Research Article

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Chemical Screening and Antibacterial Activity of Crude Extracts of *Chlorella* sp. in Culture

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ABSTRACT

In this study, the antibacterial activity of methanol and acetone extracts of *Chlorella* sp. was examined. The chemical contents of the extracts were clarified by GC-MS analysis. Antibacterial activity of *Chlorella* sp. extracts was determined as a minimum inhibitory concentration by broth microdilution method against *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213. It was found that methanol and acetone extracts of *Chlorella* sp. showed antibacterial activity against *B. subtilis* ATCC 6633 (625 µg/ml and 1250 µg/ml, respectively), *E. faecalis* ATCC 29212 (>5000 µg/ml and 1250 µg/ml, respectively), *E. coli* ATCC 25922 (>5000 µg/ml), *P. aeruginosa* ATCC 27853 (>5000 µg/ml), *S. aureus* ATCC 29213 (2500 µg/ml) at the specified concentrations. In the chemical analysis of the extracts, it was determined that the fatty acids were in high amounts, 33,22%, and 40,41%, respectively, in the methanol and acetone extracts. Among the alternative methods to show activity against pathogenic microorganisms, algae can be a good natural resource. This study showed that *Chlorella* sp. contains high fatty acids and has the potential as an antibacterial agent of natural origin.

Keywords: Antibacterial activity, *Chlorella*, GC-MS

1. Introduction

Microalgae are a potential raw material source in various industrial fields due to the different biomolecules they contain. Valuable biomolecules such as FAME (fatty acid methyl esters), vitamins, proteins, sterols and pigments can be obtained at high concentrations, depending on the species and culture conditions [1-3]. Microalgae, which is a sustainable resource, can be used as a natural coloring agent and enriching additive in the food industry [4-7]. It is also accepted as a raw material source in the production of fertilizers in agriculture, feed additives in livestock, bioenergy [8-10]. In addition, microalgae are also utilized in cosmetic products such as shampoo and face cream [11,12]. Pharmaceutical applications focus on purposes such as supplements, drugs, and drug delivery [13,14].

Microalgae are organisms that have developed abilities to adapt to environmental conditions such as temperature changes, light, pH, and humidity and have the capacity to live with little water, nutrients, or carbon dioxide [15-17].

Chlorella sp. is a commercially produced green microalgae which can be found in marine and inland waters, commonly. The potential to be produced in high density and its biochemical composition are the reasons of this microalgae widely used for different industrial purposes. It is shown that this species may contain up to 40% lipid content [18]. Also, a study was conducted with *Chlorella vulgaris* shows that 10% EPS (exopolysaccharide) production was possible [19]. Studies indicate that *Chlorella* EPS has anti-cancer effects against colon cancer cells and has anti-asthmatic properties [19,20]. Wide range of antioxidants such as ascorbic acid, α-tocopherol, chlorophylls and carotenoids are found in microalgae species [21]. Studies have reported that *Chlorella* contains 2.5% chlorophyll a and 0.4% carotenoids [22-24].

Importance of microalgae production is already understood by industrial producers. It is estimated that global market of microalgae is more than 3 billion USD already and, Spirulina which is the most produced microalgae would reach to 2 billion USD in next 5 years [25,26]. Also, it is indicated that approximately 5000 tons of microalgae biomass produced yearly only for nutraceutical and pharmaceutical purposes [27]. The biochemical composition of microalgae contains many biomolecules that may have a positive effect on health. For instance, it is stated that bioactive phenolic compounds derived from microalgae obtained from species such as *Chlorella*, Spirulina and *Scenedesmus* can provide antidiabetic, antioxidant and anticancer benefits [27].

2. Material and Methods

2.1. Microalgal culture condition

Chlorella sp. (freshwater) strain was obtained from Ege University, Faculty of Fisheries. *Chlorella* sp. was cultured in 5 L flasks at 18 ^{0}C temperature and was illuminated continuously at 80 µmol.m^{-2}.s^{-1}. BG-11 [28] media (initial pHs were arranged to 7.5) was used for *Chlorella* sp. cultures. Mixing of the cultures was provided by air passing through a 0.2 µm syringe filter without any CO_2 addition. The microalgae were harvested at the stationary phase.

2.2. Preparation of extracts

The dried *Chlorella* sp. material was powdered. It was extracted by maceration using methanol and acetone solvents. The extraction process was repeated for 8 hours x 3 days. At the end of the process, the fractions were combined and dried under reduced pressure.

2.3. Gas chromatography-mass spectrometry (GC-MS)

The derivatization method adopted in our previous study was used for the preparation of the samples, before the GC-MS analysis of *Chlorella* sp. extracts [29]. The methoxymation procedure was performed before trimethylsilyl (TMS) derivatization because it was used to prevent multiple chromatographic peaks of sugars on the chromatogram *via* reacting with the carbonyl groups of the sugars and blocking the ring formation [30]. Before the methoxymation procedure, methoxyamine hydrochloride (MOX) (Sigma-Aldrich, Germany) was prepared freshly in pyridine (25 mg/ml). After that 50 µL of MOX solution was added to 1 ug extracts and oximation was carried out at 30°C for 90 minutes. The next step of derivatization, TMS derivatization was completed using 50 µL of N,O-Bis(trimethylsilyl)trifluoroacetamide + 1% Trimethylchlorosilane (Sigma-Aldrich, Germany) and held the samples in the incubator for another 45 min at 70°C.

Agilent 6890 model Gas Chromatography coupled with Agilent 5973N model mass selective detector (Santa Clara, USA) was used for analysis. Restek RTX-5MS (30 m × 0.25 mm i.d. × 0.25 µm) gas chromatography capillary column was used as a stationary phase (Bellefonte, USA). Gas chromatography grade (ultra-pure) helium at a rate of 1.5 mL/min was used as the carrier gas. The injection port, ion source, quadrupole, and transfer line temperatures were maintained at 280°C, 230°C, 150°C, and 280°C, respectively. The GC oven program was held at 50°C for 2 min, and then increased to 280°C at 4°C/min and held for 10 min. The total analysis time was 70 min. The mass range was 50-550 m/z and the scan rate was 0.45 scan per second in full scan mode. Electron ionization was carried out using 70 eV ionization energy. Compounds were determined and identified using Mass Hunter software (Qualitative Analysis B.07.00) and the NIST Mass Spectral Library.

2.4. Antibacterial activity

The antibacterial activity of the *Chlorella* sp. extracts was tested against *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213. Antibacterial activity was determined as a minimum inhibition concentration (MIC) by broth microdilution method in accordance with the European Society for Antimicrobial Susceptibility Testing recommendations [31]. Two-fold dilutions of the active substance to be tested were prepared in cation-adjusted Mueller Hinton Broth. A 1:100 dilution of the bacterial suspension with 0.5 McFarland standard was added to the dilutions in equal amounts. Plates were incubated for 18-20 hours at 35 ± 2°C. Ciprofloxacin and DMSO were used as controls.

3. Results and Discussion

Antibacterial activity of *Chlorella* sp. extracts was determined as a MIC value by broth microdilution method and it was found that the methanol extract of *Chlorella* sp. showed more efficacy against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 29213 with 625 µg/ml and 2500 µg/ml concentrations, respectively. Acetone extract of *Chlorella* sp. was found more effective against *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212, and *S. aureus* ATCC 29213 with 1250 µg/ml, 1250 µg/ml, and 2500 µg/ml concentrations, respectively. Antibacterial activity results were shown in Table 1.

Shaima et. al [32] showed that the methanol extract of *Chlorella* sp. UKM8- ME had antibacterial activity against methicillin-resistant *S. aureus* (390 µg/ml), *S. aureus* (1250 µg/ml), *B. subtilis* 2 (2500 µg/ml), *E. coli* (2500 µg/ml), *E. faecalis* (2500 µg/ml) strains similar to the results of this study, at different concentrations. According to Santhosh et. al [33], the antibacterial activity of *Chlorella* sp. SRD3 extracts by well diffusion method and as a MIC value against different pathogens, the maximum inhibition zone was evaluated in the ethyl acetate extract against *B. megaterium* (26 ± 0.6 mm), followed by methanol extract (23 ± 1.2 mm). The chloroform extract was found more effective against *E. faecalis* (15 ± 1.1 mm), in addition, the hexane extract showed efficacy most against *Serratia marcescens* (15 ± 1.2 mm). Moreover, the methanol extract had no activity against *E. coli*, *P. aeruginosa,* and *S. aureus* as an inhibitory concentration, and was found effective against *B. subtilis* (100 µg/ml) and *E. faecalis* (25 µg/ml). The ethanol extract activity results were 25, 1.5, 50, 25, and 25 µg/ml for *B. subtilis*, *E. faecalis*, *E. coli*, *P. aeruginosa*, and *S. aureus*, respectively. Hussein et. al [34] detected that *Chlorella vulgaris* extract had antibacterial activity against both Gram-

Table 1. Antibacterial activity results for tested extracts as MIC.

Extracts/Standard	Minimal inhibition concentrations (µg/ml)				
	S. aureus ATCC 29213	E. faecalis ATCC 29212	E. coli ATCC 25922	P. aeruginosa ATCC 27853	B. subtilis ATCC 6633
methanol extract	2500	> 5000	> 5000	> 5000	625
acetone extract	2500	1250	> 5000	> 5000	1250
Ciprofloxacin	0.25	0.5	0.0078	0.25	0.125

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negative (*Enterobacter* sp. - 25 mm, *Proteus* sp. - 24 mm, *E. coli* -15 mm) and positive bacteria (*S. aureus* - 20 mm, *Lactobacillus acidophilus* - 18 mm, *Streptococcus pyogenes* - 15 mm) except *Klebsiella* sp. by agar well diffusion method.

Figures 1 and 2 show the major compounds identified in *Chlorella* sp. extracts by GC-MS. The analyses show 63 and 38 compounds (Table 2), respectively, in methanol and acetone extracts. Methanol extract of *Chlorella* sp. contains 0.39% ketoaldonic acid, 0.56% nucleoside, 0.58% ketone, 0.62% amine, 0.73% terpene, 0.75% amide, 0.87% phytosterol, 1.01% fatty acid ester, 1.45% acid, 1.51% carboxylic acid, 2.44% carbamide, 3.39% alkylglycerol, 4.18% nucleobase, 7.75% amino acid, 10.59% glucosylglycerol, 12.05% sugar alcohol, 17.91% sugar, 33.22% fatty acid.

However, the acetone extract of *Chlorella* sp. contains 0.14% fatty alcohol, 0.36% alkaloid, 0.48% acid, 0.69% phytosterol, 0.79% nucleobase, 1.43% glucosylglycerol, 1.63% carboxylic acid, 1.94% ketone, 2.18% sugar, 5.14% amine, 5.77% sugar alcohol, 8.41% acylglycerol, 8.89% diol, 10.47% amide, 11.27% alkylglycerol, 40.41% fatty acid.

This study examined the antibacterial activity of methanol and acetone extracts of *Chlorella* sp. In addition, the chemical contents of the extracts were clarified by GC-MS analysis. The methanol extract of *Chlorella* sp. showed more efficacy against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 29213 with 625 µg/ml and 2500 µg/ml concentrations, respectively. Acetone extract of *Chlorella* sp. was found more effective against *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212, and *S. aureus* ATCC 29213 with 1250 µg/ml, 1250 µg/ml, and 2500 µg/ml concentrations, respectively. In the chemical analysis of the extracts, it was determined that the fatty acids were in high amounts, 33.22%, and 40.41%, respectively, in the methanol and acetone extracts. The antimicrobial properties of fatty acids have been known, and they are effective against pathogens, including multi-drug resistant bacteria (MDRB). Algae-derived natural compounds are thought to be useful in identifying promising drug candidates [35-37].

4. Conclusions

Algae extracts have many different bioactive compounds and these compounds are used in the characterization of algae and may also be responsible for the activity seen in microorganisms. Bacteria develop resistance to antibiotics by various mechanisms as a result of inappropriate and uncontrolled

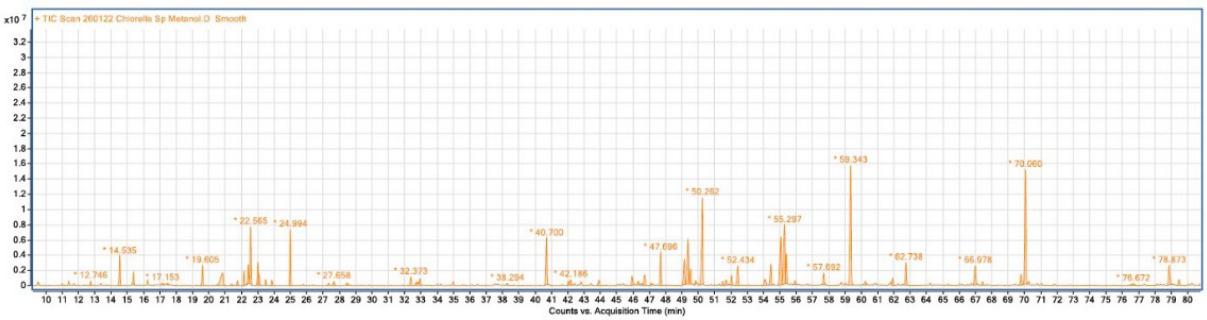

Figure 1. GC-MS chromatogram of *Chlorella* sp. methanol extract

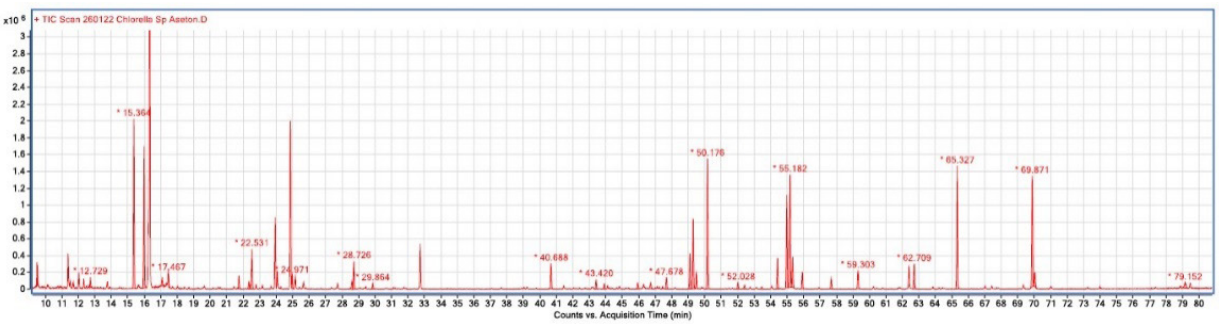

Figure 2. GC-MS chromatogram of *Chlorella* sp. acetone extract

Table 2. Compounds identified by GC-MS in *Chlorella* sp. methanol and acetone extracts

#	RT (min)	Identified Compounds	Area Sum % in Methanol Extract	Area Sum % in Acetone Extract	Classification
1	8.37	Serotonin	0.31	4.31	Amine
2	9.52	Ethylene glycol	-	0.51	Diol
3	12.75	Lactic Acid	0.25	0.46	Carboxylic acid
4	14.54	Alanine	1.83	-	Amino Acid
5	15.38	(2S,3R)-3-[(4E,7E)-Nona-4,7-dienoyl]-N,N-bis(trimethylsilyl) oxirane-2-carboxamide	0.75	9.94	Amide
6	15.99	Pinacol	-	8.38	Diol
7	17.09	Pentanamide, N-[2-(indol-3-yl)] ethyl-	-	0.53	Amide
8	17.15	γ-Hydroxybutyric acid	0.17	-	Carboxylic acid
9	17.47	Aceburic acid	0.12	0.53	Carboxylic acid
10	19.61	Valine	1.35	-	Amino Acid
11	20.86	Urea	2.44	-	Carbamide
12	21.39	Serine	0.13	-	Amino Acid
13	21.75	Ethanolamine	0.31	0.83	Amine
14	22.15	Leucine	0.93	-	Amino Acid
15	22.41	Phosphonic Acid	1.2	0.48	Acid
16	22.57	Glycerol	3.9	2.75	Sugar alcohol
17	23.00	Proline	1.51	-	Amino Acid
18	23.07	Isoleucine	0.63	-	Amino Acid
19	23.48	Glycine	0.4	-	Amino Acid
20	23.87	Succinic acid	0.33	-	Carboxylic acid
21	24.99	Uracil	3.88	0.79	Nucleobase
22	27.35	Threonine	0.16	-	Amino Acid
23	27.66	Thymine	0.3	-	Nucleobase
24	28.48	Aspartic Acid	0.15	-	Amino Acid
25	28.61	Propionic acid	-	0.64	Carboxylic acid
26	28.73	3-Methyl-4-(4-methylsulfanyl-benzylidene)-4H-isoxazol-5-one	-	1.94	Ketone
27	29.86	Epistephamiersine	-	0.36	Alkaloid
28	32.37	Pyroglutamic acid	0.66	-	Amino acid
29	32.72	γ-Aminobutyric acid	0.28	-	Carboxylic acid

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#	RT (min)	Identified Compounds	Area Sum % in Methanol Extract	Area Sum % in Acetone Extract	Classification
30	32.93	1H-Inden-1-one, 5-(dimethylamino)-2,3-dihydro-	0.58	-	Ketone
31	38.29	Ribonic acid	0.11	-	Carboxylic acid
32	40.70	Xylitol	3.52	1.83	Sugar alcohol
33	42.04	Phosphoric acid	0.25	-	Acid
34	42.19	2-Keto-l-gluconic acid	0.39	-	Ketoaldonic acid
35	43.92	Myristic Acid	0.48	0.35	Fatty acid
36	45.95	Pentadecanoic acid	-	0.48	Sugar
37	46.32	D-(-)-Fructose	1.02	-	Sugar
38	46.52	D-Altrose	0.12	-	Sugar
39	46.71	Allose	1.16	0.49	Sugar
40	47.12	Pentadecanoic acid	0.17	-	Fatty acid
41	47.70	Sorbitol	2.61	0.88	Sugar alcohol
42	49.15	Linoelaidic acid	2.29	2.55	Fatty acid
43	49.31	Norlinolenicacid	-	4.93	Fatty acid
44	49.38	Arachidonic acid	3.84	-	Fatty acid
45	49.53	Palmitoleic acid	1	1.15	Fatty acid
46	49.83	Gluconic acid	0.25	-	Carboxylic acid
47	50.26	Palmitic acid	8.61	9.11	Fatty acid
48	51.51	8,11-Octadecadienoic acid, methyl ester	0.3	-	Fatty acid ester
49	51.71	9,12,15-Octadecatrienoic acid, methyl ester	0.4	-	Fatty acid ester
50	52.03	Heptadecanoic acid	0.77	0.43	Fatty acid
51	52.43	Myo-Inositol	2.02	0.31	Sugar alcohol
52	53.46	1-Octadecanol	-	0.14	Fatty alcohol
53	54.09	Phytol	0.73	-	Terpene
54	54.46	Mead acid	1.73	2.15	Fatty acid
55	55.07	Linoleic acid	5.14	6.59	Fatty acid
56	55.30	α-Linolenic acid	6.69	9.42	Fatty acid
57	55.40	Vaccenic acid	2.23	2.08	Fatty acid
58	55.94	Stearic acid	0.27	1.17	Fatty acid
59	57.69	1-O-Tetradecylglycerol	0.97	0.75	Alkylglycerol

#	RT (min)	Identified Compounds	Area Sum % in Methanol Extract	Area Sum % in Acetone Extract	Classification
60	59.34	Glyceryl-glycoside	10.59	1.43	Glucosylglycerol
61	60.25	1-O-Pentadecylglycerol	0.29	-	Alkylglycerol
62	61.92	Uridine	0.56	-	Nucleoside
63	62.74	1-O-hexadecylglycerol	1.85	1.92	Alkylglycerol
64	65.33	1-Monopalmitin	-	8.6	Alkylglycerol
65	66.98	Maltose	2	-	Sugar
66	67.43	Batyl alcohol	0.28	-	Alkylglycerol
67	69.79	Turanose	0.98	-	Sugar
68	69.87	Glycerol monostearate	-	8.41	Acylglycerol
69	70.06	Trehalose	10.67	1.69	Sugar
70	76.67	Linoleic acid, isopropyl ester	0.31	-	Fatty acid ester
71	78.87	α-D-Lactose	1.96	-	Sugar
72	79.15	Ergosterol	-	0.69	Phytosterol
73	79.48	Dehydroergosterol	0.52	-	Phytosterol
74	80.83	Ergost-8(14)-en-3-ol, (3β)-	0.35	-	Phytosterol

use of antibiotics. As a result of this resistance, antibiotics become ineffective against bacteria. This is an important public health problem worldwide, and alternative treatment methods are being investigated. Among the alternative methods that will show activity against pathogenic microorganisms, algae can be a good natural resource. This study showed that *Chlorella* sp. contains high fatty acids and has the potential as an antibacterial agent of natural origin.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Statement of Contribution of Researchers

Concept – K.C.T., G.Ç.E., Ş.Y., M.E.K., M.M.H.; Design – K.C.T., G.Ç.E., Ş.Y., M.E.K., M.M.H.; Supervision – K.C.T., M.E.K., M.M.H.; Resources – K.C.T., G.Ç.E., Ş.Y., M.E.K., M.M.H.; Materials –K.C.T., G.Ç.E., Ş.Y., M.E.K., M.M.H.; Data Collection and/or Processing – K.C.T., M.E.K., M.M.H.; Analysis and/or Interpretation – K.C.T., M.E.K., M.M.H.; Literature Search – K.C.T., G.Ç.E., Ş.Y., M.E.K., M.M.H.; Writing – M.M.H.; Critical Reviews – K.C.T., G.Ç.E., Ş.Y., M.E.K., M.M.H.

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