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Neihaya Z. Heikmat
Biology Department, College of Science, Mustansiriyah University, Baghdad, Iraq.
neihaya_2008@yahoo.com

Ghaidaa H. Abd Ali
Biology Department, College of Science, Mustansiriyah University, Baghdad, Iraq.

Shurooq L. Al-Razaq
Biology Department, College of Science, Mustansiriyah University, Baghdad, Iraq.

Nada Z. Mahdi
Biology Department, College of Science, Mustansiriyah University, Baghdad, Iraq.

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Biofilm Inhibitory Potential of *Westiellopsis prolifica* Extract Against Some Pathogenic Microorganisms

Abstract- *Resistance of microorganisms to many kinds of antibiotics push towards using natural products to eliminate the biofilm. In this study, 7 different species of algae were identified, 3 species of Chlorophyta and 3 of Cyanophyta. Westiellopsis prolifica is considered a potent organism. Fourier transform infrared spectroscopy (FTIR) was determined 13 clear bands, and the main bands were carbohydrate (1200- 900 cm−1), protein (1660 and around 1540 cm−1) and lipid bands (1740 cm−1). Extracellular crude acetone extract from W. prolifica better than hexane extract and more efficient on negative gram bacteria than positive gram bacteria. Antibiofilm was conducted by Congo red Agar and Microtiter plate against bacterial isolates (Staphylococcus aureus, Bacillus subtilis, Streptococcus sp., Shigella sp., Proteus sp. and Pseudomonas aeruginosa) and two fungi (Aspergillus niger and Candida albicans). The highest inhibition was against Streptococcus sp. and the remaining ratio of biofilm was 0.7 %, while lower inhibition 33 % against C.albicans. The GC-MS analysis of the purified extract has identified many active compounds, mainly were 4-Trimethyl-1-hexene, Octadecane, Ethylene-14-Pentadecane, n-Hexadecanoic acid, Octadecadienoic acid, Octadecadienoyl chloride, and Phytol.*

Keywords- *Algal extract, Antibiofilm, GC-MS analysis, Pathogenic microorganisms, Westiellopsis prolifica.*

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1. Introduction

Pathogenic bacteria have resistance to a broad type of antibiotics, and this affects human health, so huge anxiety has required finding novel antibacterial materials [1]. Microalgae cultivate effectively at any environmental conditions (in freshwater– to severe salinity) and can live with this requirement. The wide spectrum of the microalgae distribution makes the algae a unique requirement for the production of a novel cheap metabolite, which involves in blue biotechnology [2]. The main attention in microalgae especially cyanobacteria is anti-biofilm property, the ability to change their metabolism according to environmental conditions, also, microalgae is recognized to be an important resource for biologically active compounds which play physiological effect for itself and their surroundings [3]. Cyanobacteria which are a great source for the useful metabolites, the primary or secondary metabolites might have a therapeutic activates such as antibacterial, antifungal properties, and antiviral diseases, antitumor and anti-inflammatory as well as enzyme inhibiting and cytotoxic [4]. Biologically active compounds formed by algal cells and include vitamins, amino acids, enzymes, growth regulators, pigments, toxins, proteins, fats, lipids, carbohydrates, phenol and flavonoids [5]. Many of these products are accumulating in the biomass of microalgae (Intracellular); nevertheless, in some cases, these metabolites are excreted during growth in the medium and these are known as exometabolites (extracellular) [6]. Fatty acids, pigments, polyphenol, carbohydrate were considered as the most important groups of antimicrobial agent set up in freshwater microalgae [4]. The cyanobacteria cell produces fatty acids as a protection mechanism of the algal population against predators and when exposure to pathogenic bacteria in its environment. Fatty acids have bactericidal effects against a wide variety of bacteria [7]. The damaging effects of fatty acids on bacterial membrane, causing a reduction of the nutrient intake, a reduction of the cellular respiration and a cell leakage [8]. Because of their rapid growth rate and huge biodiversity, cyanobacteria and microalgae present many benefits as antimicrobial materials

producers [9]. Cell extracts and active components of many types of algae exposed to have a wide range of antimicrobial activities in vitro [10]. So this study aimed to examine the efficacy of the extracellular crude extract of W. prolifica isolated from the local environment, and test its ability for inhibiting microbial biofilm, includes clinical isolates of S. aureus, B.subtilis, Streptococcus sp., Shigella sp., Proteus sp. and P.aeruginosa, as well as C. albicans and Aspergillus sp. In addition to investigating the active metabolites of purified extracts by GC-MS apparatus.

2. Materials and Methods

I. Microalgae Collection

Algal samples collected during January and February of 2017 from different locations in Baghdad city. Samples were marked with the date and location and then transported to the laboratory immediately. The isolates were grown in BG-11 medium under suitable condition (268 µE/m²/s, 25±2 oC, 16:8 light: dark, for 10-14 day). The isolate was harvested at their exponential phase of growth, which is the 28th day [11], then isolates were recognized by different taxonomic and morphological approaches as in [12].

II. Extraction of bioactive metabolites

According to the method of ref. [13] with some modification, one gram of extracellular W.prolifica powder was extracted with 250 ml of 95% acetone solvent using a Soxhlet extraction apparatus at 60 ºC for 3-4 h until the solvent become colorless. The crude extract was dried by rotary evaporator at 40 ºC. The extract weighted and put in a temperature of -20°C. Repeat the same step using a hexane solvent.

III. FTIR analysis

FTIR-8400S (Shimadzu-Japan) was used to analyze the main compounds in cyanobacteria samples; this analysis was carried out at the Department of Chemistry, Mustansiriyha University, then and before measurement, the powder of algae extract was mixed with potassium bromide (KBr) and hard-pressed in tablets. The samples were analyzed in transmission mode at 400-4000 cm-1 wave number range [14].

IV. Clinical microorganisms

Eight species of pathogenic bacteria and fungi were used to study the antibiofilm activity to algal extract. Six isolates of bacteria and two fungi were obtained from microbiology laboratories of AL- Diwaniyah Teaching Hospital and Al-Kindy Teaching Hospital in Iraq, and they included: S. aureus, B. subtilis, Streptococcus sp. Shigella sp., Proteus sp., and P. aeruginosa, in addition to A. niger and C.albicans, were grown in Brain- heart infusion broth for bacteria and Sabouraud's Dextrose Broth for fungi.

V. Biofilm production

a. Congo red method

Each bacteria and fungi isolate inoculated at Congo red agar, then incubated aerobically at 37 ^{o}C for (24-48) h. for bacterial isolates, while fungi incubated for 48-72 h. at 28 oC. A positive result is indicated by presence black colonies with a dry crystalline consistency. The weak slime producers usually remain pink. A darkening of the colonies, with the absence of a dry crystalline colonial morphology, indicates an indeterminate result [15].

b. Microtiter plate method

About 200 µl of each bacterial and fungi suspensions were put in Microtiter plate. Control wells contained 200 µl of Brain heart infusion broth and 200 µl of Sabouraud's Dextrose Broth. The microtiter plate was sealed with parafilm and incubation for 24 h and 48 h at 37oC and 28C for bacteria and fungi respectively. Then the well washed with PBS (pH 7.2) to remove nonattached cells, after that drying at room temperature, and added 200 µl of crystal violet (0.1%) then incubated for 10min. Attached cells were rinsed with PBS three times and allowed to dry at room temperature, then 200 µl of 95% ethanol was used twice. Finally, the absorbance of each well measured by using ELISA Reader at 600 nm [16].

VI. Effect of crude extract of W. prolifica on biofilm

a. Method of Congo red agar

The crude extract of W.prolifica (1ml) added to the Congo red agar, and leave it at room temperature to dry. Each bacterial and fungal isolates were streaked on plates and incubated aerobically for 24 to 48 h at 37^{o}C and 48 to 72 h at 28 oC respectively [17].

b. Microtiter plate method

About 100 µl of each bacterial and fungi suspensions were added to Microtiter plate together with 100 µl of an extracellular extract of W. prolifica. Control wells contained 180 µl of the brain, heart infusion broth, and 20 µl of bacterial suspensions and 180 µl of Sabouraud's Dextrose Broth with 20 µl of fungi. So, same steps of the previous method completed.

Percentage of inhibition of biofilm was calculated as mentioned in [16]:

% of Inhibition of biofilm formation=

$$\frac{\text{OD of(+ve) control}-\text{OD of treatment}}{\text{OD of(+ve)control}-\text{OD of(}-\text{ve)control}} \times 100$$

The microtitre-plate antibiofilm assay estimates the percentage of fungal and bacterial biofilm reduction, relative to control wells, which were set at 100% to show the absence of crud extracts. In contrast, negative percentage results display no inhibitory activity of crude extracts on biofilm [18].

VII. Purification of the crude extract by solid adsorption chromatography

Extracellular acetone extract obtained from W. prolifica analyzed by silica gel chromatography. Partial purification extract of W. prolifica using open glass Column (2.5×21) cm filled with silica gel (200g) especially for column chromatography. The residue was dissolved and mixed with 5 ml acetone at room temperature for one hour. The column rinsed with acetone for 30 min., then on the top of the column, put a thin layer (about 1cm) of sand. The surface of silica gel bed was applied with acetone extract after separated to a small amount. About 50 ml of each mobile phase (first, Hexane: benzene 25ml: 25ml, followed by the mobile phase 50, 25: 25, 25: 25 and 50 were eluted. At a flow rate of 0.4 ml/min, each eluting solvent was collected in separate flasks. Finally, solvents were evaporated by using a rotary evaporator [19].

VIII-Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

Purified fractions of algal extract were analyzed by gas chromatography in the Ministry of Industry/ Industrial Research and Development Authority/ Ibn al-Bitar Research Center. A high-temperature column of GC-MS contained (Inert cap 1MS; 30 m × 0.25 mm id × 0.25 μm film thickness). The initial column temperature was placed at 100 °C and the injector and detector temperatures were placed at 280 °C. About 5 μL of sample injected into the column and ran using split (1:10) mode after 1 min, and the oven temperature was raised to 225 °C. The helium carrier gas was programmed to maintain a constant flow rate of 17.5 ml/min. The compounds were recognized by comparison of their mass with NIST library [20].

IX-Evaluation of the purified extract on biofilm

Microtiter plate method was chosen to evaluate the activity of purified extracts on biofilm formation. Same previous steps of the antibiofilm experiment were done, but here five groups of purified fractions of algal extracts were used and added to a suspension of microorganisms.

X-Statistical Analysis

SAS program was used to identify the different parameters in this study [21]. LSD (ANOVA) and Chi-square tests were used to compare between percentage and means of the current study.

3. Result and Discussion

I. Isolation and identification of algae

About 7 different species of algae were identified, 3 species belong to Chlorophyta and 3 species belong to Cyanophyta, from this group the genus of Westiellopsis prolific is considered as potent organism.

II. Extraction Method

Two types of solvents (acetone and hexane) were studied for extraction the crude extract of W. prolifica by using Soxhlet method, and the acetone solvent was more efficient compared to the hexane solvent as seen in Figure 1.

III. FTIR analysis of extracellular W. prolifica crude extracts

FTIR was used to identify and determine 13 clear bands (Figure 2), and the main three bands were carbohydrate at (1200- 900 cm−1), protein (1660 and 1540 cm−1) and lipid bands (1740 cm−1) as in (Table 1). FTIR spectra showed the functional groups of macromolecules of the algal biomass, including (C=O, P=O, C–H, CH2, CH3, C–O–C, O–H, and N–H). This analysis used in many studies to determine the differences in macromolecules components of microalgae; also, it used as a fingerprint for biochemical components of the algae [22].

Figure 1: Acetone and hexane extracellular extract of Westiellopsis prolifica

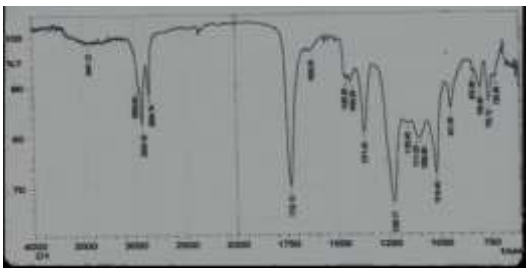

Figure 2: FTIR spectral of Crude extracellular extract of W. Prolifica

Table 1: Main bands of biochemical standards found in the FTIR spectra of W. Prolifica

Band	Main peak (cm^{-1})	Wave number range (cm-1)	Typical band assignment from the literature
1	1095.6	1099-1072	Carbohydrate (C-O-C) of polysaccharides Nucleic acid (and other phosphate-containing compounds) (>P=O) stretching of phosphor di esters
2	1111.03	1134- 1174	Carbohydrate (C-O-C) of polysaccharides
3	1155.4	1134 - 1174	Carbohydrate (C-O-C) of polysaccharides
4	1226.77	1191 -1356	Nucleic Acid (other phosphate-containing compounds
5	1371.43	1357 - 1423	Protein (CH2) and (CH3) bending of methyl Carboxylic acid (C-O) of COO- groups of carboxylates
6	1435.09	1425 - 1477	Protein (CH2) and (CH3) bending of methyl Lipid (CH2) bending of methyl
7	1456.3	1481 - 1585	Protein amide II band Mainly (N-H) bending and (C-N) stretching
8	1626.05	1583 - 1709	Protein amide Mainly (C=O) stretching
9	1732.13	1763 - 1712	Cellulose – Fatty acids (C=O) stretching of esters
10	2854.74	2809 - 3012	Lipid - Carbohydrate Mainly (CH2) and (CH2) stretching
11	2924.18	3809 - 3012	Lipid - Carbohydrate Mainly vas(CH2) and (CH2) stretching
12	2955.04	3809 - 3012	Lipid - Carbohydrate Mainly vas(CH2) and (CH2) stretching
13	3441.12	3029 - 3639	Water (O-H) stretching Protein (N-H) stretching (amide A

IV-Biofilm formation on Congo red agar

Method of Congo red agar was used to identify biofilm production by testing microorganisms through changing the color and texture of the medium. The black color of colonies interpreted as positive biofilm producing isolate in contrast with red colonies which interpreted as negative biofilm producing as showed in Figure 3. Each bacteria and fungi isolated in this study produce biofilm by Congo red agar method, and details of biofilm production shown in Table 2.

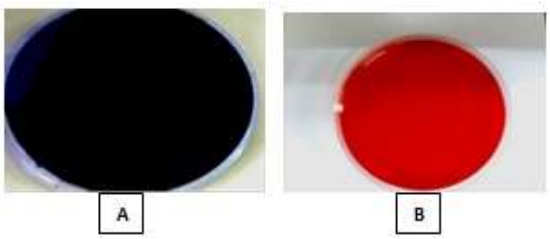

Figure 3: Biofilm Production on Congo red Agar. A-Positive result, B-Negative result

Table 2: Biofilm production of Microbial Isolates

Biofilm production					
Gram + ve bacteria		***Gram -ve bacteria***		**Fungi**	
S. aureus	++ +	*P. aeruginosa* +++	Ve++ +	*C. albicans*	+
Streptococcus sp.	++	*Shigella sp.*	+	*A. niger*	+ *
B. subtilis	++	*Proteus sp.* +++			

* +++ = Strong, ++= Moderate, += Weak

The pigment of the Congo red medium was used directly to distinguish the production of exopolysaccharide in components that forming biofilm texture, so it is simple to use and explain the results, which based on color produced by colonies [23].

V- Antibiofilm test

a. Congo red agar

The results in Table 3 presented that the crude extracellular acetone extract from W.prolifica better than hexane extract in the antibiofilm activity, and revealed that the acetone extract has efficacy against biofilm for all isolates studied, it showed very strong antibiofilm activity against Streptococcus sp , B.subtilis, Proteus sp. (+++) and antibiofilm activity was strong against S. aureus, Shigella sp. (++). While anti-biofilm activity was moderate (+) to C. albicans , but it was very weak against A. niger and P. aeruginosa. In contrast, the hexane extract displayed little efficiency against biofilm for all studied isolates (Figure 4) .

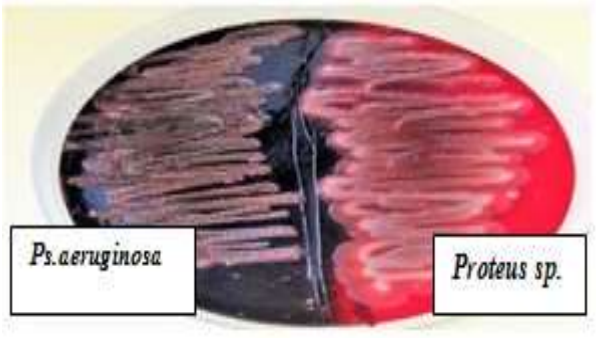

Figure 4: Anti-biofilm activity of the crude extract on Congo red agar

Table 3: Antibiofilm activity of crude extracellular extract of W.prolifica

Antibiofilm activity of extracellular		
Microorganisms	**Acetone extract**	**Hexane extract**
S. aureus	++	+
Streptococcus sp.	+++	Trac
B. subtilis	+++	+
P. aeruginosa	Trace	Trace

Shigella sp.	++	+
Proteus sp.	+++	+
C. albicans	+	++
A.niger	Trace	Trace

Trace=very weak, Moderate= +, strong = ++, very strong= +++

The best anti-biofilm activity was against C. albicans (++), While it was very weak to A. niger, P. aeruginosa and Streptococcus sp., but antibiofilm activity was moderate (+) against B. subtilis, Shigella sp. and Proteus [24] showed that black colonies indicated biofilm formation in the medium, while pink colonies in existence of algal extract, indicating the ability to form biofilm in tested bacteria and fungi isolates of the study, and this because exopolysaccharides is not formed in the medium which contains the extract.

b. Antibiofilm in Microtiter plate

Table 4 showed a significant difference between both extracts (acetone, hexane), and it showed significant differences among bacterial and fungal isolate. Also revealed no significant differences between Shigella sp., P.aeruginosa, A. niger in both crude extracts. The highest biofilm inhibition of acetone extract against Streptococcus sp. reached to 0.7%, while lower biofilm inhibition 33% against C. albicans. On the other side, the hexane extract has less efficiency; it recorded the highest biofilm inhibitory in Shigella sp. 4.7% and lower biofilm inhibitions against C.albicans reached 45.5%.

The variation and difference in the results because of the differences in the membrane structure and components of the cell wall [25]. The extracellular polymeric substances of biofilm formation by microbes showed a huge role in the adhesion and development of the rigidity of it, and EPS increased bacterial resistance towards antibiotics and insensitive environmental conditions [26].

Decreased the number of EPS in the bacterial cell will cause easy contact between bacterial with antimicrobial materials produced by algae, and could lead to the reduction of biofilm. Decreased the bacterial adhesion will influence on the bacterial population inside the biofilm matrix and also maintain the inhibition of biofilm-forming by bacteria [27].

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VI- GC-MS analysis of Extracellular Extract after Purification with (Hexane 1:1 Benzene)

Chemical compounds were present and identified by W. prolifica as shown in Figure 5.

The highest area of the screened compounds was which belonged to Dibutyl phthalalate- Di-n-octyl phthalalate Bis (2-ethyl hexyl) phthalate 89.73% as shown in Table 5.

VII- GC-MS analysis of Extracellular Extract after Purification (Group2=Benzene 50 ml):

Chemical compounds were present and identified of W. prolifica as shown in Figure 6. In group 2 of (Benzene 50 ml) of W. prolific, the highest area of the screened compounds was which belonged to Hexadecane as shown in Table 6.

Table 4: The effect of crude extract of W.prolifica On microbial biofilm activity

Microorganisms	Remaining of biofilm percentage% Acetone	Hexane	Chi-Square – $\chi 2$
S. aureus	2.9	12.9	4.273 *
Streptococcus sp.	0.70	30.2	9.512 **
B. subtilis	13.5	44.4	9.074 **
Proteus sp.	8.5	23.9	6.025 **
Shigella sp.	4.4	4.7	0.073 NS
P. aeruginosa	11.8	11.6	0.008 NS
C.albicans	33.0	45.5	4.59 *
A. niger	9.4	10.0	0.167 NS
Chi-Square–$\chi 2$	9.05 **	11.62 **	---

*** (P<0.05), ** (P<0.01), NS: Non-Significant.**

Figure 5: GC- Mass analysis of extracellular extract (Group1=Hexane with Benzene)

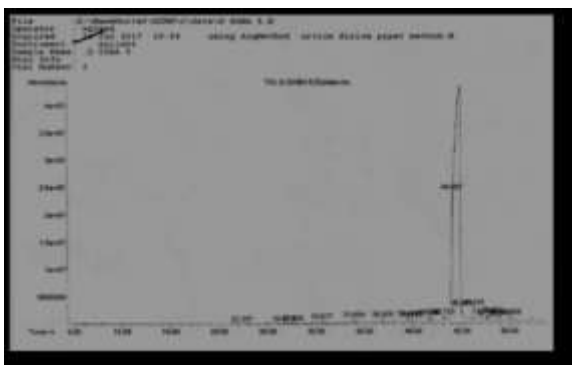

Figure 6: GC- Mass analysis of extracellular extracts in Group2 (Benzene 50 ml)

The various active compounds found in this study, which include saturated and unsaturated fatty acids as well as other compounds have high effective against microbes, especially bacteria and fungi.

The mechanism of fatty acids action work to lead many cellular goals, still though cell membrane damage which goes ahead to cell leakage and decreases the nutrient uptake, in addition to inhibiting cellular respiration [31].

Consistent with the effect of Westiellopsis sp on microbes (positive and negative bacteria and fungi).W .prolifica, consist of many fatty acid after purification by using GC-Mass such as Saturated fatty acid (Tetracosanoic 2.55%, Hexadecanoic 18.9%) and Unsaturated fatty acid Octadecenoic (Oleic acid) 22.9% and other active compounds such as 9,12-octadecadienoic acid, 9-Octadecenoic acid, Tetracosanoic acid, Benzenecarboxylic acid dioctyl ester, Benzenecarboxylic acid bis 2-ethyl hexyl, 2,6,11-Trimethyldodecane (Farnesol), 2,6,10-Trimethyl-14-pentadecene, 2,4-Di- Tert. Butylcyclohexanecarboxylic acid, Stigmast-5-en-3-ol (β-Sitosterol), Cholest-5-en-3-ol, 1,6-Anhydro-deoxyglucopyranose,1,5-Anhydro deoxyhexodilulose and 9-Octadecenal [32].

VIII-Evaluation of the purified extract on microbial biofilm

The purified extracellular acetone extract gave five groups of purified elutions as follows: Group1(Hexane 25 ml +Benzene 25 ml); Group2 (Benzene 50 ml); Group3 (Ethyl acetate 25 m l+ Benzene 25 ml); group 4 (Ethyl acetate 25 ml+ methanol 25 ml) and Group5 (Methanol 50ml).

The results in Table 7 showed that Group2 (Benzene 50 ml) gave better results than other groups against testing fungi and bacteria. In Group 1 the highest biofilm inhibition against A.niger reached to10 %, while Group 2 showed the highest biofilm inhibition against E.coli reached to 11.8. In group 3 the highest biofilm inhibition against C. albicans reached to 13%. Group 4 exhibited the highest inhibition percentage of remaining biofilm 6 % against S.aureus, while in group 5 The highest biofilm inhibition against C.albicans reached 7.1%.

Because of Cyanobacteria extracts generally have a mixture of different kinds of bioactive products with different properties, the purification of these extracts remains a large problem in methods of identification and characterization of them. The common practice in isolation of these bioactive compounds is silica gel chromatography, which should be used to obtain pure compounds and then used for the determination of structure and biological activity [28].

Ref. [29] Explained that some species of algae produce secondary metabolites that contribute to their defense in the middle of the environment in addition to their production compounds used in the pharmaceutical industries such as 2–(N, N-Dimethyl hydrazine) Cyclohexane carbonitride which was used against Antiprotoscolices.

Cyanobacteria and red algae produce the most main Phycobiliproteins pigments (PBPs). However, PBPs such as phycocyanin, phycoerythrin, and allophycocyanin, the effect on bacteria and fungus. As well exhibited that C-phycocyanin (C-PC) from Westiellopsis sp. and Spirulina platensis had been demonstrated to inhibit the growth of Pseudomonas aeruginosa, Bacillus subtilis, S. aureus. Klebsiella pneumonia and Xanthomonas sp. [30].

4. Conclusion

Cyanobacteria are a great source for the useful metabolites, it has therapeutic activities such as antibacterial, antifungal properties, and the genus of Westiellopsis prolifica is considered a potent organism. Extracellular crude acetone extract from W. prolifica was better than hexane extract and more efficient on clinical gram-negative bacteria than gram-positive bacteria. The purified extract has many active compounds when analyzed by GC-MS. Active compounds from these algae may be used as an anti-biofilm substance in different applications.

5. Acknowledgment

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Table 5: Active Compounds present in purified extracellular extracts (Group1=Hexane with Benzene)

No.	Rt.	Area%	Name of compound	Biological Activity
1	23.783	0.98	Hexadecane Tetracosane	Antibacterial and Antifungal antioxidant,
2	27.511	1.14	Pentadecanoic acid	Antibacterial and Anticandidal
3	30.005	1.26	1,2-Benzenedl carboxylic acid	Antibacterial and Antifungal
4	31.760	0.60	Bis-2-methylpropy)ester	Antibacterial and Antifungal
5	33.803	0.80	Dibutyl phthalalate	Antimicrobial and pharmacological Activity
6	34.345	0.97	Tridecyne	Antibacterial
7	83.128	0.86	2-methyl Hexacosane	Antibacterial and Antifungal
8	41.549	0.51	Tetracosane	Antibacterial activity
9	44.066	89.73	phthalalate Bis(2-ethyl hexyl) phthalate	Antimicrobial Activity and pharmacological Activity
10	46.070	0.92	3,5-Cholestenol	Antimicrobial

Table 6: Active Compounds of extracellular extracts from W. prolifica Group2 (Benzene 50 ml)

No.	**R.t.**	**Area%**	**Name of compound**	**Biological Activity**
1	22.3	0.77	Tridecyne	Antibacterial
2	26.6	0.44	Hexadeca mehyl	Antibacterial
3	27.5	0.99	Pentadecanoic acid	Antibacterial and Anticandidal
4	30.5	0.99	Octadeca methyl-	Antimicrobial
5	33.8	1.25	phathalic	Antimicrobial
6	36.8	0.88	Benzoic acid,	Antifungal
7	39.44	0.95	Octadeca m ethyl	Anti-cancer
8	39.85	0.58	2- methyl hexacosan	Antibacterial and Antifungal
9	41.53	1.18	Tetracosane	Antibacterial
10	41.8	1.14	Tetracosane	Antibacterial
11	43.15	0.96	Pentadecadien-1-ol	Antimicrobial
12	44.00	0.78	Hexacosane	Antimicrobial
13	45.01	1.12	Diiso octyl phthalate	Antibacterial
14	45.228	2.92	Hexadecane	Antimicrobial
15	47.600	0.96	2- methyl hexacosane	Antibacterial

Table 7: The remaining biofilm percentage of purified extracellular extract for W.prolifica

	Remaining of biofilm percentage%					
Microorganism	Group 1	Gro 2	Gro. 3	Gro. 4	Gro. 5	Chi-Square–$\chi 2$
S. aureus	33.0	33.1	36.8	-	87.6	11.53 **
Streptococcus sp.	3.0	14.0	96.8	-	-	14.78 **
B.subtilis	44.8	14.8	58.4	-	26.5	10.43 **
Proteus sp.	45.1	78.5	-	91.6	-	14.07 **
Shigella sp.	30.0	36.2	18.9	84.3	54.9	13.69 **

P. aeruginosa	1.50	4.0	40.0	6.5	-	9.51 **
C. albicans	28.3	8.6	18.8	9.4	30.1	9.04 **
A. niger	0.12	0.6	0.13	0.04	0.09	0.026 NS
*(-) means no inhibitory biofilm rate						
** (P<0.01), NS: Non-Significant.						

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