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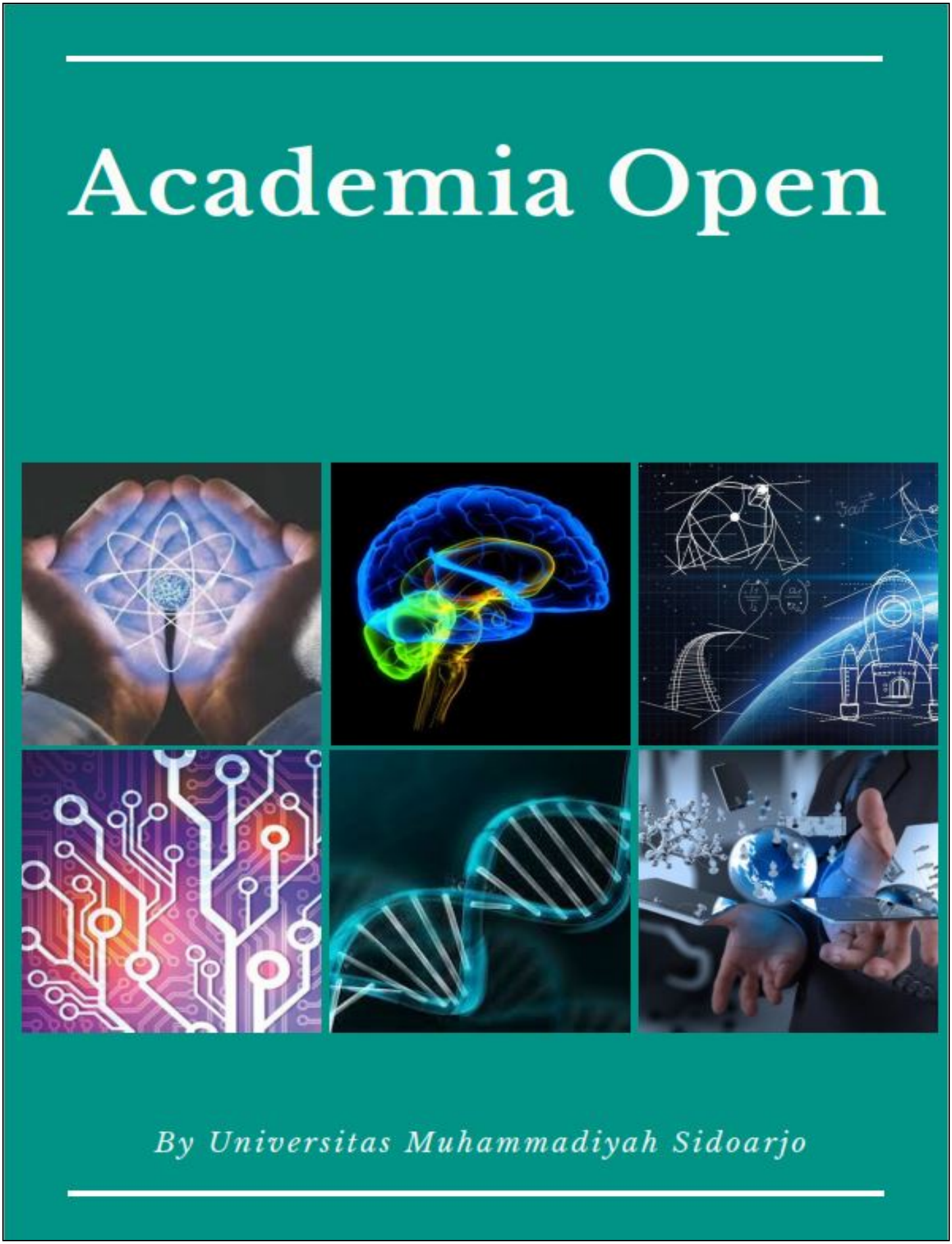

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Cladophora spp. Extracts Show Remarkable Antibacterial Potential Against Pseudomonas aeruginosa

Ekstrak Cladophora spp. Menunjukkan Potensi Antibakteri yang Luar Biasa Terhadap Pseudomonas aeruginosa

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Abstract

Background: Bacterial resistance, influenced by genetic processes and adaptive strategies, necessitates the discovery of novel antibacterial agents, especially from natural sources. **Specific Background:** Pseudomonas aeruginosa, a notorious pathogen in urinary tract infections (UTIs), demonstrates considerable resistance to conventional therapies, necessitating alternative therapeutic approaches. **Knowledge Gap:** Research indicates that while natural sources like Cladophora spp. offer antibacterial agents, their effectiveness in combating P. aeruginosa resistant strains remains underexplored. **Aims:** This study aims to evaluate the antibacterial potential of Cladophora spp. algae extracts against Pseudomonas aeruginosa isolated from UTIs, utilizing solvent extraction, MIC determination, disc diffusion assays, and GC-MS analysis to identify bioactive compounds. **Results:** All extracts, including those prepared with water, ethanol, and hexane, demonstrated inhibitory effects on P. aeruginosa. The hexane extract exhibited the most significant activity, with a mean zone of inhibition of 13.0 ± 0.7 mm at a concentration of 50%. GC-MS analysis identified several bioactive compounds potentially responsible for these effects. **Novelty:** This study is among the first to investigate Cladophora spp. as a source of antibacterial agents specifically targeting P. aeruginosa, providing new insights into the potential of algae-based therapeutics. **Implications:** Cladophora spp. holds promise as a source of novel antibacterial compounds, with potential for multidrug-resistant infections treatments. Further research is needed for clinical application.

Highlights:

1. Hexane Extract: Most effective against Pseudomonas aeruginosa.
2. Novel Source: Cladophora spp. shows potential as antibacterial agent.
3. GC-MS Findings: Identified key bioactive compounds.

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Introduction

P. aeruginosa, along with other members of the pseudomonad family, is widely distributed in various settings, including water, soil, and plants. It is also not a prominent component of the normal resident microbial flora, known as the microbiome, in healthy individuals, including the gastrointestinal or vaginal tracts [1]. Nevertheless, P. aeruginosa is an opportunistic human pathogen that primarily affects individuals with cancer or AIDS, patients with impaired immune systems due to surgery, or burn wounds, cystic fibrosis, or infections of the blood, skin, eyes, or genitourinary tract [2]. Specifically, hospitalized patients are the group of people who are most vulnerable to infections by this organism. where one of the most prevalent nosocomial infections is P. aeruginosa. Thus, anywhere between 10% and 15% of nosocomial infections globally are caused by P. aeruginosa. [3]. Urinary tract infections are typically caused by P. aeruginosa in a medical setting, frequently in conjunction with a urinary catheter. Unless there is an underlying urological problem, such as blockage, recent instrumentation, or neurogenic bladder, P. aeruginosa rarely results in community-acquired infections [4]. It is not unexpected that P. aeruginosa infections cause a great deal of illness and death because this microbe can quickly evolve antibiotic resistance, adapt to new environments, and produce a wide range of virulence factors [3]. One way P. aeruginosa stays alive in hospitals longer than other possible infections is because it has an inherent resistance to many routinely used antibiotics in hospitals [1]. So, the treatment of this pathogen or others has become a significant concern for healthcare systems globally due to the bacteria's growing resistance to antimicrobial medicines [5]. In order to tackle this problem, it is necessary to conduct a search for novel antibiotics derived from natural sources and to design antibiotics that specifically target hitherto unexplored biological pathways [6]. Over the past few decades, there has been an increasing interest in an alternate method to antibiotics, which involves using natural compounds that inhibit the growth of hazardous bacteria [7]. Multiple studies have suggested that algae, with their high diversity and rapid development rate, may be able to produce a broad variety of chemical compounds with unique biological characteristics [8], [9]. As a result of adapting to their complex environments, algae have developed these bioactive compounds [10]. Where its production is considered to be a response to ecological pressures such as competition, predation deterrence, and reproduction [11]. So, biotic factors, such as plants and microbes, as well as abiotic factors, including temperature, pH, salinity, and light intensity, play a role in the formation of secondary metabolites by algae [12]. Thus, Algae have emerged as an appealing reservoir of antibacterial substances owing to their rapid proliferation and extensive range of species [8].

Cladophora spp. is a widely distributed alga that can be found in large quantities in many marine and freshwater environments, and it is possibly the most prevalent macroalgae in freshwater ecosystems globally [13]. While it is relatively easy to identify the genus, distinguishing between distinct species of Cladophora can be challenging due to the significant number of variations within the genus. Therefore, the differentiation of species necessitates a careful investigation of minute characteristics. [14]. Cladophora possesses a diverse range of bioactive compounds, rendering it a suitable substance for use in the field of medicine. This genus has been recognized as an abundant and sustainable reservoir of biologically active secondary metabolites, such as alkaloids, polysaccharides, flavonoids, cyclic phlorotannins, peptides, sterols, quinones, lipids, and glycerol [15]. Factually, these compounds possess a wide range of biological properties including antioxidant, antidiabetic, antibacterial, and antitoxic [16]. Studies have shown that extracts derived from Cladophora possess antibacterial properties, suggesting their capacity to effectively combat bacteria, fungi, and viruses. This presents potential applications in the fields of anti-infection, wound healing, and disinfection [17]. However, given the paucity of data on the antibacterial efficacy of Cladophora spp. against Uropathogenic P. aeruginosa, the present study aimed to investigate the potential of antibacterial activity of Cladophora spp. extracts against P. aeruginosa derived from urinary tract infections (UTIs).

Methods

Algae Samples Collection and Processing

The Algal Samples (Cladophora spp.) 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 [18], 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 Cladophora spp. 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 using Ultrasonic Bath, which was modified from [19].

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Preparation of Stoke Solution and Working Extracts

The stoke solution of algal extract was prepared according to [20] 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 extracts 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 [21]. 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.

Minimal inhibitory concentration (MIC) determinations of Cladophora spp. Extracts

The MIC of algal crude extracts was determined using the broth microdilution method according to [22]. 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 [23]. The determinations were performed in triplicate.

Antibacterial Activity Testing of Cladophora spp. Extracts Using Disc Diffusion Assay

The antimicrobial activity of Cladophora spp. extracts were 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 (Mohammed et al., 2021). 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.

Result and Discussion

Result

MIC determination of Cladophora spp. extracts of P. aeruginosa

The results showed that the lowest inhibitory concentration was in the Hexanal extract of Cladophora, as its MIC was 0.01 mg/ml, and sub-MIC was 0.005 mg/ml. As for other algal extracts, the MIC was 0.12 mg/ml and the sub-MIC was 0.06 mg/ml. (Table 1-Fig. 1).

Concentration mg/ml & Growth Case							

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Type of Extract	0.25	0.12	0.06	0.03	0.01	0.005
Clado-Water	-Ve	-Ve	+Ve	+Ve	+Ve	+Ve
Clado-Ethanol	-Ve	-Ve	+Ve	+Ve	+Ve	+Ve
Clado-Hexane	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve

Table 1. *Minimal inhibitory concentration (MIC) of Cladophora spp. extracts*

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

Figure 1. *MIC of Ethanolic extract of Cladophora spp. of P. aeruginosa*

The activity of Cladophora spp. extracts against P. aeruginosa

The current study showed that the highest inhibition zone of Cladophora against P. aeruginosa was recorded in Hexanal extract at a concentration of 50% with a mean of 13.0±0.7 mm. The results also showed the highest inhibition zone of Ethanolic extract of Cladophora was recorded at a concentration of 100% with a mean of 12.5±0.7 mm. Whereas, the highest inhibition zone of the aqueous extract of Cladophora was recorded at a concentration of 100% with a mean of 11.0±1.4. The results also showed 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).

	Cladophora sp. Extracts Concentrations & Inhibition Zone/ mm				ANOVA Test	
	100%	75%	50%	25%		
Type of Extract	Mean & Std	Mean & Std	Mean & Std	Mean & Std	Sig.	LSD
Water	11.0±1.4	11.0±0.0	7.50±0.7	6.00±0.0	0.007	0.696
Ethanol	12.5±0.7	12.0±1.4	10.5±0.7	9.50±0.7	0.024	0.418
Hexane	12.0±0.0	12.5±0.7	13.0±0.7	11.5±0.7	0.138	NS

Table 2. *Activity of Cladophora extracts against P. aeruginosa*

(A)=Water, (B)=ethanol, (C)=hexane

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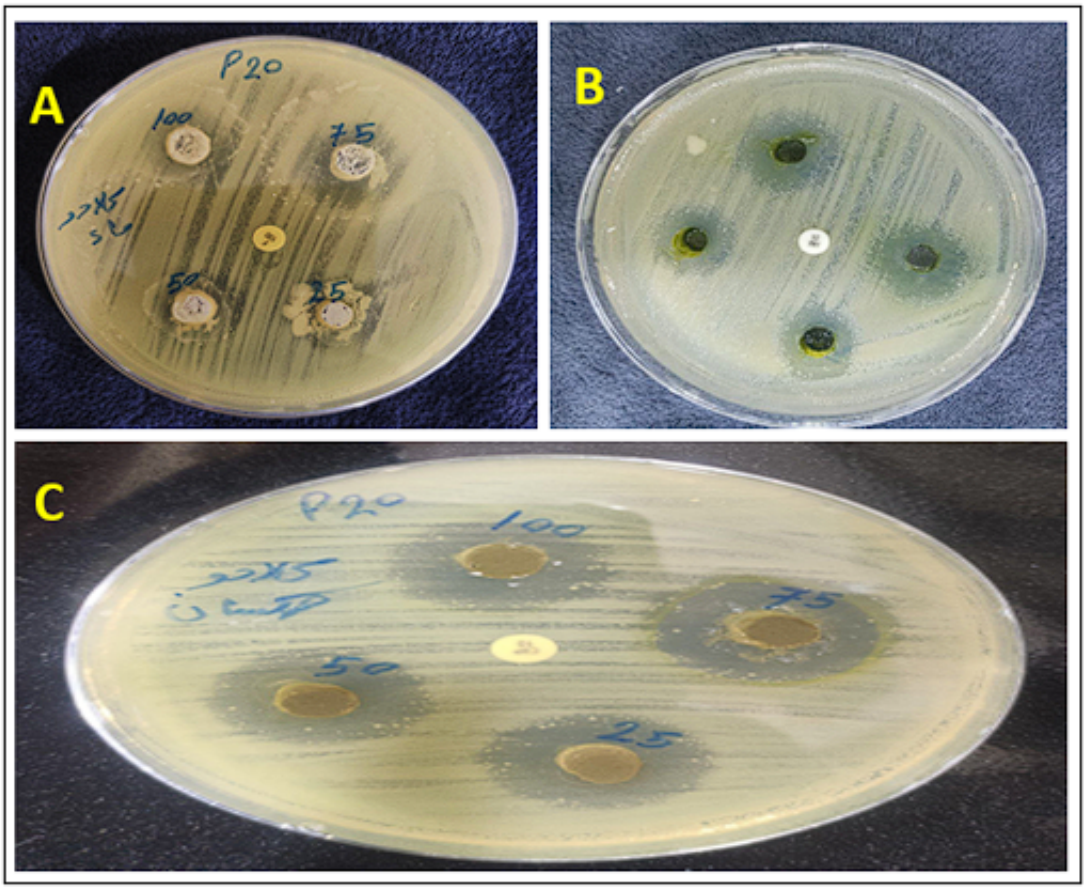

Figure 2. *Activity of Cladophora spp. extracts against P. aeruginosa*

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 Cladophora spp. algae showed the presence of several active compounds as shown in Table 3.

Aqueous Extract of Cladophora Spp.				
Peak	R. Time	Area%	Chemical name	Formula
1	2.097	6.24	Hexaldehyde	C6H12O
6	19.329	31.44	Ethylheptanoic acid	C9H18O2
7	21.15	35.99	9-Oxabicyclo[6.1.0]nonane, cis-	C8H14O
8	21.32	5.21	5-Dodecene, (E)	C12H24
16	26.32	8.06	9-Octadecenoic acid (Z)-, hexyl ester (Oleic acid, hexyl ester)	C24H46O2
Total		86.94		

Table 3. *Active compounds for extracts of Cladophora spp. using GC-MS*

Ethanolic extract of Cladophora spp.				
1	3.46	4.52	Dimethyl Sulfoxide	C2H6OS
2	17.13	6.64	3-Ethylheptanoic acid	C9H18O2
5	18.24	4.27	Oxalic acid, dibutyl ester	C10H18O4
7	19.33	6.63	n-Hexadecanoic acid (Palmitic acid)	C16H32O2
9	24.62	75.26	L-Ornithine, N2,N5-bis(trifluoroacetyl)-, 1-methylpropyl ester	C13H18F6N2O4

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Total	97.32		

Table 4.

Hexanal Extract of Cladophora spp.				
1	2.09	80.58	Acetic acid, butoxyhydroxy-, butyl ester	C10H20O4
2	17.09	7.24	Isoamyl nitrite	C5H11NO2
3	17.93	4.28	2-Pentanone, 4,4-dimethyl-	C7H14O
4	19.29	7.90	Isobutyl nitrite	C4H9NO2
Total		100		

Table 5.

(A)=Water, (B)=ethanol, (C)=hexane

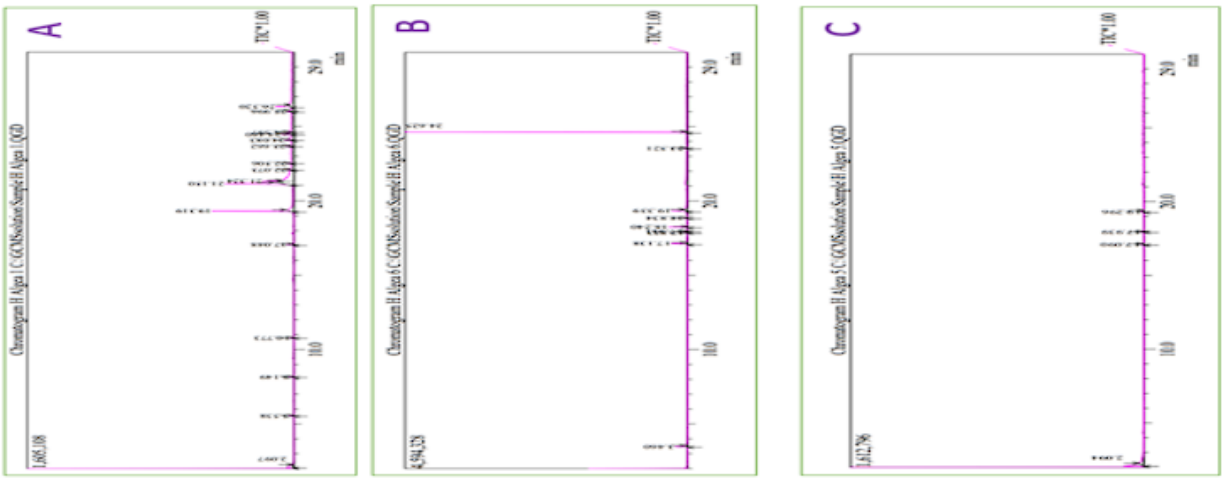

Figure 3. *Active compounds for Cladophora spp. using GC-MS*

Discussion

In their ability to create a wide range of secondary metabolites with antibacterial properties, the macroalgae are broadly screened to isolate drugs or bioactive substances all over the world [24]. Accordingly, the present study was focused on screening water, ethanol, and hexane extracts of Cladophora spp. algae for the potential of antimicrobial activity on Uropathogenic P. aeruginosa. The findings indicated that the extracts had antimicrobial activity against the bacterium isolates under investigation. Furthermore, the results demonstrated that the hexane extract showed the highest efficacy against P. aeruginosa with an inhibition zone of 13.0±0.7 mm. These results aline with the Egyptian study prepared by Abdel-Raouf et al.)2018), they observed that the maximum biological activities of Cladophora algae extracts were against P. aeruginosa among the other types of negative bacteria (E. coli and K. pneumonia). Al-Khafaji & Saeed,. (2024), in Iraq, also found a similar inhibitory effect of Cladophora extracts against P. aeruginosa, with an inhibition zone of 11 mm. Whilst, another study done in Iran by Saadatmand et al.(2011) came to a different conclusion, their experiment found that P. aeruginosa was the most resistant bacteria to kill with Cladophora extracts among all other bacteria species, where it completely resisted the extract, without inhibition zones. Furthermore, Soltani & Khoshrooei. (2014), also found P. aeruginosa was found to be the most resistant among all bacteria (without zones of inhibition) of Cladophora extracts.

Conflicting results on the effectiveness of Cladophora algae extracts against bacterial species between this study and the other studies may be attributed to several reasons. One of these is that different species of Cladophora can produce different types of antibacterial compounds. This is especially true given the large number of species within the genus Cladophora. Like Algae, there is strain variation among bacterial isolates as well, with some strains perhaps being more susceptible to the antimicrobial compounds of Cladophora extracts than others. Additionally, the efficacy of the extraction procedures, the type of extraction solvent utilized, the season at which samples were collected, and other factors may account for the observed difference in the antibacterial activity of algal extracts [28].

In this study, the findings also indicated that GC-MS analysis of Cladophora extracts revealed a diverse range of

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constituents as shown in (Table 3). Among all these compounds, the antibacterial action may be attributed to fatty acids, including octadecenoic acid, ethylheptanoic acid, and palmitic acid. This is confirmed by Bintari & Risandiansyah,)2019), which identified palmitic acid as a fatty acid in the ethanol extract of Cladophora Spp. which had antibacterial activity against several bacterial species, including Pseudomonas. Ismail et al.(2018) also detected the anti-bacterial activity of algae-isolated fatty acids and found that the activity found in the seaweed samples seems to be caused by palmitic acids. The current study's identification of octadecenoic acid (Oleic acid) in Cladophora extract is corroborated by the prior work of Al-Khafaji & Saeed, (2024) where they found that octadecenoic acid is one of the active compounds present in Cladophora extract. Stabili et al.) 2011) also noted in their study that since palmitic acid and Oleic acid are the most abundant among the fatty acids of Cladophora extract, it is likely responsible for the antibacterial activity. Preliminary studies by Bozzini et al. (2023) have shown that ethylheptanoic acid exhibits some antimicrobial activity against certain bacterial strains. Moreover, the current study, like others, identified Acetic acid, butoxyhydroxy-, butyl ester from hexanal extract of the Cladophora as the most potent antibacterial compound. Simply, the potential antibacterial efficacy of this compound may be attributed to its constituent acetic acid, as supported by the findings of Ryssel et al. (2009), where their study demonstrated that even low concentrations of acetic acid can exhibit potent antimicrobial activity against various bacterial strains, including A. baumannii and P. aeruginosa. Fan et al.(2024) reported that Hexaldehyde (Hexanal) is a natural aldehyde compound derived from plants and algae, which has been proven to possess antimicrobial activity against some bacteria such as E. coli and Pseudomonas. Supporting earlier findings, the screening GC-MS analysis of Cladophora for a study by JV, (2019) confirm that Cladophora algae extracts are rich in bio-actives such as saturated and unsaturated fatty acids, sterols, terpenoids, and phenolic compounds. Furthermore, the present study identified several additional compounds within Cladophora extracts. However, a comprehensive evaluation of their effectiveness is lacking in the existing literature or has not been adequately researched. Thus, the present results encourage elucidating the potential direct and/or indirect antibacterial activity of these compounds. So, further investigation is necessary to elucidate their potential therapeutic properties.

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 the human pathogen P. aeruginosa is an opportunistic human pathogen that primarily affects nosocomial patients, the use of the algal extract could be useful in the production of new molecules against resistant pathogens. In this work, the extracts obtained from Cladophora spp. were screened to determine their ability to inhibit the Uropathogenic bacterium P. aeruginosa. As a result, three extracts from Cladophora 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. Thus, the present results encourage the elucidation of the potential direct and/or indirect antibacterial 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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