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Cladophora fracta var. *intricata'*nın Yağ Asitleri, Vitamin ve Antioksidan Özellikleri

Tuğba DEMİRİZ YÜCER[1*], Köksal PABUÇCU[2]

Öne Çıkanlar:

- *Cladophora fracta* var. *intricata*'da Miristik asit, Palmitik asit, Palmitoleik asit, Oleik yağ asitleri ve A, C, E vitaminleri yüksek oranda bulunmuştur

Anahtar Kelimeler:

- *Cladophora fracta* var. İ*ntricata*
- Antioksidan
- Yağ asitleri
- Vitamin A-C-E

ÖZET:

Bu çalışmada kültüre alınan (CFI) *Cladophora fracta var. intricata*'nın yağ asitleri, vitaminleri ve antioksidan özellikleri araştırılmıştır. CFI, Tokat Yeşilırmak nehrinin bentik habitatlarından izole edilmiş ve daha sora aksenik kültüre alınmıştır. Yapılan antioksidan analiz sonuçlarına göre CFI'da DPPH serbest radikal giderme aktivitesi, FRAP ve TEAC indirgeme gücü açısından doza bağlı bir aktivite görülmüş ancak IC_{50} değerlerinin üzerinde kaldığı için anlamlı bulunmamıştır. CFI'da C14:0 Miristik asit, C16:0 Palmitik asit ve C16:1 Palmitoleik asit, C18:1 N9C Oleik yağ asitleri ve A, C, E vitaminleri yüksek bulunmuştur. Antioksidan analiz sonuçlarına göre CFI'nin yağ asidi ve vitamin değerlerinin gıda ve ilaç sektöründe kullanımı açısından dikkat çekici olduğu tespit edilmiştir. İncelenen alg takson, düşük kontaminasyon özelliklerine sahip olduğundan kültür ortamlarında rahatlıkla kullanılabilir.

Fatty acids, Vitamins and Antioxidant Properties of *Cladophora fracta* var. *intricata*

Highlights:

- In *Cladophora fracta* var. *intricata*, Myristic acid, Palmitic acid, Palmitoleic acid, Oleic fatty acids and vitamins A, C, E were found to be high

ABSTRACT:

In this study investigations of the fatty acids, vitamins and antioxidant properties of cultured (CFI) *Cladophora fracta var. intricata*. CFI was isolated from benthic habitats of the Tokat Yeşilırmak River (Tokat) and axenic cultured. According to the antioxidant analysis results, a dose-dependent activity was observed in CFI in terms of DPPH free radical scavenging activity, FRAP and TEAC reducing power, but it was not found to be significant as it remained above IC_{50} values. In CFI, C14:0 Myristic acid, C16:0 Palmitic acid and C16:1 Palmitoleic acid, C18: 1 N9C Oleic fatty acids and vitamins A, C, E were found to be high. According to antioxidant analysis results, CFI was found to be notable for the use of its fatty acid and vitamin values in food and pharmaceutical. Since it has low contamination properties, the algal taxon examined can be easily used in culture media.

Keywords:

- *Cladophora fracta* var. *intricata*
- Antioxidant
- Fatty acids
- Vitamins A-C-E

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INTRODUCTION

Algae are organisms that can live in all aquatic environments on earth, and that contain various pigment molecules such as chlorophyll, carotenoid, xanthophyll, and they are important for aquaculture (Rose et al., 1994). Algae are the main producers of oxygen needed by all living things on earth (Baytaşoğlu et al., 2014). Algae, which are primary producers, form the first ring of the food chain (Rose et al., 1994). Algae have been used by humanity in different areas since ancient times. With the help of their valuable metabolites stored inside the cell; they are used as preventive medicine, food support, animal feed, fertilizer for soil structure improvement, natural food colouring and cosmetics in the industry (Göksan et al., 2003). As they contain carbohydrates, proteins, fatty acids, vitamins, minerals, phycobilin and various metabolites in the cell, they are used by humans as essential nutrient support. Algal species containing important antioxidants such as ascorbic acids, carotenoids, tocopherols, flavonoids, and retinoids are important for protection from various degenerative diseases (Baytaşoğlu et al., 2014).

Cladophora fracta (O.F.Müller ex Vahl) Kützing 1843

C. fracta form large, light green masses of irregularly branched filaments. The branches usually show slight bending. The cells are slightly swollen, irregularly shaped. In some species it is cylindrical. The diameter of the main axis is around 60-120 μm. They can be 1-3 times longer than this. Terminated filaments are 20-40 μm in diameter. The length of the cells is 3-6 times their diameter. Chloroplasts show a discoid structure and contain prenoids. Sexual reproduction is diplohaplont and isomorphic; Zoospores emerge from the rupture of the thallus. This form of asexual reproduction is also seen (Prescott, 1970; Guiry and Guiry, 2021).

Cladophora fracta var. *intricata* (Lyngbye) van den Hoek 1963

Cladophora fracta var. *intricata* shows less branching than *C. fracta*. It is also lighter green in color. This taxa, belongs to the Cladophora phylum and Cladophoraceae family. Its taxonomic categorization is as follows (Guiry and Guiry, 2021).

Kingdom: Plantae
Subkingdom: Viridiplantae
Phylum: Chlorophyta
Subphylum: Chlorophytina
Class: Ulvophyceae
Order: Cladophorales
Family: Cladophoraceae
Genus: Cladophora

This article aimed to investigate the fatty acids of *Cladophora fracta var. intricata* (CFI), their antioxidant activity and vitamin content.

MATERIALS AND METHODS

Isolation and Culture Of Algae

CFI was brought to Tokat Gaziosmanpaşa University Micro Algae Culture Laboratory with plastic containers in water samples taken from Tokat Yeşilırmak River benthic habitats and isolated by mechanical isolation method. It was then transferred to Eppendorf tubes under an inverted microscope and taken into the liquid culture and incubated at 26 ° C (155 μmol / m^2 /h, l: D period) in Allen, BG11 liquid growth environments, Sanyo MLR 351 climatic cabinet (Lobban et al., 1988; Andersen, 2005). After reaching a certain volume density, it was harvested and stored in the culture collection in the freezer at -86 °C for further inspection. Related sources have been used in the diagnosis of algae

(Prescott, 1970; Lund, 2002; Guiry and Guiry, 2021). After 5 gr-CFI was extracted from the culture collection in 150 mL of solvent (1:1methanol+methylene chloride), fatty acid, vitamin analysis and antioxidant activity tests were performed on the samples.

Antioxidant Activity Tests

It is difficult to accurately determine the antioxidant activity of natural products with a single test. Many tests have been proposed to assess the antioxidant activity of such products. In this study, three tests were performed to determine the antioxidant activity of the used algae species.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity method

Free radical (DPPH: 2,2-diphenyl-1-picrylhydrazyl) removal activity of microalgae was performed according to the Liyana-Pothirano method (Liyana *et al*., 2005). Stock Solutions (1mg/mL) were prepared from microalgae extracts and compounds were used as standard (BHT, BHA, α-tocopherol). Then, different concentrations (10-80µg/mL) of samples and standards were added to 1 mL of DPPH solution, and the final volume was completed with ethanol to 4 ml. For positive control, ethanol was formed from 3 mL of ethanol+1 mL of DPPH solution and ethanol was used. After the samples were incubated for thirty minutes in a light-free environment, the absorbance was measured at 517 nm. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a free radical, taking an electron or hydrogen radical to become a stabildiamagnetic molecule. This method is based on the fact that the DPPH solution, which is dark violet-coloured, and its decolouring when there is an electron transfer by any molecule in the system, and this colour change is measured using a UV spectrometer. The more the colour lightens and the lower the solution absorption is, the greater the free radical removal activity is. Free radical (DPPH) activity was calculated using the following formula (Re et al., 1999; Elmastaş and Gülçin, 2006; Günal Köroğlu et al.,2021; Nurjanah et al., 2021).

$$\text{DPPH Scavenging activity}(\%) = \left(\frac{A_{517}\text{ of control} - A_{517}\text{ of sample}}{A_{517}\text{ of control}}\right) x100 \quad (1)$$

Iron (III) Ion Reducing Antioxidant Power (FRAP) activity

The total reduction power of CFI extracts was determined using the Oyaizu method (Oyaizu, 1986). After different concentrations of algae extracts and standards were pipetted into tubes, 2.5 mL phosphate (KH_2PO_4) buffer (0.2 M pH: 6,6) and 2.5 mL potassium ferricyanide $K_3FE(CN)_6$ (1%) solutions were added to it. After thoroughly vortexed, it was incubated in a 50°C water bath and washed for 20 minutes. Then 2.5 mL of 10% trichloroacetic acid (TCA) solution was added to this mixture and centrifuged at 3000 rpm for 10 minutes. After 2.5 mL of the centrifuged mixture was taken and 0.5 mL of 1% Iron (III) chloride ($FeCl_3$) solution was added and vortexed, their absorption was read blind at 700 nm using UV-Vis spectroscopy (Re et al., 1999; Elmastaş and Gülçin, 2006).

Trolox Equalling Antioxidant Capacity (TEAC or ABTS) activity

It is based on the inhibition of the absorption of the radical cation (ABTS) by antioxidants. Cation radical (ABTS) removal activity was performed according to the method proposed by Re (Re *et al.* 1999). 0.1 M pH: 7.4 PO_4^{3-} buffer, 2mM ABTS and 2.45 mM potassium persulfate ($K_2S_2O_8$) solution were prepared for free radical (ABTS) removal activity. $ABTS^{\cdot+}$ and $K_2S_2O_8$ Solutions (1:2) were mixed as $ABTS^{+}$ - $K_2S_2O_8$ and incubated in darkness for 6 hours. In different concentrations (2,5-5-10 µg / mL), samples and standard solutions were taken and 1 ml of $ABTS^{\bullet+}$ - $K_2S_2O_8$ solutions were added to it. A phosphate buffer was added to make the total volume of 4 ml. The mixture was strongly vortexed and incubated for 30 minutes, and spectrophotometric measurement was performed under room conditions, at 734 nm. The application was repeated for three repetitions. % cation free radical removal

activity of samples and standard was calculated by the following formula suggested by Re et al., (1999), Elmastaş and Gülçin, (2006).

$$\text{TEAC (\%)} = \left(\frac{\text{Abs control}-\text{Abs sample}}{\text{Abs control}}\right) \text{x}100 \quad (2)$$

Abs control: Absorbance of buffer PO_4^{3-} + ABTS + - $K_2S_2O_8$ solution

Abs sample: PO_4^{3-}buffer + ABTS + - $K_2S_2O_8$ solution + extract / standard absorbance

Determination of Fatty Acid Composition

Dried CFI samples were crushed into powder in a garlic press and 1 gram from each sample was used for fatty acid analysis. In the determination of fatty acids, saponification, methylation, extraction processes were performed and washing steps were processed as defined below. At the 1st stage, 1 ml was added to each tube from a solution of pure water, NaOH + CH_30H for the breakdown of cells, and rinsed for 5-10 seconds. Then the test tubes were left in 100°C boiling water for 5 minutes, rinsed in warm water for 10 seconds, and incubated again in 100°C boiling water for 25 minutes to release the fatty acids.

At the 2nd stage, 2 ml of the second solution (- 6N HCl+CH_3OH for methylation) was added to the test tubes and rinsed for 5-10 seconds. It was then incubated in a water bath of 80ºC for 10 minutes and cooled in ice for 2 minutes.

At the 3rd stage, 1.25 ml of the third solution (methyleterhexane + MFBE) was placed in cooling tubes and rinsed for 10 minutes. Fatty acids, the organic liquid at the top and the other one at the bottom, were separated from the acidic phase and passed into the organic phase.

At the 4th stage, 3 ml of the fourth solution (NaOH + deionized pure water) was added to each tube and rinsed for 5 minutes. After incubating at room temperature for 10 minutes, the samples were analyzed by transferring them to gas chromatography tubes with a pastor pipette. Methyl esters of fatty acids were carried out by FID (Flame Ionization Detector) and gas chromatography with an automatic injector. 100-meter HP-88 capillary column was used for analysis. In the gas chromatograph, the injector block temperature was set to 210 °C and the detector block temperature was set to 230 °C. The flow rate of the carrier gas (helium) was 40mL / min (Paquot, 1979).

Vitamin Analysis

Fat-soluble CFI vitamins were extracted with hexane: chloroform (3:1) after being removed from the freezer to be studied in HPLC. Then, under vacuum, the solvent was removed and injected into the C18 column (150x4.6 mm ID,5µm Wackosyl) and the column temperature was kept constant at 50°C. Acetonitrile: methanol (1:1) was used as the mobile phase. The flow rate was programmed as 1ml/min and a DAD detector was used as a detector. Calibration graphs were drawn using α-tocopherol and β-carotene as standard, and vitamin amounts were calculated (Moreno and Salvadó, 2000).

RESULTS AND DISCUSSION

(CFI) *Cladophora fracta* var. *intricata* (Lyngbye) C. Hoek 1963, is a species of branched fibrous algae from the order Chlorophyta and the Cladophorales ordo. It usually spreads in fresh and salt waters. It was first identified in fuursöe Lake litoral (Sjaelland, Denmark) (Hoek, 1963; Guiry and Guiry, 2021).

Antioxidant Activity Results

In diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity analyses, the free radical removal activity of CFI was measured to be 1339.20 and its effect was found to be low compared to that of Trolox, BHA and BHT (Table 1).

CFI has been observed to have properties lower (0.24) than BHA and BHT standards, which results in Iron (III) ion reducing antioxidant power (FRAP) activity (Table 1). In terms of Trolox equal antioxidant capacity (TEAC or ABTS) activity results, CFI was found to have a relatively low rate of Cation radical removal activity than the standards and the results are given in Table 1.

Table 1.CFI antioxidant analysis results

ALGAL TAXA	DPPH IC_{50} (µg extract/mL) average values	ABTS IC_{50} (µg extract/mL) average values	FRAP µmol TE/mg extract average values
CFI (*Cladophora fracta* var. *intricata*)	1339.20 ± 126.27	66.88 ± 1.73	0.237 ± 0.012
TROLOX	5.68±0.14	5.38±0.12	-
BHA (Butylated hydroxyanisole)	5.78± 0.13	5.48±0.17	4.35±0.167
BHT (Butylated hydroxytoluene)	7.67±0.12	6.89±0.27	3.87±0.112

Fatty Acids

In the fatty acid determination of CFI, the ratios of C16:0 Palmitic Acid, C16:1 Palmitoleic acid, C18:1 N9C Oleic acid and C14:0 Myristic acid were found to be high when ranked based on their % density (Table 2).

Vitamins

In the vitamin analyses of CFI, the proportions of vitamins C, E and A were examined. According to the data obtained; vitamin C was found to be 39.03 mg/kg, Vitamin E was found to be 5.19 mg/kg and vitamin A was found to be 19.25 mg/kg and results are presented in Table 2.

Table 2. Fatty acid ratios and vitamin analysis of CFI

FATTY ACIDS	**%**
C10:0 Caprinic acid	1.34
C11:0 Undecanoic acid	0.23
C12:0 Lauric acid	0.60
C14:0 *Myristic acid*	**10.29**
C14:1 Myristoleic acid	0.15
C15:0 Pentadecanoic acid	0.72
C15:1 Cis-10-Pentadecanoic acid	0.01
C16:0 *Palmitic acid*	**29.66**
C16:1 *Palmitoleic acid*	**20.77**
C17:0 Heptadecanoic acid	0.55
C17:1 cis-10-Heptadecanoic acid	0.60
C18:0 Stearic acid	6.07
C18:1 n9c *Oleic acid*	**10.78**
C18:2n6c Linoleic acid	2.20
C18:3n6 gama Linoleic acid	0.61
C20:0 Arachidic acid	1.98
C18:3n3 alfa Linoleic acid	3.19
C20:1 cis-11-eicosenoic acid acid	0.20
C20:2 cis11,14-eicosadienoic acid	0.33
C20:3n6	0.42
C21:0 Dihomo-gamma-linolenic acid	0.32
C22:0 Behenic acid	1.34
C22:1n9 Erucic acid	1.21
C20:3n3	0.15
C20:5n3 Eicosatrienoic acid	1.80
C23:0 Tricosanoic acid	1.65
C24:0 Lignoceric acid	1.39
C24:1 Nervonic acid	0.48
C22:6n3	0.94
VITAMINS	**mg/kg**

Vitamin C	39.03
Vitamin E	5. 19
Vitamin A	19. 25

The use of metabolites in the structures of aquatic organisms in the pharmaceutical sector is increasing day by day. Metabolites derived from algae make significant contributions to human health, and these organisms are good sources for the compounds used in new drug discoveries. Algae are of great importance in terms of the phenolic components, pigments, vitamins, lipids, minerals and proteins they contain. Algae are an important source of natural antioxidants, which can be used as a substitute for synthetic antioxidants (Michalak and Katarzyna, 2015). During the breakdown of nutrients, the oxygen used turns into various free radicals and damages the body. As these radicals are unstable, they can easily react with certain groups of substances in the body and cause some damage (Burtis and Ashwood, 1999). Antioxidants help minimize these damages by neutralizing free radicals (Gökpınar et al., 2006).

According to the antioxidant analysis results, a dose-dependent activity was observed in CFI in terms of DPPH free radical scavenging activity, FRAP and TEAC reducing power, but it was not found to be significant as it remained above IC_{50} values (Table 1). CFI, a subspecies of *Cladophora fracta*, has been found to have less activity than *C. fracta* and some other species of Cladophora.

Kartal et al., (2009) researched *Cladophora glomerata* and *C. fracta*, C fracta was found to have 2 mg/ml concentration of fracta, 2.4±0.91%, C. glomerata was found to have 0.5, 1 and 2 mg/ml concentration, 4.4±0.78%, 6.4±0.28% and 8.8±0.01%, respectively; both algal species were found to have performed good antioxidant activity and *C. glomerata* was found to be more effective than *C. fracta*.

Some research conducted on *C. glomerata*, phenolic substance content and DPPH activity have been investigated. When the total phenolic substance content was examined, it was determined that the liquid extract was 0.025 ± 0.004 mg GAE/G and the methanol extract was found to be 0.032 ± 0.003 mg GAE/g. According to the IC50 (µg / mL) value in the DPPH free radical removal activity test, *C. gloremerata* appears to have good antioxidant activity. DPPH radical removal activity of water extract was 39.69 ± 2.17 µg/mL and methanol extract was found to be 29.92 ± 2.56 µg/mL (Akköz, 2009).

Zbakh et al., (2014) conducted a study and examined *C. prolifera* (Roth) Kutzing' antibacterial, cytotoxic and antioxidant properties of *C. prolifera* (Roth), and they performed ABTS testing and the activity of the extract at 200 µg/ml of concentration was compared with Trolox. The inhibition percentage of Trolox was found to be 97.7% while the inhibition percentage of *C. prolifera* extract was measured as 70.32%. The obtained results have shown that prolifera has both antibacterial activity and strong antioxidant potential.

Krish and Das, (2014) conducted research and examined antimicrobial and antioxidant effects of *C. rupestris* extracts obtained with different solvents (methanol, ethanol, ethyl acetate). In the DPPH method, concentrations of 1-5 mg/ml of extracts were used and in particular, the highest activity was to be 78% in methanol extract at a concentration of 5 mg/ml.

Lezcano et al., (2018) researched *C. surera*, and they used water and methanol as solvents in their research. In the tests, the DPPH method was used for antioxidant and the Folin Ciocalteu method was used for total polyphenolic substance content. Antioxidant activity test results revealed that methanol extract was 10.66±0.22% and water extract was 17.73±0.24%. Similarly, water extract (2.69±0.14 µg gallic acid/ mg extract) was more effective than methanol extract (0.64±0.06 µg gallic acid/ mg extract) in total polyphenolic substance results.

In the fatty acid analysis of CFI, it was found to contain many types of fatty acids. Among them, C16:0 Palmitic acid was found to be 29.66%, C16:1 palmitoleic acid was found to be 20.77%, C18:1 N9C oleic acid was found to be 10.78%, C14:0 myristic acid was found to be 10.29% and to be higher than others (Table 2). In another study conducted on the fatty acids of *Cladophora fracta*, oleic acid was found to be 46% and Palmitic acid was found to be 15.6%. Palmitoleic acid was found to be 1.54% (Karan and Erenler, 2018). In the study of Pabuçcu et al., (2018), among the unsaturated fatty acids of *C. fracta*, Oleic acid was found to be 9.77% and linoleic acid was 1.68%.

Palmitic acid in CFI was found to be higher than C. fracta, while oleic acid was found to be lower. Palmitoleic acid was very high in CFI, while it was to found be low in C. fracta. High levels of palmitoleic acid in CFI indicate that its anti-cancer properties may be high (Itoa et al., 1982). Vitamin C (Ascorbic acid) is an important molecule that supports the antioxidant system in the body and it also increases the body's immunity. This vitamin also acts as a cofactor in enzymatic reactions (Padayatty et al., 2003). In vitamin analysis of CFI, vitamin C was found to be relatively high at 39.03 mg/kg (Table 2).

Vitamin E is an important fat-soluble vitamin that prevents many types of cancer. It acts in the rapid regeneration of cells, rapid healing of wounds and removal of toxins from the body. Toxic substances accumulated in the body due to both radiation and drugs are removed from the body with the help of vitamin E (Annette et al., 2019). In vitamin analysis of CFI, vitamin E was found to be 5.19 mg/kg (Table 2). Vitamin A is a vitamin that improves the quality of nutrition and is usually found in higher proportions in organisms living in aquatic environments. This vitamin strengthens the immune system and helps regulate cell function. It is also a biomarker for child development (Tanumihardjo, 2011). In the vitamin analysis of CFI, vitamin A was found to be 19.25 mg/kg (Table 2).

CONCLUSION

In the study, it was found that CFI has several similarities with close species in some characteristics, but it also has some unique characteristics. As a result of the characteristics examined, CFI is a pharmaceutically important organism and it needs to be examined in a more detailed way in the future.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author’s Contributions

The authors declare that they have contributed equally to the article.

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