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ANTIMICROBIAL CHARACTERISTICS OF BIOACTIVE COMPOUNDS BY A THERMOTOLERANT MICROALGA *CHLORELLA VULGARIS* ISOLATED FROM THERMAL SPRINGS OF TURKEY

Nur Koçberber Kılıç[1], Elif Safran[1], Gönül Dönmez[1]*

Address(es): Nur Koçberber Kılıç
[1]Department of Biology, Faculty of Science; Ankara University, 06100, Ankara, TURKEY, +90312 2126720.

*Corresponding author: nrkilic@ankara.edu.tr

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ABSTRACT

This study presents bioactive character of 10 thermal microalgae that were isolated from thermal springs (Turkey). Strain A was the most successful microorganism with the maximum antimicrobial activity. Strain A was identified as *C. vulgaris* according to its 18S rDNA. The effect of nitrogen concentrations (0.5 g/L, 1.0 g/L, and 1.5 g/L) and temperatures (30 °C and 45 °C) on bioactive compounds production by thermotolerant *C. vulgaris* was studied. The highest antimicrobial effect was found in the biomass when microalga was cultivated in media with 1.0 g/L nitrogen at 30 °C. On the other hand, stress conditions like 45 °C and media with 1.5 g/L nitrogen, microalgae produced more efficient bioactive compounds then at 30 °C in media with 1.5 g/L nitrogen. *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 1026 were affected the most by bioactive compounds of *C. vulgaris.* In addition, chloroform was the most efficient solvent to obtain effective extracts from *C. vulgaris*. Pigments and polar compounds had important role for higher antimicrobial activity under harsh conditions like 45 °C and media with 1.5 g/L nitrogen. In addition, caffeic acid having bioactive character was firstly shown for *C. vulgaris* in the current study.

Keywords: bioactive compounds, antimicrobial activity, *Chlorella vulgaris*, thermotolerant, stress condition

INTRODUCTION

Microalgae harvesting energy from sunlight, need only minimal amount of nutrients, fix nitrogen and CO_2 and achieve a superior growth rate by utilizing different carbon sources can be found in all around the world. These microorganisms have found usage in areas like human health, industry, and biofuel generation and raised increased interest in recent years. On the other hand, they can produce bioactive compounds having great potential in several biological applications related to human health. It was known that bioactive compounds had several useful properties like antimicrobial, anticancer, antidiabetic, etc. **(Gamal, 2010)**. Such compounds could be chlorophyll, carotene, phenolics, proteins, and fatty acids **(Maadane *et al.*, 2017; Rao *et al.*, 2010)**. Among microalgae, *Chlorella* genus has been investigated having biological effects in human, animal, and microorganisms **(Kitada *et al.*, 2009; Priya, 2012; Plaza *et al.*, 2012; Ibrahim *et al.*, 2015; Syed *et al.*, 2015; Jayshree *et al.*, 2016; Chaidir *et al.*, 2016; Alzahrani *et al.*, 2019)**. In a previous study performed by **Kitada *et al.* (2009),** with different separation methods, extracts of *C. vulgaris* had variable antimicrobial activity against bacteria. **Priya (2012)** showed that acetone extracts of *C. vulgaris* had antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, and *Klebsiella pneumonia*. In another study done with *C. vulgaris*, different methods [pressurized liquid extraction (PLE) and ultrasound-assisted extraction (UAE)] methods were used to obtain microalgal extracts; with PLE extraction and using ethanol, effective bioactive compounds were determined like carotenoids, chlorophylls, sterols, phytols and fatty acids against microorganisms **(Plaza *et al.*, 2012)**. **Ibrahim *et al.* (2015)** demonstrated that ethanol extracts of *C. vulgaris* had antimicrobial effect against Gram-positive and Gram-negative bacteria with inhibition zones ranged 6-14 mm. In another study showed that acetone, ethanol, and chloroform extracts of *C. vulgaris* had bioactive character against bacteria related to its phytochemical compounds **(Syed *et al.*, 2015). Jayshree *et al.* (2016)** studied with two microalgae as *C. vulgaris* and *Chlamydomonas reinhardtii* and they found that methanol extracts of *C. reinhardtii* had better bioactive property than extracts of *C. vulgaris*. **Chaidir *et al.* (2016)** performed trials with *Chlorella* sp. and found that extracts of the microalga had antimicrobial activity to *E. coli*, *Staphylococcus aureus*, *Salmonella typhii*, *Bacillus cereus.*

The purpose of the study was to find the bioactive compounds produced by a thermotolerant *C. vulgaris* isolated from hot springs. For this, different nitrogen concentrations and temperatures were studied to search the most effective bioactive compound produced by *C. vulgaris.* Different solvent types were also used to find the most effective extract from the tested microalga. Our main aim was to explore the optimum conditions for the most effective bioactive compound by *C. vulgaris* that could be found usage area in biotechnological applications. It is more advantageous to study with a microorganism that can tolerate extreme conditions. Such organisms can produce more stable compounds capable of using biotechnological applications. To our knowledge, there is no report that investigated the thermotolerant *C. vulgaris* with the approach of the current study.

MATERIAL AND METHODS

Microalgae, conditions of growth

Water samples from thermal springs (Haymana and Kızılcahamam, Turkey) were inoculated on Petri dishes containing BG11 with agar (12 g/l) **(Rippka, 1988)** (pH 7.5) and incubated at 45 °C under 2400 lux continuous illumination. Isolated algal cells were purified under aseptic conditions by streaking the cells repeatedly on BG11 agar. These purified microorganisms were inoculated to liquid BG11. These cultures were checked for bacterial contamination. Microalgae were grown in 250 ml Erlenmeyer with 100 ml of media, at 30 °C for 14 days under 2400 lx continuous light intensity at a growth chamber (BINDER, model: KBW 400 (E5.1), S.no: 15-13640, Tuttlingen, Germany).

Selection of microalgae and its identification

Ten different microalgal strains were inoculated in BG11 and microalga having the most effective bioactive compound was determined. Further experiments were done with it. The selected microalga was identified by its 18S rDNA gene. DNA of microalga were amplificated using PCR (2 ml of genomic DNA, 0.4 mM deoxynucleotide triphosphate, 1.25 units of Taq DNA polymerase). Forward primer was F5' *CTTGGTCATTTAGAGGAAGTAA* and reverse primer was R5' *TCCTCCGCTTATTGATATGC*. PCR was adjusted to 95 °C for 5 min, 30 cycles of 95 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 45 sec. DNA sequencing analysis was done with Bigdye Cycle kit v3.1 and ABI 3130XL Genetic Analyzer. Phylogenetic analysis of the nearly complete data was performed by BLAST.

Effect of nitrogen concentration and temperature on bioactive compound production by the selected microalga

Production of bioactive compounds by microalgae was affected from different environmental conditions. Thus, such compounds with different bioactive character could be produced under harsh media. In this context, bioactive compound production by the selected microalga under different media was studied regarding different nitrogen concentrations (0.5 g/L, 1.0 g/L, 1.5 g/L), and temperatures (30 °C and 45 °C). Microalgae were grown in BG11 under 2400 lx continuous light intensity at a growth chamber with an incubation period for 14 days.

Effect of different solvent on effectiveness of bioactive compound

The efficiencies of bioactive compounds prepared by using different solvents can be different. Therefore, ethanol, methanol, hexane, chloroform, Tris-HCI (pH:8; 0.5 M), and water were tested. All the chemicals were purchased from Merck, Germany.

Preparation of bioactive compounds

After incubation for 14 days, cultures were centrifugated (MPW-351R, Warsaw, Poland) at 10 000 rpm for 5 min, supernatant was removed, and biomass was collected. Biomasses were freeze-dried (Millrock Technology, Inc., Kingston, NY 12401, USA) for overnight. Then, dried biomass (1 gram) was exposed to 3 ml ethanol. To obtain algal extract, the mixture was incubated for 1 hour. It was centrifuged for 10 000 rpm (5 min) and supernatant was used as algal extract. Extracts were kept at 4 °C and analysed within 2 days (**Rao *et al*., 2010; Pradhan *et al*., 2012)**.

Determination of bioactive compound effectiveness

Antimicrobial activity was determined to understand effectiveness of bioactive compound. For this purpose, disc diffusion method was used **(Murray *et al.*, 1995)**. Two *Escherichia* species (*Escherichia coli* 0157:H7 ATCC 35150, and *E. coli* ATCC 25922), three *Staphylococcus* species *(Staphylococcus aureus* ATCC BAA 976, *S. aureus* ATCC 25923, and *S. aureus* ATCC 1026), *Brochothrix thermosphacta* ATCC 11509, *Bacillus subtilis* ATCC 6633, and *Enterobacter cloacae* ATCC 700323 were used as standard bacterial strains. Nutrient Broth was used to cultivate bacteria (24 h). They were inoculated uniformly by using sterile cotton swab onto Nutrient Agar to determine the antibacterial activities of the extracts. A 40 µl of extracts was applied to a sterile disc (40 µl/per disc)**.** The impregnated discs were put on to the plates using sterile forceps properly spaced at equal distance. They were incubated for 24 h (30 °C). The zone of inhibition was determined in diameter (mm).
The chlorophyll (a + b) in the media was found by observing optical absorption at 646.6 and 663.6 nm **(Porra *et al*., 1989)**.

HPLC-DAD analysis of the extracts

HPLC analysis was performed by using an Agilent 1260 Liquid Chromatograph with a DAD to understand the pigment composition. C18 column (150mm×3.9mm, 4 µm particle size) in a (zorbax) from ACE was used for the separation. As a mobile phase the mixture of solvent A and solvent B was used. Solvent A (methanol/ammonium acetate 0.1N; 7:3) and solvent B (methanol) at 0.9 mL/min was applied according to the following step gradient: 25% B, changing to 50% in 1 min, and finally 100% B at minute 20 at 450 nm. The peaks were identified by comparing with the data presented in the literature.
Polar compounds were determined with HPLC (Agilent 1260 Liquid Chromatograph with a DAD). A mixture of solvent A (H_2O/CH_3COOH, 95:5) and solvent B (acetonitrile 100%) was used as mobile phase. Mobile phase was applied with a flow rate as 0.9 mL/min at 280 nm. Due to the following step gradient lasting for 30 min, starting from 1% B, changing to 2% B at 6 min, increasing to 100% B at 20 min and holding 100% B constant for the 30-min run. The peaks were identified by comparing with the data reported in the literature.

Analysis of fatty acids by GC-MS

To analyse the fatty acid methyl ester analysis for the extracts having the highest bioactive property, extracts were mixed with 0.1 M KOH in methanol, and for transesterification, hexane was added. A 1 µL sample was taken from the upper phase. GCMS-QP2010 Ultra gas-chromatograph (Shimadzu, Japan) was used to analyse the methylated fatty acids. The condition of GCMS-QP2010 Ultra analysis was flame ionization detector (FID-250 °C); column SP-2560 (100 m × 0.25 mm × 0.20 µm, Sigma-Aldrich); carrier gas He. Peaks were identified by the chromatogram of a mixed fatty acid methyl ester standard (37 Comp. FAME Mix 10 mg/mL in CH_2Cl_2; Supelco, USA).

RESULTS AND DISCUSSION

The microalga having the most effective bioactive compound

Ten different microalgal strains (Strain A, Strain B, Strain C, Strain D, Strain E, Strain F, Strain G, Strain A1, Strain A2, and Strain A3) were tested in these experiments. The data were summarized in Table 1. All microalgal isolates had antimicrobial activity to *B. subtilis* ATCC 6633, *B. thermosphacta* ATCC 11509, *E. coli* 0157:H7 ATCC 35150, *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 25923. Among them, extracts of Strain C were not effective to *E. coli* ATCC 25922 and *E. cloacae* ATCC 700323; extracts by Strain G and Strain A3 had no antimicrobial activity against *S. aureus* ATCC 1026. According to the results from these experiments, ethanol-extracts of Strain A had the most effective antimicrobial activity against *S. aureus* ATCC 1026 and *S. aureus* ATCC BAA 976 with an inhibition zone as 14 mm. In addition, Strain D and Strain A2 extracts had effective bioactive properties against *E. coli* ATCC 25922 with inhibition zones as 12.5 mm. At the end of these trials, further experiments were carried out with the microalga (Strain A) with the most effective antimicrobial activity.

Table 1 Antimicrobial activity [inhibition zone (mm)] of bioactive compounds obtained from microalgal species (T: 45 °C; N concentration: 1 g/L; illumination: 2400 lx; incubation period: 14 d)

Bacteria	Strain									
	A	**B**	**C**	**D**	**E**	**F**	**G**	**A1**	**A2**	**A3**
B. subtilis ATCC 6633	12±2.0	6.5±1.5	9±1.0	10±0.5	12±1.0	8±1.0	7±2.0	10±1.0	7±2.0	9±1.5
B. thermosphacta ATCC 11509	12±1.5	12±1.0	8±0.5	9±1.5	11±0.5	8.5±1.5	7±1.5	8±1.5	6.5±1.0	8.5±1.0
E. coli 0157:H7 ATCC 35150	11.5±1.0	11.5±1.0	10±0.5	10±1.0	12±1.0	10±0.5	8±1.0	6±2.0	10±1.0	7±1.0
E. coli ATCC 25922	11±1.0	7±1.0	-	12.5±1.0	10±1.5	10±1.5	6±2.0	8±1.5	12.5±0.5	8±1.5
E. cloacae ATCC 700323	12±1.0	11±1.0	-	8±2.0	7±0.5	11±1.5	7±1.5	10±1.0	8±1.0	7±1.0
S. aureus ATCC BAA 976	14±1.5	6.5±1.5	7±1.5	10±1.5	9±2.0	10±0.5	6±1.5	7±1.5	8±1.5	9±0.5
S. aureus ATCC 25923	12±0.5	6.5±1.5	9±0.5	8±2.0	9±2.0	11±1.5	6±2.0	9±1.5	12±1.0	8±1.0
S. aureus ATCC 1026	14±1.5	12±0.5	8±2.0	11±1.0	10±1.0	8.5±1.0	-	9±0.5	7±2.0	-

Identification of the Selected Microalga

Strain A was identified by amplification and sequencing of its 18S rDNA gene. BLAST search was performed to find the phylogenetic analysis of the nearly complete sequence data. The microalga had a >99 % similarity to *Chlorella vulgaris* according to the alignment and further analysis in ARB database. The microalga was submitted to NCBI Gen-Bank with accession number as MN 114114.

Effect of different nitrogen concentrations on bioactive compound effectiveness under 30 °C and 45 °C

Under 30 °C, the most effective bioactive compound was obtained from extracts by microalga cultivated in media with 0.5 g/L nitrogen to *S. aureus* ATCC 1026 and *S. aureus* ATCC 25923 with inhibition zones as 11 mm and 10.5 mm, respectively (Figure 1a). On the other hand, at 45 °C, effectiveness of bioactive compounds was higher than it was at 30 °C against only one bacterium as *S. aureus* ATCC BAA 976 (inhibition zone: 12 mm) produced by *C. vulgaris* grown in media with the same nitrogen concentration. Under 45 °C, antimicrobial activity was found same against *S. aureus* ATCC 1026 and less than it was at 30 °C against other bacteria tested.
The extracts obtained from the biomass when the microalgae were grown in medium with 1 g/l of nitrogen at 30 °C, showed the maximum antimicrobial activity to *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 1026 with 14 mm inhibition zones (Figure 1b). Extracts produced by *C. vulgaris* grown in the same media at 45 °C, had lower antimicrobial activity than it was 30 °C. Extracts from microalgal biomass obtained under 45 °C, had the most effective activity to *E. coli* 0157:H7 ATCC 35150 (inhibition zone: 11.5 mm).

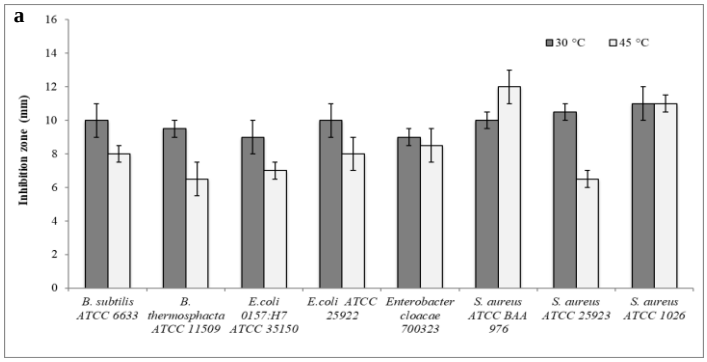
When *C. vulgaris* was cultivated in media with 1.5 g/L at 30 °C and 45 °C, antimicrobial activity of extracts was shown in Figure 1c. The highest activity (inhibition zone: 11 mm) was observed against *S. aureus* ATCC 1026 with 30 °C-extracts, while it was 12 mm with 45 °C-extracts towards to the same bacterium. In these experiments, it was observed that extracts obtained in media at 30 °C had lower bioactive property than it was with 45 °C-extracts. On the other hand, antimicrobial activity had its highest value in samples obtained in media with 1 g/L nitrogen at 30 °C.

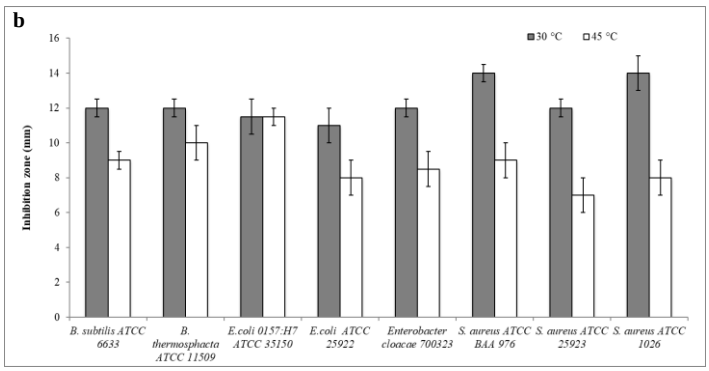
When antimicrobial activities of extracts obtained from algal biomass grown at 30 °C and 45 °C were compared, it was concluded that 30 °C-extracts had higher bioactive character. Further raising the temperature to 45 °C made a decrease of microalgal growth, and later the microalgal cells died. This result was celarly followed as the color of the cells changed from green to brown, and as a result the chlorophyll content decreased, as well. Chlorophyll of the microalga were 2.2 µg/ml, 3.3 µg/ml, 1.9 µg/ml when *C. vulgaris* was cultivated in media under 0.5 g/L, 1.0 g/L, and 1.5 g/L nitrogen concentrations at 30 °C, respectively. On the other hand, when *C. vulgaris* was cultivated at 45 °C in BG11 including 0.5 g/L nitrogen, chlorophyll content was 0.6 µg/ml; in media having 1.0 g/L nitrogen, it was 0.7 µg/ml, and under 1.5 g/L nitrogen conditions chlorophyll amount was 0.4 µg/ml.

In addition to this, bioactive compounds affected *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 1026 at most. According to these results, further experiments were done with using *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 1026 bacteria.

Different environmental properties such as temperature and the nitrogen content in the media are factors that affect microalgal growth and affect the effectiveness of bioactive substances obtained from microalgae. For this purpose, to understand that affect, the concentration of nitrate in media was diminished to half and increased to half of the standard media, while the illumination was kept the same during the trials.

At the end of these trials, the effect of nitrogen concentration and the temperature on antimicrobial activity were evaluated together, increasing the amount of nitrogen had more effect than the increase in temperature on the antimicrobial effect. In addition, the highest bioactive character was found in the biomass obtained from the microalgae grown at 30 °C and 1 g/L nitrogen containing medium. The variation of efficiency of bioactive character with these parameters is due to the fatty acids, polar compounds and pigments that are produced in microalgae under different ambient conditions. **Converti *et al.* (2009)** also showed that growth of *C. vulgaris* decreased as the temperature increased. In the same study, no change in the fatty acid composition was observed by changing the nitrogen content from 0.375 g/L to 1.5 g/L. In the current study, with an increase in temperature, growth of the *C. vulgaris* decreased. On the other hand, the extracts prepared from biomasses obtained by increasing the nitrogen concentration from 0.5 g/L to 1.0 g/L were found to have more effective bioactive substances. This case may be due to fatty acids having more antimicrobial activity in that media.





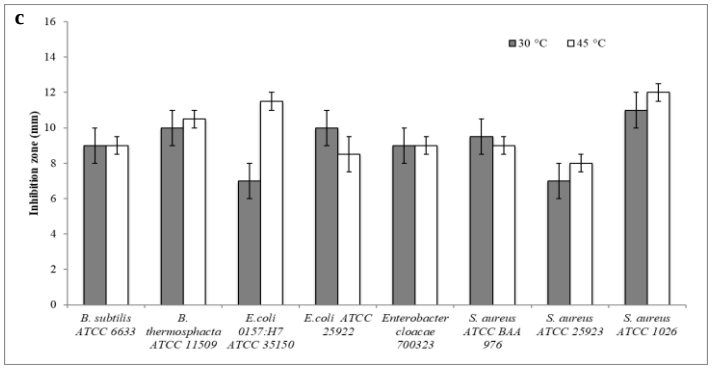


Figure 1 Antimicrobial activities [inhibition zone (mm)] of bioactive compounds by *C. vulgaris* (a) 0.5 g/L nitrogen (b) 1.0 g/L nitrogen (c) 1.5 g/L nitrogen (T: 30 °C and 45 °C; illumination: 2400 lx; incubation period: 14 d)

In addition to this subject, *C. vulgaris* produced more effective bioactive compounds when grown in media with 1.5 g/L nitrogen at 45 °C then in media with same nitrogen but at 30 °C.

In the current work, with an increase in temperature growth of the microalga decreased. **Converti *et al.* (2009)** also reported that *C. vulgaris* was adversely affected by the increase in temperature above 30 °C and growth was not observed at 38 °C. In the same study, with the reduction of nitrogen, the composition of fatty acids was obtained in a similar amount and was not affected by this parameter, only the temperature affected the content of fatty acid, which was only for one fatty acid as linolenic acid. Results showed that antimicrobial activity of the tested alga can only be induced by high nitrogen concentrations and temperature.

Effect of different solvents

In Figure 2 antimicrobial effectiveness of bioactive compounds obtained with ethanol, methanol, chloroform, Tris-HCI, hexane, and water were shown. These extracts were tested against *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 1026 bacteria which microalgal extracts had the highest antimicrobial activity under tested conditions.

In these trials, extracts obtained with ethanol and chloroform showed the highest antimicrobial activity against the tested bacteria. Inhibition zones with chloroform-extracts were 17 mm to *S. aureus* ATCC BAA 976 and 16 mm to *S. aureus* ATCC 1026. On the other hand, bioactive compounds prepared with ethanol solvent, had 14 mm inhibition zones to the tested bacteria.

Methanol and Tris-HCI extracts had similar antimicrobial character, while hexane and water-extracts had no bioactive character to the tested bacteria.

Pigment content of the microalgae can be affected from used solvents. Previous studies on this subject vary considerably. **Kitada *et al.* (2009)** found that ethanol was better than acetone in the study they investigated the isolation of *C. vulgaris* microalgae pigments with different separation techniques. **Syed *et al.* (2015)** also supported that ethanol was a better solvent then acetone. On the other hand, **Plaza *et al.* (2012)** used different solvents like ethanol, hexane, and acetone; they found that acetone was to be the best solvent to get more *Chlorella* carotenoids. **Ibrahim *et al.* (2015)** used ethanol as an extraction solvent; they found that *C. vulgaris* extracts formed 11 mm inhibition zone against *S. aureus*. However, in the present study, chloroform was the most effective organic solvent to have more efficient extracts from *C. vulgaris*; and chloroform extracts of *C. vulgaris* had 17 mm inhibition zone against *S. aureus* ATCC BAA 976.

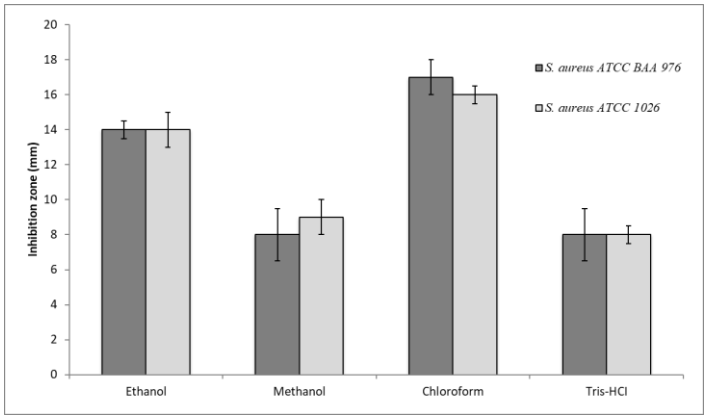


Figure 2 Antimicrobial activity of bioactive compounds obtained with different solvents to *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 1026 (T: 30 °C; N concentration: 1 g/L; illumination: 2400 lx; incubation period: 14 d)

HPLC and GC analysis

Analysis of fatty acids by GC-MS

Chloroform-extracts of *C.vulgaris* having the maximum bioactive character were analysed to find the fatty acid profile. In Table 2 fatty acid definition, the peak area contribution, and the concentration of fatty acids were shown. Stearic (26.9%), caprylic (14.9%), palmitic (14.5%) and lauric acids (10.9%) were found to be the major fatty acids due to the chromatographic area as nearly 70% in total. Similar results were found in previous studies. **Converti *et al.* (2009)** showed that *C. vulgaris* produced more saturated fatty acids like palmitic acid (C 16:0) when temperature raised above 30 °C. Researchers also concluded that unsaturated fatty acids could only be produced when temperature was lower than 20 °C (**Ahn *et al.*, 2016**). The results of our study are also supported by these previous works.

Table 2 Fatty acid identification in chloroform-extracts of *C. vulgaris* by GC-MS analysis

ID	Retention time (min)	Fatty acids	%
1	13.39	Caprylic acid (C8:0)	14.90
2	16.99	Lauric acid (C12:0)	10.9
3	26.60	Palmitic acid (C16:0)	14.5
4	27.46	Palmiteloic acid (C16:1)	4.07
5	29.59	Cis-10-Heptadecanoic acid (C17:1)	4.03
6	30.34	Stearic acid (C18:0)	26.9
7	30.60	Elaidic acid (C18:1n9t)	1.69
8	31.52	Oleic acid (C18:1n9c)	5.59
9	32.30	Linolelaidic acid (C18:2n6t)	1.68
10	33.30	Linoleic acid (C18:2n6c)	3.3
11	35.34	Heneicosanoic acid (C21:0)	3.7
12	39.30	Cis-13,16 docosadienoic acid (C22:2)	1.7
13	39.9	lignoceric acid (C24:0)	3.3
14	44.27	Cis-4,7,10,13,16,19 docosahexaenoic acid (C22:6)	3.7

HPLC-DAD analysis of the extracts

In *C. vulgaris* chloroform-extracts, it was determined that main component of pigments was 19'butanoyloxyfucoxanthin according to the highest chromatographic area. It was followed by β-carotene, and lutein (Table 3).

Microalgae had pigments like carotenoids (carotenes) and xanthophyll (lutein, astaxanthin, fucoxanthin etc.) which are bioactive compounds having antimicrobial, antioxidant, anti-inflammatory properties (**Koller *et al.*, 2014; Encarnação *et al.*, 2015; Falaise *et al.*, 2016**). In the current study, high amount of fucoxanthin following by β-carotene, and lutein were found as main carotenoid having bioactive property.

Previous studies showed that *C. vulgaris* contained pigments such as xanthophyll, lutein, a-carotene and b-carotene (**Plaza *et al.*, 2012; da Silva vaz *et al.*, 2016**). Of these, fucoxanthin having the highest chromatographic area in the current study was previously reported that it had antimicrobial activity against *Listeria monocytogenes* (**Rajauira and Abu-Ghannam, 2013**). Furthermore, fucoxanthin is thought to be a good candidate to develop new drugs (**Peng *et al.*, 2011**). Antimicrobial, antioxidant, anti-inflammatory characters of β-carotene and lutein were also studied by other researchers (**Plaza *et al.*, 2012; de Morais *et al.*, 2015**). Table 4 summarized analyses of polar compounds in *C. vulgaris* chloroform-extracts. **Rodriguez-Meizoso *et al.*, (2010)** mentioned that HPLC analysis of polar compounds (280 nm) in microalgal extracts might be connected to phenolic compounds having bioactive character**.** Chromatogram of polar compounds showed that 12 peaks were obtained; of these, 7., 8., and 9. peaks (Retention time: 21.73, 22.07, 22.57, respectively) had higher intensity from other peaks. **Zhang *et al*. (2013)** demonstrated that these compounds may be related to caffeic acid according to their retention time. It was previously mentioned that *C. vulgaris* contained phenolic compounds; these compounds included phenolic acids such as caffeic acid. Caffeic acid was previously studied for its antimicrobial activity to *E. coli* (**Meyuhas *et al.*, 2015 Matejczyk *et al.*, 2018**). To the best our knowledge, caffeic acid having antimicrobial effect was firstly shown in thermotolerant *C. vulgaris* in the current study.

Table 3 Pigment composition in *C. vulgaris* extracts obtained with chloroform, 450 nm.

ID	Retention time (min)	Compound	Area	Reference
1	10.41	Zeaxanthin (RT:10.39; 448 nm)	109.88	**(Plaza *et al.*, 2010)**
2	10.75	Luteoxanthin (RT: 9.64; 446 nm)	155.77	**(Deli *et al.*, 2014)**
3	10.92	Antheraxanthin (RT: 11.37; 447 nm)	109.28	**(Deli *et al.*, 2014)**
4	11.19	Chlorophyll c2 (RT: 11.01; 452 nm)	9.76	**(Rodriguez *et al.*, 2002)**
5	11.46	Chlorophyll c1 (RT: 11.72; 448 nm)	7.25	**(Rodriguez *et al.*, 2002)**
6	17.36	19'butanoyloxyfucoxanthin (RT: 17.27; 446 nm)	575.84	**(Rodriguez *et al.*, 2002)**
7	19.36	Diainoxanthin (RT:18.8; 448 nm)	103.67	**(Louda *et al.*, 2008)**
8	20.18	Alloxanthin (RT:20.8 ; 454 nm)	472.87	**(Louda *et al.*, 2008)**
9	21.43	β-carotene (RT:22.43; 452 nm)	536.93	**(Plaza *et al.*, 2010)**
10	24.07	Lutein (RT:24.3; 445 nm)	383.61	**(Serive *et al.*, 2017)**
11	25.00	Crocoxanthin (RT: 24.80; 446 nm)	188.41	**(Mendes *et al.*, 2007)**
12	31.54	α-carotene (RT: 31.93; 444 nm)	454.02	**(Deli *et al.*, 2014)**
13	34.56	β-ε carotene (RT: 35.39; 452 nm)	323.18	**(Rodriguez *et al.*, 2002)**

Table 4 Polar compound composition in *C. vulgaris* extracts obtained with chloroform

ID	Retention time (min)	Area
4	19.68	3904.9
5	19.84	3896.5
6	21.29	2609.1
7	21.73	6537.2
8	22.07	4091.2
9	22.57	5016.3
10	22.83	2890.3
11	23.72	77.7
12	24.34	59.5
13	24.70	141.9
14	25.81	1435.4
15	28.72	2432.6

CONCLUSION

Antimicrobial activities of bioactive compounds in *C. vulgaris* were studied in the current study. When bioactive properties examined it was concluded that antimicrobial activity of the tested alga can only be induced by high nitrogen concentrations and temperature. The highest antimicrobial activity was found to *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 1026. In addition to this, antimicrobial activity increased with using different solvents rather than ethanol. Chloroform-extracts had higher antimicrobial activity than extracts obtained with other solvents tested. Of the fatty acids identified, stearic acid, fucoxanthin from pigments and caffeic acid as a simple phenol from polar compounds were found with their highest amount in chloroform-extracts of the *C. vulgaris*. It can be stated that fucoxanthin and caffeic acid were responsible for more effective antimicrobial activity at conditions like 45 °C and media with 1.5 g/L nitrogen. Thus, thermotolerant *C. vulgaris* is an advantageous biomaterial to be used in several biotechnological applications due to its unique properties.

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