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Evaluation of Anti-Biofilm Activity of *Enteromorpha* Spp. Against Uropathogenic *Pseudomonas aeruginosa*

Hussain Adnan Hussain[1], Ahmed. S. Alashoor[2], Ezat H. Mezal[3]
Department of Biology, Collage of Science, University of Thi-Qar, Thi-Qar, 64001, Iraq[1,2].
Department of Microbiology, Collage of Nursing, University of Thi-Qar, Thi-Qar, 64001, Iraq[3].
Corresponding Author Gmail: biologist_huss@sci.utq.edu.iq

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Abstract

Background*: Besides the well-known intrinsic and acquired genetic mechanisms involved in the bacterial resistance phenomenon, bacteria also exhibit an adaptive strategy that consists of the formation of a strongly structured cell assembly named "biofilm providing increased protection against conventional treatments. Thus, to overcome this, searching for new antibiofilm agents, especially of natural origin.*

Objective: *To highlight the ability of Enteromorpha extracts to prevent biofilm formation of Pseudomonas aeruginosa isolated from urinary tract infections.*

Methods: *Enteromorpha sp. extracts were prepared by successive maceration in three solvents; namely, distilled water, ethanol, and n-hexane by using Ultrasonic Bath. The MIC and sub-MIC of each extract were also determined using the broth microdilution method. The antibacterial activity of the extracts was performed using the method of measuring the zone of inhibition by disc diffusion agar method. The antibiofilm activity was performed using the microtiter plates method. The bioactive compounds in each extract were also identified using a GC-MS device.*

Results: *All extracts showed an inhibitory ability on the growth of P. aeruginosa, and the highest inhibition zone was recorded in Hexanal extract at a concentration of 100% with a mean of 14.5±0.7mm. A significant reduction in biofilm formation was recorded when bacteria were treated with concentrations below the MIC of the extracts, with an inhibition rate reached 96% for the ethanolic extract. Several active chemical compounds in each extract were also identified using GC-MS.*

Conclusion: *These findings present new insights into the ability of the green alga Enteromorpha Spp. as a valuable source of antibacterial and antibiofilm agents for the pharmaceutical industries*

key words: *Enteromorpha Spp; Pseudomonas aeruginosa; anti-biofilm; antibacterial.*

INTRODUCTION

Antibacterial activity refers to substances that have the ability to eradicate or restrict the proliferation of bacteria while maintaining a non-harmful effect on the surrounding tissue. [1]. The extensive utilization of antibiotics to combat bacteria has been prevalent, however, ongoing research is being conducted to restrict their usage due to the proliferation of antibiotic-resistant bacterial strains resulting from their extended or incorrect use [2, 3]. Antibacterial resistance can arise from alterations in the molecular composition of antibiotics, reduced antibiotic penetration, efflux pumps, alterations in the activity of antibiotics, and cellular adaptability resulting from exposure to antibiotics [4]. In addition to the widely recognized inherent and acquired genetic processes

implicated in bacterial resistance, bacteria also demonstrate an adaptive approach characterized by the development of a highly organized cellular community known as a "biofilm" [5]. Biofilms are structured communities of microorganisms that stick to each other and a surface. They are surrounded by a matrix made up of extracellular polymeric substances (EPS), which mostly consist of exopolysaccharides, proteins, nucleic acids, and minerals [6]. *Pseudomonas aeruginosa* biofilm exhibits the characteristic feature of persistent long-term infection and progression from colonization to infection, which can result in mortality, especially in immunocompromised individuals [7]. Where P. aeruginosa is one of the most common infections in healthcare settings[8]. Hence, it is responsible for 10–15% of nosocomial infections globally [9]. This pathogen typically induces nosocomial urinary tract infections, frequently linked to the presence of a urinary catheter. Community-acquired infections caused by *P. aeruginosa* are rare unless there is an underlying urological problem such as obstruction, recent instrumentation, or neurogenic bladder [10]. It is not surprising that *P. aeruginosa* infections are associated with significant morbidity and mortality, given its ability to adapt to environmental changes swiftly, rapidly develop antibiotic resistance, and create several virulence factors [9]. Furthermore, the capacity of this bacterium to develop biofilm not only enhances its inherent and acquired resistance but also serves as a protective shield against host immune responses and anti-Pseudomonas drugs [11]. To address this issue, searching for new antibiotics from nature and developing antibiotics that target new biological pathways [12]. In recent decades, there has been a growing interest in an alternative approach to antibiotics, which involves the utilization of natural substances that restrict the proliferation of harmful bacteria [13]. Due to their fast growth rate and great biodiversity, multiple studies have suggested that algae have the potential to produce a wide range of chemical compounds that possess diverse biological properties, where these components can impede the growth of detrimental bacteria, or eliminate them [14, 15].

*Enteromorpha S*pp. is a globally distributed genus of macroscopic green algae [16]. It is well known for its wide distribution in marine, freshwater, and brackish environments throughout the world [17]. Previously, Enteromorpha and Ulva were believed to be independent categories, but the molecular data has shown that they should not be classified as separate genera. Thus, the taxonomic name of Enteromorpha is presently considered to be a synonym for Ulva [18]. The Ulva genus comprises approximately 40 species, including Ulva prolifera (formerly known as Enteromorpha prolifera), Ulva linza (Enteromorpha linza), and Ulva intestinalis (Enteromorpha intestinalis), and the distinguishing between these species can often be challenging [19]. *Enteromorpha* has garnered growing interest in the realms of maritime environment conservation and marine bioresources [20]. Where it has a significant impact on the carbon cycle, and it frequently leads to the production of the 'green tide', which results in ecological and indirect economic harm [21]. Economically, the green algae genus Enteromorpha possesses significant economic potential for commercial utilization due to its abundant and diverse chemical composition. It is highly recommended for human consumption due to its numerous beneficial components, including minerals, protein, essential fatty acids, vitamins, and fiber [22]. On the other hand, Enteromorpha spp. contain a variety of secondary metabolites such as flavonoids, terpenoids, and alkaloids. These compounds have the potential to exhibit antidiabetic, anti-inflammatory, antioxidant, antibacterial, antineoplastic, and antiviral effects, as well as influence the immune system [23]. Regarding antibacterial activity, Enteromorpha spp exhibits significant potential as a natural source of antibacterial agents, as earlier studies have confirmed its exceptional antibacterial characteristics [24]. However, given the paucity of data on the anti-biofilm efficacy of *Enteromorpha* sp. against Uropathogenic P. *aeruginosa* isolates, the present study aimed to investigate the potential of anti-biofilm activity of *Enteromorpha* extracts against *P. aeruginosa* derived from urinary tract infections (UTIs).

MATERIALS AND METHODS

Algae Samples Collection and Processing

The Algal Samples (*Enteromorpha* sp.) were collected in March 2022 from the marshes of Chaibayish, Thi Qar, Iraq. Algal samples were handpicked and washed thoroughly with water to remove all the impurities,

sand particles, and epiphytes. Harvested algae were stored in sterile polyethylene bags for transport to the laboratory. Then the algae species were identified according to [25], and with the assistance of Dr. Ruaa Jaafar Khudair and Dr. Ahmed Shaker Al-Ashour; Professor of Phycology in the College of Science, University of Thi-Qar. After that, the samples were washed well with tap water and then with sterile distilled water and dried in air at room temperature for four days. The dried samples were cut into small pieces and ground with a blender into a powder to be analyzed and examined for their therapeutic effects. Fresh algae samples were immediately processed for their antimicrobial activity, and the remaining algae powder was stored in sterile plastic containers for later use.

Preparation of Algal Extract

The extracts of *Enteromorpha* were prepared by using three solvents; water, ethanol, and hexane. This method is based on Ultrasonic baths that use cavitation bubbles induced by high-frequency pressure (sound) waves to agitate the liquid by use Ultrasonic Bath, which was modified from [26].

Preparation of Stoke Solution and Working Extracts

The stoke solution of algal extract was prepared according to [27] by dissolving 1 g of extract in 10 ml of DMSO and thus becoming the solution concentration is 100 mg/ml, then the concentrated solution of each extract was filtered using a Millipore filter with a diameter of (0,22) mm. Different concentrations (100%, 75 %, 50 %, 25 %, 12 %, 6 %, 3 %) of algae extract were then prepared

Gas Chromatography-Mass Spectrometry Analysis (GC-MS)

Chemical compounds of the Algal extracts were identified by comparing the spectra with known compounds stored in the (NIST library,2005) by using a Shimadzu GCMS-QP2010 Ultra Gas Chromatograph Mass Spectrometer in the Department of Environmental and Water Technology, Environmental Research Center in Baghdad.

Isolation and identification of bacterial isolates

All urine samples from UTIs associated with various medical conditions of both genders and ages were taken at the Al-Nasiriya Teaching and Al-Hussain Teaching Hospitals in Thi-Qar province, Iraq. The specimens were examined microscopically for the presence of pus cells, RBC, and casts.10ml of urine was transferred to a sterilized centrifuge tube and centrifuged at 2000 rpm for 10 min to get bacterial pallet. After centrifugation, a loopful of inoculums was taken and streaked on the sterilized MacConkey agar medium. All plates were incubated at 37 C for 24-48hrs. Then the plates were examined after overnight incubation to quantify the organisms present. The colony count was evaluated and organisms were identified by conventional methods and antimicrobial testing was done according to Kirby Bauer's method on all isolates, and the bacterial isolates that were used in this study were identified using the Biomerieux VITEK®2 system.

Antibacterial Activity Testing of *Enteromorpha* sp. Extracts Using Disc Diffusion Assay

The antimicrobial activity of *Enteromorpha* sp. extracts was determined as described below. Briefly, as a qualitative test, the agar disk diffusion technique (Kirby-Bauer Method) has been used to assay algal extracts for their antimicrobial activities. The bacterial isolates (P. *aeruginosa*) were prepared from 24 hours of old cultures in nutrient broth. A microbial broth culture adjusted to 0.5 McFarland turbidity standard has been prepared. All of the bacterial strains suspension were spread evenly using a sterile cotton swab into Mueller Hinton agar (MHA) plates. Algal extracts were serially diluted with dimethyl sulfoxide (DMSO) to obtain diluted concentrations in the range of 100%, 75%, 50%, and 25% mg/ml. In this method, on each plate, four wells of 5mm diameter were made using a sterilized cork borer, and nearly 100 µL of each extract was pipetted onto each well in the plate [28]. After 24 hours of incubation at 37˚C, the inhibition zone from the edge of the disc to the inner margin of the surrounding bacterial growth was measured in mm by using a graduated scale

and recorded. The determinations were performed in triplicate. DMSO was used as a control for inhibiting bacterial growth.

Minimal inhibitory concentration (MIC) determinations of *Enteromorpha* sp. Extracts

Determination of Minimum inhibitory concentrations (MIC) of *Enteromorpha* sp. crude extracts was done using the broth microdilution method [27]. The sample concentration range was prepared from the stock solutions by dilutions in sterile broth. Four appropriate concentrations (25 %, 12 %, 6 %, and 3% mg/ml) of each extract were prepared from the serial dilutions method in the DMSO. From each concentration, 100 µL was transferred into the sterile Eppendorf Tube and then 100 µL of the overnight grown cultures, adjusted to (0.5 in the McFarland scale) was added to each tube. Then pipetted up and down to ensure that the bacteria were mixed with the extract. All samples were marked and incubated for 24 hours at 37°C. Some tubes were reserved for a positive bacterial growth control (DMSO without any extract added) and a negative sterility control (no inoculum added). The MIC was determined by growing the mixture on a nutrient agar medium and incubating it for 24 hours at 37°C. The lowest concentration of the extract that inhibited microbial growth after 24 h incubation was measured as MIC. After incubation, the MIC values were observed at least in duplicate as the lowest concentration of the extracts that produced complete suppression of bacterial growth, as well as (sub-MICs) were determined as concentrations lower than the MIC values [29]. The determinations were performed in triplicate.

Biofilm Formation (adherence) Assay

In vitro, the ability of isolated bacteria (P. *aeruginosa*) to form biofilm was determined using the microtiter plates method essentially as described by [30] with slight modification. Briefly, the isolates' overnight cultures were diluted (0.05 in the McFarland scale) in brain heart infusion broth (BHI). Subsequently, 100 µL overnight culture was added per well of 96-well polystyrene microtiter plates (in the presence and absence of sub-MIC of algal extracts) and covered the plates, then incubated for 24 h at 37 ºC. Unattached bacterial cells were then removed from the culture medium by rinsing the microtiter plate and submerging it in distilled water in a rising tray three times to eliminate free bacteria, and the biofilm layer was fixed by air-drying by using a Rotary evaporator. The fixed biofilm layer was stained with 150 µL of 0.1 % (w/v) crystal violet at room temperature (RT) for 15 minutes (this dye stains the cells but not the polystyrene). Then the excess crystal violet dye was washed out, and this was followed by washing the samples three times with distilled water. After drying in the open air, the appearance of a visible film on the walls of the microplates and the bottom of the walls was considered an indication of biofilm production. The crystal violet was then removed from the wells with 200 µl of ethanol 99% and acetone solution [7:3 (v/v)] per each well to detach the fixed cells (biofilm) from the well, then pipetted up and down to ensure that the biofilm was well solubilized, and the plate was left at room temperature for 10 min without shaking. Then the optical density of each well was measured in the plate at 630 nm using a microplate reader [Bio Tek Instruments]. Four control wells were added to each test batch; Wells containing bacteria only, wells containing extract concentration only, wells containing elution (99% ethanol and acetone solution), and blank wells. This spectrophotometric method measures the total biofilm biomass, including bacterial cells and extracellular matrix. Each test was performed in triplicate, and the mean absorbance standard deviation was computed for all assays. The percent inhibition of biofilm formation was calculated using the ratio between the values of OD630nm wells with and without the extracts according to [31].

RESULTS

MIC determination of *Enteromorpha* sp. extracts of P. *aeruginosa*

The results showed that the lowest inhibitory concentration was in the Ethanolic extract of *Enteromorpha,* as its MIC was 0.06 mg/ml, and sub-MIC was 0.03 mg/ml. It is followed by the the aqueous and hexane extract of *Enteromorpha,* as its MIC was 0.12 mg/ml and sub-MIC was 0.06 mg/ml (Table 1-Fig. 1).

Table 1. Minimal inhibitory concentration (MIC) of *Enteromorpha* sp. extracts

Concentration mg/ml & Growth Case						
Type of Extract	**0.25**	**0.12**	**0.06**	**0.03**	**0.01**	**0.005**
Entero-Water	-Ve	-Ve	**+Ve**	**+Ve**	**+Ve**	**+Ve**
Entero-Ethanol	-Ve	-Ve	-Ve	**+Ve**	**+Ve**	**+Ve**
Entero-Hexane	-Ve	-Ve	**+Ve**	**+Ve**	**+Ve**	**+Ve**

(+Ve) =There is bacterial growth, (-Ve) There is no bacterial growth

Figure 1. MIC of Ethanolic extract of *Enteromorpha* sp. of P. *aeruginosa*

The activity of *Enteromorpha* Spp. extracts against P. *aeruginosa*

The results of the current study showed the highest inhibition zone of *Enteromorpha* against P. *aeruginosa* was recorded in Hexanal extract at a concentration of 100% with a mean of 14.5±0.7. The results also showed significant differences at the level of <0.05 for the growth inhibition process of P. *aeruginosa,* according to the concentrations of the aqueous extract. Whereas the results showed the highest inhibition zone of the Ethanolic extract of *Enteromorpha* was recorded at a concentration of 25% with a mean of 11.0±2.8, and the highest inhibition zone of the Aqueous extract of *Enteromorpha* was recorded at a concentration of 100% with a mean of 13.0±0.0. The results also showed no significant differences at the level of <0.05 for the growth inhibition process of bacterial isolates according to the concentrations of the two extracts (Ethanolic and Aqueous).

Table 2. Activity of *Enteromorpha* Spp. extracts against P. *aeruginosa*

	***Enteromorpha* sp. & Inhibition Zone/ mm**		**extracts**	**Concentrations**	**ANOVA Test**	
	100%	**75%**	**50%**	**25%**		
Type of Extract	Mean & Std.	Mean & Std.	Mean & Std.	Mean & Std.	Sig.	LSD
Water	13.0±0.0	12.5±0.7	11.5±0.7	11.5±0.7	0.158	NS
Ethanol	11.0±0.0	10.5±0.7	10.0±0.0	11.0±2.8	0.881	NS
Hexane	14.5±0.7	12.5±0.7	11.5±0.7	10.5±0.7	0.019	0.557

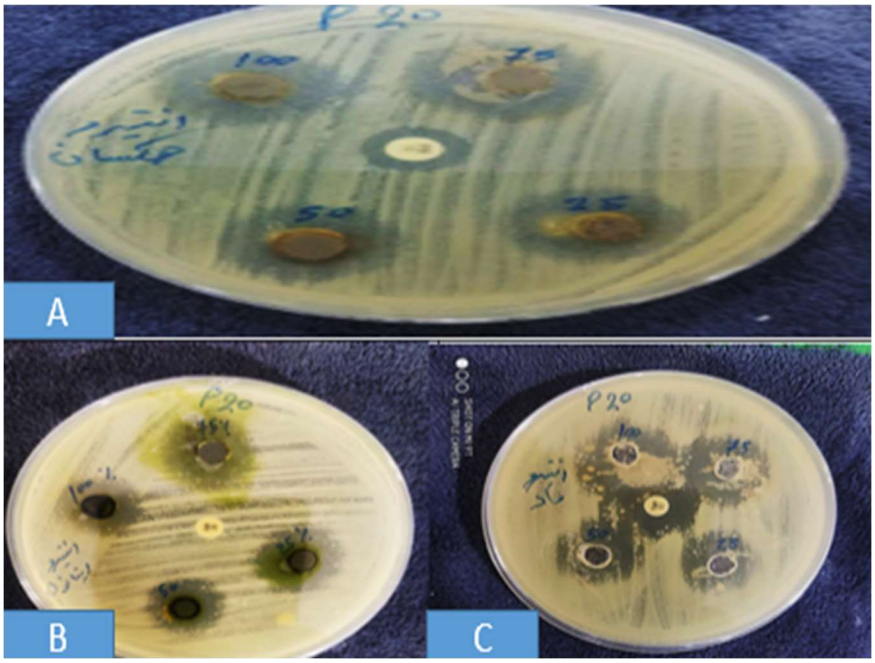

Figure 2. Activity of *Enteromorpha S*pp. extracts against P. *aeruginosa* (**A**=Entero-hexane, **B**= Entero-ethanol, **C**= Entero-water)

Inhibitory effect of *Enteromorpha* sp. on biofilm formation in P. *aeruginosa*

All strains of P. *aeruginosa* were tested for biofilm formation. The results of the current study showed that out of the 20 tested P. *aeruginosa* isolates, 14 isolates were biofilm producers while 6 isolates did not produce. The highest biofilm yield (2.49±0.25) was recorded for strain No.20 (P20) which was then selected

for treatment with algae extracts.

The current study showed a significant decrease in biofilm formation when P. *aeruginosa* was grown in the presence of the sub-MICs of algal extracts. The results recorded a maximum reduction of 96% with the Ethanolic extract of *Enteromorpha*, while a minimum reduction was in the Aqueous extract of *Enteromorpha* with a percentage of 52%. The results also showed a significant difference at <0.05 in biofilm inhibition level before and after treatment with all extracts (Table 3-Fig. 3).

Table 3. Inhibitory effect of *Enteromorpha* Spp. extracts on biofilm formation in P. *aeruginosa*

Type of Extract	No.	Mean & Std.	Inhibition Percentage	LSD Sig.
Before (P-20)[1]	21	2.49±0.25	0.00%	
After Entero-Water[2]	12	1.191±0.01	52%	0.001[1:2,3,4, 2:3,4]
After Entero-Ethanol[3]	12	0.105±0.00	96%	
After Entero-Hexane[4]	12	0.182±0.03	93%	NS[3:4]
ANOVA Sig.		**>0.001**[**]		

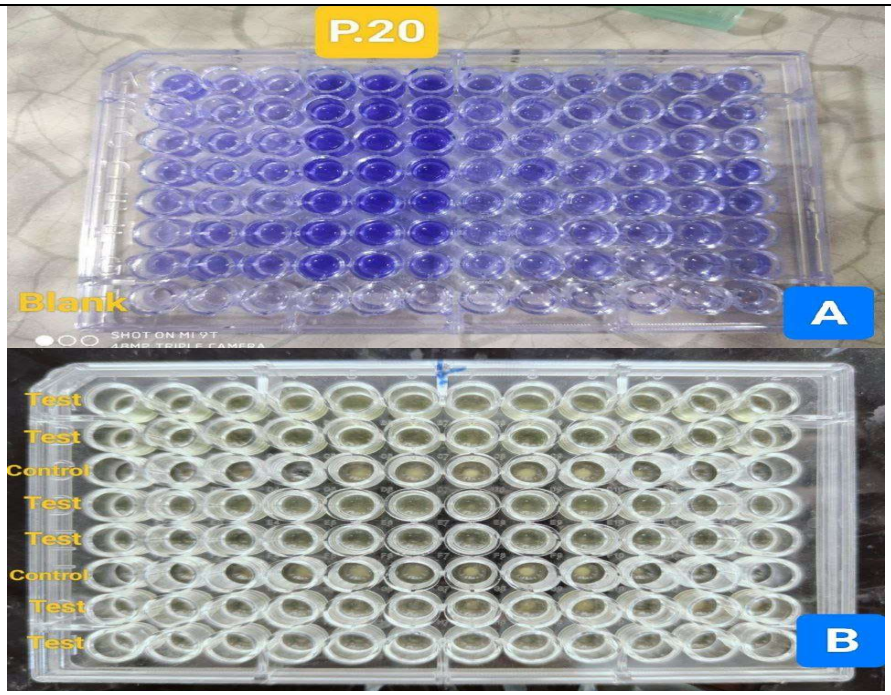

Figure 3. Inhibitory effect of *Enteromorpha* sp. extracts on biofilm formation in P. *aeruginosa* (**A**= Highest biofilm-forming isolate of *P. aerugenosa*, **B**= After treatment with algal extracts)

The active compounds for algal extracts

The chemical analysis using a GC-MS device for extracting active compounds for the aqueous, ethanol, and hexane extracts of Enteromorpha algae showed the presence of several active compounds as shown in Table 4.

Table 4. Active compounds for extracts of *Enteromorpha* Spp. using GC-MS

Ethanolic Extract of *Enteromorpha*				
Peak	**R. Time**	**Area%**	**Chemical name**	**Formula**
2	17.13	6.31	3-Ethylheptanoic acid	C9H18O2
4	18.24	4.55	Glycyl-l-phenylalanine, N-isobutoxycarbonyl-, isobutyl ester	C21H34N2O4
8	19.35	14.03	n-Hexadecanoic acid (Palmitic acid)	C16H32O2
11	21.13	4.99	5-Dodecene, (E)-	C12H24
15	24.64	63.35	Pentanoic acid	C5H10O2
Total		**93.23**		
Aqueous Extract of *Enteromorpha*				
1	2.05	22.89	2,2-Dihydroxymalonic acid	C3H4O6
2	17.0	8.65	(3-Propyl-2-oxiranyl)methanol	C6H12O2
3	17.8	3.82	Oxalic acid, cyclobutyl heptyl ester	C13H22O4
4	19.28	30.52	tert-Butyl acrylate	C7H12O2
5	21.09	11.89	Pentanenitrile, 4-methyl-	C6H11N
6	27.61	22.23	Ergosta-5,24-dien-3.beta.-ol	C28H46O
Total		**100**		
Hexanal Extract of *Enteromorpha*				
1	2.09	73.23	2,2-Dimethylhexane	C8H18
2	17.09	6.87	(3-Propyl-2-oxiranyl)methanol	C6H12O2
5	19.29	9.01	2-Pentanone, 4,4-dimethyl-	C7H14O

6	21.09	5.15	2,3-Dimethylsuccinonitrile	C6H8N2
Total		**94.26**		

DISCUSSION

Enteromorpha Spp are broadly screened to isolate drugs or bioactive substances all over the world, they can produce a great variety of secondary metabolites characterized by a broad spectrum of antimicrobial activities [32]. Accordingly, the present study was focused on screening water, ethanol, and hexane extracts of *Enteromorpha* Spp for the potential of antimicrobial activity on Uropathogenic P. *aeruginosa*. Different concentrations of the extracts of the studied green algae exhibited antibacterial activity and they inhibited most of the tested bacteria. The maximum biological activities against P. *aeruginosa* were observed with the inhibition zone reaching 14.5 mm.

These results are consistent with the results of another study conducted by Swathi et al [33], where they found that *Enteromorpha* extracts have an inhibitory effect against P. *aeruginosa* , with an inhibition zone of up to 13 mm. The study of the antibacterial activity of *Enteromorpha* against multidrug-resistant and beta-lactamase-producing isolates conducted by [34] also showed that *Enteromorpha* extracts had antibacterial activity for all bacterial isolates studied, including P. *aeruginosa* , where they found the highest inhibitory activity against P. *aeruginosa* (14 mm) , where their findings mirrored the current results. While Soltani & Khoshrooei, [32] reported that P. *aeruginosa* to be the most resistant among all bacterial species tested (without inhibition zones) of *Enteromorpha* extracts, which disagrees with our findings where P. *aeruginosa* was affected. Our findings on the antibacterial effects of *Enteromorpha* extracts might not always match those of other studies for a few reasons. One reason is the extraction process itself, where different solvents can pull out different bioactive compounds from the algae, and these compounds may have varying levels of antimicrobial activity. Another reason, as mentioned in a study by Munir *et al.*[35], the antimicrobial activity of algae can depend on the season the samples were collected. Finally, the resistance of the bacteria itself can also influence the results, where bacteria can develop resistance to antimicrobial compounds, and this resistance can vary between strains, as pointed out by Swathi *et al.* [33].

On the other hand, the organization of bacteria in biofilms is one of the adaptive resistance mechanisms providing increased protection against conventional treatments. Thus, the search for new antibiofilm agents for medical purposes, especially of natural origin, is currently the object of much attention [36]. So, algae extracts and their metabolites such as sulfated polysaccharides (fucoidan), carotenoids (zeaxanthin and lutein), lipid and fatty acids (γ-linolenic acid and linoleic acid), and phlorotannins can inhibit the cell attachment, reduce the cell growth, interfere in quorum sensing pathway by blocking related enzymes, and disrupt extracellular polymeric substances [37]. Great interest has been focused on the search for synthetic and natural alternatives to conventional antibiotics to overcome the strong ability of "superbug" P. *aeruginosa* to form deleterious biofilms that override antibiotherapy [38].

In this vein, in the present study, the extracts of macroalgae *Enteromorpha* sp. were explored for their potential antibiofilm activity against this pathogen. The results of the current study showed that there was a significant decrease in biofilm formation of P. *aeruginosa* in the presence of the sub-MICs of *Enteromorpha* extracts, with an inhibition percentage of biofilm reaching up to 96% inhibition. This finding is similar to another study by Yuvaraj & Arul. [39] in India investigated the anti-biofilm properties of extracts from E. lactuca and E. reticulata against P. *aeruginosa* using a microtiter plate method, where their results revealed that these extracts effectively reduced total biofilm formation by P. *aeruginosa* . Also, research by LewisOscar et al. [40] showed that an algal extract significantly exhibited the amount of extracellular polymeric substances

(EPS) produced by P. *aeruginosa* , by about 88%." Where EPS, in turn, plays a significant role in the attachment and biofilm-forming ability of bacteria. It has also been reported by Prabhakaran et al[41] that different extracts from seaweeds such as Ulva, exhibited anti-biofilm formation activity against *Pseudomonas* spp, and this inhibitory activity was related to the main functional groups, including hydroxyl, amino, carbonyl, and phosphoryl functionalities, aliphatic extracts (fatty acids), and NH2 (amide I and II) extracts.

In particular, lipids and fatty acids possess antimicrobial and anti-biofilm activities [37]. Where recent studies suggest that lipids exhibit antibiofilm activity against bacteria and fungi at lower concentrations than their corresponding MIC [42]. A recent study by Rima et al.[36] reinforces this concept. They investigated *Enteromorpha* extracts against P. *aeruginosa* biofilms and found a significant reduction (over 80% inhibition). Interestingly, their results identified hexadecanoic acid (palmitic acid), a saturated fatty acid abundant in *Enteromorpha* as a potential key player in this anti-biofilm effect.

The current finding also aligns with other research suggesting the importance of specific compounds within algae extracts. El Zawawy et al. [43] investigated green algae extracts from the Egyptian coast and found similar results. Their study linked the anti-biofilm ability to fractions like n-hexadecenoic acid, detected using GC-MS analysis. They proposed that these fractions downregulate bacterial genes essential for biofilm formation (hyphal-specific genes). Additionally, Alreshidi et al. [44] reported that an algae extract rich in n-hexadecanoic acid exhibited remarkable anti-biofilm activity (over 82%). They suggested the extract disrupts biofilm formation through a chemical interaction between its components and specific bacterial receptors. As well as, Santhakumari et al. [45] reported that the crude algal extract successfully inhibited the initial attachment, interrupted the biofilm structure, and therefore prevented bacterial biofilm formation. Where the algal extract can interfere with the quorum sensing pathway by blocking the protease and gelatinase, which were positively regulated by the quorum sensing. They also reported that the anti-quorum sensing activity of the algal extract was related to the presence of hexadecanoic acid in the extract, and molecular docking revealed its potential for antagonistic binding to the acyl-homoserine lactone (AHL) receptor protein. LewisOscar *et al.*[46], also reported that palmitic acid, a fatty acid found in some algae, can disarm P. *aeruginosa* 's biofilm-forming abilities. It appears to achieve this by turning down the activity of a specific gene (abaR) in the bacteria. This gene is involved in the production of N-acyl-homoserine lactones (signaling molecules), which P. *aeruginosa* uses to communicate and coordinate biofilm formation (quorum sensing). By reducing these signaling molecules, palmitic acid disrupts P. *aeruginosa* 's ability to form biofilms. These combined findings highlight the potential of algae extracts, particularly those containing hexadecanoic acid (Palmitic acid) is a saturated fatty, as a promising strategy to combat P. *aeruginosa* biofilms. Further supporting the role of algal lipids as antibiofilm, Cepas et al. [47] research demonstrated that certain lipids, including polyunsaturated fatty acids (PUFAs) like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), possess antimicrobial and anti-biofilm activities.

Besides lipids, the presence of bioactive substances in algae extracts such as hydrocarbons, phenols, esters, 1-Heptacosanol, and Octadecyl chloride prevent the biofilm formation via the inhibition of bacteria growth, quorum quenching, and disruption of biofilm [42]. Furthermore, Powell et al.[48] have demonstrated the ability of oligosaccharides derived from the algae to decrease P. *aeruginosa* biofilm biomass by disrupting its Extracellular polymeric substances (EPS) network. The function of the EPS is not limited to providing a protective barrier against exogenous factors, it also ensures nutrition, hydration, and intercellular interaction within the biofilm [49].

There's a challenge in comparing biofilm studies due to the many factors that can influence results. These factors include the extraction methods, the specific strain of bacteria being studied, and the conditions used to grow the biofilm (e.g., environmental factors, inoculum preparation, and nutrients). On top of that, biofilm experiments often involve a lot of variation in how they're set up and measured. This can include things like the surrounding environment, how the bacteria are prepared, and how the biofilm itself is quantified. Some

measurement methods, especially those relying on color changes (like Cresyl Violet staining), can be particularly prone to variation. Because of all this variability, directly comparing data from different biofilm studies can be very difficult [50].

Finally, as the QS communication system is a key factor in bacterial biofilm formation, the active extracts discovered in this study may potentially act on this complex system and/or on other factors regulated by QS, such as the production of rhamnolipids. This biosurfactant, controlled by the rhl QS system, is involved in the different stages of biofilm formation, particularly in the mediation of cell dispersion [49]. Thus, the present results encourage towards elucidating the potential direct and/or indirect anti-QS activity of these extracts.

CONCLUSION

The antibacterial activity of algal extract holds new prospects in the evolving biotechnologies relative to both the exploitation of marine biomasses and the research of new natural active compounds. Indeed, since P. aeruginosa is an opportunistic human pathogen that primarily affects nosocomial patients, the use of the algal extracts could be useful in the production of new molecules against resistant pathogens. In this work, the extracts obtained from *Enteromorpha* Spp. were screened to determine their antibacterial and antibiofilm activity against Uropathogenic *P. aeruginosa*. As a result, three extracts from *Enteromorpha* Spp. (water, ethanol, and hexane) were chosen. When the bacteria were treated with these extracts using the disk diffusion test, it was observed that there was an inhibitory effect of these extracts on the tested bacterial isolates. Regarding the biofilm test, the findings were promising, where extracts from *Enteromorpha* at sub-MIC were able to prevent biofilm formation of *P. aeruginosa* by as much as 96%. Thus, the present results encourage the elucidation of the potential direct and/or indirect antibacterial and antibiofilm activity of these extracts. Considering these promising outcomes, additional tests are planned to unravel the potential mechanism of action of the chosen active extracts, namely by molecular analysis. Moreover, it would be intriguing to delve deeper into the examination of the chemical composition of the active extracts with the aim of isolating exceptionally potent compounds. In summary, the results of this study provide a foundation for potential future uses of algae as a preventative measure against MDR bacteria.

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