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Evaluation the Effect of *Chlorella vulgaris* and *Oscillatoria limnetica* Fatty Acid Extracts on Four-Gram Negative Pathogenic Bacteria

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Abstract

Antibiotic resistance has become concern for the scientific community worldwide. As a result, using natural chemicals has become an essential rather than an option in an attempt to decrease the harm caused by bacterial illnesses, which can be fatal due to the difficulties of treating them. In this study, two distinct species of microalgae (Chlorella vulgaris and Oscillatoria limnetica) were isolated from the surrounding aquatic environment. The antibacterial and antibiofilm properties of the hexane extract of these two microalgae and Chlorella vulgaris fatty acids were investigated in vitro against (Pseudomonas aeruginosa, Acinetobacter baumannii, Proteus mirabilis, and Klebsiella pneumoniae), which were clinically isolated from 100 burn and surgical patients, and diagnosis by some biochemical tests, and have been tested for resistance to antibiotics. The results showed that crude hexane extract of Chlorella vulgaris and Oscillatoria limnetica had a high effect as antibacterial and antibiofilm. Also, Chlorella vulgaris fatty acids extract showed superiority as an antibiofilm and as an antibacterial when applied at two concentrations (10 and 100 mg/ml). The important chemical components for all extracts of microalgae have been identified by Gas Chromatography-Mass Spectrometry.

Keywords: Antibacterial, Antibiofilm, Antibiotic resistance, Fatty acids, Microalgae.

Introduction

Antibiotic resistance is regarded as one of the most serious threats to human health worldwide, and antibiotic overuse exacerbated the situation, multidrug-resistant (MDR) bacteria are becoming more difficult to treat, due to the drugs used to treat them becoming less effective. This resistance is the result of several mechanisms used by the bacterial cell to get rid of the drug without affecting it. The most important of these mechanisms are the modification of the antibiotic target site, changes in the permeability of a bacterial cell, active pumping of the antibiotic out of the cell, and enzymatic inactivation. [1-3]. As a result, it became important to turn to natural substances present in many plants and microalgae to offset the widespread use of antibiotics in the treatment of numerous diseases [4-6]. Focusing on microalgae more than other organisms is logical because of its various features, which have attracted researchers to it. Fast biomass development, the ability to modify biochemical composition in response to cultivation circumstances , and the ability to produce a wide range of biologically active compounds [7]. The production of antibacterial substances contributes directly or indirectly to the ability of green microalgae to survive under harsh conditions [8]. Therefore, it has been modified in a lab to produce the chemicals that are responsible for its antibacterial and pathogen activity. Microalgae and the products they produce have been utilized in the treatment of numerous illnesses, including heart, kidney, and bladder disease, as well as the inclusion of their extracts in the formulation of numerous drugs used in the medical industry. Because of this, pharmacology has recently categorized microalgae as a novel source [9,10]. Fats, proteins, lipids, phenol, carbohydrates, enzymes, flavonoids, toxins, amino acids, growth regulators, and pigments are among the bioactive chemicals produced by microalgae; these active compounds are deposited in the intercellular of microalgae (biomass) [11]. The capacity of microalgae to do this can be related to their ability to produce a variety of secondary metabolites that are physiologically active against diverse pathogenic bacterial strains [12]. *C. vulgaris* is a species of green microalga, mostly used in Japan as a nutritional supplement or as a protein-rich food additive [13], it contains bioactive ingredients such as polyunsaturated fatty acids (PUFAs), polysaccharides , photosynthetic pigments , and

phenolic compounds, which considered to be an essential functional food [14,15]. The antitumor, anti-inflammatory, antioxidant, and antibacterial effects of *Chlorella* extracts have been proven [16-19]. The second microalgae in the current study are *O. limnetica* A species of freshwater cyanobacteria belonging to the genus *Oscillatoria*. It is a facultative anaerobic organism, therefore when hydrogen sulfide is available or when anaerobic conditions are present, it uses hydrogen sulfide as a source of hydrogen for photosynthesis; when aerobic conditions are present, it uses water as a substitute [20]. Its proven success in removing the organophosphorus herbicide glyphosate from the environment is crucial for bioremediation [21], and its application in Copper and Zinc Bio removal [22]. Some species of bacteria and fungi can be effectively eliminated by their intracellular and extracellular extracts [23]. In the current study, only the fatty acids of *C. vulgaris* were extracted for several reasons, it has easy and adaptable culture conditions and resistance to interfering factors. easily obtained large biomass in a short period [24]. And depending on the reviewer and previous studies, as this microalga is distinguished by its rich content of fatty acids, and fats. A study done by Teh, *et al*., observed, the fat content reached 63.6% of the dry weight, in addition to several types of fatty acids [25]. The study mentioned above supports other studies such as those done by Jahromi, *et al.*, which proved the same previous result that this microalga contains a large amount of fats and fatty acids of various kinds [26]. It is also known that the ability of fatty acids to inhibit many pathogenic microorganisms, according to the study by Toshkova-Yotova, *et al*., proved the ability of fatty acids extracted from algae to inhibit pathogenic microbes, whether bacteria or fungi, as well as the success of these compounds as antitumor substances. A lot of studies about the capacity of fatty acids and their uses as pharmaceutical products [27]. It is noteworthy that species are the most famous in the research field, whether it is pharmaceutical, food, environmental, or industrial [28]. All these reasons made choosing this species to extract fatty acids more than others. The aim of this study is to isolate two species of microalgae (*C. vulgaris* and *O. limnetica*) from the local environment. and then analysis of hexane extracts of these microalgae and *C. vulgaris* fatty acids partially purified by GC-MS. After that, tested the inhibiting of bacterial pathogenesis as an antibacterial and an antibiofilm, against *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Proteus mirabilis*, and *Klebsiella pneumoniae*, which were isolated from burn and surgical patients.

Materials and Methods

Samples collection of microalgae

Samples were taken between January to April of 2022 in various parts of Baqubah city. One hundred ml of sample was collected in a sterile container that was labelled with the time and place of the sample collection. Then they were transferred right away to the lab, where they were incubated under the proper conditions (16 h of light and 8 h of darkness) at 25±2°C and 268 E/m2/s light intensity [29]. According to Desikachary and Prescott the samples were identified as typical algal classification systems [30,31].

Preparation and sterilization of media and harvested biomass

In this study, a modified Chu-10 from adopted culture media was employed. For major nutrients (macronutrients) and minor nutrients (micronutrients), stock solutions of each salt were made. In order to raise the pH of the medium Chu-10 to 6.4, a few drops of sodium hydroxide and hydrochloric acid (0.01N) were added to Adjust the pH of the media. The media were then sterilized in an autoclave at 121°C, 1.5 Bar for 15min according to Al-Abboodi [24]. And harvested biomass culture by centrifuging it for 10 minutes at 4000 rpm after a month [32] then dried in the oven at 38-40°C, later on, these samples were weighed and stored in the refrigerator [33].

Preparation of organic algal extract

According to the method of Elnabris, the method used to prepare crude algal extracts has been modified as follows: A Soxhlet extraction device was used to extract five grams of powdered microalgae with 250 ml of absolute hexane (95%) at 60 °C for three to four hours until the solvent became colorless [34]. Hexane was used as a solvent because it is useful for extracting fatty acids and lipids, which are the target component for extraction and are abundant in these microalgae.

The partially purified of fatty acid from chlorella vulgaris

The fatty acids were partially purified using the method demonstrated by Najafabadi, *et al.*, [35].

Gas chromatography-mass spectrometry (GC-Ms) analysis

These experiments were carried out at the Basra Oil Company to identify the active compounds in microalgae extracts using GC-Ms (Agilent). That were identified through matching mass spectra to library spectra [36].

Table 1. GC/MS conditions. [37]

Instrument	Agilent 5977A GC Mass Spectrometer
Analytical Column	Agilent HP-5MSUI 30 m × 0.25 mm, 0.25 µm column
Injection volume	1µl
Pressure	11.933 psi
Temperature	Transfer line temperature 100–350 °C Ion source temperature 150–350 °C Quadrupole temperature 106 -200 °C
Carrier Gas	He 99.99%
Injector Temperature	250 ˚C Scan Range: m/z 25-1000
Injection Type	Split less Injection

Pathogenic bacteria isolation

Pseudomonas aeruginosa, Acinetobacter baumannii, Proteus mirabilis, and Klebsiella pneumoniae (Gram negative) were from 100 patients in Baqubah Teaching Hospital from the burn and surgical. Specimens were cultured immediately on blood agar and MacConkey agar, then plates incubated aerobically for 24 hr. at 37°C [38].

Identification of bacterial isolates

The identification was done by examining the characteristics of cultural morphology by cultured them on the media and some biochemical test (Oxidase reagent, Catalase reagent, Blood agar, Eosin Methylene Blue (EMB) agar, MacCkonky agar, Cetrimide Agar, Urea Agar Base) [39].

Antibiotics susceptibility test (Disc diffusion)

The sensitivity test procedure was done according to (CLSI, 2022).

Antibacterial activity by Agar well diffusion method

A suspension was prepared by picking five to six colonies from the nutrient agar culture plate of ~1 mm diameter from a 24-hour, then inoculated in 5 mL of sterile saline, and its turbidity was adjusted to 0.5 McFarland standards visually. A sterile cotton wool swab was moistened in the adjusted inoculum suspension, and then, excess fluid was rinsed by rolling the swab on the inside surface of the tube above the fluid surface. Muller-Hinton agar (MHA) surface was streaked to make a lawn of the isolate. Holes were made with a diameter of 5 mm in the culture media by using sterilized cork borer. By micropipette adding one hundred µl from algal extract to each hole, with three replicates. After then, incubate the dishes at 37 °C for 24 h. The effectiveness of each replicate was determined by measuring the diameter of the inhibition zone around each hole [38].

Biofilm formation

The following was done with the micro-titer plate method: The study's bacterial isolates were cultivated for 24 hours at 37°C on nutrient agar medium. A Brain- heart infusion broth medium was used to transfer about 2-4 colonies, and the turbidity of the medium was compared to the turbidity of the MacFarland solution. Transfer two hundred µl of the bacterial suspension from each isolate, in three duplicates, on a flat polystyrene plate that has been drilled with 96 holes using a micropipette. Several pits received additions of brain-heart infusion broth without being infected as a negative control. The plate was then covered and incubated in the incubator for 24 hours at 37°C. After incubation, the plate cover was removed, the pits containing the liquid cultures were gently emptied 2-4 times with distilled water for washing, added two hundred µl of methanol for 10 minutes, and adding two hundred µl of crystal violet for 15 minutes. After the dyeing was finished, 2-3 washes with distilled water were used to remove any remaining dye. and 200 µl of 100% ethanol are added. Finally, by using manual ELISA Microplate Reader (BioTek), the absorbance was measured at a wavelength of 630 nm [39].

Antibiofilm activity of algal extract

The same method mentioned above (Biofilm formation) was used except for the third step, equal amounts of suspension and algal extract are added in a ratio of 1:1. The inhibition of biofilm percentages of crud extracts for each pathogenic bacterium was calculated as described (mathematical equation by Namasivayam, and Roy [40].

$$\textit{Inhibition of biofilm formation}\ \% = \frac{OD\ control - OD\ ot\ tretment}{OD\ control} \times 100$$

Results and Discussion

Identification of microalgae

One Chlorophyta (*C. vulgaris*) and one Cyanophyta (*O. limnetica*) species were identified. As Figure 1 (A- *C. vulgaris* while B – *O. limnetica*).

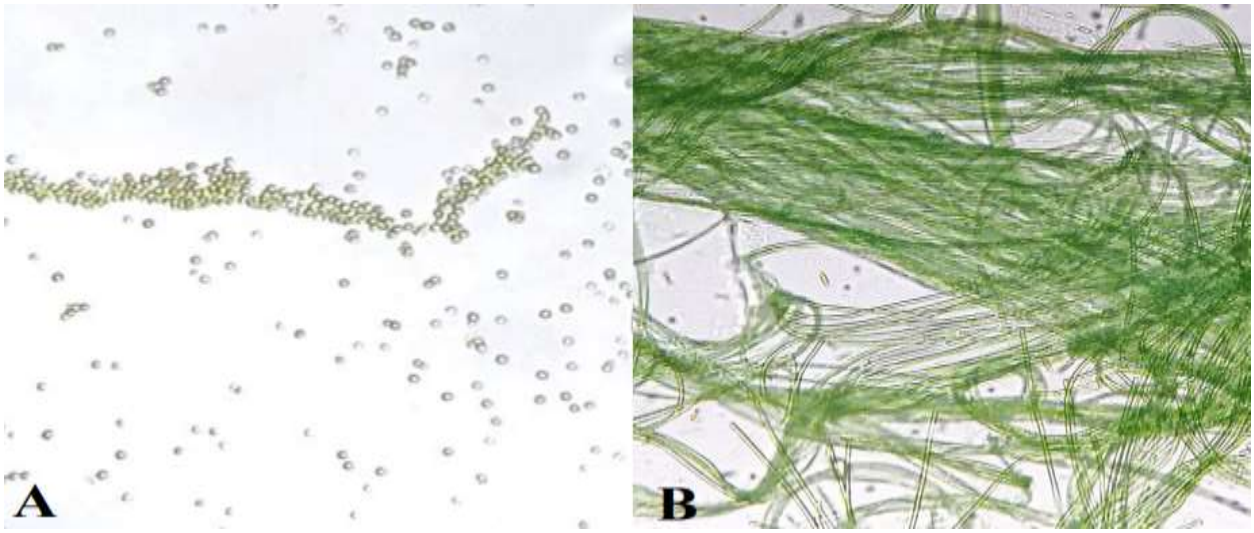

Figure 1. (A- *C. vulgaris*, B – *O. limnetica*).

Among the various species of microalgae that were discovered. Where were able to obtain pure isolates and it was possible to proliferate them into biomass. Two species of microalgae one Chlorophyta species (*C. vulgaris*) and one Cyanophyta species (*O. limnetica*) were isolated for this study, after preparing media and ideal growth conditions. The wide diversity of microalgae in freshwater is due to several reasons, including moderate temperatures during the collection of samples, which range between 25-30 °C, it is the ideal temperature for growth, in addition to environmental factors that influence the growth of algae, such as light intensity, pH levels, and others [24,41]. Also, the most important reason is the large increase in pollutants in fresh water, the most important of which are nitrates and phosphates, which are the main catalysts for the growth and reproduction of all algae in general. This huge number of different species of algae is an effective bioindicator that indicates a high increase in pollutants, as algae is an environmental indicator of more value than other physical and chemical indicators [42,43].

The GC-Ms analysis

After analyzing the Hexane extracts of *C. vulgaris*, *O. limnetica* and *C. vulgaris* fatty acids by GC/MS, it was found that they contain many active compounds, which are summarized in Figures (2-4) and Tables (2,3) for *C. vulgaris* and *O.limnetica* respectively.

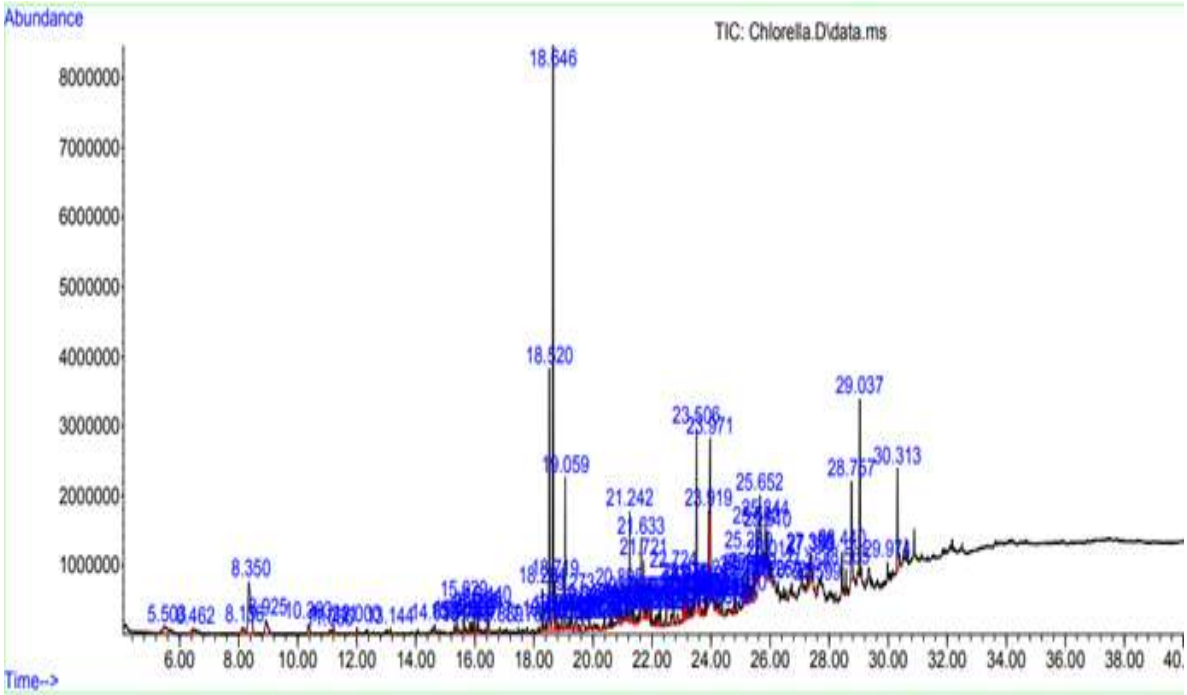

Figure 2. The GC-Ms Analysis of *C. vulgaris*

Table 2.GC-MS analysis of C. *vulgaris*

No.	Chemical class	Compounds	Molecular formula	Area Pct	Biological activity	Ref
1	Phenol	Butylated Hydroxytoluene	$C_{15}H_{24}O$	2.8961	Antioxidant	(44)
2	Alkanes	Eicosane	$C_{20}H_{42}$	3.9368	Antifungal, Antitumor, Antibacterial, Larvicidal, Cytotoxic, Antimicrobia	(45)
3	Fatty Acid	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	4.5793	antimicrobial activity	(46)
4	Long-Chain Fatty Acids	9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$	4.4432	anti-inflammatory activity	(47)
5	Long-Chain Fatty Acids	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	2.6827	Antibiofilm	(48)
6	Phenolic Compounds	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	3.6204	antibacterial activity	(49)
7	Fatty Acid	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{25}H_{46}O_6$	2.5608	Anticancer, antimicrobial	(50)

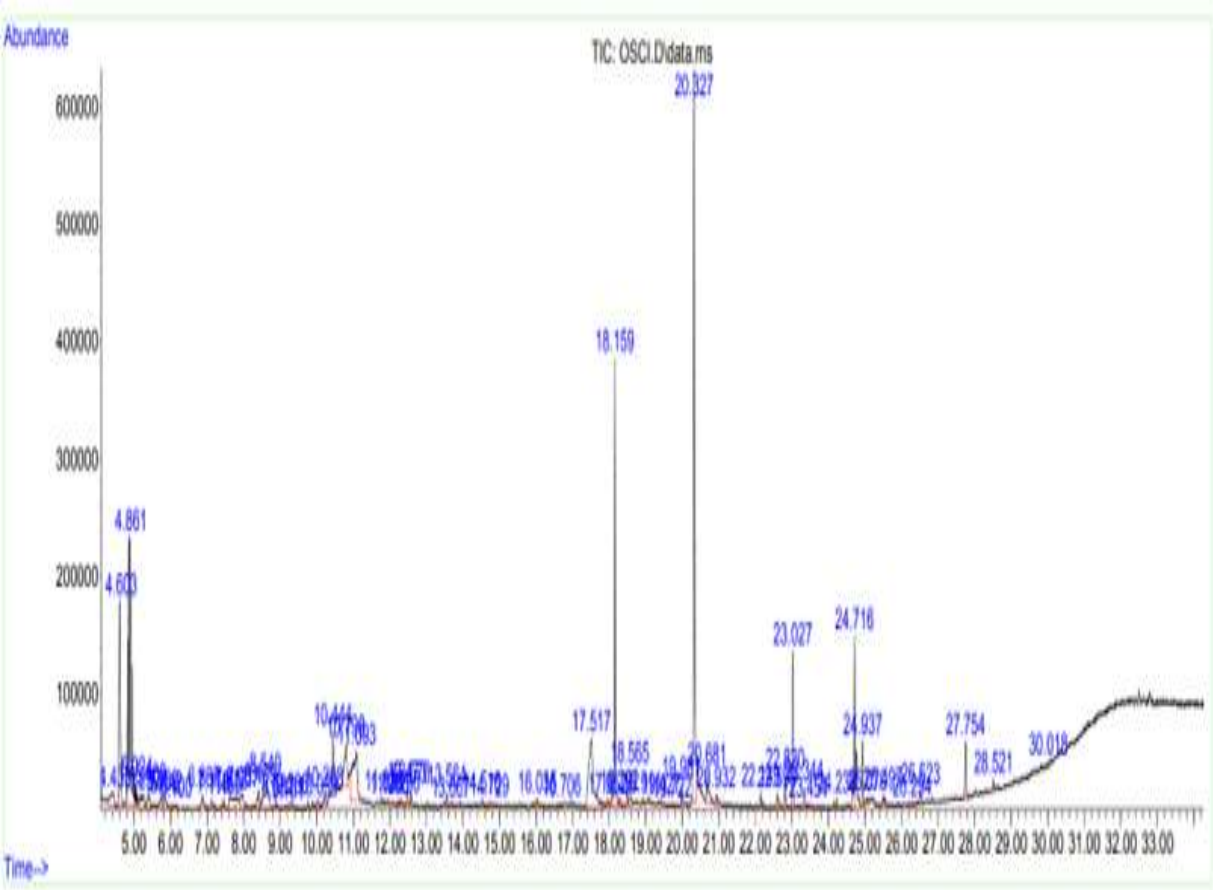

Figure 3. The GC-Ms Analysis of *O. limnetica.*

Table 3. GC-MS analysis of *O. limnetica.*

No.	Chemical class	Compounds	Molecular formula	Area Pct	Biological activity	Ref
1	Nitrogen Compounds -> Hydroxyl amines	Diethyl hydroxylamine	$(C_2H_5)_2NOH$	1.75649	antioxidant, antitumor, antimicrobial, anti-inflammatory, antiallergic, antialtering and antiatherosclerosis	(51)
2	Aromatic Solvents	Ethylbenzene	C_8H_{10}	31.2658	cytogenetic damage	(52)
3	Aldehydes	Benzaldehyde	C_7H_6O C_6H_5CHO	17.955	antibacterial activities.	(53)
4	Fatty Acid Methyl Esters	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	13.8026	antibacterial activity against Gram-positive and Gram-negative bacteria.	(54)
5	Fatty Acid Methyl Esters	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	9.633	antibacterial	(55)
6	Fatty Acid Methyl Esters	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	6.097	Antimicrobial and cytotoxic	(56)
7	diterpenoid and a long-chain primary fatty alcohol.	Phytol	$C_{20}H_{40}O$	1.654	cytotoxic, antioxidant, autophagy- and apoptosis-inducing, antinociceptive, anti-inflammatory, immune-modulating, and antimicrobial effects.	(57)

The GC-Ms Analysis of Partial purification of *C. vulgaris* fatty acid appeared three types of fatty acids: Dodecanoic acid, 9-Eicosene, (E)-, and n-Hexadecanoic acid. These compounds are characterized by their high ability to inhibit bacteria, whether they are Gram-positive or Gram-negative. A study conducted by Duraisamy, and Selvaraju, suggested that assume n-hexadecanoic acid has antimicrobial activities, by increasing oxidative stress inside the cell by inducing ROS [58]. While Andrew and Valerie believed that the primary target of fatty acid action as an antibacterial is the cell membrane, where FFAs disrupt the electron transport chain and oxidative phosphorylation. FFA effect can also occur via the inhibition of enzyme activity, reduction of nutrient uptake, production peroxidation, and auto-oxidation degrade products, or direct lysis of bacterial cells, in addition to interfering with cellular energy production [59]. Shown in Figure 4.

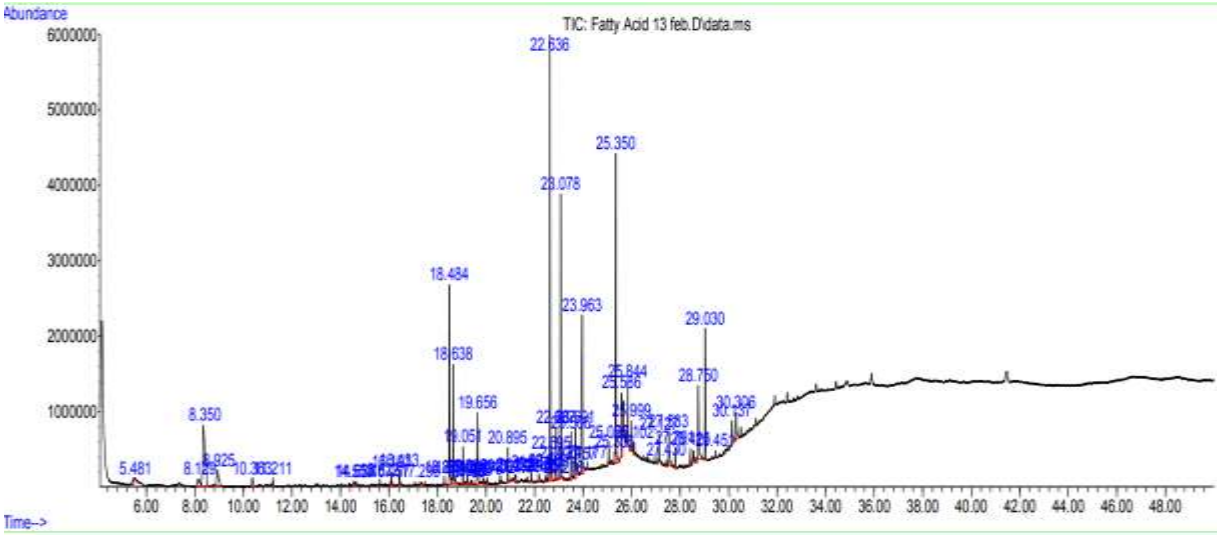

Figure 4. The GC-Ms analysis of Partial purification of *C. vulgaris* fatty acid

Isolation and identification of bacteria

One hundred specimens of different age groups for both genders from clinical sources (burns and wounds) were collected, between the beginning of April 2022, to the middle of July 2022 from patients of Baqubah Education Hospital in Diyala. 22 isolates of *Pseudomonas aeruginosa*, 12 isolates of *Acinetobacter baumannii*, 12 isolates of *Proteus mirabilis*, and 10 isolates of *Klebsiella pneumoniae* have been identified; the other samples were no growth.

***Pseudomonas aeruginosa* diagnosis**

The features of their cultural morphology have been examined by growing them on a MacConkey agar medium in order to the identification. The colonies were pale due to their inability to ferment lactose [60], but when cultivated on cetrimide agar medium, the colonies had an appearance that was either greenish-yellow or greenish-blue, due to producing their pigments Pyoverdine and Pyocyanin [61]. Also, the colonies indicated their capacity for blood hemolysis on Blood Agar. Other characteristics are gram staining (negative), shape (rods), flagella (single flagella), catalase (+ve), and oxidase (+ve) [38,62].

***Acinetobacter baumannii* diagnosis**

The isolates of *Acinetobacter baumannii*, when cultured on MacConkey agar medium, showed small colonies, circular, regular, smooth, and pale, and some of them appeared sticky and were not fermented for lactose sugar, while they appeared on blood agar medium in the form of gray colonies that did not hemolysis blood because they did not produce the enzyme hemolysin. And the results of the microscopic examination showed that bacterial cells are small, spherical rod-shaped, regular, in pairs or may be single, gram-negative [39,63].

***Proteus mirabilis* diagnosis**

The isolates of *Proteus mirabilis* were identified as convex, tiny, pale, circular, smooth-edged colonies that could not ferment lactose and had a unique fishy odor by cultivating the bacteria on a MacConkey agar medium and evaluating their morphological features. It has the usual swarming and slimy spreading layer on Blood Agar [38]. The isolates were Gram-negative, single or short-chain, coco-bacilli, and red in color, according to Garrity. They also had catalase and urease responses that were positive, but not oxidase reactions [64]

***Klebsiella pneumoniae* diagnosis**

The bacteria were cultured on MacConkey agar medium, and the colonies appeared mucoid, round, large, pink in color, and lactose-fermenting, as opposed to blood agar, which displayed non-hemolytic grey-white, mucoid colonies [65].

Antibiotic susceptibility of isolated bacteria (Disc diffusion)

Antibiotics susceptibility test was conducted for four types of pathogenic bacteria (*Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Proteus mirabilis*, *Klebsiella pneumoniae*). The sensitivity was done against eleven types of different groups of antibiotics for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* while fourteen for *Proteus mirabilis* and *Klebsiella pneumoniae* to detecting the sensitivity of isolates or their resistance to the antibiotics circulating in health institutions, these antibiotics were chosen for their frequent use in treating bacterial infections. In recent decades, the effectiveness of various commonly used antibiotics in preventing the spread of or treating a variety of human and animal infections has reduced, and recently the problem of antibiotic resistance has become an international problem, threatening global health and increasing the spread of bacterial infections and dangerous epidemics [17,66]. As figure 5 Antibiotic susceptibility of isolated bacteria (Disc diffusion)

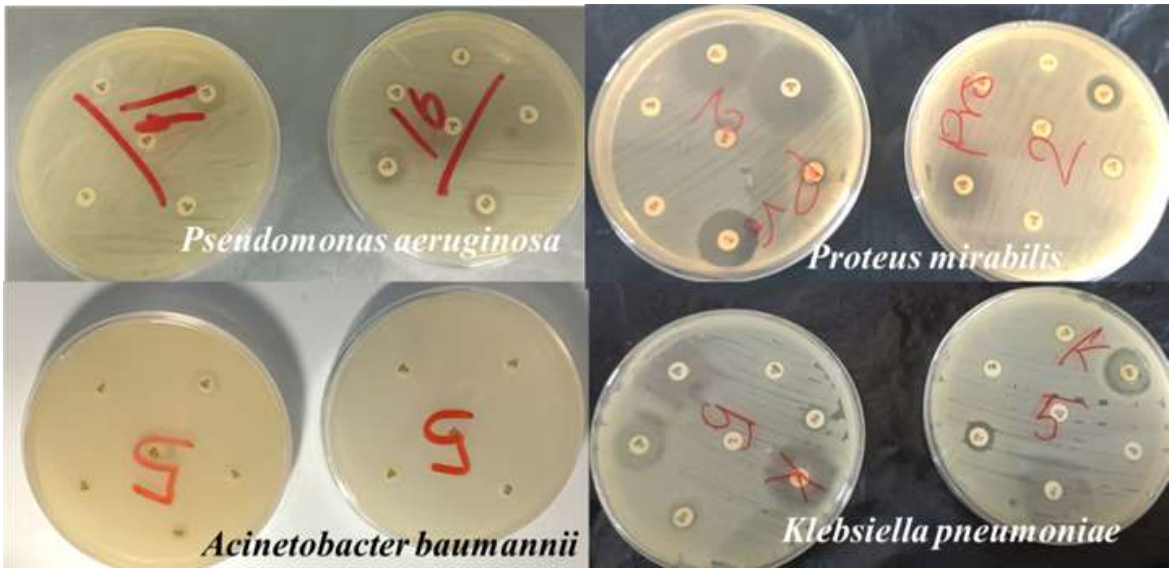

Figure 5. Antibiotic susceptibility of isolated bacteria

The *P.aeruginosa* gave antibiotic resistance as follows : Piperacillin 72.8% , Piperacillin – tazobactam 31.8% , Ceftazidime 91%, Cefepime 81.8% , Imipenem 50%, Meropenem 50%, Gentamicin 59.09%, Tobramycin 54.54%, Amikacin 54.54%, Ciprofloxacin4.5%, Levofloxacin 9%. as shown in (Figure 6)

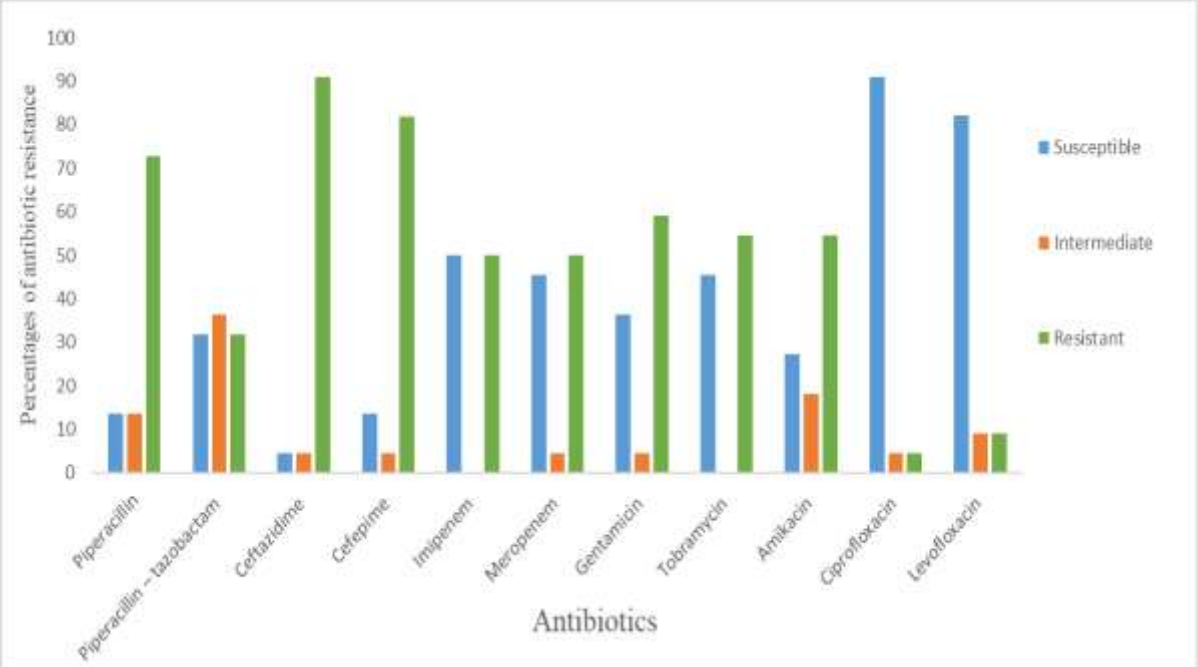

Figure 6. The percentages of antibiotic resistance of *Pseudomonas aeruginosa*

According to previous studies, *P. aeruginosa* resistance to antibiotics is caused by a number of processes, such as its capacity to alter membrane permeability, and it produces wide-narrow beta-lactamase enzymes, biofilm development, and Efflux pumps. In addition, it has its own R-resistance plasmids that carry several antibiotic-resistance genes [67,68].

While the *Acinetobacter baumannii* gave antibiotic resistance as follows: 100 % for Piperacillin, Piperacillin – tazobactam, Ceftazidime, Cefepime, Imipenem, Trimethoprim – sulphathiazole, Gentamicin, Amikacin, Ciprofloxacin, Levofloxacin and 50 % for Tetracycline. As shown in (Figure 7)

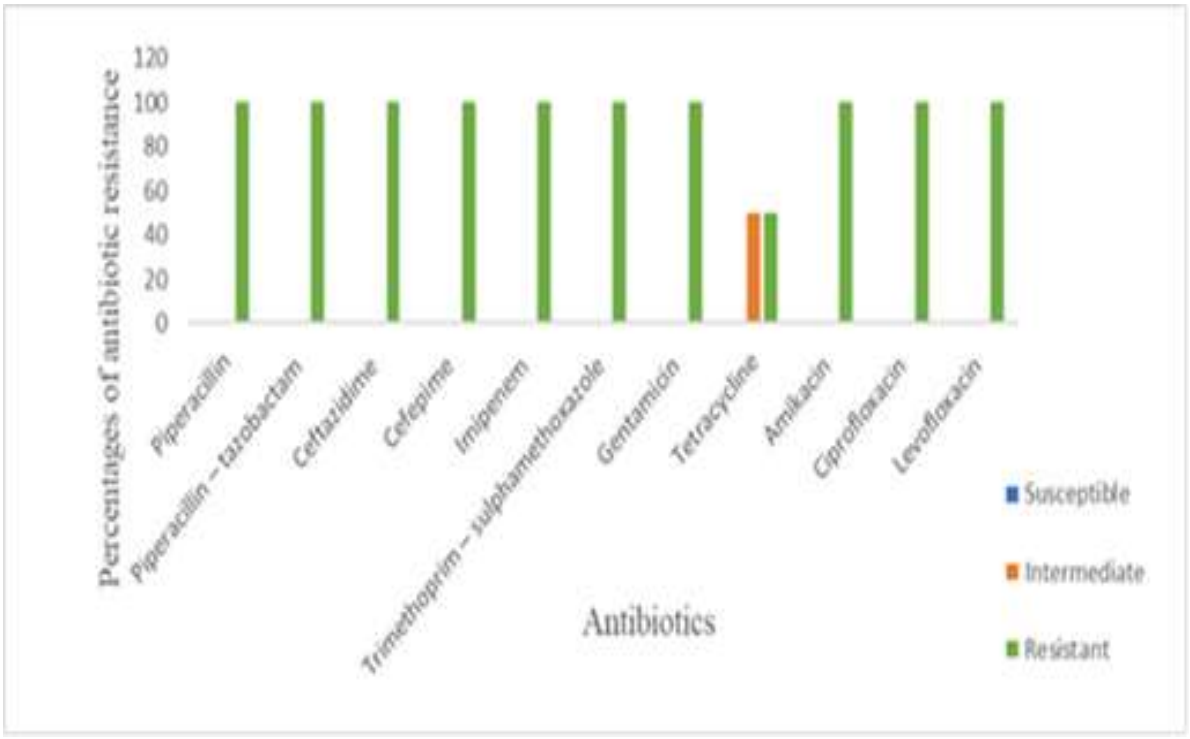

Figure 7. The percentages of antibiotic resistance of *Acinetobacter baumannii*

The production of broad-spectrum beta-lactamases and aminoglycoside enzymes, such as phosphotransferases, acyltransferases, and nucleotidyltransferase, by *Acinetobacter baumannii* contributes to its resistance. By reducing the channels in the outer membrane, one may additionally decrease the concentration of antibiotics inside the cell. [69,70].

Also, *Proteus mirabilis* gave antibiotic resistance as follows: Trimethoprim – sulphamethoxazole 50%, 100% for Nitrofurantoin, Clarithromycin, Vancomycin, Tobramycin, Tetracycline, Penicillin 92%, 75% for Tobramycin and Ampicillin, Trimethoprim 67%, Gentamicin 66%, Chloramphenicol 33%, Azithromycin 25%, 17% for Imipenem and Meropenem. As shown in (Figure 8).

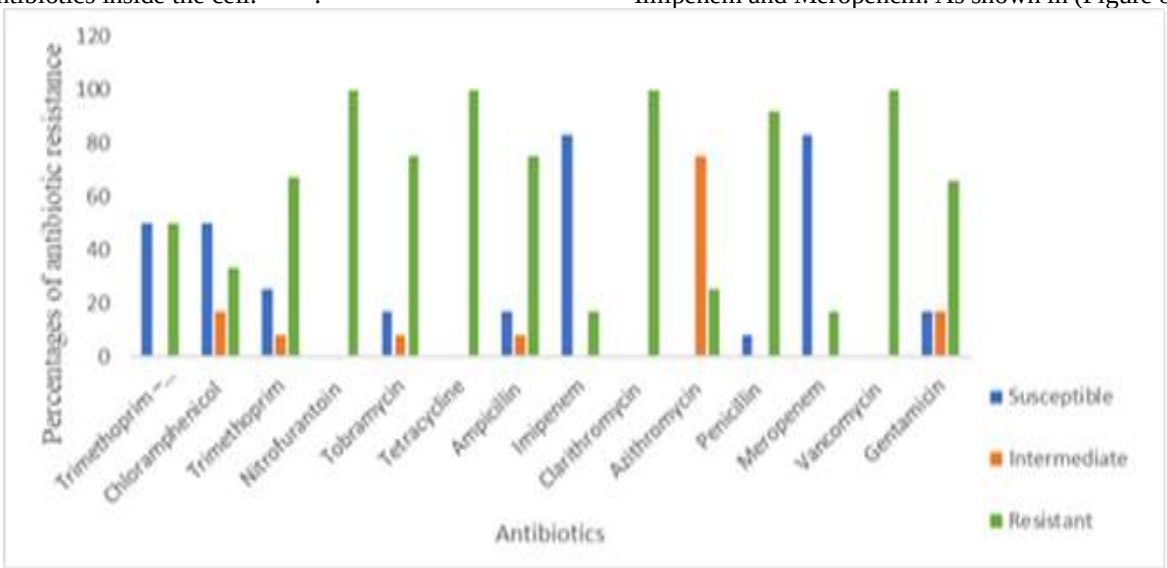

Figure 8.The percentages of antibiotic resistance of *Proteus mirabilis*

Furthermore, *Klebsiella pneumoniae* gave antibiotic resistance as follows: 100% for Trimethoprim – sulphathiazole, Nitrofurantoin, Ampicillin, Imipenem, Penicillin, Vancomycin, 90% for Tobramycin and Meropenem, 80% for Tetracycline and Clarithromycin, Gentamicin 70% Trimethoprim 60%, Chloramphenicol 30%, Azithromycin 50% (Figure 9).

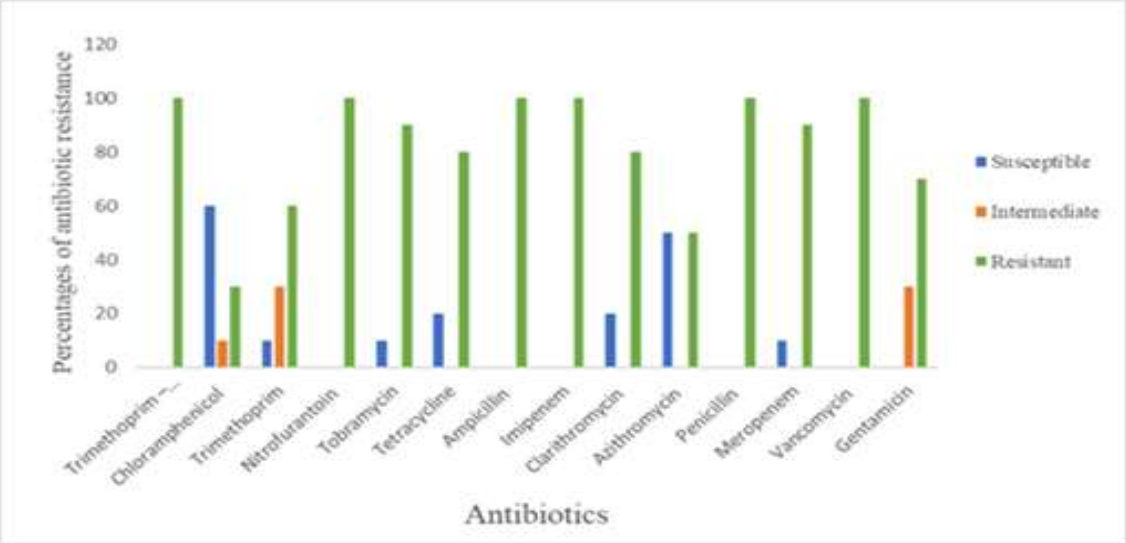

Figure 9. The percentages of antibiotic resistance of *Klebsiella pneumoniae*

Proteus mirabilis and *Klebsiella pneumoniae* are members of the Enterobacteriaceae family and are resistant due to their capacity to produce beta-lactamase enzymes and their resistance through a number of mechanisms, such as reducing the permeability of the antibiotics into the cell, as well as analyzing the antibiotics by beta-lactamase enzyme, and the other by reducing affinity to the enzyme Binding Proteins for Penicillin [71,72]. In this study, confirmed that the excessive use of antibiotics is one of the most important causes of common resistance in health care centers and hospitals.

Antibacterial activity of C. vulgaris and O. limnetica extracts

The Figures 10,11,12,13,14 below describes the effect of hexane extract of *C. vulgaris* and *O. limnetica* on the isolate's bacteria, and Figure 15, Table 4 explained the effect of *C. vulgaris* fatty acids on (*Pseudomonas aeruginosa, Acinetobacter baumannii, Proteus mirabilis* and, *Klebsiella pneumoniae*) with two concentrations.

Figure 10. Inhibition zone of *C. vulgaris* and *O. limnetica* extracts on bacteria isolates

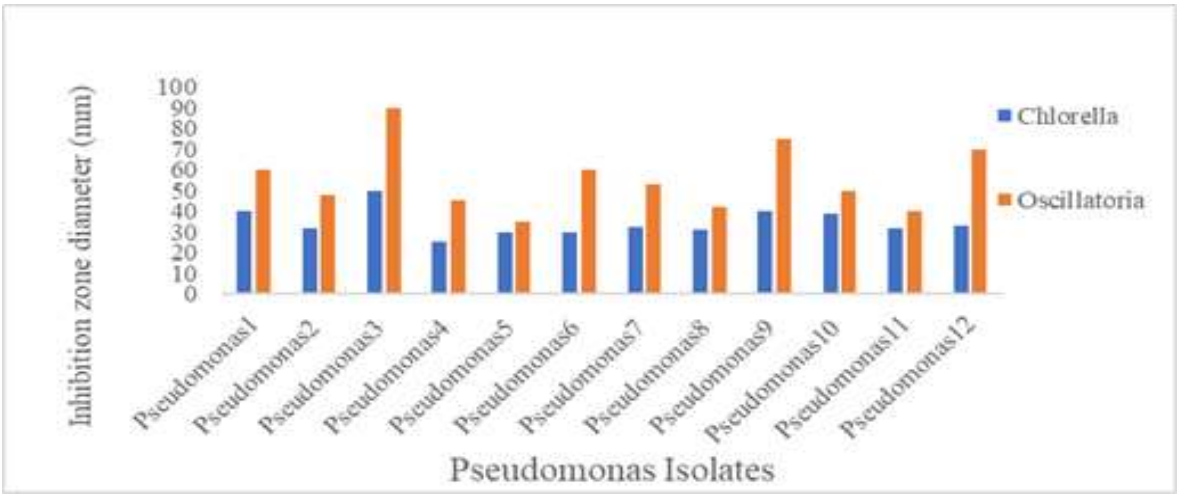

Figure 11. The effect of hexane extract of *C. vulgaris* and *O. limnetica* on the isolates *Pseudomonas aeruginosa*

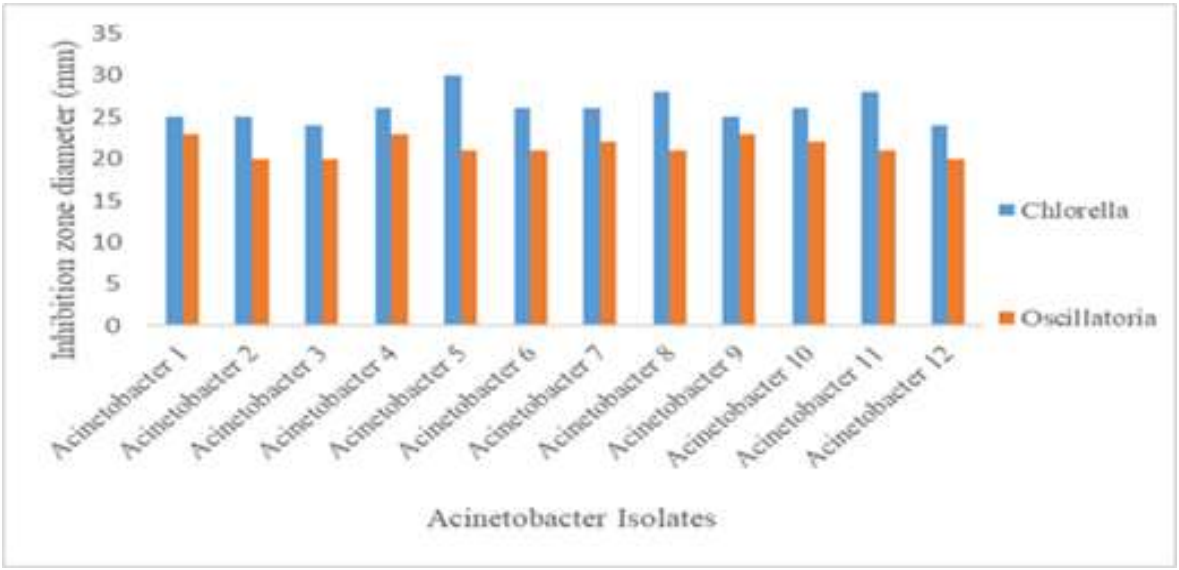

Figure 12. The effect of hexane extract of *C. vulgaris* and *O. limnetica* on the isolates *Acinetobacter baumannii*

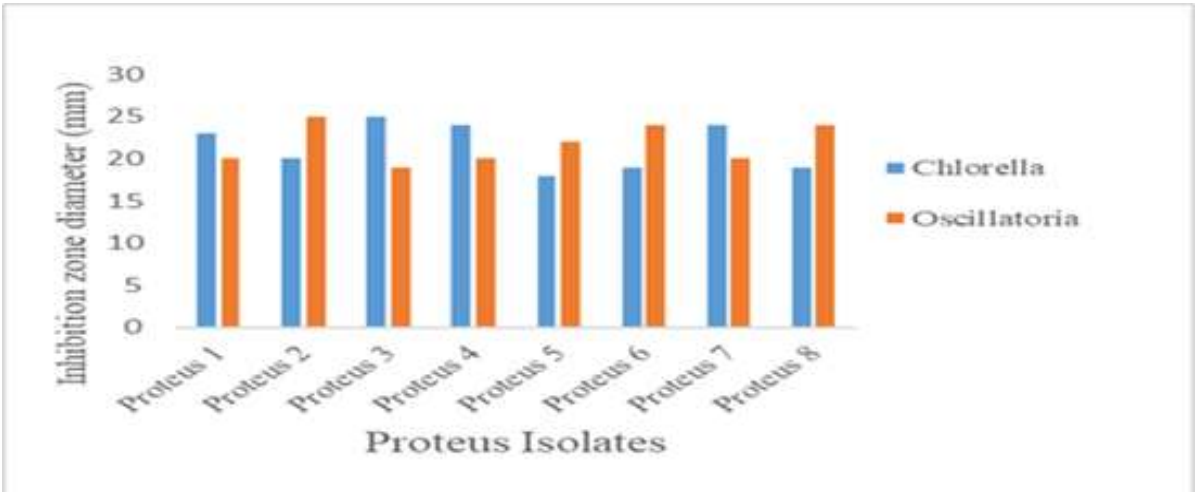

Figure 13.The effect of hexane extract of *C. vulgaris* and *O. limnetica* on the isolates *Proteus mirabilis*

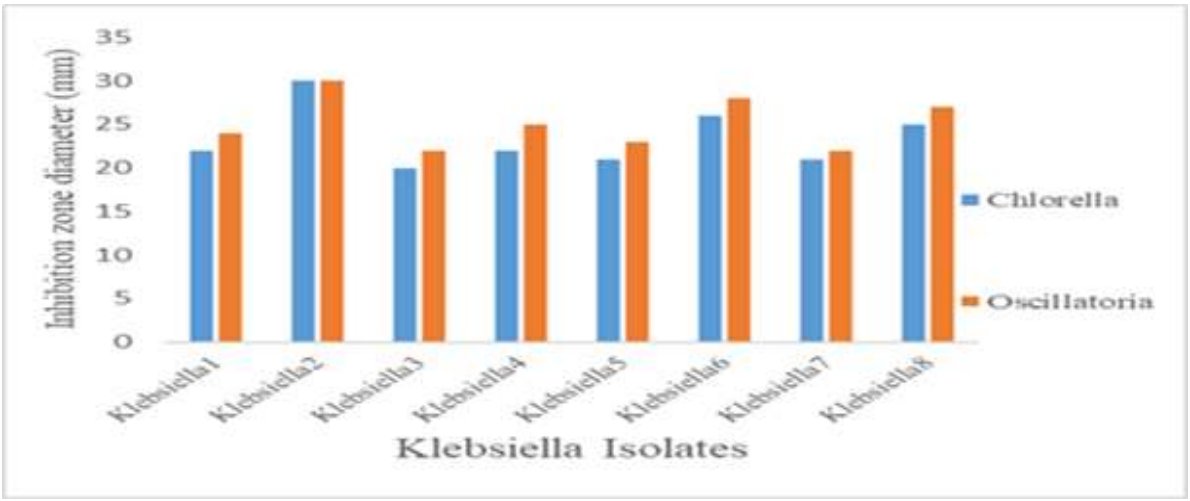

Figure 14. The effect of hexane extract of *C. vulgaris* and *O. limnetica* on the isolates *Klebsiella pneumoniae*

Table 4.The effect of *C. vulgaris* fatty acid on bacterial isolates

Inhibition zones of *C. vulgaris* Fatty Acids (mm)		
Bacterial species	**Concentration 100mg/ml**	**Concentration 10mg/ml**
Acinetobacter baumannii	45	35
Pseudomonas aeruginosa	60	50
Proteus mirabilis	23	20
Klebsiella pneumoniae	30	38

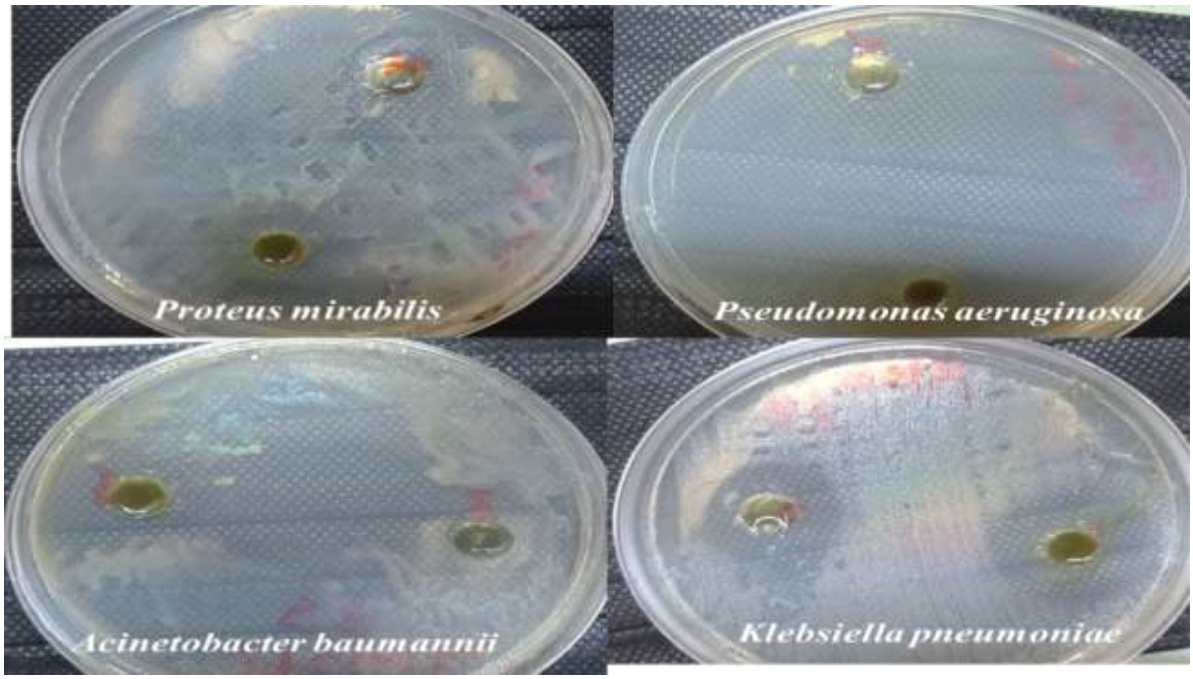

Figure 15. The effect of *C. vulgaris* fatty acid on bacterial isolates

The results demonstrated that the utilized microorganisms clearly responded to the microalgal extract, most of the chemicals that were identified from microalgae have antibacterial properties [73]. The present study, all isolates of bacteria that resisted to antibiotics were inhibited by the crude extract of two microalgae.

The response of Gram-negative bacteria due to the layers surrounded by the cell wall may be few, allowing the extract to permeate, as well. The algae extract has an inhibitory effect against the negative bacteria (Gram stain) by inhibiting the Enzyme of phosphatase protein, which plays an important role in the process of inserting substances into the body of the organism [74]. The inhibition due to the richness of both microalgae (*O. limnetica* and *C. vulgaris*) in secondary metabolite compounds [75]. It has been demonstrated that *C. vulgaris* has a significant amount of fatty acids with considerable antimicrobial activity [76]. As a result of their effects

on cell membranes, fatty acids' mode of action primarily interferes with cellular respiration by reducing food absorption and increasing the path for the entry of hazardous substances [10].

Antibiofilm activity of C. vulgaris and O. limnetica extracts

All bacterial isolates were examined for biofilm formation, and they were found that 91% of the isolates *Pseudomonas*, 42% of *Acinetobacter*, 33.34% of *Proteus* and 40% of *Klebsiella* are biofilm-forming. After that, the sensitivity of the extract as an anti-biofilm was examined for biofilm-forming isolates, and the inhibition percentage was calculated according to the aforementioned mathematical equation. The average inhibition percentage reached 83.4% for *chlorella* extract and 83% for *Oscillatoria* extract with *Pseudomonas* Isolates (Figure 16), 86.2% for *chlorella* extract and 85.5% for *Oscillatoria* extract with *Acinetobacter* (Figure17). While 49.7% for *chlorella* extract and 46.7% for *Oscillatoria* extract with *Proteus* (Figure 18) and 43.7% for *chlorella* extract and 34.35% for *Oscillatori*a extract with *Klebsiella* (Figure 19). While (Figure20) showed the effect of *C. vulgaris* fatty acids on biofilm formation with two concentrations.

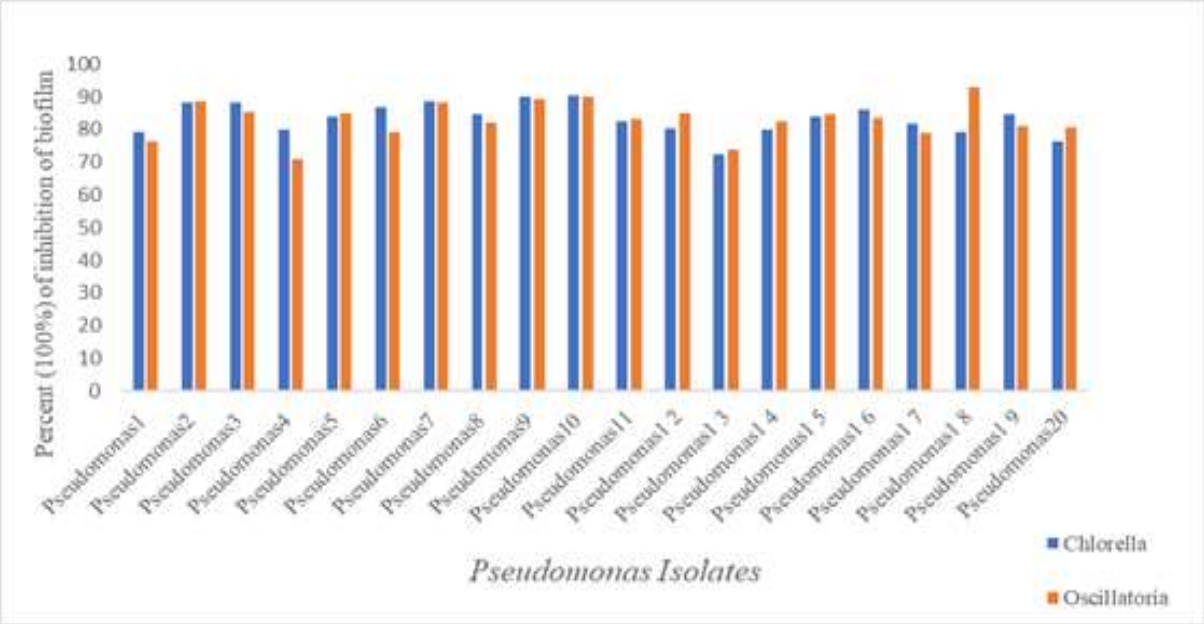

Figure 16. The Percent inhibition of biofilm with crude extract of *C. vulgaris* and *O. limnetica* on *Pseudomonas* Isolates.

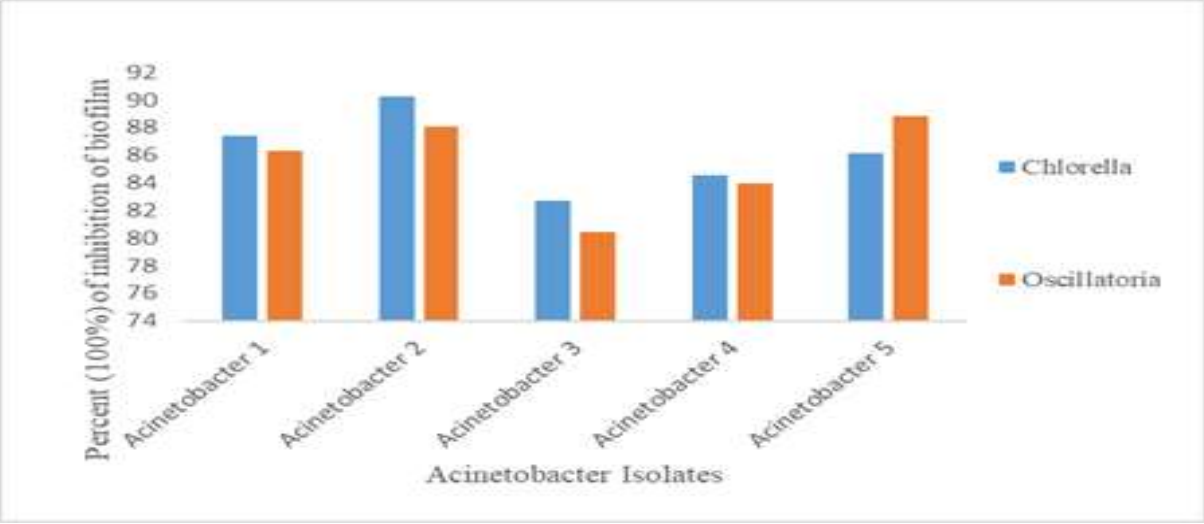

Figure 17. The Percent inhibition of biofilm with crude extract of *C. vulgaris* and *O. limnetica* on *Acinetobacter* Isolates

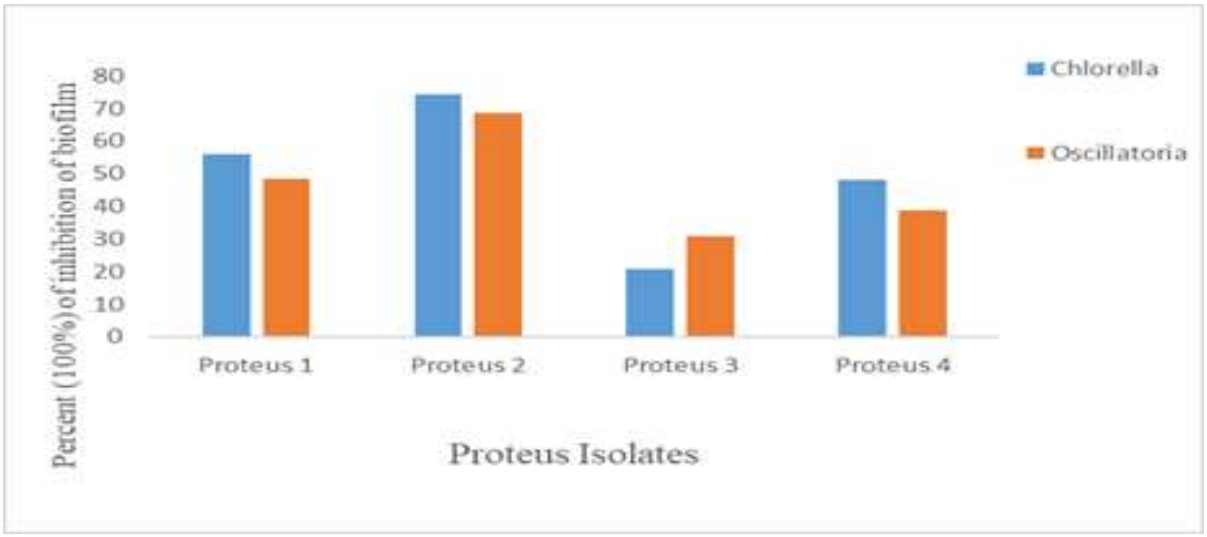

Figure 18. The Percent inhibition of biofilm with crude extract of *C. vulgaris* and *O. limnetica* on *Proteus* Isolates

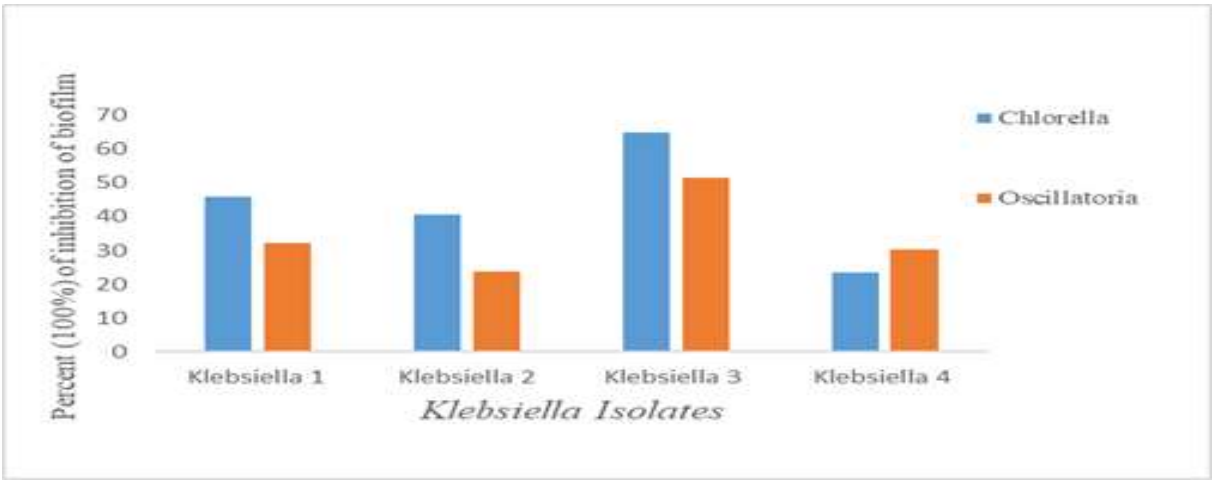

Figure 19. The Percent inhibition of biofilm with crude extract of *C. vulgaris* and *O. limnetica* on *Klebsiella* Isolates

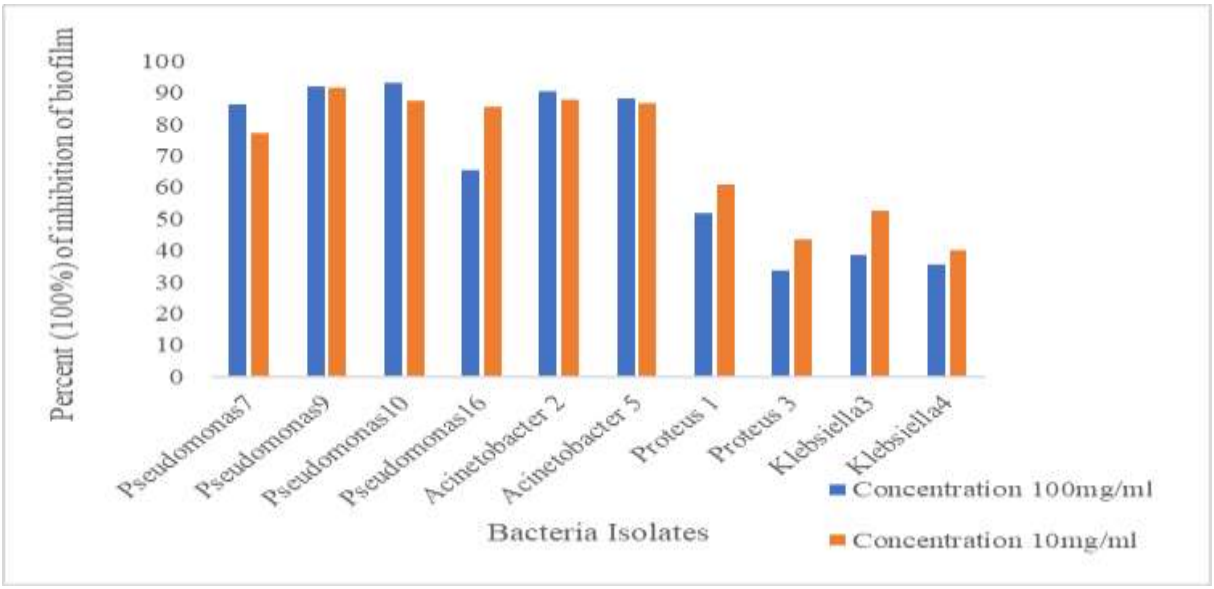

Figure20. The effect of *C. vulgaris* fatty acids on biofilm formation with two concentrations

Biofilms are defined as microbial communities of surface-attached cells embedded in a self-produced extracellular matrix and are crucial in infectious illnesses [77,78]. Microorganisms' production of extracellular polymeric substances (EPS) has a crucial role in the development of biofilm stiffness and powerful adhesion [79]. So, the most current research indicates that microalgae extract significantly reduce the pathogenic cells' resistance by altering the thickness and size of the biofilm [80]. Numerous fatty acids derived from *chlorella* that have antibacterial properties have been found. The free fatty acids made by microalgae appear to have the ability to eliminate or limit the growth of some gram-positive and gram-negative bacteria. Microalgae and bio compounds have demonstrated anti-biofilm capabilities in addition to antibacterial properties, which are crucial for the management of infectious disorders [81,82].

Conclusion

Microalgae extracts and their derivatives have proven to contribute as projects for alternative therapies, for suggested a source of pharmaceutical compounds in the pharmaceutical industries, with the global increase in antibiotic resistance. Where, the results of this study that the hexane extract of two species of microalgae, *Chlorella vulgaris, Oscillatoria limnetica*, and *Chlorella vulgaris* fatty acids, has a powerful effect as antibiofilm and antibacterial on isolates that were clinically isolated from burn and surgical patients. This strong effectiveness of the extracts is due to the rich content of active compounds such as phenols, terpenoids, and alkaloids, and the relatively high content of fatty acids and lipids, to which the antimicrobial activity is attributed. These bioactive compounds were detected by GC-MS technique.

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Conflict of Interest

The authors have no conflict of interest.

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Ethics Statements

We confirm as authors that the submitted manuscript is by the ethical considerations (Helsinki Ethical Principles) and that we have received ethical approval from the related institution (Diyala Health Directorate /Baqubah Teaching Hospital) with code number 10380 on 16/3/2022. All participants and healthcare professionals were also made aware of the study's objectives. patients were advised of the advantages and that there were no health concerns associated with the study. The participants' voluntary participation in the study gave them the right to withdraw at any moment, without having to give a reason or have any negative effects on their treatment or health. Through the coding of the data, precautions were taken to preserve anonymity. Participants were also informed that the data collected would only be used for the study, and oral consent was acquired from participants.

Author Contribution

The authors confirm their contribution to the paper as follows: study conception and design: ZN, AG. Authors; data collection: ZN. Author; analysis and interpretation of results: ZN. Author, AG. Author. NH. Author; draft manuscript preparation: All authors reviewed the results and approved the final version of the manuscript.

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تقييم تأثير مستخلصات الأحماض الدهنية *Chlorella vulgaris* و*Oscillatoria limnetica* على أربعة أنواع من البكتيريا المرضية السالبة لصبغة غرام

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الخلاصة

أصبحت مقاومة المضادات الحيوية مصدر قلق للمجتمع العلمي في جميع أنحاء العالم. نتيجة لذلك، أصبح استخدام المواد الكيميائية الطبيعية أمرًا ضروريًا وليس خيارًا في محاولة لتقليل الضرر الناجم عن الأمراض البكتيرية، والتي يمكن أن تكون قاتلة بسبب صعوبات علاجها. في هذه الدراسة، تم عزل نوعين متميزين من الطحالب الدقيقة (Chlorella vulgaris و Oscillatoria limnetica) من البيئة المائية المحيطة. تمت دراسة الخواص المضادة للبكتيريا والغشاء الحيوي لمستخلص الهكسان لهذه الطحالب الدقيقة، والأحماض الدهنية *Chlorella vulgaris* في المختبر ضد (*Pseudomonas aeruginosa*، *Acinetobacter baumannii*، *Proteus mirabilis* و *Klebsiella pneumoniae*)، والتي تم عزلها سريريًا من ١٠٠ مريض من مرضى الحروق والجراحة وتشخيصها ببعض الفحوصات البيوكيميائية، واختبار مقاومتها للمضادات الحيوية. أظهرت النتائج أن مستخلص الهكسان الخام من *Chlorella vulgaris* و*Oscillatoria limnetica* له تأثير كبير كمضاد للبكتيريا ومضاد حيوي. كما أظهر مستخلص الأحماض الدهنية *Chlorella vulgaris* تفوقًا كمضاد حيوي وكمضاد للبكتيريا عند استخدامه بتركيزين (١٠ و١٠٠ مجم / مل). تم التعرف على المكونات الكيميائية الهامة لجميع مستخلصات الطحالب الدقيقة بواسطة كروماتوغرافيا الغاز - مطياف الكتلة.

الكلمات المفتاحية: مضادات البكتيريا، مضادات الغشاء الحيوي، مقاومة المضادات الحيوية، الطحالب الدقيقة.