by using 16S rRNA gene sequence. By Applying the Biosystem 16S ribosomal RNA sequence, the tested bacterial isolate number (9) revealed a similarity of 98% with *Staphylococcus aureus* sub sp. *aureus* JH1, figure (1).

16S ribosomal RNA sequence of *Staphylococcus aureus* subsp. *aureus* JH1

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Query 39    GACGGGTGGTGTGTA-AAGATCCGGGAACGTATTCACCGTAGGAGCTGATCTACGATTA  96
Query 97    CTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTGAGAACAACTT 156
Query 157   TATGGGATTTGCATGACCTCGCGGTTTAGCTGCCCTTTGTATTGTCCATTGTAGCACGTG  216
Query 217   TGTAGCCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTG  276
Query 277   TCACCGGCAGTCAACCTAGAGTGCCCAACTTAATGATGGCAACTAAGCTTAAGGGTTGCG 336
Query 337   CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC 396
Query 397   TGTCACTTTGTCCCCCGAAGGGGAAGGCTCTATCTCTAGAGTTTTCAAAGGATGTCAAGA 456
Query 457   TTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGT  516
Query 517   CCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAAT  576
Query 577   GCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGG 636
Query 637   CGTGGACTACCAGGGTATCTAATCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAG 696
Query 697   TTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTGCGCATTTCACC 756
Query 757   GCTACACATGGAAATTCCACTTTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCAATGACC 816
Query 817   CTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCGCCTACGCGCGCTTTA 876
Query 877   CGCCCAATAATTCCGGATA-CGC-TGCCACCTACGTATTACCGCG-CTGCTG-CACGTAG  932
Query 933   T-AGC-GTG-CTTTCTGAT-AG-TAC-GTC-AGACGTGCACAGT-AC-TACACGTTTGTC  983
Query 984   CT-CC-TA-TA-C-GAGTTT-ACGAGC-GAAC-CCT-CATCACTCA-GCG-CGT-GCTC-  1030
Query 1031  GTCAGCCTT-CGCC-AT-GCT-A-GAT-C--TACTGCTGC-TCC-GTAG-AGTCTGGACC  1079
Query 1080  G-GTTTCAGTTC-AGTG-G-C-GAT-ACCCT-TCAG-TCG-C
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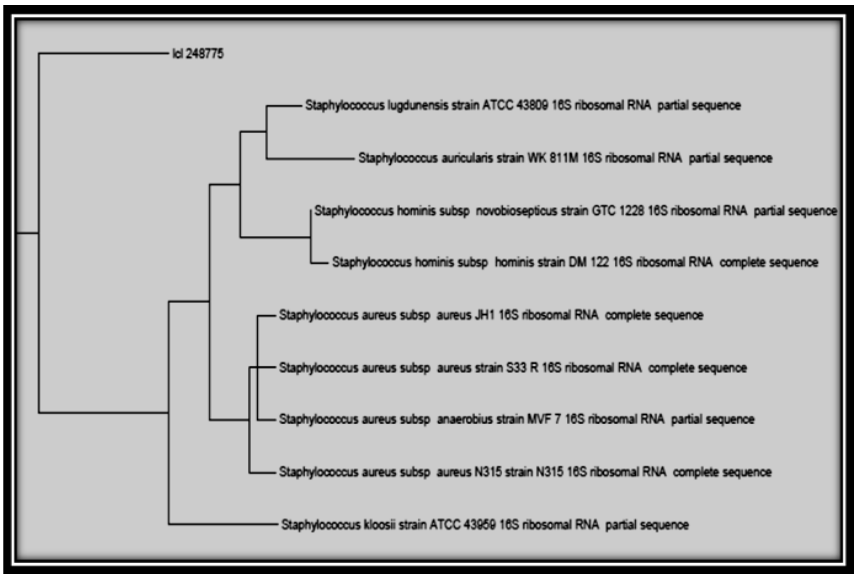

Figure (1): Nucleotide sequence of 16S rRNA of *Staphylococcus aureus sub sp aureus JH1.*

Antimicrobial activity of *Saragassum latifolium, Ulva lactuca and Jania rubens* against the tested bacterial isolates:

The antibacterial activity of *Saragassum latifolium, Ulva lactuca and Jania rubens* were tested against the most multi-drug resistant bacterial isolate (number 9) compared with the control, table (3).

Saragassum Latifolium extract appeared the highest antibacterial activity (12 mm), both *Ulva lactuca* extract and *Jania rubens* appeared the same antibacterial activity (10 mm). The negative control (DMSO) didn't show any antibacterial activity.

Table (3): The antibacterial activity of the *Saragassum latifolium, Ulva lactuca and Jania rubens* against bacterial isolate number (9)

Isolates	Diamter of inhibition zone of *Saragassum latifolium*	Diamter of inhibition zone of *Ulva lactuca*	Diamter of inhibition zone of *Jania rubens*	Diamter of Inhibition zone of DMSO
Bacterial isolate number (9)	12 ± 0.11	10 ± 0.12	10 ± 0.14	0 ± 0.0

Characterization of the purified material extracted from *Saragassum latifolum.*

UV spectra of the antagonistic material:

In this work, the ultraviolet spectrum of some compounds is recorded in conjunction with other spectral data such as infrared as an attempt to deduce the compounds which caused inhibition to the growth of bacterial isolate. Ultraviolet spectra tend to act as complimentary or even supplementary evidence to infrared. Before measuring the ultraviolet spectrum, the

different pigments and impurities were removed by filtration using charcoal, then the ultraviolet spectrum of the purified antagonistic material isolated from *Saragassum latifolium* was carried out in pure acetone.

Figure (2) shown that there are four absorption peaks at 344nm, 394 nm, 436 nm and 666 nm. This indicating that it does not contain any conjungation or aromatic ring

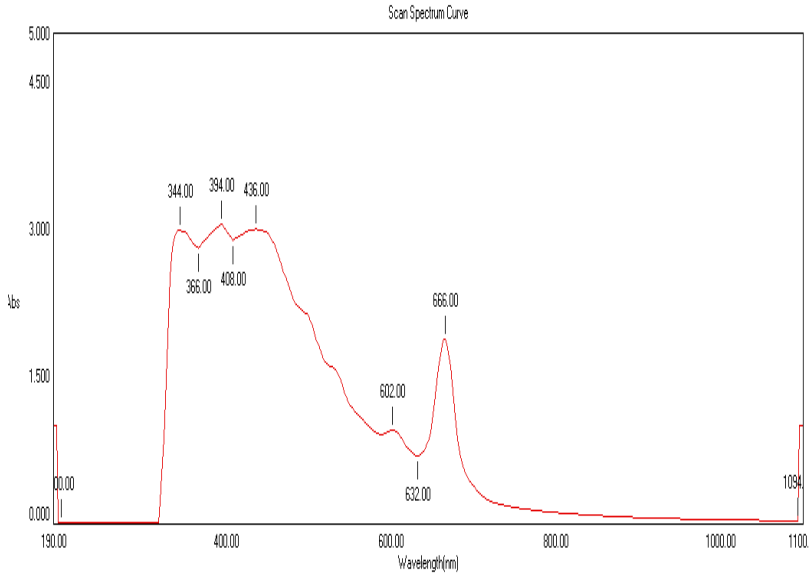

Figure (2): UV of antagonistic material produced by *Saragassum latifolium* after purification by charcoal

The infrared spectrum (IR) of the antagonistic material:

In the molecular diagnosis of vibrational frequencies, it is extremely useful to refer to the tabulated values of the various functional groups and their associated characteristic group frequency ranges. The spectrum was subdivided into different regions , figure (3), as the following:

Absorption in the 3422 region: This region, comprise one band due to the stretching vibration of the OH group or NH2 group.

Absorption in the 2854-2925 region: This region, comprise two bands due to the stretching vibration of the CH group.

Absorption in the 1627-1708 region: This region, comprise two bands due to the stretching vibration of the C=0 group.

Absorption in the 1050-1462 region: This region, comprise one band due to the stretching vibration of the C-N group at 1050 cm-1. In addition, three bands due to the stretching vibration of the N-O group at 1208 cm-1, 1263 cm-1 and 1462cm-1.

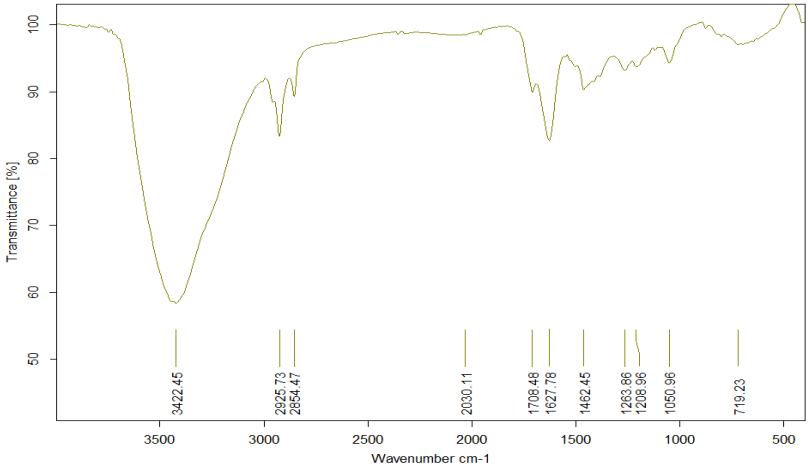

Figure (3): IR spectra of the purified antagonistic material produced by *Saragassum latifolium*.

GC-MS of the antagonistic material: The algal extract of *Saragassum latifolium* obtained by acetone were subjected to GC-MS analysis to determine the main component especially the antimicrobial agents. The GC-MS chromatogram shown in Figure (4) revealed four main peaks.

The first peak (22.405): May be n-hexadecanoic acid (45.158%).

The second peak (26.987): May be docosanoic acid 1,2,3-propanetriyl ester (24.856%).

The third peak (32.184): May be hexanedioic acid, dioctyl ester (17.126%).

The fourth peak (35.305): May be docosanoic acid 1, 2, 3-propanetriyl ester (12.861%).

From GC-MS, IR and UV we found that *Saragassum latifolium* contain long chain saturated fatty acids which responsible for antibacterial activity. These fatty acids may be free such as n-hexadecanoic acid or found in ester form such as docosanoic acid 1,2,3-propanetriyl ester and hexanedioic acid, dioctyl ester.

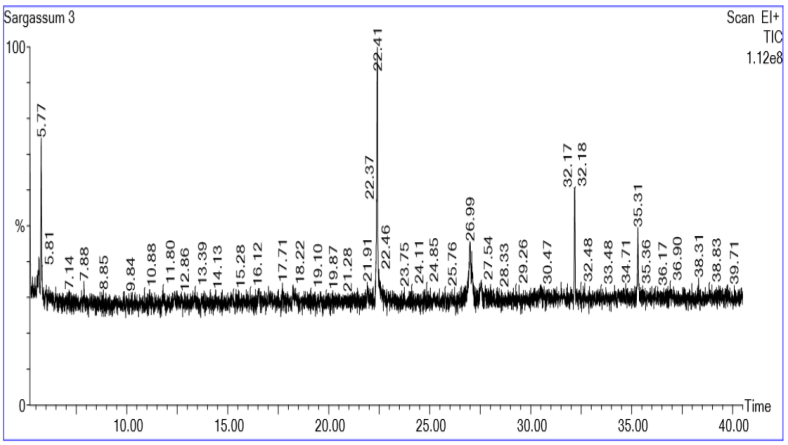

Figure (4): GC Mass spectra of the antagonistic material produced by *Saragassum latifolium.*

The suggested chemical structure of purified antagonistic material isolated from *Saragassum latifolium:* Figure (5)

- Chemical formula: C69H13406
- Molecular weight : 1059.7987
- Chemical structure:

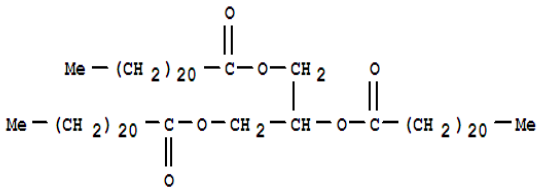

Figure (5): The molecular formula of the active component of *Saragassum latifolium* extract.

DISCUSSION

Antibiotic susceptibility of the collected isolates to seventeen antibiotics was studied using the disc diffusion method.The resistance of these bacterial isolates ranged from 25% to 100%. Results showed that the highest resistance of all bacterial isolates was recorded by ampiciline and amoxicillin (100%) and the lowest resistance was recorded by imipenem (25%).

S. aureus (Bacterial isolates number 9 and 17) were the most resistant to seventeen tested antibiotics. These results were in agreement with these obtained by *(Gad et al., 2007 ; Al-Zubaydi et al., 2008).*They found that gram positive bacteria were the major bacteria in wound and dermal infection, among which *S.aureus* ranked the first among the gram positive bacteria.

In this study, the three studied seaweed namely *Saragassum latifolium , Ulva lactuca and Jania rubens* showed antibacterial activity against the tested bacteria with varying degrees, brown algae (*Phaeophyta*) represented in *Saragassum latifolium* showed the strongest antibacterial activity, this result is in accordance with the data obtained by*(Caccamese et al. 1985),* as they have reported that brown algal extracts showed higher activity than the extracts of red algae, also *(Viachosi et al., 2001)* reported that extracts of the *Phaeophyta* exhibited the highest level of antibacterial activity, followed by the *Rhodophyta* and then the *Chlorophyta*.

According to the obtained data from GC-MS, IR and UV we found that *saragassum latifolium* contain long chain saturated fatty acids which responsible for antibacterial activity, These fatty acids may be free such as n-hexadecanoic acid or found in ester form such as docosanoic acid 1,2,3-propanetriyl ester and hexanedioic acid, dioctyl ester. These results were agreement with *(El Shoubaky and Salem 2014)* who illustrated that acetone extracts of tested brown seaweeds algae such as *Hormophysa triquetra (H. triquetra) and Padina pavonica (P.pavonica)* showed strong antimicrobial activity against both gram positive and gram negative bacteria. The tested algae was rich in fatty acids which may be saturated or unsaturated fatty acids, the antimicrobial activity of fatty acids has been more attributed to long chain unsaturated fatty acids such as palmitoleic, oleic and linolenic acids.

CONCLUSION

Thirty two bacterial isolates were randomly collected from patients attending the outpatient clinic of the dermatology of Tanta university hospital. Bacterial isolates were grown on nutrient agar medium and tested for determination of the level of susceptibility to antibiotics and to be compared with some algal extracts. Results were summarized as following:

Among the tested bacteria isolates number (9) and (17) recorded the highest multi-drug resistance, so bacterial isolate number (9) was subjected to molecular identification by using 16S rRNA gene sequence. By Applying the Biosystem 16S ribosomal RNA sequence,

the tested bacterial isolate number (9) revealed a similarity of 98% with *Staphylococcus aureus* subsp. *Aureus*.

Treatment the tested bacteria by algal extract, we found that *Saragassum latifolium* recorded the highest inhibition zone of (12 mm),

From GC-MS , IR and UV we found that *Saragassum latifolium* contain long chain saturated fatty acids which responsible for antibacterial activity, These fatty acids may be free such as n-hexadecanoic acid or fonud in ester form such as docosanoic acid 1,2,3-propanetriyl ester and hexanedioic acid, dioctyl ester.

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دراسه بعض الامراض الميكروبيه الجلديه ومعالجتها ببعض المستخلصات من بعض انواع الطحالب البحريه

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في هذه الدراسه تم عزل اثنان وثلاثون عزله بكتيريه من جلد وجروح المرضى بمستشفى طنطا الجامعي العام قسم الجلديه واختبرت حساسيتها للمضادات الحيويه والطحالب البحريه المستخدمة. العزلات ارقام (9) و (17) هم اكثر مقاومه للمضادات الحيوية لذا تم تعريف العزله البكتيريه رقم (9) باستخدام بى سى ار واتضح انها استافيلوكوكس اوريس .تم دراسه تاثير بعض مستخلصات الطحالب البحريه على العزله البكتيريه واتضح ان طحلب ساراجسم لاتيفوليم هو الافضل تاثيرا على العزله البكتيريه. تم عمل تحليل كميائي بالمعمل المركزي بطنطا للتعرف علي المواد الكميائيه الموجوده في مستخلص طحلب ساراجسم لاتيفوليم واتضح ان هذا النشاط المقاوم للبكتيريا يرجع الى احتوائه على مجموعه من الاحماض الدهنيه.