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Evaluation of antimicrobial activities of microalgae Scenedesmus dimorphus extracts against bacterial strains

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ABSTRACT

The study was conducted to analyze the existence of bioactive phytochemicals extracts in green alga *Scenedesmus dimorphus* and their antimicrobial role. Various solvents such as methanol, ethanol, N-hegzane and diethylether were used for extraction. The extracts of of *Scenedesmus dimorphus* were tested against two Gram - positive bacteria (*Bacillus subtilis* and *Micrococcus luteus*), a Gram - negative bacterium (*A. hydrophila*) and *Escherichia coli* by the agar well diffusion method. Four different methanolic, ethanolic, hexane and diethylether extracts showed effective inhibition against different bacterial pathogens. Diethylether extract was very effective against bacterial strains compared to other extracts. Methanolic extract effectively inhibited *A. hydrophila* in comparison with other extracts, while ethanolic extract did not have any inhibitory effect on the bacterium. Methanolic, ethanolic, hexane and diethylether extracts were analyzed by GC mass. The most abundant compounds in methanolic extract of *S. dimorphus* included esters, plasticizer compound and terpens, while in the ethanolic, N-hexane and diethylether extracts, the most abundant compounds were found to be plasticizers, hydrocarbon and esters. These results indicate the presence of promising antimicrobial compounds in the examined algal species. Further phytochemical studies are required to elucidate the structutre and detailed activities of these compounds. So, we achieved antimicrobial activity in the methanolic, ethanolic, hexane and ether extracts of green microalgae against some pathogenic bacteria as well as employing GC mass autogram for *S. dimorphus* extracts for preliminary detection of active constituents.

Key words: *Scenedesmus dimorphus*, Antibacterial, antimicrobial, *Aeromonas hydrophila*, *Bacillus subtilis.*

INTRODUCTION

Microalgae has become a very popular source of antibacterial agents and offer numerous advantages for antimicrobial studies due to their huge biodiversity and fast growth rate (Pulz & Gross 2004). Microalgae are new source of structurally - novel and biologically - active compounds in the pharmaceutical industry (Patra *et al*. 1944; Ely *et al.* 2004 and Tuney *et al.* 2006). Some algae produce anti-oxidative, anti-inflammatory and anti-cancer compounds (Justo *et al.* 2001). The cell extracts and active constituents of different algae have been shown to uphold antibacterial activity against Gram-positive and Gram-negative bacteria (Borowitzka *et al.* 1992). Algal biomass and compounds have an extensive range of potential applications from animal feed in aquaculture to human nutrition and health products (Borowitzka 1988; Soltani *et al.* 2005; Skulberg 2006). Bacterial resistance to antibiotics has become a major problem in health care and has become even more problematic to treat newly-evolving pathogenic bacteria (Sieradzki *et al.* 1999). Numerous studies suggest that algae can

produce hundreds to thousands of diverse chemical compounds with different biological activities. These substances can inhibit the growth of microorganisms or eradicate them. There is a continuous need to discover new antimicrobial compounds with chemical structures and novel mechanism of action because of the development of resistance to the antibiotics (Bhagavathy *et al.* 2011). Among the major bioactive constituents of algae with demonstrated antimicrobial potential, proteins, polysaccharides, polyunsaturated fatty acids (PUFAs), especially EPA and DHA, amino acids, and antioxidants (polyphenols, flavonoids, and carotenoids) are the most important ones (Senthilkumar & Sudha 2012; Al-Saif *et al.* 2014). Aquatic microorganisms and algae produce a pool of underinvestigated secondary metabolites and are potential sources of drug-like compounds to inhibit pathogens (Dussault 2016). Several recent studies have been revealed that seaweed and algae are potential sources that can be used as antimicrobial products (Rabia *et al.* 2013; Al-Saif *et al.* 2014; Maftuch 2016). Large number of literature have been released about compounds derived from algae with antibacterial activity, such as acrylic acid, halogenated aliphatic compounds, terpenes (Ming 2017). However, the identification of compounds directly responsible for the antimicrobial potential of algae is still a relatively incipient field of research, mainly owing to the new kinds of compounds found in recent years (Amaro *et al.* 2011, Pina- Perez 2017). In this study, we investigated the antibacterial activity of methanolic, hexane, diethylether, and ethanolic extracts of *S. dimorphus* along with their antibacterial compounds that were extracted using specific eluent. The bacterial species challenged, belong to a type of pathogenic bacteria that easily invade the freshwater and brackish water fish. So by testing in vitro results of an extract of *S. dimorphus*, we get pertinent information about power of its extract against freshwater and brackish water bacteria.

MATERIALS AND METHODS

Culturing and Growth of Algal organisms

Green unicellular alga *Scenedesmus dimorphus* is a typical freshwater species belonging to class Chlorophyceae and order Chlorococcales. The species was isolated from a earthen freshwater pond and maintained and cultured in the laboratory under appropriate culture conditions. The Zehnder medium was used throughout the maintenance and experimental period (Kotai 1972; Schlosser 1994). A 1000 ml - conical flasks containing 5 ml growth medium were inoculated with known number of cells (5×10^4 cell.mL^{-1}) at 30°C temperature on shelves illuminated by fluorescent tubes (light intensity of 2500 lux). No aeration was provided. The cultures were hand shaken every day to prevent algal cells settle down. The algae were cultured for 10 days and harvested.

Extraction of bioactive metabolites

The dried biomass was sonicated with liquid nitrogen and then was extracted with 96% methanol and 95% ethanol (Cowan 1999 and Zheng *et al.* 2011). Extracts were centrifuged at 100rpm for 10 min and further concentrated in vacuum under reduced pressure. The stock solutions of extract were prepared in DMSO at 50 mg.ml^{-1} for evaluation of antimicrobial activity (Hussain *et al.* 2011; Jelodarian *et al.* 2013).

Preparation of antimicrobial agents

Methanolic, ethanolic, hexane and diethyl ether extracts were tested against a panel of microorganisms including gram-ve, *Aeromonas hydrophila* and *Escherichia coli* and gram +ve *B. subtilis* and *Micrococcus luteus*. Stock cultures were maintained on tryptic soy broth and nutrient agar medium at 40°C, then sub-cultured in nutrient broth at 37°C prior to antimicrobial test. Ethanolic, methanolic, N-hexane and diethylether extracts were dissolved in 5ml DMSO, impregnated with 50µl of algal extracts and introduced to the upper layer of the seeded agar plate. The plates were incubated overnight at 37°C. Negative controls were prepared by using DMSO. Penicillin was used as positive reference standard. After incubation, the clear zone

around the discs were measured and expressed in mm as a measure of inhibitory effects.

GC/MS Analysis of *Scenedesmus dimorphus* crude extract

Methanolic, ethanolic, N-hexane and diethyl ether extracts of *S. dimorphus* were analyzed by GC/MS. The GC/MS analyses were performed by a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, HP-5MS fused silica capillary column (15 m, 0.251mm, 0.1 mm film thickness). An electron ionization system with ionization energy of 118 eV was used for GC/MS detection. Helium gas was used as carrier at a constant flow rate of 3 $mL.min^{-1}$. The temperature increased from 50°C to 120°C. The injector and MS transfer line temperature were set at 300 °C. Quantification of all identified components was examined using a percent relative peak area. Tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

RESULTS

Methanolic extracts of *S. dimorphus* were tested against Gram - positive bacteria including *B. subtilis*, *M. luteus* as well as Gram - negative species such as *A. hydrophila* and *E. coli.*

The extract of *S. dimorphus* effectively inhibited *B. subtilis*, *M. luteus, A. hydrophila* and *E. coli* with inhibition zones of 13, 13.3, 21.3 and 11 mm respectively. Ethanolic extracts of *S. dimorphus* inhibited the growth of *M. luteus*, *E. coli* and *B. subtilis* with inhibition zones of 15.66, 13.66 and 11.66 mm. However, there was no effective inhibition or control on *A. hydrophila*.

N- hexane extracts of *S. dimorphus* inhibited the growth of *M. luteus* and *B. subtilis* with inhibition zones of 13 and 12.66 mm; respectively.

It also inhibited the growth of *A. hydrophila* and *E. coli* only with smaller inhibition zone about 11.33 and 7 mm; respectively. Diethylether extract of *S. dimorphus* inhibited the growth of *M. luteus*, *B. subtilis*, *A. hydrophila* and *E. coli* with inhibition zones of 23.6 , 18, 21.6 and 22.66 mm; respectively.

The diethylether extract of *S. dimorphus* effectively inhibited the growth of *M. luteus* with an inhibition zone of 23.6 mm.

Since bacterial fish diseases like saddleback disease, erythrodermatitis, red spot disease, fin rot, furunculosis and vibriosis are caused by Gram-negative bacteria, the results showed that these eukaryotic algae produce active secondary metabolites which can inhibit proliferation of fish pathogens.

GC mass Analysis of *S. dimorphus* methanolic crude extract

The chemical composition of ethanolic, methanolic, N-hexane and diethylether extracts of *S. dimorphus* determined by GC mass are shown in Fig. 1. The spectrum analysis revealed the presence of 4 distinct peaks. Noteworthy, among all the examined organisms, Gram - positive *B. subtilis* and Gram - negative *A. hydrophila* displayed maximum susceptibility to metabolic extract of *S. dimorphus*. The results of GC mass analyses confirmed that all extracts of *S. dimorphus* had moderate inhibitory effects against the examined microorganisms.

Table 1. Antibacterial activities of ethanolic, methanolic, N-hexane and diethylether extracts of *S. dimorphus* against Gram - positive and Gram - negative bacteria presented by inhibition zone diameter (mm).

Algal extracts	Inhibition zone diameter of bacterial strains			
	Gram-positive		Gram-negative	
	B. subtilis	***M. luteus***	***E. coli***	***A. hydrophila***
Ethanol	11.66	15.66	13.66	0
Methanol	13	13.33	11	21.33
Hexane	12.66	13	7	11.33
Diethylether	18	23.6	22.66	21.6

Table 2. GC/MC mass analysis of ethanolic crude extract of *S. dimorphus.*

Name of compound	Group	Molecular formula	Peak area %
Limonene	hydrocarbon	$C_{10}H_{16}$	98
Eicosane	hydrocarbon	$C_{20}H_{42}$	96
Hexadecane	hydrocarbon	$C_{16}H_{34}$	95
1,2-Benzenedicarboxylic acid	Carboxylic acid	$C_8H_6O_4$	95
Diethyl Phthalate	ester	$C_{12}H_{14}O_4$	95
Methyl dihydrojasmonate	ester	$C_{13}H_{22}O_3$	98
Hexamethyl-pyranoindane	hetroxyl	C18H26O	96

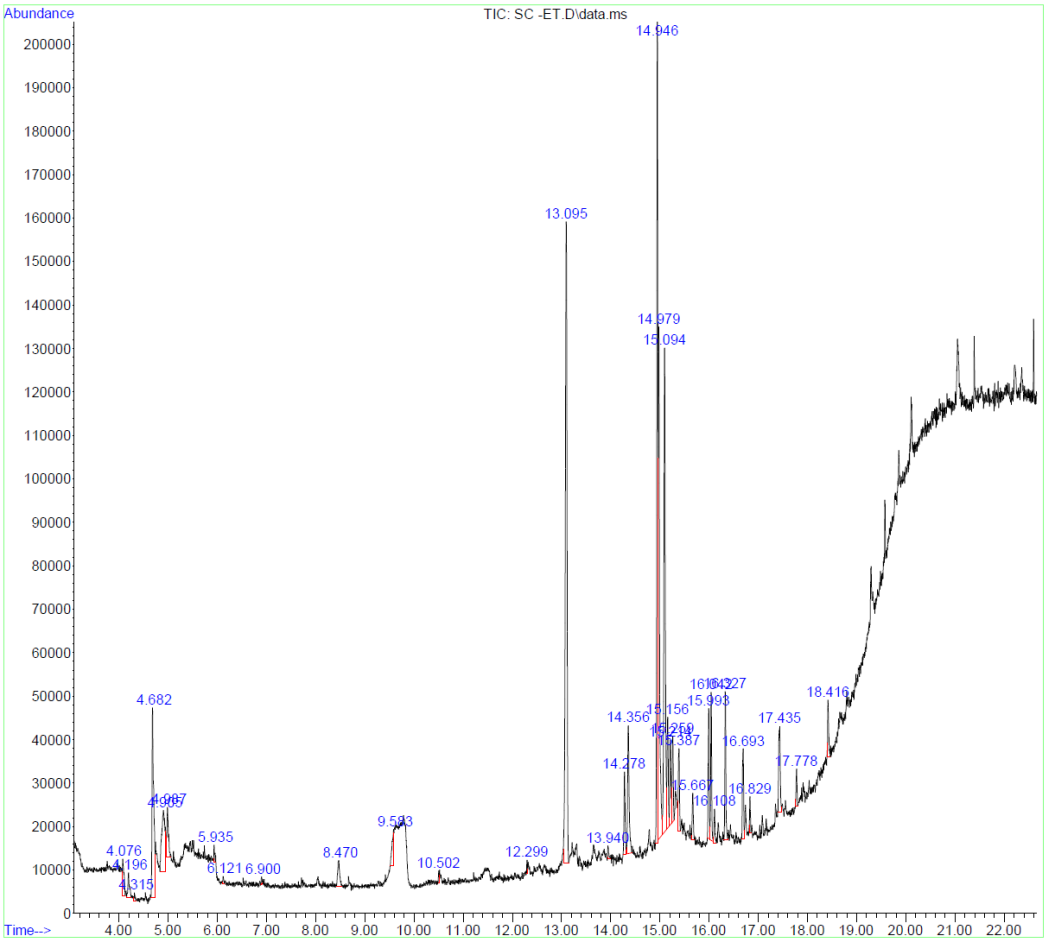

Fig. 1. Gas chromatographic profile of the major constituents in ethanolic extract of *S. dimorphus.*

Table 3. GC mass analysis of methanol crude extract of *S. dimorphus.*

Name of compound	Group	Molecular formula	Peak area (%)
d-Limonene	Terpen	$C_{10}H_{16}$	98
Azulene	Hydrocarbon	$C_{10}H_8$	96
Phenol	Acyclicditerpenealcohol	C_6H_6O	95
Methyl dihydrojasmonate	Ester	$C_{13}H_{22}O_3$	98
Heptadecane	hydrocarbon	$C_{17}H_{36}$	95
Hexadecanoic acid	Ester	$C_{16}H_{32}O_2$	99
Hexamethyl-pyranoindane	Hetroxyl	C18H26O	96
9,12-Octadecadienoic acid (Z,Z)-	Carboxylic acid	$C_{18}H_{32}O_2$	99
8-Octadecenoic acid	Carboxylic acid	$C_{18}H_{34}O_2$	99

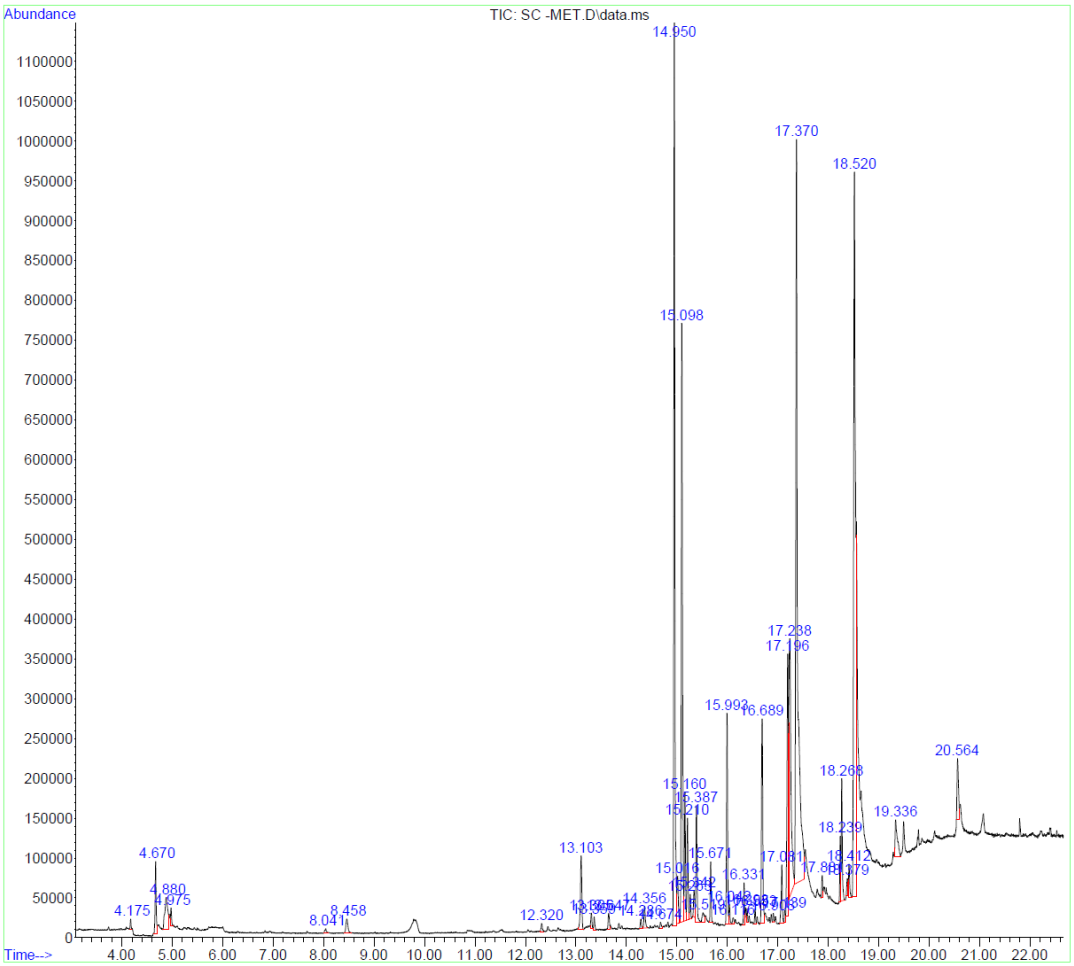

Fig. 2. Gas chromatographic profile of the major constituents in methanolic extract of *S. dimorphus*.

Table 4. GC mass analysis of hexane crude extract of *S. dimorphus*.

Name of compound	Group	Molecular formula	Peak area (%)
Limonene	Hydrocarbon	$C_{10}H_{16}$	98
Diethyl Phthalate	Ester	$C_{12}H_{14}O_4$	98
Dihydro methyl jasmonate	Acyclicditerpenealcohol	C_6H_6O	98

Table 5. GC mass analysis of diethylether crude extract of *S. dimorphus*.

Name of compound	Group	Molecular formula	Peak area (%)
Cyclopentaneacetic acid	Ester	$C_{13}H_{22}O_3$	89
Diethyl Phthalate	Ester	$C_{12}H_{14}O_4$	98
Dihydro methyl jasmonate	Acyclicditerpenealcohol	C_6H_6O	98
sane	Hydrocarbon	$C_{20}H_{42}$	92

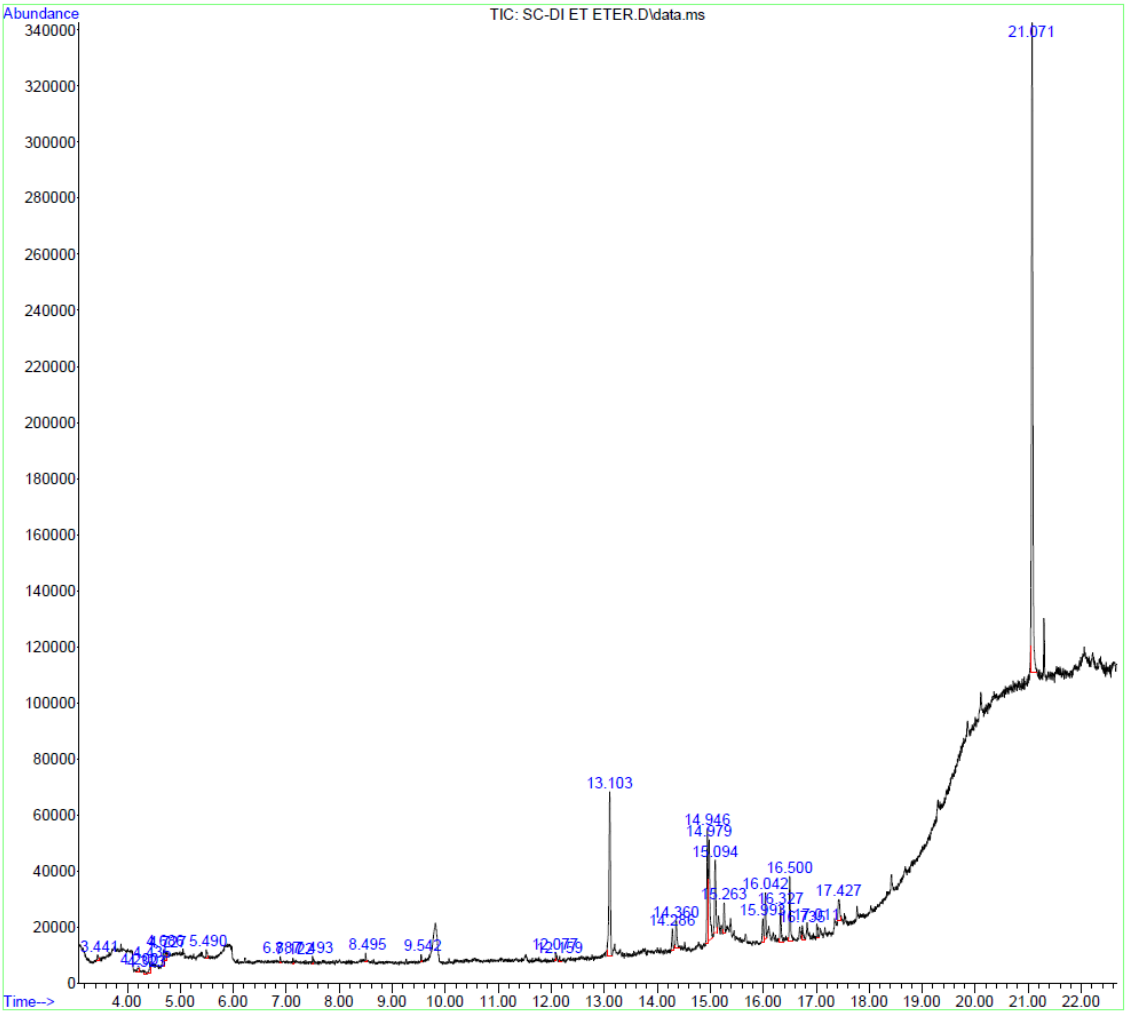

Fig. 3. Gas chromatographic profile of the major constituents in *S. dimorphus* N-hexane extract.

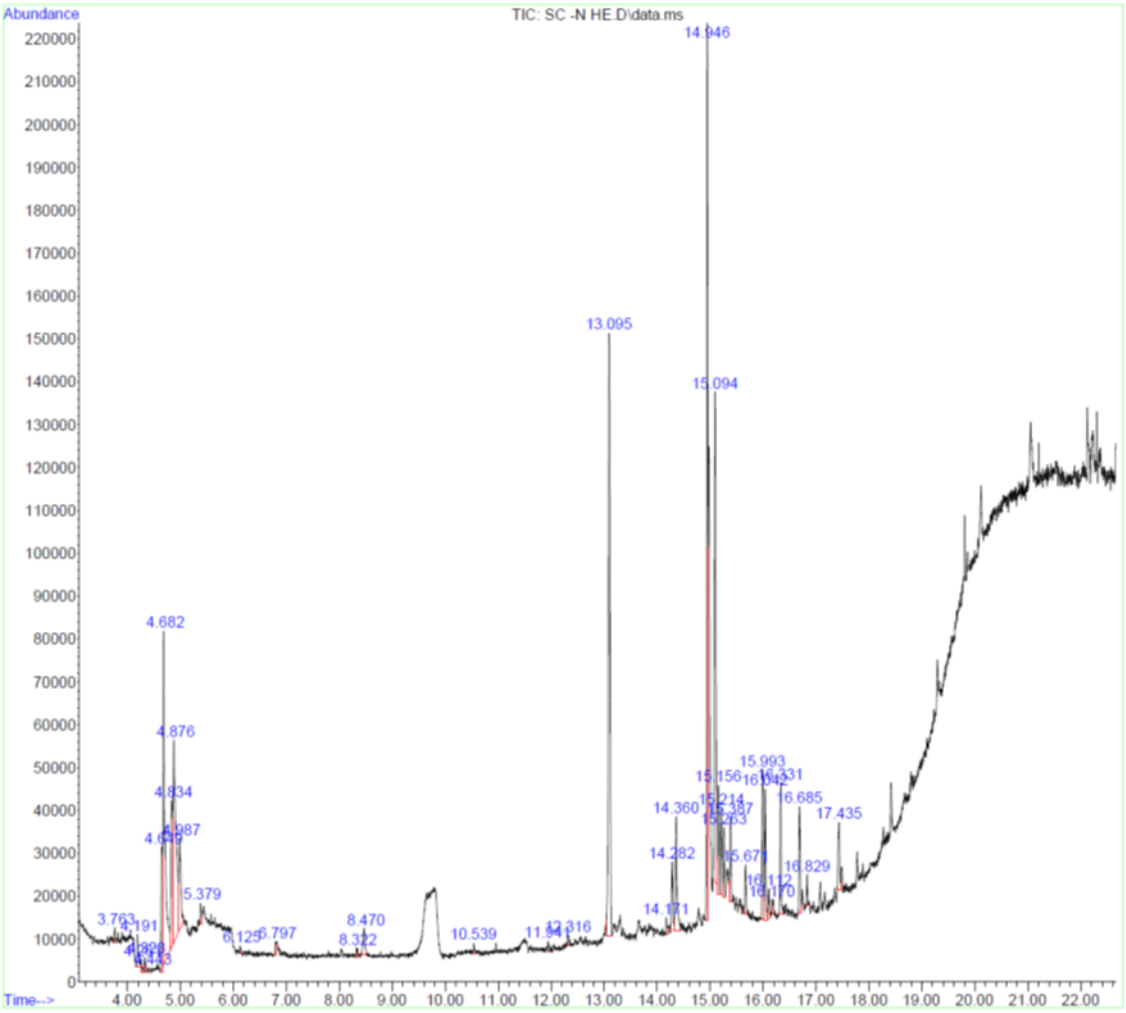

Fig. 4. Gas chromatographic profile of the major constituents in diethylether extract of *S. dimorphus.*

DISCUSSION

Emerging resistance to antibiotics has raised serious concerns regarding the next source of new chemical entities that can meet the challenge of continually emerging drug resistance. Although considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antimicrobial compounds, nature still remains the richest and the most versatile source for new antibiotics (Prasanna 2016). *Scenedesmus* spp. have been reported to produce antimicrobial substances, which from the pharmaceutical's point of view are a good source of new bioactive compounds. Some recent studies reveal seaweed and algae as a potential source of antimicrobial products (e.g. Alghazeer *et al.* 2013; Mendes *et al.* 2013; Al-Saif *et al.* 2014). The antibacterial activities of *S.dimorphus* extracts against selected bacteria varied depending on the species and nature of solvents used for extraction. Methanolic extract of *S. dimorphus* showed the highest inhibitory on *A. hydrophila*. Inhibition zone with diameter > 15 mm has strong inhibitory activity, diameter 9–14 mm has moderate inhibitory activity and diameter < 8 mm has weak inhibitory activity (Genovese *et al.* 2012; Maftuch 2016). Methanolic, N-hexane and diethylether extracts of *S. dimorphus* exhibited inhibitory activities against *A. hydrophila* which is in accordance with Guedes *et al.* (2011), Padmini *et al.* (1986), Prashantkumar *at al.* (2006). In addition, all *S. dimorphus* extracts had clear antibacterial activities against *B. subtilis,* in agreement with Ordog *et al.* (2004), Ghasemi *et al.* (2004) and Desbois *et al.* (2009).

Methanolic extract of *S. dimorphus* effectively inhibited the growth of *B. subtilis* and *A. hydrophila* similar to Ostensvik *et al.* (1998) who reported effective inhibitory of ethanolic extracts of *S. dimorphus* on *B. subtilis.* Looking at the results of several previous studies on antimicrobial activities of algae extracts one may approve that the methanolic extract was more effective against the selected bacterial strains in this study.

Methanolic extracts of *S. dimorphus* inhibited *A. hydrophila,* while ethanolic extract practically did not have any control on *A. hydrophila,* although imposed some inhibition on *B. subtilis* which is agreement with Ghasemi *et al*. (2004) who reported inhibitory effects of *Scenedesmus* sp. on *B. subtilis* and *M. luteus*. It suggests that antimaicrobial activity varies with algae speceis and extracts from different *Scenedesmus* species inhibit bacterial strains with different efficiency. In our study methanolic extract of *S. dimorphus* strongly inhibited the growth of *A. hydrophila* and the diameter of inhibition zone was 21.3 mm. Ethanolic extract inhibited the growth of all bacterial strains examined except for *A. hydrophila* which is in contrast with Kulik (1995) and Rizk (2006).

It is assumed that larger zones of inhibition produced by N-hexane reflect the non-polar nature of the active components (Moreau *et al*. 1984). Ether extracts of *S. dimorphus* in present study showed remarkable antibacterial activity, compared to other solvents. Beena & Krishnika (2011) examined antibacterial activity of *Scenedesmus* sp. isolated from a natural pond against three pathogenic bacteria with different solvent. The methanolic extracts showed much better antimicrobial activity and the extent of inhibition depended on both algal species and the solvents used for extraction (Prakash *et al.* 2011; and Radhika *et al*. 2012). Diethylether was by far the best solvent for extraction of effective antimicrobial materials against bacterial strains in this study except for *A. hydrophila.* Methanolic extract strongly inhibited the growth of these bacteria. Ethanolic and N-hexane fractions seemed to be specific, particularly against the examined Gram-positive bacteria. Some studies on the effectiveness of extraction methods highlighted that methanol extraction yields higher antimicrobial activity than N-hexane and ethyl acetate (Rosell 1987; Moreau 1988; Sastry 1998), whereas other reports emphasized that chloroform is better than methanol and benzene (Febles *et al.* 1995). It is clear that using organic solvents always provides a higher efficiency in extracting compounds for antimicrobial activities compared to water-based methods (Masuda 1977; Lima-Filho 2002). According to our results, diethylether caused better halozones than methanol, N-hexane, and ethanol. GC mass analysis indicated the presence of Limonene (a poly unsaturated fatty acid), Eicosane and Hexadecanoic acid may be reasons for antimicrobial effects of *S. dimorphus* extract. Desbois *et al.* (2008) detected the presence of poly unsaturated fatty acid in the methanolic extract of microalgae and reported that a polyunsaturated fatty acid hexadecatrienoic acid n-4 is active against Gram +ve and Gram -ve bacteria and also is highly active against multidrug - resistant *S. aureus*. Tuney *et al*. (2006) also indicated that antimicrobial effect could be related to volatile compounds in the samples such as hydrogen peroxide, terpenoid, and bromoether as well as volatile fatty acid compounds. The most abundant compounds in methanolic extract included terpens, phenol, ester, carboxylic acid; in ethanolic extract, hydrocarbon, carboxylic acid and ester; while in N-hexane and diethylether extracts the most abundant components were Acyclicditerpenealcohol, hydrocarbon and ester. These results are compatible with Kannan *et al*. (2010) who explained that the important compounds identified as antimicrobial agents from *S. dimorphus* included terpenes, carbohydrates and phenols. These results are an indication to the presence of promising antimicrobial compounds in methanolic, ethanolic, hexane and diethylether extracts of *S. dimorphus*. Our goal in this study was to reveal the biological production of bioactive compounds by some green algae. The

study confirmed that *S. dimorphus* possesses biologically - active compounds. In this study the used solvents were capable to extract diverse biological compounds from the microalgae with antimicrobial activities

CONCLUSION

Effective antimicrobial potential has been demonstrated against several bacterial strains by microalgae compounds. However, some contradictions may be found in the literature as a result of the different strains used in the determination of antimicrobial activity, different methods of extraction, and different ranges of algal material concentration used in the assays. The promising results of bacterial growth inhibition and inactivation by algal compounds urges further phytochemical studies to elucidate the components responsible for antimicrobial activity of these extracts against various bacteria. On the other hand, the development of algal culturing methods and the use of modern technology in breeding will decrease the cost of microalgae production and increase availability of resources for extraction of antimicrobial agents. We claim based on our study that *S. dimorphus* could be used as a potential source of antibacterial compounds against pathogenic strains if extracted effectively with suitable solvents.

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ارزیابی فعالیت ضدمیکروبی عصاره‌های ریزجلبک *Scenedesmus dimorphus* بر گونه‌های باکتریایی

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چکیده

فعالیت ضدمیکروبی عصاره‌های ریزجلبک *Scenedesmus dimorphus* علیه دو باکتری گرم مثبت *Micrococcus luteus* و *Bacillus subtilis* و دو باکتری گرم منفی *Escherichia coli* و *Aeromonas hydrophila* با استفاده از روش انتشار در آگار ارزیابی شد. برای این منظور عصاره‌های اتانولی، متانولی، ان هگزانی و دی‌اتیل‌اتری استخراج شد. نتایج نشان داد که عصاره اتری در برابر باکتری‌های مورد مطالعه از عصاره های دیگر موثرتر بود. عصاره متانولی دارای خاصیت مهارکنندگی علیه باکتری *A. hydrophila* بود، در حالی که هیچ یک از غلظت‌های عصاره اتانولی خاصیت مهاری بر روی این باکتری نشان ندادند. برای شناسایی ترکیبات موجود در عصاره‌های اتانولی، متانولی، هگزانی و دی‌اتیل‌اتری گاز-کروماتوگرافی انجام شد و نتایج نشان دادند که بیشترین ترکیبات در عصاره‌ها، هیدروکربن‌ها و استرها بودند. این نتایج نشانه‌ای از حضور ترکیبات ضدمیکروبی امیدوارکننده را در گونه جلبکی مورد مطالعه نشان داد که می‌تواند به عنوان دارو در کنترل و مهار بیماری‌ها استفاده شود. هدف از این بررسی نشان دادن فعالیت ضدمیکروبی عصاره‌های متانولی، اتانولی، هگزان و اتری ریز جلبک سبز *S. dimorphus* در برابر برخی از باکتری‌های بیماری‌زا بود و علاوه بر این، گاز کروماتوگرافی عصاره *S. dimorphus* برای تشخیص اولیه ترکیبات فعال در مهار فعالیت باکتری‌ها استفاده شد.

*مولف مسئول