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# Effect of antibacterial substance extracted from brown algae on bacteria isolated from wastewater

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**ABSTRACT**

This study shows the antibacterial activity of three selected brown marine algae from Al-Shuqaiq province's red sea coast, Saudi Arabia. The three selected algal species were identified as *Sargassum denticulatum*, *Sargassum filipendula*, and *Padina gymnospora* and evaluated for their potential antibacterial bioactivity against 11 isolates of bacteria isolated from the wastewater collected from swage station of Khamis Mushait area. Three different algal extracts were prepared using methanol, ethanol, and diethyl ether. All algal extracts prepared with the diethyl ether were the most effective among all solvents used for the preparation of extracts in this study. The diethyl ether extract for *S. denticulatum* followed by *S. filipendula* were the most effective targeted marine algae against wastewater isolated bacteria. The *S. denticulatum* diethyl ether extract gave the highest inhibition zone diameter (33, 45, and 25 mm) bacterial isolates 5b, 12b, and 14b, respectively. Therefore, the bacterial isolates 5b, 12b, and 14b subjected to identification based on sequencing and phylogenetic analysis of 16S rRNA gene. The phylogenetic analysis for the three isolates 5b, 12b, and 14b indicated that they are closely relative with *Bacillus cereus*, *Bacillus amyloliquifaciens*, and *Chryseobacterium cucumeris*, respectively. The major constituents of *S. denticulatum* diethy ether extract fraction were confirmed by gas chromatography-mass spectrometry (GC–MS) analysis, Fourier-transform infrared spectroscopy, and nuclear magnetic resonance (NMR) ($^{1}$H and $^{13}$C-NMR) spectroscopy. A total of six fatty acid esters were recognized in *S. denticulatum* extract by the retention time and the fragmentation pattern data of GC/MS analysis. The identified fatty acid esters were, methyl 13-methyl pentadecanoate, methyl 9-(Z)-octadecenoate, methyl 15-methylheptadecanoate, 2,3-dihydroxypropyl 9-(Z)-octadecenoate, methyl 13-docosenoate, and 9-(Z)-octadecenyl hexadecanoate.

*Keywords:* Wastewater; Antibacterial bioactivity; Sargassum; Padina; 16S rRNA gene; FTIR; GC–MS; NMR

## 1. Introduction

Wastewater is a significant alternative source of irrigation water. It will be highly polluted by microbes if this wastewater is not disinfected or treated. Therefore, there are a number of risks associated with the use of wastewater to irrigate crops [1,2]. A wide variety of algal bioactive secondary metabolites such as antifeedants, cytotoxic and antihelmintics agents, including polyalkaloids, cyclic peptides, phlorotannins, lipids, polysaccharides, quinones, diterpenoids, glycerols, and sterols has been reported antimicrobials [3]. In most cases, the extracts generated

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using organic solvents appear more effective [4,5] possibly because the inhibition mechanisms are partially due to the hydrophobic existence of certain components, such as fatty products [6,7]. The higher antibacterial activity of polar extracts was confirmed by many researchers [8–10]. The algal extracts have been used for various diseases such as hypertension, cough, tumor, diarrhea [11,12].

For antibacterial compound production against *Staphylococcus Aureus*, *Escherichia coli*, *Klebsiella pneumonia*, and *Enterococcus faecalis* as a preventive and healing agent. Seaweed species such as *Gracilaria*, *Calorpha*, and *Hydroclothres* have been screened for six bacterial pathogens such as *Enterobacter aerogenes*, *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Streptococcus faecalis* [13]. *Gracilaria edulis* ethanol extract inhibited the growth of all tested organisms with the exception of *B. cereus* and *E. aerogenes* [14]. Methanol extracts from 32 macroalgae from Morocco's Atlantic and Mediterranean coasts were evaluated [15]. *Sargassum*, *Ulva lactuca*, *Padina gymnospora*, and *G. edulis* antibacterial activities have been screened for human bacterial pathogen [16]. The present study was conducted for screening and evaluating the effectiveness of diethyl ether, methanol, and ethanol extracts of selected seaweeds, collected from the Red Sea coast of Saudi Arabia, as antibacterial agents against the most active bacterial species isolated from wastewater collected from sewage station of Khamis Mushait area.

## 2. Material and methods

### 2.1. Brown marine algae collection and extraction

The selected brown marine seaweeds, used in this study was collected from the coast of the Al-Shuqaiq province of Saudi Arabia during February 2018. The algal species were recognized in the Aleem and Coppejans studies which are based on the phenotype and reliance systems recorded [17,18]. The algae gathered were then carefully cleaned with tap water followed by washing three times with distilled water to remove the impurities. The seaweeds were dried by putting them in an electric oven at a temperature of 50°C for 2 d. The dried algae were grounded using the electrical mixer until they became a fine powder.

The powdered samples were then stored until use in a dark place. The active ingredients of selected brown seaweeds were extracted from dried brown algae, according to Motamedi et al. [19] and Okemo et al. [20] with some modification, using a net dry weight about 10 g. Ethanol 95%, methanol, and diethyl ether were used as an extraction solvents. One gram of the powdered seaweeds was homogenized with 40 mL of previously mentioned organic solvent each separately. The algal mix with solvent shaked for 60 min at 25°C using shaker incubator at 70 rpm. The extracts were centrifuged for 10 min at 5,000 rpm to get rid of the cellular residue. intercellular extracts were incubated at 30°C throughout the night until dry to remove the organic solvent. The weighted crude extracts were suspended at a final concentration of 50 mg/mL in dimethyl sulfoxide (DMSO) and stored in a refrigerator.

The purification of algae extract was carried out by Si gel vacuum liquid chromatography using different solvents of increasing polarity from petroleum ether (PE) to MeOH to yield three fractions based on thin layer chromatography and high performance liquid chromatography analysis. These fractions were further purified by reversed-phase column chromatography (CC) over Lobar LiChroprep RP-18 with a MeOH–$H_2O$ gradient (from 10:90 to 90:10). Identification of long chain fatty acid methyl esters (FAMEs) was achieved by gas chromatography/electron impact mass spectrometer (GC/MS). Structures were confirmed by 1H- and 13C-nuclear magnetic resonance (NMR) spectral data and also by comparison with spectral data reported earlier for analogous compounds.

### 2.2. Bacterial isolation and its morphological study

The nutrient agar medium was used for isolation of different kind of bacteria from wastewater. One milliliter of wastewater, which collected previously from Khamis Mushait area, was spread on the nutrient agar plate. The plates were incubated at 37°C for 24 h. Isolation of these bacteria until get single colony had been done by using serial dilution method and streaking method. The pure bacterial colonies were sub cultivated in nutrient agar slants, incubated at 37°C to attain vigorous development. In order to differentiate the bacteria whether it belongs to gram positive or negative; the gram stain was done according to Aneja [21].

### 2.3. Determination of antibacterial activity

Antibacterial operations from chosen algal extracts were screened against 11 bacterial isolates collected from wastewater. The bacterial isolates were cultivated at a temperature of 37°C in nutrient agar medium [22]. Stock crops were retained at 4°C in the nutrient medium and regularly sub-cropped. Antimicrobial activity was performed *in vitro* using the method of agar well diffusion [23]. Briefly Small inoculums are spread evenly onto nutrient agar with a sterile cotton swab of each of the prepared bacterial suspensions. Plates were kept at room temperature to allow the bacterial inoculums to be absorbed. A 6 mm diameter sterile cork borer was used to make four equidistant wells on each night agar plate. Wells were loaded with 100 μL of the corresponding tested marine algal extracts. All tested plates were carried out in duplicate. Measuring the inhibition area around each well stated the antimicrobial activity. Mean diameter values were calculated from each assay's duplicate runs. As a negative control, the effectiveness of the algal extracts was contrasted with DMSO.

### 2.4. Molecular identification of isolated bacteria

#### 2.4.1. Amplification of 16S rRNA gene and DNA sequencing

Three bacterial isolates (5b, 12b, and 14b) were selected for farther molecular characterization according to its algal extract sensitivity and the gram stain. Following the manufacturer's instructions, the bacterial genomic DNA was extracted using GenElute™ bacterial genomic DNA kit (Sigma Aldrich®, Germany). The bacterial rDNA gene was PCR amplified using universal 16srRNA primers. Identification of

the bacterial isolates 16S rRNA gene were carried out based on DNA sequencing and bioinformatics evaluation by GATC Company Agilent Technologies, Bruker (USA) using ABI 3730xl DNA sequencer. The sequences obtained were compared with those deposited in the NCBI GeneBank database. BLASTn was subjected to the 16 s rRNA gene sequences.

#### 2.4.2. Phylogenetic analysis

The sequence alignment and the Phylogenetic analyses were performed among the selected bacterial isolates (5b, 12b, and 14b isolates) and their phylogenetically closest isolates deposited in NCBI GeneBank databases by the Mega 6 software package.

### 2.5. Chromatographic analysis using GC–MS

Chromatographic analysis using GC–MS was performed (Agilent Technologies 7890B GC Systems combined with 5977A. The capillary column was used (HP-5MS capillary; 30.0 m = 0.25 mm, ID = 0.25 μm film) and the carrier gas was helium at a flow rate of 1.8 mL/min with 1 μL injection. The sample was analyzed with the column initially held at 40°C for 3 min after injection, then the temperature increased to 300°C with a 20°C/min heating platform with a hold of 2.0 min. Split-free injection was performed at 300°C. The range of MS scans was (m/z): 40–500 units of nuclear mass, atomic mass unite (AMU) under ionization by electron effect (EI) (70 eV). The reaction is performed by adding 100 μL of Silylation agent:BSA.N, Obis (trimethylsilyl) acetamide (BSA+) sample quantity after extraction and heating for 2 h in a water bath at 70°C and then injecting it into GC/MS under the circumstances above. Mass fragmentation with the NIST mass spectral search program for the National Institute of Standards and Technology (NIST)/Environmental Protection Agency (EPA)/National Institutes of Health (NIH) mass spectral library version 2.2 (June 2014) determined the constituents.

### 2.6. Fourier transform infrared analysis

The main functional groups of the constituents of the most bioactive algal extracts, for *S. denticulatum*, *S. filipendula*, and *P. gymnospora* were characterized by Fourier transform infrared (FTIR) spectrometer, using a attenuated total reflectance diamond crystal/the Agilent Cary 630 FTIR (USA) instrument in the range 4,000–500 $cm^{-1}$. The resolution was 1 $cm^{-1}$ and 15 scans.

### 2.7. NMR analyses

$^{1}H$- and $^{13}C$-NMR spectra for the most bioactive algal extracts were measured on a Bruker model 500 MHz ultra shield NMR spectrometer in deuterated methanol ($CD_3OD$) using tetramethylsilane as an internal standard. The chemical shift values are recorded as $\delta_{ppm}$.

## 3. Results and discussion

### 3.1. Algal identification and extraction

A broad range of bioactive secondary metabolites are known to generate from marine brown algae and several compounds have been obtained from them [3]. The present study demonstrates the three selected brown marine algae collected from Al-Shuqaiq province, Saudi Arabia Red Sea coast, were identified as *Sargassum denticulatum*, *Sargassum filipendula*, and *P. gymnospora* [17,18] as shown in (Fig. 1). Three different extracts were prepared using methanol, ethanol, and diethyl ether were assayed for anti-bacterial activity against gram-positive, gram-negative bacteria isolated from the wastewater. According to earlier reports, anti-bacterial behavior depends on algal species,

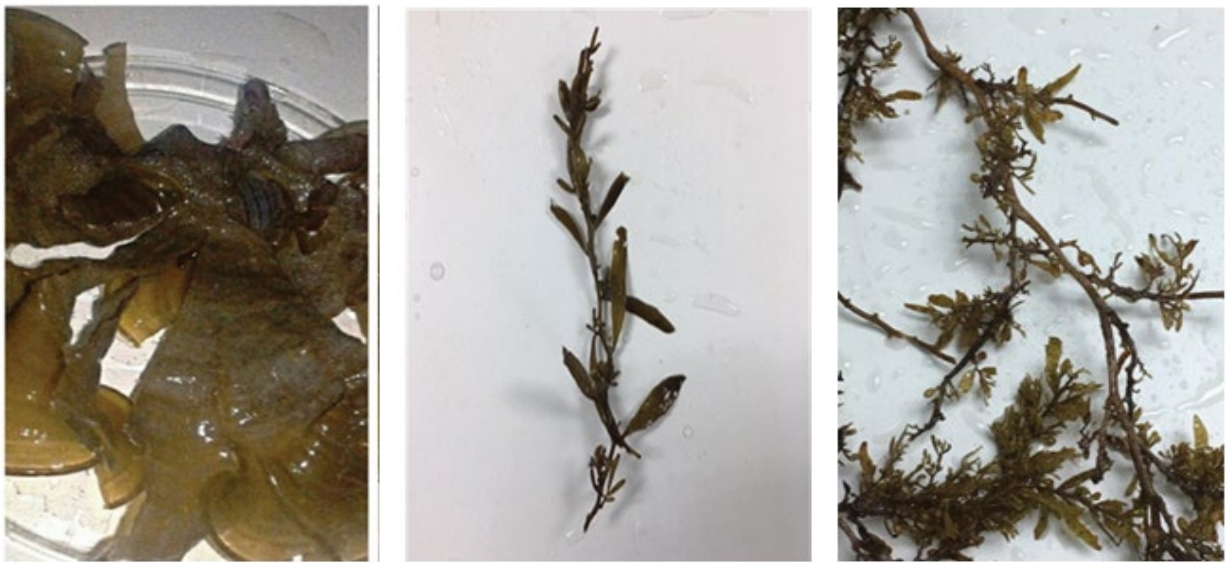


Fig. 1. Indicate the brown algae under study (a) *Padina gymnospora*, (b) *Sargassum denticulatum*, and (c) *Sargassum filipendula*.

the tolerance of the tested bacteria, and the efficiency of the extraction method [24]. It is evident from the outcomes of this research that the organic solvents always have higher efficiency in extracting anti-bacterial compounds compared to DMSO as a negative control.

### 3.2. Bacterial isolation and its morphological study

In the present study, 11 bacterial isolates were isolated and named as 1b to 8b, 11b, 12b, and 14b on nutrient agar medium. The colonies of the bacterial isolates chosen were characterized by their morphological features such as colonial size, shape, color, and nature. Furthermore, Gram's staining has screened all the isolates to verify whether they belong to the positive or negative gram. The morphological characteristics details as shown in Table 1. The bacterial isolates 1b, 3b, 4b, and 12b, were found to be gram-positive long chains bacilli; 5b and 6b are gram-positive short rods. Whereas 2b, 14b, and 7b are gram-negative short rods and 11b is gram-positive cocci. From our results, it seems that the *Bacillus* was the dominant genus found in the wastewater sample.

### 3.3. Determination of antibacterial activity

The three collected algal species, *S. denticulatum*, *S. filipendula*, and *P. gymnospora*, were evaluated for their potentials for antibacterial bioactivity. Table 2 revealed that the three algal extracts prepared with the diethyl ether were shown to be the most effective extracts among all solvents used for the preparation of extracts in this study. The diethyl ether extracts for *S. denticulatum* followed by *S. filipendula* were the most effective algal extract against wastewater isolated bacteria. The *S. denticulatum* diethyl ether extract gave the highest inhibition zone diameter (33–45 mm) with the gram-positive bacterial isolates 5b and 12b, respectively, and (25 mm) diameter with 14b isolate gram-negative bacteria. Whereas, *S. filipendula* diethyl ether extract gave the largest halos (25 mm) against 14b and 12b bacterial isolates and 22 mm diameter with 5b. Therefore, the bacterial isolates 5b, 12b, and 14b were subjected for farther genetic molecular identification by DNA sequencing. Further from our obtained results, it was observed that *P. gymnospora* extract prepared with diethyl ether could record higher inhibitory activities against isolates number 1b, 5b, and 11b with inhibitory zone diameter 18, 20, and 16 mm, respectively. On the other hand, the *S. filipendula* and *P. gymnospora* ethanol and methanol extracts achieved halos not superior of inhibition zone. However, the DEMSO negative control did not record antibacterial activity against all isolated bacteria. Despite of the number of studies found that methanol is more effective [25,26], but also ethanol [27], acetone [28,29], water [8], ethyl ether [6], and ethyl acetate [30] were tried. However, the antibacterial activity of algal extracts depends on algal species, extraction method effectiveness, and concentration of extraction [13]. For instance, the diethyl ether extract of *Sargassum fusiforme* and ethanol extract of *Sargassum vulgare* showed more inhibitory activity against multidrug resistant bacterial strains [31]. Compared to other references [32] who stated that chloroform: methanol is one of the primary alternatives for extracting effective antibacterial products from brown algae species, whereas methanol extracts from *S. vulgare* have no antibacterial activity against the development of pathogenic bacteria. [15]. Our diethyl ether extracts of *S. denticulatum*, *S. filipendula*, and *P. gymnospora* were found to be the most efficient extracts against most of the wastewater isolated bacteria.

Therefore, we directed our attention to analyze the chemical constituents of diethyl ether extract using GC–MS, FTIR, and NMR spectral analyses.

### 3.4. Molecular identification of isolated bacteria

Nucleotide sequence alignments were obtained with BLASTn and DNASTAR Lasergene 11 Core Suite software revealed that the 16 s rDNA of 5b isolate shared 98.99% sequence identities with the *Bacillus pacificus* strain MCCC-1A0618, *B. cereus* NBRC-15305, and ATCC 14579 strain and *Bacillus paramycoides* MCCC 1A04098 strain. In addition, the 12b isolate sequence alignment showed that it shared 99.07% sequence homology with *Bacillus amyloliquifaciens* NBRC-15535 and MBA 1034 strain (data not shown). The 16 s rDNA gene of the 14 b Isolate shared 99.79% with the *Chryseobacterium cucumeris* strain GSE06.

Table 1
Morphological characteristics of isolates obtained from wastewater bacterial isolates from, Khamis Mushait area

| No. of isolate | Characterization of Colony | | | Cell features | |
|---|---|---|---|---|---|
| | Color | Size (mm) | Nature of colony | Gram stain | Shape |
| 1b | White | 4 | Rhizoid shaped, smooth | Gram positive | Long chains *Bacilli* |
| 2b | Cream | 2 | Round shaped, shiny | Gram negative | Short rods |
| 3b | Cream | 4 | Irregular shaped, smooth | Gram positive | Long chains *Bacilli* |
| 4b | Cream | 3 | Rod shaped, smooth | Gram positive | Long chains *Bacilli* |
| 5b | Cream | 2 | Round shaped, shiny | Gram positive | Short rods in chain |
| 6b | White | 1 | Rod shaped, smooth | Gram positive | Short rods |
| 7b | Cream | 2 | Round shaped, shiny | Gram negative | Short rods |
| 8b | White | 4 | Rod shaped, smooth | Gram positive | *Bacilli* |
| 11b | Yellow | 2 | Spherical shaped, shiny | Gram positive | *Micrococcus* |
| 12b | Cream | 3 | Rod shaped, smooth | Gram positive | Long chains *Bacilli* |
| 14b | Golden Yellow | 1 | Round shaped, shiny | Gram negative | Short rods |

Table 2
Antibacterial activity of certain brown algae extracts against wastewater-isolated bacterial inhibition of growth expressed as mm diameter of inhibition zone

| Algal species | Organic solvents | No. of isolate obtained from wastewater | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1b | 2b | 3b | 4b | 5b | 6b | 7b | 8b | 11b | 12b | 14b |
| *Padina gymnospora* | | | | | | | | | | | | |
| | Ethanol | 15 | 0 | 10 | 18 | 0 | 6 | 5 | 9 | 4 | 0 | 0 |
| | Methanol | 10 | 0 | 7 | 13 | 0 | 4 | 0 | 12 | 0 | 0 | 0 |
| | Diethyl ether | 18 | 3 | 13 | 14 | 20 | 6 | 0 | 12 | 16 | 15 | 10 |
| | DMSO | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Sargassum denticulatum* | | | | | | | | | | | | |
| | Ethanol | 15 | 0 | 12 | 20 | 15 | 0 | 13 | 0 | 0 | 35 | 0 |
| | Methanol | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 33 | 0 | 0 | 0 |
| | Diethyl ether | 30 | 15 | 20 | 25 | 33 | 25 | 0 | 32 | 30 | 45 | 25 |
| | DMSO | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Sargassum filipendula* | | | | | | | | | | | | |
| | Ethanol | 0 | 0 | 9 | 20 | 18 | 7 | 9 | 15 | 10 | 19 | 0 |
| | Methanol | 0 | 0 | 0 | 12 | 15 | 0 | 2 | 10 | 0 | 3 | 0 |
| | Diethyl ether | 20 | 10 | 17 | 10 | 22 | 17 | 0 | 19 | 20 | 25 | 25 |
| | DMSO | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

On the other hand, the phylogenetic tree using CLUSTAL W analysis showed that 5b isolate is closely relative with *B. cereus* strain NBRC-15305, (with accession No. NR 112630.1), as shown in Fig. 2. Whereas, 12b isolate showed ancestral relationship with *B. amyloliquifaciens* strain NBRC-15535, (with accession No. NR 112685.1), as shown in Fig. 3. On the other hand, the phylogeny showed that the 14b isolate located in the same cluster with *C. cucumeris* strain GSE06, with accession No. NR 156145.1, as illustrated in Fig. 4. From our results, we can notice that the diethyl ether extract has high antibacterial potential against the spore forming gram-positive bacteria which found in treated

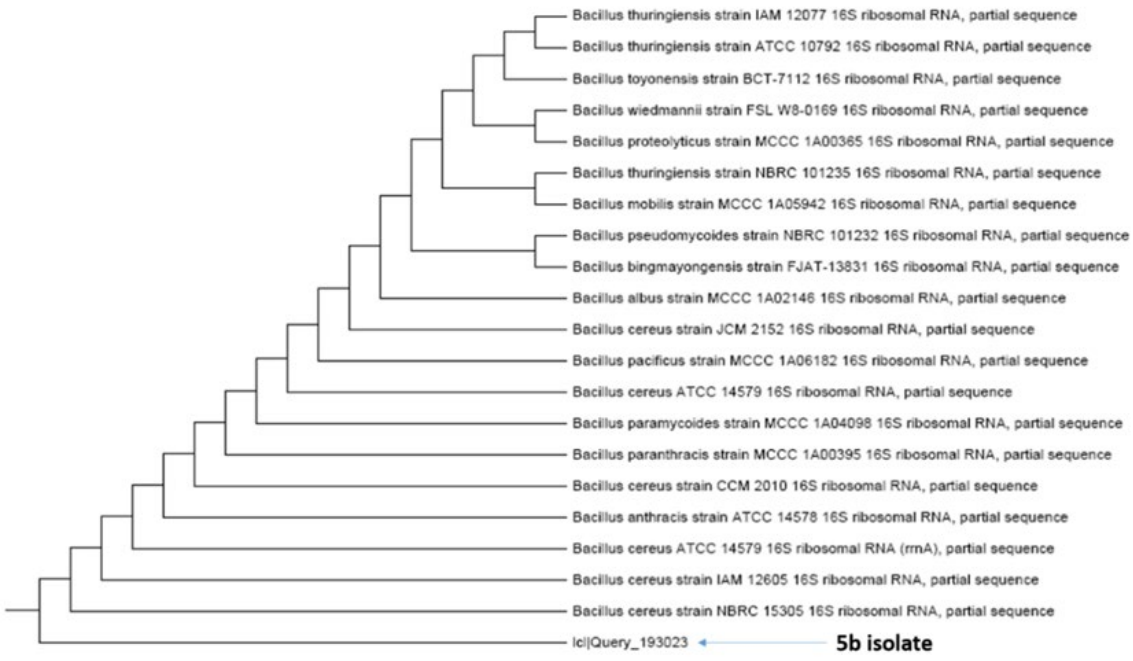


Fig. 2. Phylogenetic analysis of the 5b unknown bacterial isolate based on multiple nucleotide sequence alignments of the 16SrRNA gene and other isolates available in GenBank, instructed by CLUSTAL W using DNASTAR Lasergene 11 Core Suite Software.

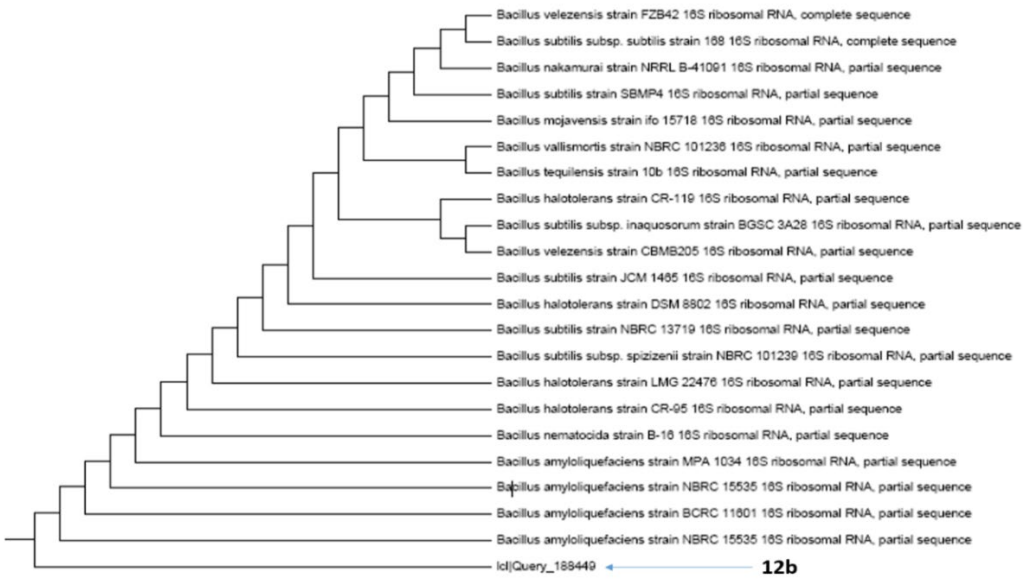


Fig. 3. Phylogenetic analysis of the 12b unknown bacterial isolate based on multiple nucleotide sequence alignments of the 16SrRNA gene and other isolates available in GenBank, instructed by CLUSTAL W using DNASTAR Lasergene 11 Core Suite Software.

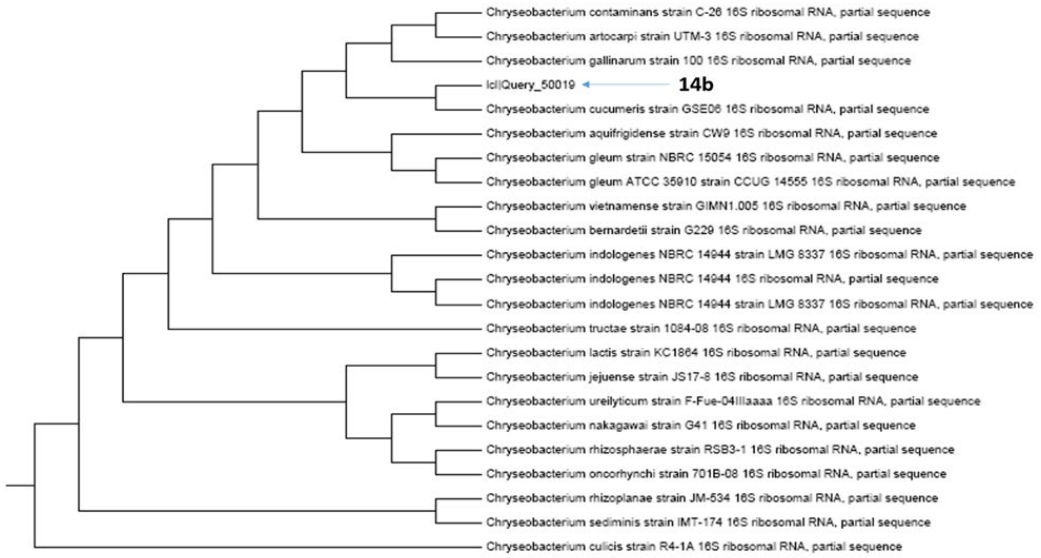


Fig. 4. Phylogenetic analysis of the 14b unknown bacterial isolate based on multiple nucleotide sequence alignments of the 16SrRNA gene and other isolates available in GenBank, instructed by CLUSTAL W using DNASTAR Lasergene 11 Core Suite Software.

wastewater such as *B. cereus* and *B. amyloliquifaciens* and the gram-negative bacteria such as *C. cucumeris*.

### 3.5. GC/MS analysis

The analyses of the chemical composition of the *S. denticulatum* diethyl ether extract using GC/MS permitted the identification of seven compounds responsible for 49.7% of the total methyl esters of fatty acid present (Table 3). Saturated fatty acids constituted 28.7% of this percentage, notably pentadecanoic, heptadecanoic, and myristic acids. Unsaturated fatty acids represented 21.0% of the total percentage of acids identified, notably oleic, and erucic acids. There are also two constituents of acetate derivatives were existed in 1.4%. The rest of the constituents of the extracts were a mixture of long chain aldehydes, acid chlorides, and thiophene derivatives.

### 3.6. FTIR analysis

FTIR spectra (Fig. 5) of fatty acids methyl esters derived *S. denticulatum* diethyl ether extract showed two characteristic peaks at 2,923 and 2,853 $cm^{-1}$ which were ascribed to stretching vibrations of $–CH_2$ and $–CH_3$, respectively.

Table 3
Fatty acids methyl esters recognized in the diethyl ether extracted from *S. denticulatum* by (GC/MS) in increasing order of retention/min (RT)

| Constituents | RT (min) | (%) | Equivalent fatty acid |
|---|---|---|---|
| Pentadecanoic acid, 13-methyl-, methyl ester | 13.0639 | 24.772 | Pentadecanoic acid |
| 9-Octadecenoic acid (Z)-, methyl ester | 13.9217 | 0.9403 | Oleic acid[a] |
| Heptadecanoic acid, 15-methyl-, methyl ester | 14.0276 | 3.4352 | Heptadecanoic acid |
| 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester | 15.4574 | 1.2592 | Oleic acid[a] |
| 13-Docosenoic acid, methyl ester, (Z)- | 15.6851 | 6.862 | Erucic acid[a] |
| Myristic acid, 9-octadecenyl ester, (Z)- | 16.7706 | 0.4971 | Myristic acid[a] |
| 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester | 16.8606 | 0.4503 | Oleic acid[a] |
| cis-7, cis-11-Hexadecadien-1-yl acetate | 17.0195 | 0.209 | |
| Z-(13,14-Epoxy) tetradec-11-en-1-ol acetate | 17.1148 | 1.2349 | |
| Total | | 49.7 | |

[a]Principle fatty acid.

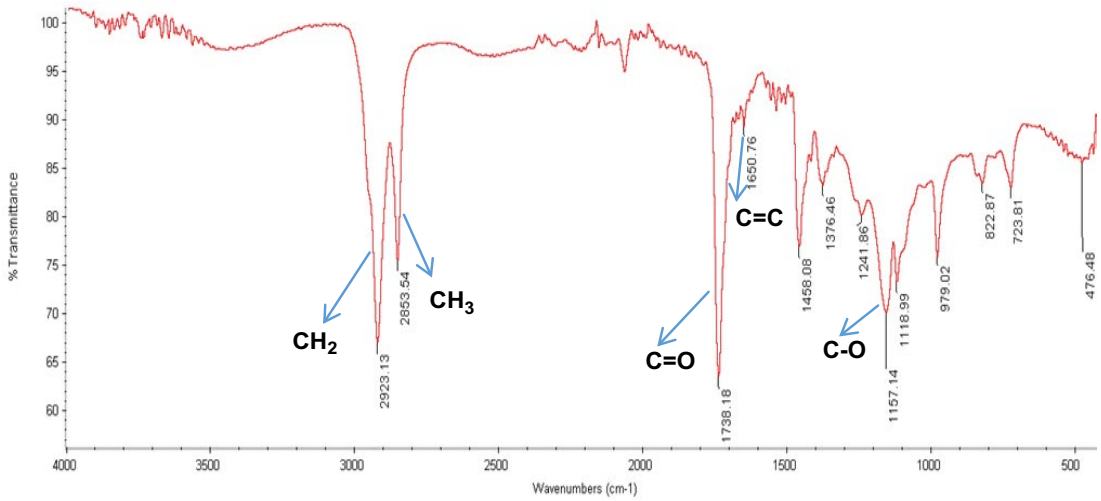


Fig. 5. FTIR spectrum of fatty acids methyl esters extracted from *S. denticulatum*.

The appearance of a sharp and strong peak at 1,738 $cm^{-1}$ confirmed the presence of an ester carbonyl group (O–C=O). The weak peak appeared at 1,650 $cm^{-1}$ was attributed to the carbon–carbon double bond (C=C). The other peaks observed in the region from 1,458 to 1,376 $cm^{-1}$ were indicative of bending vibrations for $–CH_3$ and $–CH_2$, respectively. The C–O–C absorption peak was showed at 1,157 $cm^{-1}$ which supports the existence of ester of fatty acids [33,34].

### 3.7. $^1$H-NMR analysis

The FAMEs was confirmed by $^1$H-NMR spectrum as shown in Fig. 6. The appearance of a triplet signal at δ 2.34 ppm and a single signal at δ 3.66 ppm supports the presence of α-methylene ($CH_2$) and methoxy ($–OCH_3$) protons, respectively. These two signals are the characteristic signals for the verification of methyl esters present in algae extract. The terminal methyl protons resonate at δ 0.92 ppm. A strong signal resonate at δ 1.31 ppm ascribed to methylene ($CH_2$) protons of carbon chain. The β-carbonyl methylene ($CH_2$) protons resonates at δ 1.63 ppm and the two olefinic protons displayed downfield at δ 5.29 and 5.36 ppm [33,35].

### 3.8. $^{13}$C-NMR analysis

A representative spectrum of $^{13}$C-NMR of the *S. denticulatum* diethyl ether extract is shown in Fig. 7, which displays the characteristic peaks of ester carbonyl (–COO–) and $H_3C–O$ at 173.2 and 54.1 ppm, respectively. The peaks around 131.6 and 129.3 ppm confirmed the unsaturation in methyl esters of fatty acids. Other peaks displayed at 13.0 ppm are due to the presence of terminal carbon of methyl groups and signals resonate at range 22.2–33.6 ppm are related to ($–CH_2$) carbons chain in FAMEs [34,35].

The analyses of the chemical composition of the *S. filipendula* diethyl ether extract using GC/MS permitted the recognition of five constituents accountable for

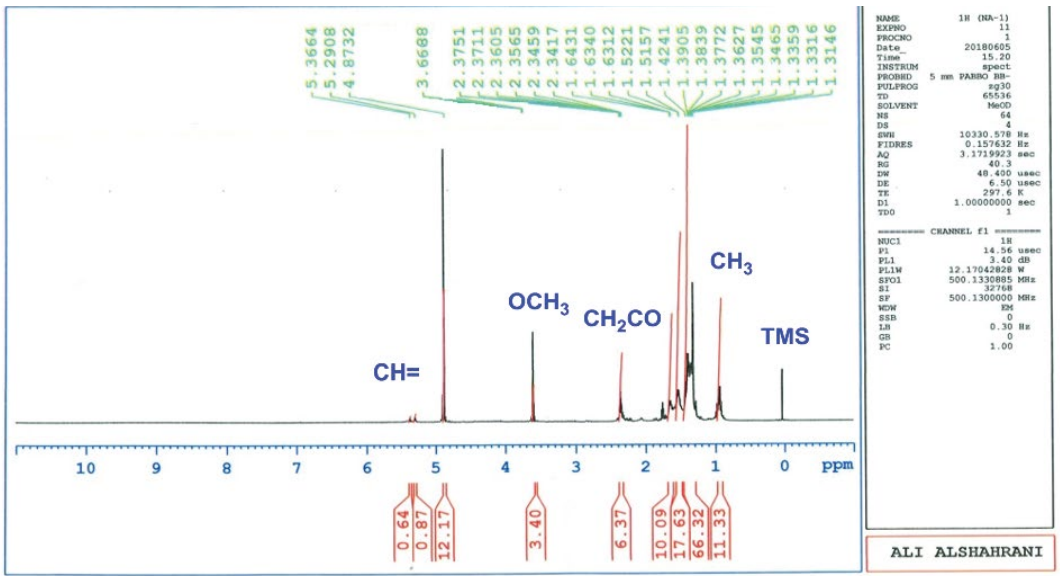


Fig. 6. $^{1}$H-NMR spectrum (500 MHz, $CD_3OD$) of unsaturated fatty acid methyl ester.

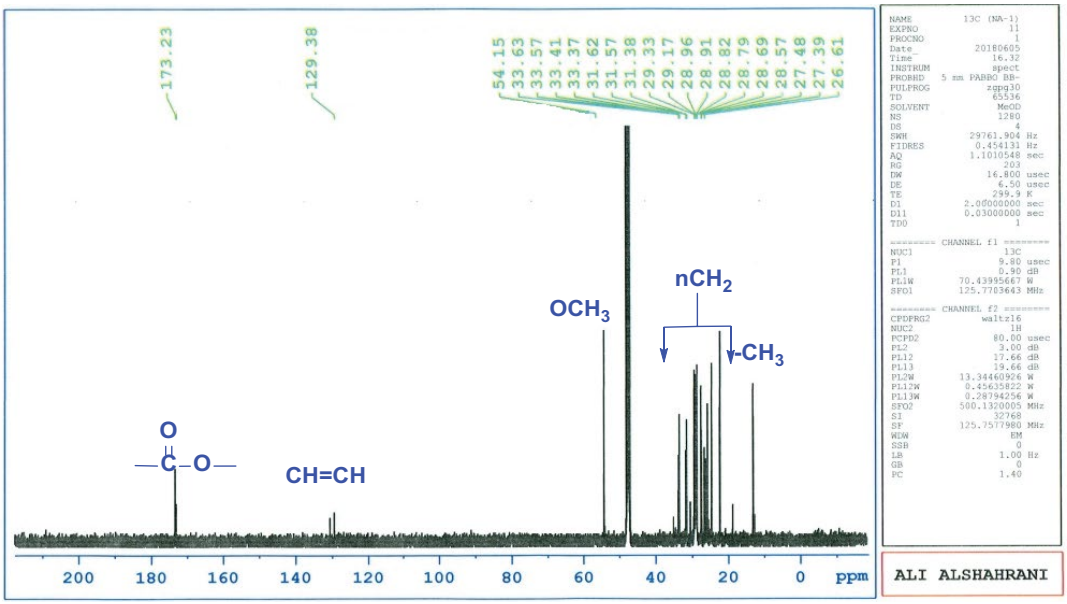


Fig. 7. $^{13}$C-NMR spectrum (125 MHz, $CD_3OD$) of unsaturated fatty acid methyl ester extracted from *S. denticulatum*.

31.6% of the total content of algae extract present (Table 4). Of this percentage, 21.27% was cis-7, cis-11-hexadecadien-1-yl acetate; Z,E-7,11-Hexadecadien-1-yl acetate represented 1.23% of the total percentage of acetates identified. E-11-Hexadecenal was existed in 4.55%. The unsaturated FAME, namely 11-octadecenoic acid methyl ester was found in 2.09% and 9,12-octadienoyl chloride, (Z,Z) existed in 2.16%. The rest of the chemical constituents of algae extract were not identified as the quality of these constituents in GC/MS analyses were low.

The contents of the *S. filipendula* diethyl ether extract was further identified by peaks related to C–H stretching vibrations (=C–H stretch at 3,187 cm$^{-1}$; C–H stretching in methyl (–$CH_3$) and methylene (–$CH_2$) groups at 2,850 and 2,919 cm$^{-1}$, respectively. Furthermore, there are two characteristic peaks showed at 1,735 and 1,715 cm$^{-1}$ assigned for two carbonyl groups of ester and aldehyde derivatives [34]. The peaks appeared at 1,647; 1,458; 1,417; and 1,375 cm$^{-1}$ were characteristic to carbon–carbon double bond (C=C), $CH_3$, $CH_2$, and CH bending vibrations, respectively [36,37]. The ether stretching group in esters (–C–O–C=O) and –$CH_2$ rocking bending vibrations were also noted at peaks (1,079–1,164 cm$^{-1}$) and 720 cm$^{-1}$, respectively [36]. All these principle peaks of FAMEs, long chain alkyl acetates and aldehydes are notably appeared in the FTIR spectrum of *S. filipendula* algae extract as shown in Fig. 8.

The analyses of the chemical composition of the *P. gymnospora* diethyl ether extract using GC/MS endorsed the identification of seven compounds liable for 84.51% of the total content of algae extract present (Table 5). Of this percentage, 57.08% were methyl esters unsaturated fatty acids, particularly oleic, erucic, and linoleic acids; methyl ester saturated fatty acid represented 11.94% of the total percentage of acids identified, notably palmitic acid. There are also linoleic acid chlorides and cis-9-hexadecenal were existed in 10.33% and 5.16%, respectively. The rest of the constituents of the extracts were a mixture of long chain aldehydes, acid chlorides, and fatty acid derivatives.

Table 4
Chemical composition of *S. filipendula* diethyl ether extract by (GC/MS) in increasing order of retention/min (RT)

| Constituents | RT (min) | (%) |
|---|---|---|
| 13-Octadecenoic acid, methyl ester | 13.906 | 2.09 |
| E-11-Hexadecenal | 15.944 | 4.55 |
| cis-7, cis-11-Hexadecadien-1-yl acetate | 16.686 | 6.99 |
| Z,E-7,11-Hexadecadien-1-yl acetate | 16.845 | 1.23 |
| cis-7, cis-11-Hexadecadien-1-yl acetate | 17.041 | 0.77 |
| cis-7, cis-11-Hexadecadien-1-yl acetate | 17.109 | 1.96 |
| cis-7, cis-11-Hexadecadien-1-yl acetate | 17.358 | 4.11 |
| cis-7, cis-11-Hexadecadien-1-yl acetate | 17.528 | 7.74 |
| 9,12-Octadecadienoyl chloride, (Z, Z)- | 17.872 | 2.16 |

The chemical constituents of the *P. gymnospora* diethyl ether extract were further confirmed by FTIR spectrum analysis. As shown in Fig. 9, there was a broad absorption band appeared at 3,391 $cm^{-1}$ characteristic for –OH group. The C–H stretching absorption vibrations of the methyl ($–CH_3$) and methylene ($–CH_2$) groups were displayed at 2,852 and 2,921 $cm^{-1}$, respectively [34]. A sharp and strong absorption band characteristic for the ester carbonyl group (–O–C=O) was showed at 1,738 $cm^{-1}$ [36]. The medium absorption band displayed at 1,648 $cm^{-1}$ was ascribed to the presence of carbon–carbon double bond (C=C) group. The absorption bands showed at 1,459; 1,417; and 1,376 $cm^{-1}$ were characteristic to $CH_3$, $CH_2$, and CH bending vibrations, respectively. The ether stretching vibration of esters (–C–O–C=O) was shown at peaks 1,058–1,162 $cm^{-1}$ [36,37].

## 4. Conclusion

From the previous data, we noticed that diethyl ether extracts possess the best results compared to ethanol and

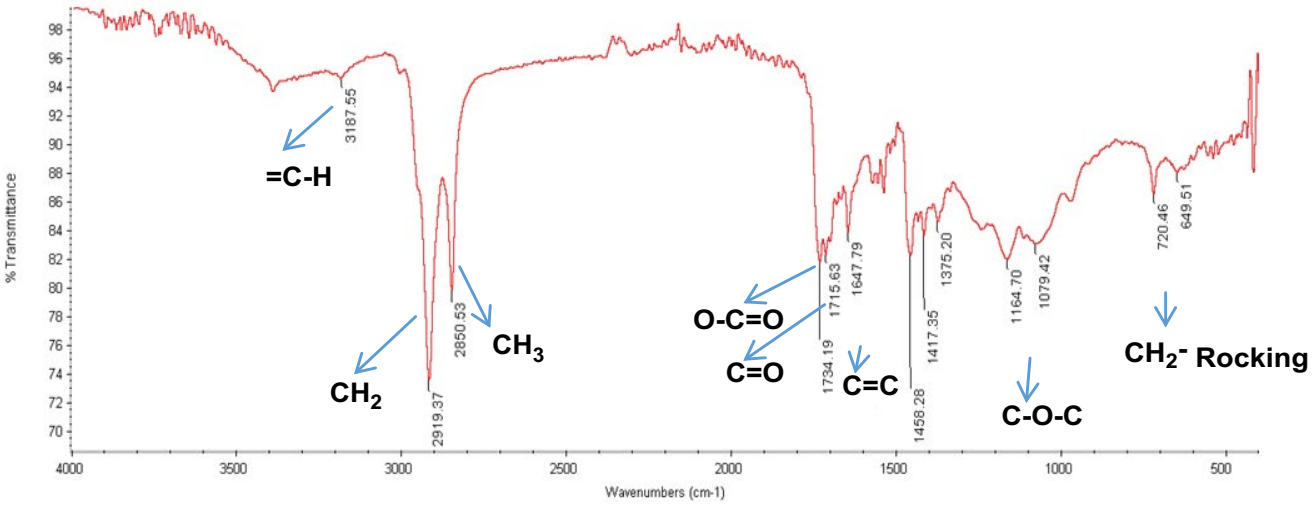


Fig. 8. FTIR spectrum of chemical constituents extracted from *S. filipendula*.

Table 5
Chemical composition of *P. gymnospora* diethyl ether extract by (GC/MS) in increasing order of retention/min (RT)

| Constituents | RT (min) | (%) |
|---|---|---|
| Pentadecanoic acid, 14-methyl-, methyl ester | 13.064 | 11.94 |
| Methyl 18-fluoro-octadec-9-enoate | 13.953 | 20.84 |
| 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester | 15.706 | 22.77 |
| 9,12-Octadecadienoyl chloride, (Z, Z)- | 16.119 | 3.91 |
| 9,12-Octadecadienoic acid (Z, Z)-, methyl ester | 16.151 | 9.49 |
| 9,12-Octadecadienoyl chloride, (Z,Z)- | 16.453 | 6.42 |
| cis-9-Hexadecenal | 16.929 | 5.16 |
| Linoleic acid ethyl ester | 17.416 | 3.98 |

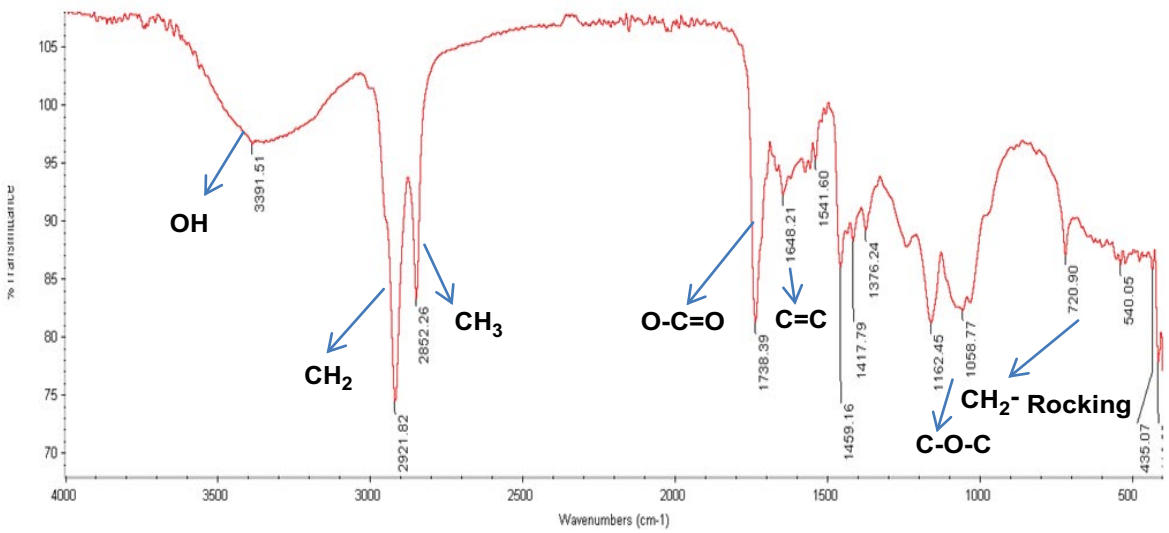


Fig. 9. FTIR spectrum of chemical constituents extracted from *P. gymnospora.*

methanol extracts, respectively. On the other hand the diethyl ether extract of *S. denticulatum* recorded inhibitory activity against most of bacterial isolates obtained from wastewater collected from sewage station of Khamis Mushait area; where it gave high inhibition zones with the three molecular identified bacterial isolates (5b, 12b, and 14b). The phylogentic analysis results indicate that the three isolates 5b, 12b, and 14b are *B. cereus*, *B. amyloliquifaciens* (gram-positive-spore forming bacteria), and *C. cucumeris* (gram-negative bacteria), respectively. Further from our obtained results, it was observed that Padina extract prepared with diethyl ether could record higher inhibitory activities against isolate number 1b, 3b, and 4b with inhibitory zone diameter 25, 18, and 20 mm, respectively, Whereas with the remaining isolates, the same extracts did not demonstrate greater inhibitory activity. The major constituents of *S. denticulatum* diethy ether extract fraction were confirmed by GC–MS analysis, FT-IR, and NMR (1H and 13C-NMR) spectroscopy and show that the fatty acid esters were the major bioactive secondary metabolites found in the diethyl ether algal extract. Therefore the diethyl ether extract of *S. denticulatum* could be used as an antibacterial bioactive substant alternative to commonly used current water chemical disinfectants, which its extensive use for long periods led to the emergence of bacterial strains resistant to them in addition to its side effects.

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