

Original Research Article

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Antibacterial Activity of Some Seaweed Extracts against Multidrug Resistant Urinary Tract Bacteria and Analysis of their Virulence Genes

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ABSTRACT

Multidrug resistant bacteria raise a serious clinical problem for treating infectious diseases worldwide. Different seaweed algae including *Ulva lactuca* (Chlorophyta), *Petalonia fascia* (Phaeophyta) and *Gelidium spinosum* (Rhodophyta) were investigated as natural sources for antibacterial compounds. Different solvents ethanol, methanol, acetone and water were used to extract the antibacterial substances from these seaweeds and were examined against urinary tract multidrug resistant bacterial isolates: *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. The ethanolic extract of *Ulva lactuca* exhibited the most significant antibacterial activity against the tested urinary tract infection (UTI) bacterial isolates. The extract was purified and chemically analyzed using UV, IR, ¹H NMR and GC-MS. The results indicated that the extract was aromatic ester derivative named Di-isooctylphthalate of molecular weight 390.56. PCR analysis of three virulence factors (adhesion, urease and hemolysin) were employed in order to genetically compare the virulence activities of the UTI isolates. The results showed that only faint PCR amplicon of hemolysin virulence gene was observed in *P. mirabilis* sensitive isolate, while two fragments were found in the multidrug and algal resistant corresponding isolates with amplicon sizes of 375 and 875 bp.

Keywords

Antibacterial activity, PCR analysis, *Ulva lactuca*, UTI infection, Virulence genes.

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Introduction

Worldwide, urinary tract infections (UTI) are among the most common infectious diseases. About 150 million people are diagnosed with UTI every year (Gonzalez and Schaeffer, 1999). A urinary tract infection is an infection involving the kidneys, ureters, bladder, or urethra. These are the structures that urine passes through before being eliminated from the body (Foxman and Brown, 2003). Virtually, an infection occurs when Gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Proteus vulgaris*) get into the urine and begin to grow. The infection usually starts at the opening of the urethra, where the

urine leaves the body and moves upward into the urinary tract, these bacteria normally live in the bowel (colon) and around the anus. The symptoms develop rapidly including fairly high fever, shaking chills, nausea and vomiting (Kalaivani *et al.*, 2016). UTI infection may complicate into an acute or chronic kidney infection (pyelonephritis) or even permanent kidney damage due to neglected or not correctly treated infection. Many classes of antibiotics were classified as typical treatments for such diseases. However, the improper and uncontrolled use of many antibiotics resulted in the occurrence of

antimicrobial drug resistance, which became a major health problem worldwide (Dethlefsen *et al.*, 2008) and forced seeking for new treatment sources.

Virulence factors are specific traits enabling pathogenic bacteria to overcome host immune system and cause various diseases (Ejrnaes, 2011). Virulence genes are located on transmissible genetic elements and/or in particular regions on the chromosome that are called pathogenicity islands (Farshad *et al.*, 2012). Pathogenicity islands directed coordinate horizontal transfer of virulence genes between strains of one species or even related species (Hacker *et al.*, 1997). UTI bacterial strains have various types of virulence factors such as adhesions, hemolysin, urease enzyme, toxins and iron uptake systems that facilitate colonization and persistence of the bacteria in the urinary tract (Oliveira *et al.*, 2011) and support their resistance system and pathogenicity to the regularly used synthetic drugs.

Nowadays, seaweeds are considered a novel source of bioactive compounds and produce a great variety of secondary metabolites exhibiting broad spectrum of biological activities (Khotimchenko *et al.*, 2002; Ramasamy and Kumar, 2009; El-Sheekh *et al.*, 2016). Many types of seaweed have been screened extensively to isolate natural drugs or biologically active substances from different habitats around the world. Several solvents with different polarities e.g. hexane, chloroform, ethanol, methanol, acetone and water were used to extract the antimicrobial material from such seaweeds. Compounds with cytostatic, antiviral, anthelmintic, antifungal, bacteriostatic and bactericidal activities have been detected in green, brown and red seaweeds with a comparable promising results with authentic antibiotics (Newman *et al.*, 2003; Manikandan *et al.*, 2011; Osman *et al.*, 2013; Ismail *et al.*, 2016).

Active antibacterial agents found in the seaweeds include terpenoid, phlorotannins, acrylic acid and phenolic compounds (Cardoso *et al.*, 2014). Also, steroids, halogenated ketone, fatty acids and alkaline, cyclic polysulphides were recorded (Cheung *et al.*, 2015; Khelil-Radji *et al.*, 2017). The present study aimed to explore the antibacterial activity of some seaweed extracts against urinary tract (UTI) infectious bacteria. To compare the activity of these extracts as natural drug sources with the standard synthetic antibiotics against multidrug resistant (MDR) isolates. Finally, to compare the virulence gene profiles of the UTI isolates and detect the effect of seaweeds treatment on their virulence activities.

Materials and Methods

Isolation and identification of bacterial isolates

The bacterial swaps were obtained from microbiology laboratories, Faculty of Pharmacy, Tanta University, Tanta, Egypt. The bacterial isolates, generally Gram negative, were cultivated on different selective media. The morphological and biochemical examinations were conducted repeatedly according to the standard methods until complete purification and identification of these bacterial isolates (Oxoid, 1981; Collee *et al.*, 1996). Eight bacterial isolates were selected of which *Escherichia coli* (3 isolates), *Klebsiella pneumoniae* (2 isolates) and *Proteus mirabilis* (3 isolates).

Antibiotic susceptibility test of bacterial isolates

The antibacterial susceptibility of the pathogenic bacterial isolates were confirmed against eight different classes of antibiotics using the modified Kirby-Bauer disk diffusion method according to the Clinical and

Laboratory Standards Institute (CLSI, 2012) on Mueller Hinton agar. The tested antibiotics were commonly used in the treatments of UTI cases and included: Carbapenems, Cephalosporins, Monobactams, Nitrofurantoin, Penicillins, Polyketide, Quinolone/ Fluoroquinolone and Sulfonamides. The plates were then incubated overnight at 37°C. The diameters of the inhibition zones were measured (Vasquez and Hand, 2004) and compared to differentiate the bacterial isolates into sensitive, intermediate and resistant and to calculate the multiple antibiotic resistances (MAR) index for each isolate (by dividing the number of antibiotics to which the isolate is resistant by the total number of tested antibiotics).

Preparation of algal extracts

Three species of seaweeds from different divisions (Chlorophyta) *Ulva lactuca*, (Phaeophyta) *Petalonia fascia* and (Rhodophyta) *Gelidium spinosum* were collected in 2016 from Abu Qir-Bay, Alexandria, Egypt (N 31°19' E 30°03'). The seaweeds were identified according to (Aleem, 1993) and (Guiry and Guiry, 2016). The different seaweeds were air dried in the shade at room temperature (25°C) and then grounded to fine powder. The powders (1:10 w/v) were soaked with different polar organic solvents (80% each of ethanol, methanol and acetone in addition to water) for 24 hrs. on a rotary shaker at 150 rpm. The extracts from three consecutive soakings were pooled together and filtered. The obtained filtrates were evaporated using rotary evaporator at 45°C until dryness, then weighed and stored at -20°C (Karthikaidevi *et al.*, 2009). The collected seaweeds were identified to be:

***Ulva lactuca* Linnaeus**

Empire: Eukaryota, *Kingdom:* Plantae, *Phylum:* Chlorophyta, *Class:* Ulvophyceae,

Order: Ulvales, *Family:* Ulvaceae *Genus:* *Ulva*.

***Petalonia fascia* (O. F. Müller) Kuntze**

Empire: Eukaryota, *Kingdom:* Chromista, *Phylum:* Ochrophyta, *Class:* Phaeophyceae, *Order:* Ectocarpales, *Family:* Scytosiphonaceae, *Genus:* *Petalonia*.

***Gelidium spinosum* (S. G. Gmelin) P.C. Silva.**

Empire: Eukaryota, *Kingdom:* Plantae, *Phylum:* Rhodophyta, *Class:* Florideophyceae, *Order:* Gelidiales, *Family:* Gelidiaceae, *Genus:* *Gelidium*.

Antibacterial activity test of seaweeds extracts

The seaweeds antibacterial activity was assayed using disk diffusion method as described in the previous section. Suspensions of the tested bacterial isolates were prepared from overnight freshly incubated bacterial cultures and mixed in sterilized saline solution (0.9% w/v NaCl) to get cell counts of 10⁶ to 10⁸ CFU/ml for each bacterial isolate (Gilbert, 1987).

Seaweed extract was prepared by re-suspending the powder (50:1 w/v) in each solvent. Previously sterilized filter paper disks were immersed in different algal extracts (20 µl) and inoculated over Petri dishes surface. The plates were incubated for 2 hours at 4°C to allow the diffusion of antibacterial substance and then incubated overnight at 37°C. Standard antibiotic discs and solvent discs were used as positive and negative controls, respectively.

The diameter of inhibition zones indicates the antibacterial activity of different seaweeds in different solvents.

Identification of sensitive bacterial isolates

Bacterial isolates which were sensitive to seaweed extracts were identified using Biomerieux VITEK®2 system. The Biomerieux VITEK®2 cards were filled with the tested bacterial suspension and manually loaded into the VITEK®2 system for identification (Thomas *et al.*, 2001).

Fractionation, purification and characterization of antibacterial crude extracts

Column chromatography and UV spectra of algal antibacterial agent

Active crude seaweed extract (5 g) was purified and fractioned by column chromatography on silica gel G EDWC, 60-200 mesh (Solomon and Santhi 2008). A glass column (3 cm x 20 cm) was packed by silica gel (30-40 g) in pure chloroform eluted with gradients of mobile phase from 30:70 % chloroform: ethanol to 70:30 % chloroform: ethanol and the collected fractions were evaporated at 40°C under vacuum. The dried fractions were dissolved in 70 % ethanol solvent and their absorption was measured using UV spectrophotometer (UV 2101/pc) at 200- 900 nm wavelength (Blunt *et al.*, 2007). The antibacterial activity of the collected fractions was examined against UTI bacterial isolates using disk diffusion method. Different active fractions with the same UV absorption were collected together, dried and subjected to subsequent analysis to determine the chemical structure of the active compounds.

Infra-red spectra (IR) of the algal antibacterial agent

The infra-red spectrophotometer (FT-IR) (Perkin Elmer 1430) was used to identify the functional groups of the antibacterial algal material (Boeriu *et al.*, 2004). The pellet

which contains sample of active compound in solid phase was mixed with KBr (FT-IR grade) and FT-IR spectra were recorded in the range of 4000- 400 cm^{-1} .

Proton nuclear magnetic resonance (¹H NMR) spectra of algal antibacterial agent

The sample was mixed with dehydrated chloroform solvent (0.04 g/ml). Different protons of the antibacterial material could be identified by their corresponding nuclear magnetic resonance (Atalah *et al.*, 2007).

Gas chromatography mass spectrometry (GC-MS)

Gas chromatography mass spectrometry (GC-MS) analysis was used to determine the chemical composition of the seaweeds antibacterial material. The acquisition parameters were done with column (30m x 250 μm x 0.25 μm film thickness) using helium as carrier gas (0.8 ml/min) with Perkin Elmer: Clarus 580/560 S model system. The GC oven temperature was programed from 60°C to 250°C at a rate of 2°C/ min. Relative area values (as a percentage of total volatile composition) were directly obtained from total ion current (TIC).

Molecular detection of the virulence factors of the bacterial strains

DNA extraction and PCR amplification of virulence factors

In order to extract genomic DNA of the multidrug resistant (MDR) bacterial isolates, which were sensitive to the seaweeds antibacterial material, boiling method was used. Before DNA extraction, the bacterial isolates were activated by overnight culturing on nutrient broth at 37°C, then it was centrifuged at 5×10^3 for 15 minutes. The resulted pellets were mixed in 100 μl of sterilized water and incubated in boiling water

for 10 min at 100°C for complete DNA extraction (Anderson *et al.*, 2004). Polymerase chain reaction (PCR) technique was used to detect three virulence factors genes including hemolysin, adhesion and urease.

Amplification of bacterial virulence genes was done in a total volume of 25 µl

containing 1 µl of bacterial suspension, 1.5 µl of each of the primers, 8.5 µl sterilized distilled water, and 12.5 µl of the red master mix (Bioline); which contained 1000 x 50 µl Reactions U Taq DNA polymerase (Sigma-Aldrich) in 1× PCR buffer containing 1.5 mM MgCl₂ (Table 1).

Table.1 Sequences of the primers used to amplify the UTI virulence genes

Primer	Sequence (5`-3`)	Amplicon size (bp)	Annealing Temp.
Hemolysin			
HpmA F	5`-GTTGAGGGGCGTTATCAAGAGTC-3`	709	55°C
HpmA R	5`-GATAACTGTTTTGCCCTTTTGTGC-3`		
Adhesin			
UcaA F	5`-GTAAAGTTGTTGCGCAAAC-3`	580	50°C
UcaA R	5`-TTGAGCCACTGTGGATACA-3`		
Urease			
UreC F	5`-CCGGAACAGAAGTTGTGGCT-3`	533	63°C
UreC R	5`-GGGCTCTCCTACCGACTTGA-3`		

PCR conditions were adjusted as following: the initialization heating step was done at 95°C for 3 min, and then a denaturation step at 95°C for 50 sec. followed by an annealing step at 55°C for 50 sec. The elongation step was performed at 72°C for 30 sec. These steps were repeated 35 times (cycles). The final elongation was performed at 72°C for 6 min. in a Thermo cycler apparatus (HVD S-96).

The PCR products were electrophoresed on 1% agarose gels. The gels were stained in ethidium bromide for 15 min. and visualized under UV-transilluminator (UVP Photo-Doc-It Imaging system). The size of the amplicons was determined by comparing them with the 1 kb DNA ladder (Thermo scientific) included on the same gel (Johnson, 1991).

Statistical analysis

All experiments were done in triplicates and the results were expressed as means ±

standard deviation (SD). Analysis of variance (ANOVA one way) was established to assess the significant variation in antibacterial activities of different seaweed extracts on the tested bacterial isolates at $P \leq 0.05$ level of significance.

Results and Discussion

Antibiotic susceptibility test of bacterial isolates

Synthetic antibiotics, by its own, constitute a global growing problem due to their many harmful side effects. Indiscriminate use of antibiotics to treat such infectious diseases increased the bacterial pathogenicity and resistance to these medications creating multidrug resistant strains (Read and Woods 2014). UTI diseases have become one of the leading causes of death worldwide especially when miss diagnosed or improperly treated.

The multi-antibiotics resistance MAR index results revealed that all tested urinary tract isolates *E. coli* (3 isolates), *K. pneumoniae* (2 isolates) and *P. mirabilis* (3 isolates) had a very high MAR index value (> 0.2) with resistance values ranged from 63% to 100% (Table 2). According to (Olayinka *et al.*, 2009), the MAR values are an indication of the degree of microbial exposure to specific antibiotics used within the community. It is usually used to test the resistance of antibiotic and to analyze health risk parameters. In this concern, they also showed high incidence multiple drug resistance (MDR) capacity of 5 to 9 antibiotic agents. The bacterium, which is simultaneously resistant to a number of antibiotics belonging to different chemical classes is indicated to be MDR (Magiorakos *et al.*, 2012).

Therefore, discovering new naturally antimicrobial compounds from seaweeds with various chemical structures and different mechanisms of action appears a promising approach. Seaweeds are abundant and famed with their healthy nutritional values around the world. Nowadays, seaweeds were re-discovered as cheap and safe sources in many medicinal and pharmacological applications (Adaikalaraj *et al.*, 2012). Regarding the calculated results of the multiple antibiotics resistance (MAR) index (Table 2), E1 isolate of *Escherichia coli*, K1 isolate of *Klebsiella pneumoniae* and P1 isolate of *Proteus mirabilis* were selected as the most multidrug resistant (MDR) bacterial isolates for testing the antibacterial activities of the seaweed extracts and for PCR analysis of the bacterial virulence genes.

Table.2 The antibiotic susceptibility test of bacterial isolates against different antibiotics

Antibiotic class	Antibiotics common name	Potency $\mu\text{g}/\text{disk}$	Bacterial isolates							
			E1	E2	E3	K1	K2	P1	P2	P3
Carbapenems	Imipenem (IMP)	10	S	I	S	S	S	R	S	S
Cephalosporin	Ceftazidime (CAZ)	30	R	R	S	R	S	R	R	R
Monobactams	Aztreonam (ATM)	10	R	R	R	R	R	R	R	R
Nitrofuratoins	Nitrofurantoin (NIT)	30	R	R	I	R	R	R	R	R
Penicillin	Amoxicillin (AMP)	25	R	R	R	R	R	R	R	R
Polyketide	Tetracycline (TE)	30	R	R	R	R	I	R	R	I
Quinolone	Nalidixic acid (NA)	30	R	R	R	R	R	R	R	R
Sulfonamides	Sulfamethoxazole (SXT)	25	R	I	R	R	R	R	R	R
Multiple antibiotics resistance (MAR) index			0.88	0.75	0.63	0.88	0.63	1.00	0.88	0.75

P=*Proteus mirabilis*, E=*Escherichia coli*, K=*Klebsiella pneumoniae*. R=resistant, S=sensitive, I=intermediate according to CLSI (Clinical and Laboratory Standards Institute).

Antibacterial activity test of seaweed extracts

Results in Table 3 showed the antibacterial activity of different seaweed extracts using different (80%) organic solvents (ethanol, methanol, acetone and water) to enable extraction of all possible active components from different seaweeds. All the extracts showed considerable activities depending on the bacterial isolate sensitivity. The ethanol

extracts of *Ulva lactuca* showed broad significant antibacterial activity than the other used solvents recording 36, 34 and 39 mm diameter of inhibition zone for *Escherichia coli* E1, *Klebsiella pneumoniae* K1 and *Proteus mirabilis* P1, respectively at $P \leq 0.05$, followed by the extract of *Gelidium spinosum* and the extract of *Petalonia fasciata*. In the same direction, methanol extracts of *Ulva lactuca* possessed the highest antibacterial activity than that of *Gelidium spinosum* and

Petalonia fascia against *E. coli* E2 (29 mm), *K. pneumoniae* K1 (26 mm) and *P. mirabilis* P2 (25 mm), respectively. The acetone and water extracts showed lower, yet significant, antibacterial activities compared to ethanol and methanol extracts. Seaweeds contain various bioactive substances which have many pharmaceutical applications. Some of these substances have bacteriostatic and bactericidal properties as reported by (Gorban *et al.*, 2003; Ismail *et al.*, 2016).

Ulva lactuca was reported to contain uniform distribution of antibiotics (Hornsey and Hide, 1974). (Fareed and Khairy, 2008) explained that *Ulva lactuca* possessed proteins or peptides responsible for the antibacterial activity, which function by binding to the microbial cell membrane and once embedded, forming pore-like membrane defects that allow efflux of essential ions and nutrients from the bacteria. The results of the present study were in accordance with (Manilal *et al.*, 2009), who reported that moderate polar solvents like ethanol showed better activity against Gram negative bacteria, which are the main cause of UTI diseases.

The ethanol extracts of green algae like *Codium adherens*, *Ulva reticulate* and *Halimeda macroloba* showed high antibacterial activity against *Staphylococcus* sp., *Propionibacterium acnes* and *Proteus mirabilis* (Karthikaidevi *et al.*, 2009; Boonchum *et al.*, 2011). Similarly, ethanolic extract of *Caulerpa sertularioides* green seaweeds showed maximum antibacterial activity against *Bacillus subtilis*, *E. coli* and *Proteus mirabilis* (Pushparaj *et al.*, 2014).

For Phaeophyceae (brown seaweeds), the ethanolic extract of *Sargassum oligocystum* and *Sargassum crassifolium* showed high antibacterial activity against *E. coli* (Francis *et al.*, 2017). Earlier, (Padmakumar and Ayyakkannu, 1997) reported that Chlorophyceae are actively growing

throughout the year, which ensure its continuous availability whereas Phaeophyceae show no active growth in certain seasons. Practically, the antibacterial activities of certain algal extract will be dependent on the tested algal species, the efficacy of the extraction method and the concentration of this extract.

In this respect, results in Figure 1 represented the means of antibacterial activities of different seaweed extracts using different solvents. Ethanol extracts of all seaweeds showed significantly high antibacterial activity compared to the other solvents. Fortunately, the ethanol extract of *U. lactuca* showed the maximum antibacterial activity with the isolates E1, K1, and P1, which were estimated to have the highest MAR index values (Table 2). Therefore, the ethanol extract of *Ulva lactuca* was subjected to further chemical analysis to identify its chemical components.

Identification of the selected bacterial isolates

The obtained results in Table 4 using the Biomerieux VITEK®2 system confirmed the morphological and biochemical identification and showed that the three bacterial isolates are *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*.

Fractionation and purification of *Ulva lactuca* antibacterial crude extract

Column chromatography

The crude ethanolic extract of *Ulva lactuca* seaweed was fractioned and purified using silica gel column chromatography. From fifteen fractions obtained, only five fractions showed antibacterial activity against the selected multidrug resistant UTI bacterial isolates (*Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*).

The recorded inhibition zones for different fractions were illustrated in Table 5. All five fractions recorded remarkable inhibition zones against the three-tested resistant pathogenic isolates especially Fr2, Fr3 and Fr8.

Determination of the chemical structure of *Ulva lactuca* antibacterial compound

UV spectrophotometer analysis

The ultraviolet absorption of the most active antibacterial fractions, which collected from column chromatography was measured using UV spectrophotometer (UV 2101 /pc) at 200-900nm wavelength. The absorption peaks of the five active fractions were of similar UV absorption at 245 nm (Fig. 2a, b, c, d and e) confirming its purity and homogeneity.

Consequently, the five fractions were collected together to form one active compound with the same estimated UV absorption peak at 245 nm (Fig. 2f) suggesting an aromatic acid esters structure of *U. lactuca* antibacterial bioactive agent.

Infra-red spectrophotometer (FTIR) analysis

The infra-red absorption spectrum was used to identify the functional groups frequencies of *U. lactuca* ethanolic extract, which constitute the active compound structures. A FT-IR spectrum was recorded in the range of 4000 to 400 cm^{-1} and the following functional groups were recorded (Fig. 3).

The strong stretching broad band (ν) of OH group was positioned at 3450cm^{-1} . The ν of CH aliphatic (CH, CH_2 and CH_3) sharp band was at 2861.84cm^{-1} .

A sharp band of C=O was positioned at 1730.8cm^{-1} . A strong sharp (ν) of C=C and C-O were positioned at 1461.78cm^{-1} and 1277.61cm^{-1} , respectively. In addition, a band (ν) of ortho-position, aromatic phenyl ring (CH, CH_2 and CH_3) was at 785.85cm^{-1} .

There was no absorbance in between the region $2220\text{-}2260\text{cm}^{-1}$ indicates that there was no cyanide group in -this extract. This result shows that *U. lactuca* extract does not contain toxic substances.

Fig.1 The mean antibacterial activities of different seaweed extracts against the multidrug resistant UTI bacterial isolates

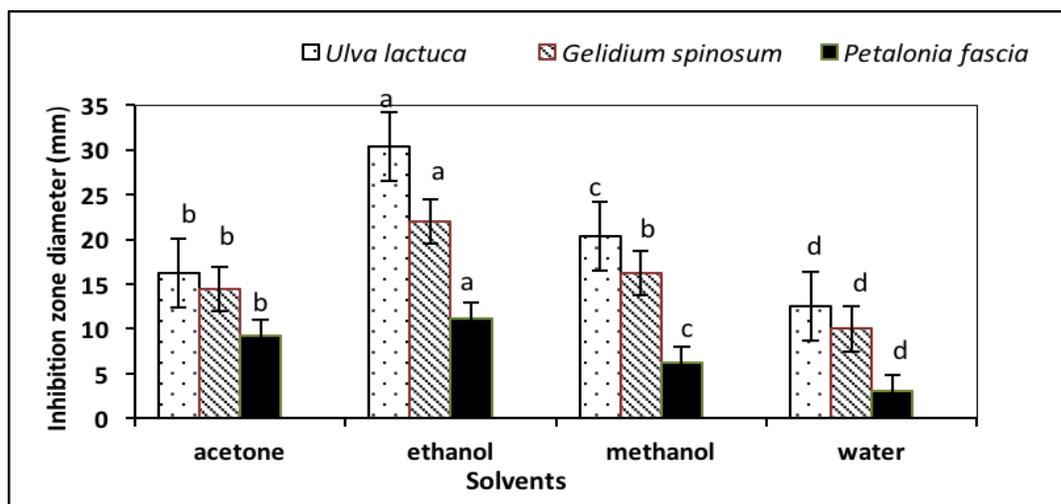


Fig.2 The UV spectrophotometer scanning of different active fractions resulted from column chromatography. a= fraction no.2, b= fraction no.3, c= fraction no.4, d= fraction no.5, e= fraction no.8 and f= total fractions collected together

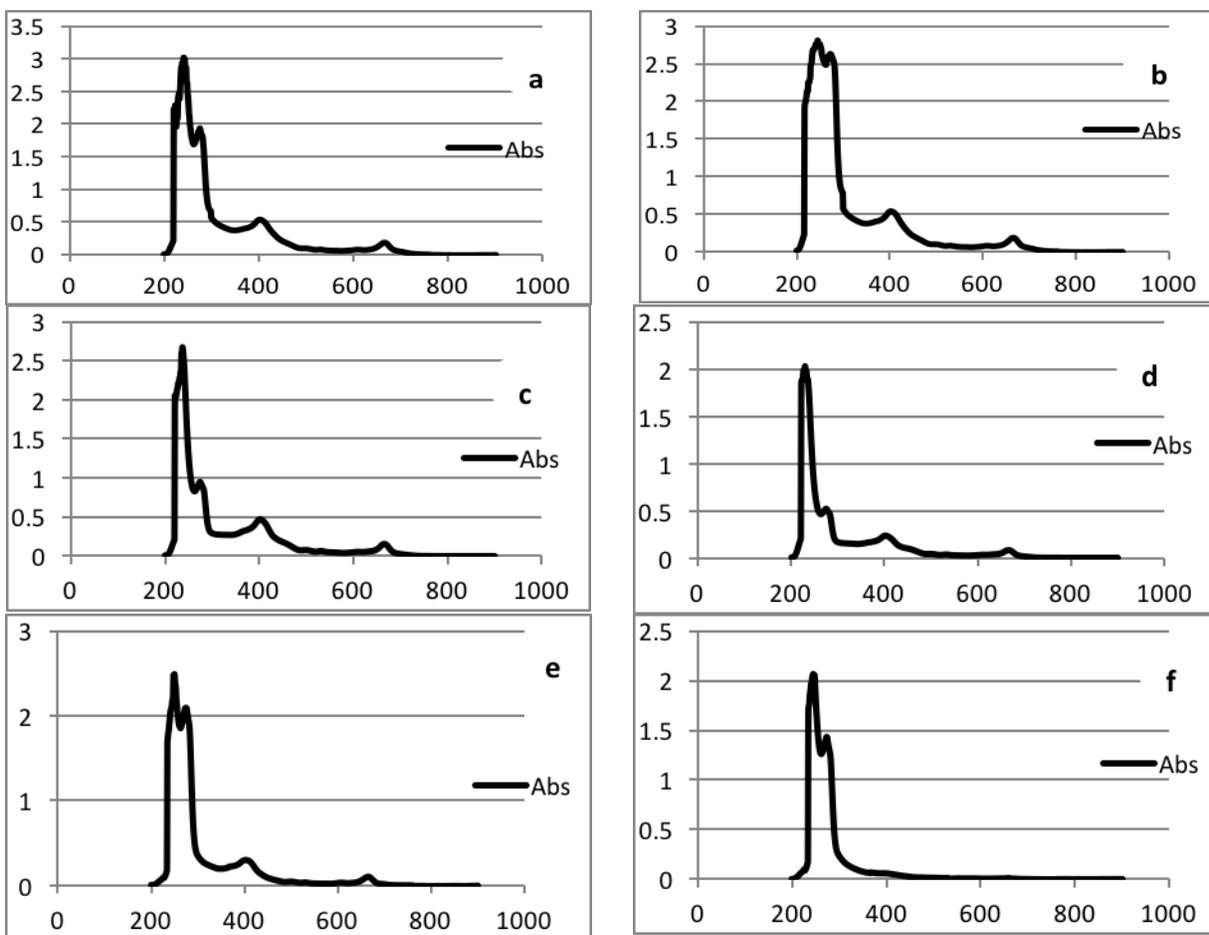


Fig.3 IR spectra of *Ulva lactuca* antibacterial bioactive compound

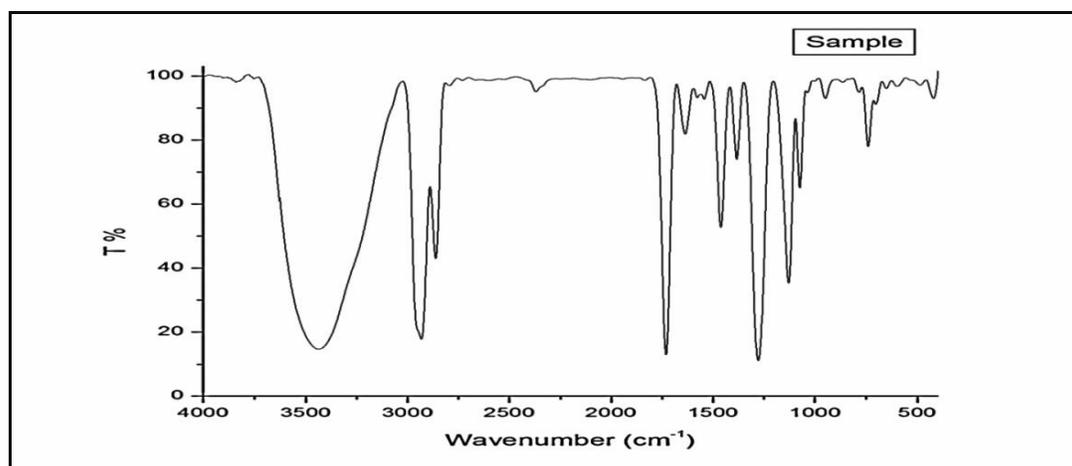


Fig.4 Proton magnetic resonance graph of *U. lactuca* antibacterial bioactive compound

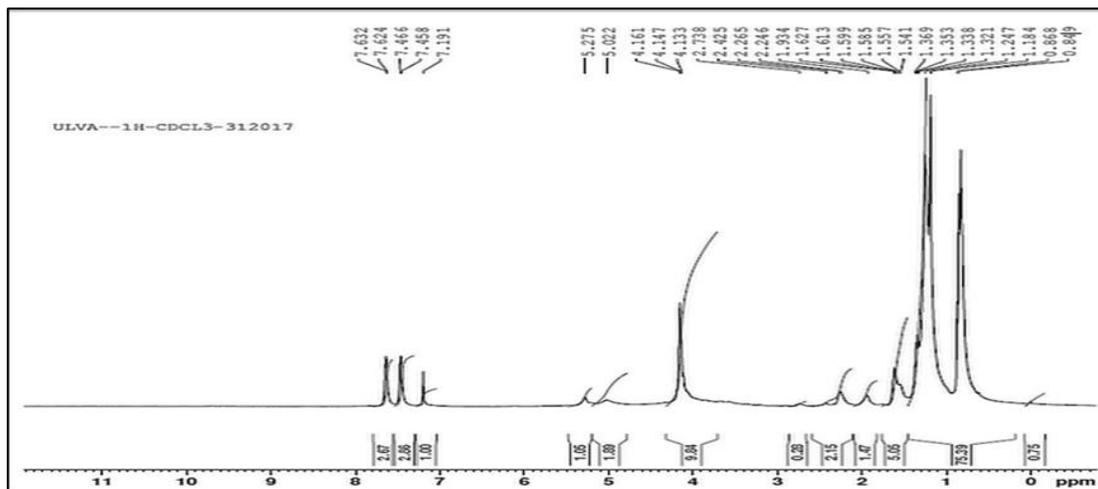


Fig.5 GC-MS analysis chromatogram of *Ulva lactuca* antibacterial bioactive compound

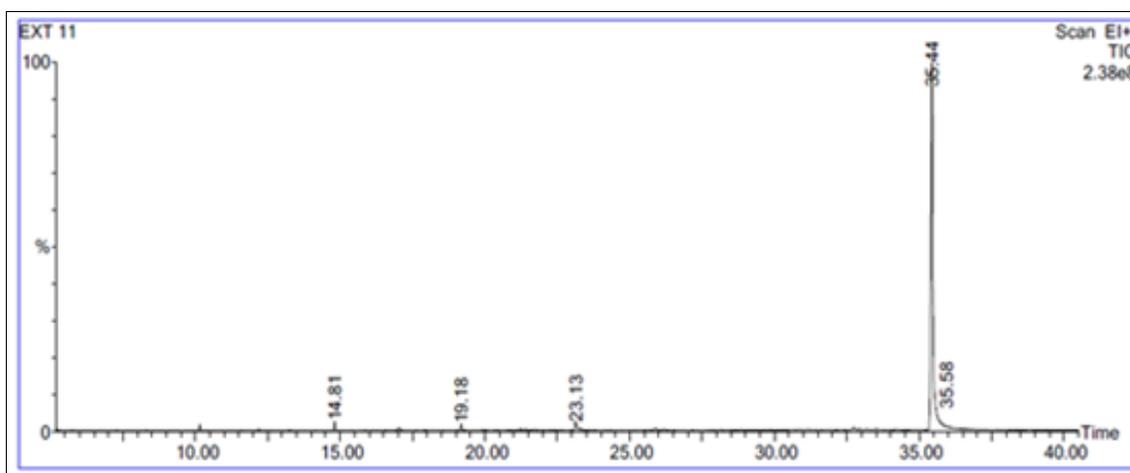


Fig.6 Chemical structure of di-isooctyl phthalate, the antibacterial bioactive compound from *Ulva lactuca* ethanolic extract

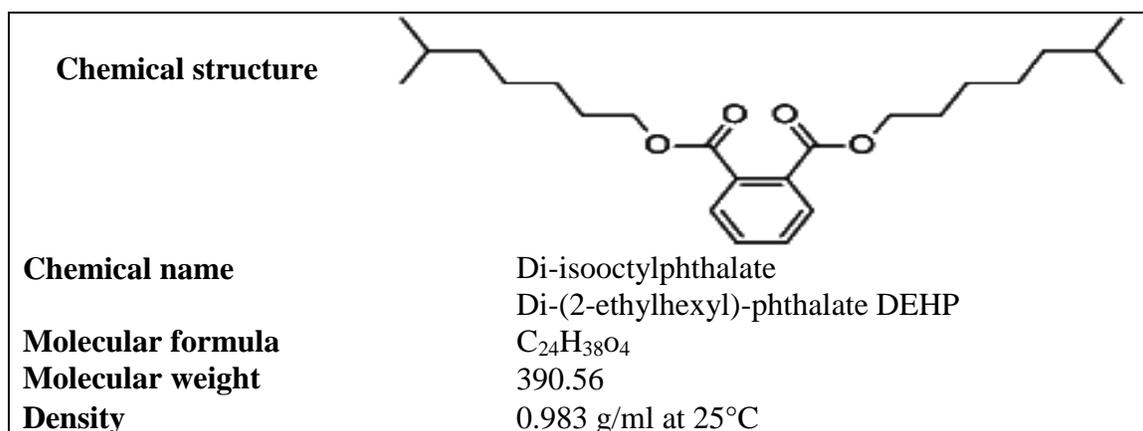


Table.3 The antibacterial activity of different seaweed extracts against the selected multidrug resistant UTI bacterial isolates

Solvents	Bacterial isolate	<i>E. coli</i>			<i>K. pneumonia</i>			<i>P. mirabilis</i>		
		E 1	E2	E3	K1	K2	P1	P2	P3	P4
Seaweeds		Diameter of inhibition zone (mm)								
Acetone	<i>Gelidium</i>	7 ^d ±0.15	6 ^d ± 0.3	3 ^e ± 0.5	10 ^c ±2.6	19 ^a ±2.5	10 ^c ±3.2	0±00	18 ^a ±0.00	15 ^b ±0.6
	<i>spinosum</i>	11 ^{bc} ±1.6	10 ^{bc} ±1.5	13 ^b ±0.9	0±00	0±00	13 ^b ± 0.03	12 ^b ±2.6	8 ^c ±1.3	17 ^a ±2.0
	<i>Petalonia fascia</i>	18 ^{bc} ± 0.1	17 ^{bc} ±0.1	14 ^c ± 0.3	20 ^b ±0.7	15 ^c ±0.2	7 ^d ± 0.06	9.5 ^d ±0.05	26 ^a ±0.03	20 ^b ±0.6
	<i>Ulva lactuca</i>									
Ethanol	<i>Gelidium</i>	20 ^c ±0.5	18 ^c ± 0.1	29 ^a ± 0.3	16 ^d ±0.4	23 ^{bc} ±0.7	22 ^{bc} ±0.3	18 ^c ±0.05	24 ^b ±0.03	26 ^b ±0.6
	<i>spinosum</i>	13±0.2	11 ^b ±1.7	14 ^a ±0.1	8 ^b ±2.6	13 ^a ±1.00	8 ^b ±3.0	10 ^b ± 0.3	10 ^b ± 0.3	13 ^a ±0.5
	<i>Petalonia fascia</i>	36 ^{ab} ±0.03	18 ^c ±0.3	30 ^b ±0.5	34 ^{ab} ±0.3	27 ^b ±0.01	39 ^a ±0.01	32 ^{ab} ±0.03	29 ^b ±0.02	28 ^b ±0.17
	<i>Ulva lactuca</i>									
Methanol	<i>Gelidium</i>	20 ^a ±0.05	13 ^d ±0.01	15 ^c ±0.01	12 ^d ±0.7	17 ^a ±0.19	19 ^a ± 0.5	15 ^c ±0.3	18 ^{ab} ±0.7	17 ^{ab} ±0.17
	<i>spinosum</i>	6 ^b ±0.4	8 ^d ±0.5	10 ^a ±0.2	8 ^d ±0.06	0±00	9 ^a ± 0.6	7 ^{ab} ±0.04	5 ^b ±0.6	3 ^c ±0.05
	<i>Petalonia fascia</i>	13 ^d ±0.06	29 ^a ±0.02	15 ^{cd} ±0.05	26 ^b ±0.1	17 ^c ±0.03	23 ^{bc} ±0.0	25 ^b ±0.02	18 ^c ±0.05	17 ^c ±0.07
	<i>Ulva lactuca</i>						1			
Water	<i>Gelidium</i>	6 ^c ±0.03	12 ^{ab} ±0.5	14 ^a ±0.3	12 ^{ab} ±0.7	10 ^b ±0.1	7 ^c ±0.5	11 ^b ±0.3	5 ^c ±0.2	13 ^a ±0.02
	<i>spinosum</i>	0±00	0±00	11 ^a ± 1.3	0±00	2 ^c ± 1.02	5 ^c ± 0.02	2 ^c ± 1.02	8 ^b ± 0.02	0±00
	<i>Petalonia fascia</i>	11 ^c ±0.02	12 ^{bc} ±0.7	13 ^b ±0.3	21 ^a ±0.5	11 ^c ±0.1	13 ^b ±0.03	11 ^c ±0.05	10 ^c ±0.03	14 ^b ±0.04
	<i>Ulva lactuca</i>									

Different letters in the same row indicate significant difference at P ≤ 0.05.

Table.4 VITEK identification of the isolated multi-drug UTI bacterial isolates

Tested organism	Bio-number	Card	Confidence	Probability %
<i>Escherichia coli</i>	0405610540526610	GN (Gram negative)	Excellent identification	97
<i>Klebsiella pneumoniae</i>	6627734773565010	GN (Gram negative)	Excellent identification	97
<i>Proteus mirabilis</i>	0017000340642210	GN (Gram negative)	Excellent identification	99

Table.5 The antimicrobial activities of the different fractions obtained from the silica gel column chromatography against seaweeds sensitive bacterial isolates

Fractions	Fr2	Fr3	Fr4	Fr5	Fr8
Bacterial isolates	Diameter of inhibition zone (mm)				
<i>E. coli</i>	10	18	11	10	13
<i>K. pneumoniae</i>	13	17	9	7	14
<i>P. mirabilis</i>	12	19	10	8	15

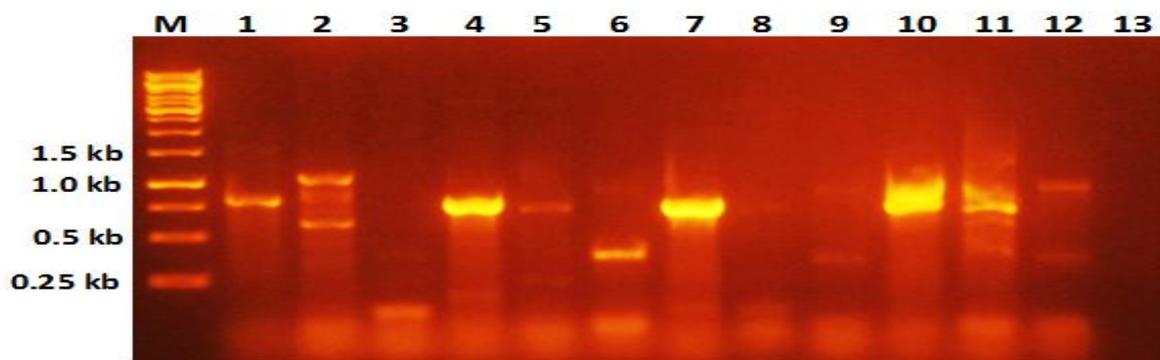
Table.6 GC-MS analysis of *Ulva lactuca* antibacterial bioactive compound

Peak	Retention Time (min)	Height	Area	Relative peak intensity %	Compound name
1	14.812	6,261,656	235,828.4	1.13	Hexadecane
2	19.179	4,590,217	187,301.5	0.9	Sulfurous acid 2 ethylisohexyl ester
3	23.131	5,819,614	337,434.7	1.62	Hexadecanoic acid ethyl ester
4	35.436	237,067,360	20,885,660	100	Diisooctylphthalate

Table.7 Number and size of PCR amplification products of UTI bacterial isolates virulence genes that was sensitive to *Ulva lactuca* extract

Bacterial isolates	<i>Escherichia coli</i>			<i>Klebsiella pneumoniae</i>			<i>Proteus mirabilis</i>			<i>Proteus mirabilis</i> (+ve control)		
Virulence gene	Adhesion	Urease	Hemolysin	Adhesion	Urease	Hemolysin	Adhesion	Urease	Hemolysin	Adhesion	Urease	Hemolysin
Primer pair	UcaAF UcaAR	UreCF UreCR	HpmAF HpmAR	UcaAF UcaAR	UreCF UreCR	HpmAF HpmAR	UcaA F UcaA R	UreCF UreCR	HpmAF HpmAR	UcaAF UcaAR	UreCF UreCR	HpmAF HpmAR
No. of amplified PCR products	1	2	2	1	2	2	1	2	1	2	2	2
Size of the PCR products	750bp	750bp 250bp	375bp 875bp	750bp	750bp 625bp	375bp 1050bp	780bp	650bp 1050bp	375 bp	750bp 125 bp	750bp 250bp	375bp 875bp

Fig.7 Agarose gel of PCR amplification products of UTI bacterial isolates virulence genes that was sensitive to *Ulva lactuca* extract.



Lane M: O'-Gene Ruler 1 kb DNA ladder as the molecular size marker. Lanes 1, 2 and 3: adhesion, urease and hemolysin genes detected in *Proteus mirabilis*. Lanes 4, 5 and 6: adhesion, urease and hemolysin genes detected in *Proteus mirabilis* (positive control strain). Lanes: 7, 8 and 9: adhesion, urease and hemolysin genes detected in *Escherichia coli*. Lanes 10, 11 and 12: adhesion, urease and hemolysin genes detected in *Klebsiella pneumoniae*. Lane 13 water: negative control (no DNA template was used).

Proton nuclear magnetic resonance (¹H NMR) analysis

Figure 4 showed the (¹H NMR) spectral analysis graph of *Ulva lactuca* antibacterial bioactive compound for accurate elucidation of its chemical structure. The characteristic signals of the compound in ¹H NMR spectrum were as following: The signal from 1.784-1.321 ppm was singlet (12 protons) of 4 CH₃ groups. The signal from 1.541-1.621 ppm was singlet (16 protons) of 8 CH₂ groups. The signal from 2.246-2.738 ppm was singlet (2 protons) of 2 CH groups. The signal from 5.022-5.275 was singlet (4 protons) of 2H₂C-O- groups. And the signal from 7.191-7.632 was multiple (4 protons) of aromatic groups.

Gas chromatography mass spectrometry GC-MS analysis

The GC-MS chromatogram of *U. lactuca* ethanolic antibacterial active compound indicated the presence of 4 peaks with different retention times and peak relative intensities as shown in Table 6 and Figure 5 and 6. All peak areas of the chromatogram

were relatively small except the parent peak at retention time of 35.436 min., corresponds to 100% relative intensity and was identified according to its molecular mass to be diisooctyl phthalate, an aromatic ester derivative compound.

Phthalate esters have been found earlier in aquatic organisms and can be potentially bioaccumulated due to their lipophilicity (Mackintosh *et al.*, 2004). Phenolic compounds are commonly found in seaweeds and have been reported to have several biological activities including antimicrobial and antioxidant activities (Yuan *et al.*, 2005). Many studies have reported the production of phthalate esters by seaweeds. Diisooctyl phthalate was isolated from the brown algae *Sargassum wightii* and showed antibacterial activity against Gram positive and Gram negative bacteria *Staphylococcus aureus*, *Proteus vulgaris*, *E. coli*, *Salmonella typhi*, *S. paratyphi*, *S. typhiridium* and *Pseudomonas aeruginosa* (Sastry and Rao, 1995).

The biosynthesis of di-(2-ethylhexyl)-phthalate (DEHP) and di-n-butyl phthalate

(DBP) have been reported by (Chen, 2004) in the red alga *Bangia atropurpurea*. According to Osman *et al.*, (2013), a high molecular weight phthalate esters derivative of broad spectrum antibacterial and antifungal activities was synthesized by *Ulva fasciata* green seaweed. The extract of *Grateloupa lithophila* seaweed was recommended as antibacterial substance for multidrug resistant (MDR) microbes including the UTI bacteria (Manikandan *et al.*, 2011). Some marine brown algae were screened by Kalavani *et al.*, (2016) and were reported to produce antagonistic bioactive compounds against UTI pathogens.

Molecular detection of the virulence factors of the bacterial strains

PCR amplification of virulence factors

Three different primer pairs (UcaAF UcaAR; UreCF UreCR; HpmAF HpmAR) were used to detect the virulence genes (adhesion, hemolysin and urease) of the tested multidrug resistant UTI bacterial isolates (*E. coli*, *K. pneumoniae* and *P. mirabilis*). Various virulence factors can be attributed to UTI pathogenicity (Tarchouna *et al.*, 2013). The Polymerase Chain Reaction technique was used to investigate the virulence genes responsible for multidrug resistance in the UTI bacterial isolates. Specific primers for three virulence genes in the bacterial isolates including *ucaA* (adhesion), *ureC* (urease), and *hpmA* (hemolysin) were selected. These three genes are responsible for the colonization of bacteria in the urinary tract.

The seaweeds sensitive UTI bacterial isolates were compared with the antibiotics and seaweed resistant isolate (positive control) carrying the virulence genes. The current results (Table 7 and Fig. 7) showed that the adhesion virulence gene was detected in all the bacterial isolates (*E. coli*, *K. pneumoniae*

and *P. mirabilis*) with rate of 95% with one amplicon (780 bp) for *Proteus mirabilis* and 750 bp for both of *E. coli* and *Klebsiella pneumoniae*, while in the positive control strain 2 PCR amplicons (750bp and faint 125bp) were observed, which indicated that the resistant strain (positive control) utilize a variety of adhesions to bind to the urinary epithelial cells to start the infection causing failure to the treatment with antibiotics and seaweeds (Le Bouguenec *et al.*, 1992; Munkhdelger *et al.*, 2017).

Regarding the *ureC* gene, which is considered as a diagnostic feature of presence of UTI bacteria due to the production of urease enzyme; the results showed that all the isolates in a rate 96.66% contained *ureC* gene. Two PCR products (750bp and 625bp) were observed in *Klebsiella pneumoniae* and in *Proteus mirabilis* (650bp and 1050bp), while two faint amplicons (750bp and 250bp) were amplified in *E. coli*. Similarly, in the positive (control) isolate the 2 faint PCR amplicons (750bp and 250bp) were amplified (Table 7 and Fig. 7). Urease enzyme works on changing pH of the urine to basic leading to the deposition of calcium and magnesium phosphate salts in the formed biofilm, which in turn leads to the formation of more complex crystalline biofilm that works to close urinary catheter and protect the bacteria from antibiotics (Stickler, 2008; Schaffera *et al.*, 2016).

HpmA gene is responsible for producing hemolysin and considered as an important virulence factor for UTI bacterial pathogenicity. The *hpmA* gene was detected in all bacterial isolates. In *Proteus mirabilis*, only very faint amplicon (375bp) was detected in this strain. Two PCR amplicons (375bp and 875bp) of *hpmA* gene were present in *E. coli* and in the positive control. Similarly, two faint PCR products (375bp and 1050bp) were observed in case of *K.*

pneumoniae. The hemolysin enzyme acts on destroying the leukocyte cells by making small holes in the membranes of leukocyte and epithelial cells. Its presence is a very important factor in providing the pathogenic bacteria with iron; and because of its cytotoxic, it leads to the destruction of the kidney tissue of the host (Cestari *et al.*, 2013; Leclercq *et al.*, 2016).

By linking the obtained results from antibacterial activities of *U. lactuca* extract with that of the PCR of the studied UTI bacterial isolates, it revealed that presence of the virulence genes (adhesion, urease and hemolysin) is a crucial factor for successful colonization of the uropathogenic bacteria and its multidrug resistance property. Seaweed derived compounds proved their ability as new natural sources for antibacterial agents, which reduce the virulence genes activity and prevent (as for *P. mirabilis*) or mitigate (as for *E. coli* and *K. pneumoniae*) the resistance and pathogenicity of these kind of bacteria.

Marine algae are one of the biggest biomass producers and proved a potential source of new and unique compounds for many applications. Many compounds derived from seaweed have bacteriostatic or antibacterial, antiviral, antitumor, anti-inflammatory and antifouling activities. In this study, an aromatic ester derivative was extracted from *Ulva lactuca* green seaweed using ethanol. The bioactive compound was chemically identified as diisooctyl phthalate and showed promising antibacterial activities against multidrug resistant UTI infectious bacteria. In addition, the virulence genes causing pathogenicity of these bacteria were detected using PCR. PCR results showed that hemolysin, urease and adhesion virulence factors were present in the treated *P. mirabilis*, *E. coli* and *K. pneumoniae* UTI isolates with high variations. Consequently, the study recommends seaweeds as promising

treatment of human diseases or as new antibacterial agents to replace synthetic antibacterial drugs.

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