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Original article

LIPIDS OF BLACK SEA ALGAE: UNVEILING THEIR POTENTIAL FOR PHARMACEUTICAL AND COSMETIC APPLICATIONS

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ABSTRACT

Background: Bulgarian Black Sea coast is rich in algae, regarding biomass and algal biodiversity. The red algae *Gelidium crinale* (Rhodophyta) and brown algae *Cystoseira barbata* (Phaeophytes) are among the most abundant species along the Bulgarian Black Sea shore. Yet information about their lipid composition is limited.

Purpose: Present study was conducted to investigate biologically active substances in two underexplored seaweed lipids. Total lipids, total phospholipids, fat soluble vitamins and carotenoids were analysed. In addition, the specific distribution of fatty acids group among the total lipids and total phospholipids were elucidated.

Material/Methods: The saponifiable lipid fraction was derivatized into fatty acid methyl esters (FAMEs) and analysed by gas chromatography–mass spectrometry (GC-MS) to identify and quantify the fatty acids. The fat soluble non-saponifiable lipids were identified by high-pressure liquid chromatography coupled with UV/Vis and fluorescence detectors (HPLC-UV-FL).

Results: Results showed that Rhodophyta and Phaeophytes have high concentrations of polyunsaturated fatty acids (PUFA), particularly from the n-3 series, thereby being a good source of these compounds. They presented a "healthy" n-6/n-3 ratio. Both seaweed species showed considerably high amounts of α-tocopherol, β-carotene and astaxanthin.

Conclusions: The study reveals that lipids from Black Sea algae have a high potential as natural sources of biologically active ingredients. They are balanced source of fatty acids and contained beneficial antioxidants, such as α-tocopherol, β-carotene and astaxanthin.

Keywords: *Cystoseira barbata*, *Gelidium crinale*, phospholipids, fatty acids or poly unsaturated fatty acids (PUFA), tocopherols, carotenoids, astaxanthin,

INTRODUCTION

Seaweeds are classified by colour into three group: Rhodophyta (red), Phaeophyta (brown) and Chlorophyta (green) macroalgae. It is known that algae are rich sources of bioactive natural products which may have a significant role in health promotion, mainly in diseases prevention and treatment. Moreover, seaweeds have been used since ancient times as food, sources of medicine, but despite their abundance, nowadays they are poorly exploited. This article focuses on the Black Sea red and brown algae species lipid composition. Algal total lipid content is usually low, but they contain a high proportion of polyunsaturated fatty acids (PUFA) combined with other interesting secondary metabolites as vitamins, pigments, proteins etc. PUFA are of the utmost importance for human metabolism. Besides their structural role, they possess other beneficial effects, like antioxidant activities, prevention of cardiac diseases, inhibition of tumour progression, anti-inflammatory, etc. Such properties are indicative of the potential of PUFA for nutraceutical and pharmaceutical purposes [1]. Algal lipids are a good source of vitamins, important not only due to their biochemical functions and antioxidant activity but also due to other health benefits [2]. The main source for long-chain PUFA is fish oil. Bearing in mind that fish is a declining resource and that there is an increasing commercial interest in such fatty acids (FA), an alternative source must be found [3]. Bulgarian Black Sea coast is rich in algae, regarding algal biodiversity and biomass, yet information about fat soluble vitamins and carotenoid content and FA composition of native species is still limited. Moreover, the lack of studies of the bioavailability of algal lipids currently limits their nutritional evaluation and assessments of possibility for pharmaceutical and cosmetic applications in Bulgaria. The objective of this study was to provide knowledge on the composition and reveal the potential of two seaweed species – *Gelidium crinale* (Rhodophyta) and *Cystoseira barbata* (Phaeophytes) from the Black Sea as alternative sources of the functional ingredients, described above.

MATERIAL AND METHODS

Sampling

Seaweeds were collected during July 2012 from the region of Cape Galata (Varna Bay), Bulgaria. All specimens were harvested manually from their respective sites and

then transported to the laboratory in wet tissue towels in an ice box. They were thoroughly cleaned with sea water, and cleaned samples were frozen and stored at -20°C prior to analysis. Voucher specimens were deposited in the Institute of Oceanology in Varna.

Total lipids, phospholipids and fatty acids analysis

Total lipids (TL) were extracted by modified method of Bligh and Dyer (1959) [4]. Phospholipids (PL) were isolated from the lipid fraction by precipitation with cold acetone. Briefly, 20 cm^3 of cold acetone (-20ºC) were carefully poured into the lipid extracts. After the precipitation of PL, the supernatant was decanted off. The precipitate was carefully washed 3 times with new portions of cold acetone. Finally obtained phospholipids were dried under a gentle stream of nitrogen. The quantification was carried out spectrophotometrically after mineralization with a mixture of perchloric acid and sulphuric acid, 1:1 (by volume) by measuring the phosphorus content at 700 nm. The calibration curve was constructed using a standard solution of KH_2PO_4.

Fatty acid compositions of algae TL and PL were determined by gas chromatographic separation of the corresponding methyl esters. Lipid fractions were methylated by base-catalyzed transmethylation according to BDS EN ISO 12966-2:2017 [5]. After centrifugation at 3500 rps and extraction with hexane FAMEs are subjected to gas chromatographic analyses. The analysis was performed by a FOCUS Gas Chromatograph with autosampler A 3000, equipped with Polaris Q MS detector (Thermo Scientific, USA). A TR-5 MS capillary column was used (Thermo Scientific, USA; 30m, 0.25mm i.d). The carrier gas was helium at flow rate 1 ml/min. Chromatographic separation was achieved by temperature range: initial temperature – 40ºC for 4 min followed by 10ºC per minute until 235ºC and final temperature reach was 280ºC for 5 min. The sample volume was 1µl. FAME peaks were identified by comparison of their retention times with authentic standards (SUPELCO F.A.M.E. Mix C4-C24) and quantified by area normalization.The amounts of FA were calculated as a percentage of total FA recovery as a mean value and standard deviation (SD) according to BDS EN ISO 12966-4:2015 [6]. Three replicate GC measurements were performed.

Fat soluble vitamins and carotenoid analysis

Astaxanthin, β-carotene, α-tocopherol, retinol eq. were determined simultaneously using HPLC/UV/FL system. Sample preparation was performed following the method described by Dobreva et al., (2017) [7]. Generally, an aliquot of the homogenized tissue (1.500±0.005g) was weighed, and 1% of methanolic L-ascorbic acid and 0.3M methanolic potassium hydroxide were added. Six parallel samples of algae tissue were prepared and saponified at 40ºC for 30 min. After cooling the lipids were extracted with n-hexane: dichloromethane. The extracts were pooled together and evaporated under nitrogen. The dry residue was dissolved in methanol and injected (20µL) into the HPLC system (Thermo Scientific Spectra SYSTEM) equipped with RP analytical column (Synergi 4µ Hydro-RP 80A pore 250x4.6 mm). Detection of astaxanthin, beta-carotene was performed by UV detector (for astaxanthin λ=474nm, for β-carotene λ =450nm). The concentration of a- tocopherol was measured by fluorescence at λ ex = 288 nm and λ em = 332 nm. The quantitation was performed by the method of the external calibration, comparing the chromatographic peak areas of the samples with those of the corresponding standards.

Chemicals

All of the chemicals used in the experiments were analytical grade and GC grade (Sharlau, Sharlab Sourcing Group, Spain). Analytical standards were purchased from Sigma-Aldrich™.

Statistical analysis

Student's t-test was employed to estimate the significance of values. Statistical significance was indicated at $p<0.05$.

RESULTS AND DISCUSSION

Lipids

Low levels of total lipids (TL) content were determined for both species. The defined TL amounts ranged between 3.3 (brown algae) to 5.1 (red algae) $g.100^{-1}$ dry weight (DW). These results are in agreement with data presented for 13 species of brown algae from the coast of Gujarat, India [8] and for brown algae from Port Underwood, New Zealand [9]. Although TL content in algae is lower than those found in marine fish, their large stock in coastal waters determine them as potential sources of functional lipids. For better assessment of this potential of algal lipid, the amounts of phospholipids (PL) were also determined. It is known that the amount of total PL in different algae species varies from 10% to 20% of the TL [10]. The total PL levels in this study are in cited range: 10.6% (red algae) to 18.4% (brown algae) of TL. These results are lower than those reported by Kendel et al. (2015) for red algae species (*S. hordalis*) – 23.7% of from Brittany, North West of France [11]. Very close amounts were found when PLs values are calculated in $mg.g^{-1}$ lipid: 5.4 for red and 6.1 $mg.g^{-1}$ lipid for brown algae. Marine PLs have many advantages compared to fish oil because they are much more stable to oxidation and can be used for pharmaceutical products. In additions, PLs in diet act as natural emulsifiers and facilitate the digestion and absorption of different lipophilic nutrients.

Fatty acid composition

The results for analysed components are presented in Table 1. The levels and the proportions of FA groups in algae vary depending on the species and the environmental factors. FA values in the present study are reported in % of TL and total PL, and in $mg.g^{-1}$ lipid due to discrepancies in expressing the values only in % since the latter could mask the inadequacies of the methods and also could be inaccurate. The total saturated fatty acids (SFA) accounted from 57% (29.7$mg.g^{-1}$lipid) for red algae to

70.63% (23.3 mg.g^{-1} lipid) for brown algae, whereas total monounsaturated fatty acids (MUFA) showed the lowest values, especially in red algae – 13.08% (4.32 mg.g^{-1} lipid). Several studies have reported that algae are rich sources of PUFA [8, 9, 10]. Our results showed lower PUFA amounts: from 16.29% (5.38 mg.g^{-1} lipid) for brown algae to 32.38% (16.51 mg.g^{-1} lipid) for red algae. The overall FA profile of TL presented a similar distribution pattern for both analysed algae species: SFA>PUFA>MUFA.

The FA distribution of total PL followed the same pattern as in TL fraction: SFA>PUFA>MUFA. When PUFAs are presented in mg.g^{-1} lipid, the low lipid *Cystoseira barbata* appears to contain almost 2 times higher amounts (1.23 mg.g^{-1} lipid) compared to *Gelidium crinale* species (0.75 mg.g^{-1}lipid). Kendel et al., (2015) reported different FA distribution in PL fraction of red algae (SFA>MUFA>PUFA) from the French coast [11]. We can suggest that the FA composition of seaweed samples collected from different location usually shows distinction because there is a close connection between FA unsaturation and environmental conditions.

The content of C_{18} and C_{20} PUFA are important characteristics of algae lipid quality. Several studies reported that Rhodophyta (red algae) contain significant quantities of C_{20} PUFA, whereas brown species (Rhodophyta) show the opposite trend [6, 9]. In contrary, the defined values in our study presented that C_{20}>C_{18} PUFA for both algae species. The content of C_{20} PUFA was 2-5 times higher than that of C_{18} PUFA in all samples, moreover, PL fraction of brown algae contained only C_{20} PUFA. The highest amounts of C_{20} PUFA (10.35 mg.g^{-1} lipid) and C_{18} PUFA (4.69 mg.g^{-1} lipid) were found in red algae TL fraction. We can suppose that red algae lipids have higher antioxidant properties compared to brown algae.

The ratios n-6/n-3 and PUFA/SFA are very important for human health. The World Health Organization (WHO) recommends that the n-6/n-3 ratio should not exceed 10 in a diet and a decrease in the human dietary n-6/n-3 PUFA ratio is essential to help prevent coronary heart disease by reducing the plasma lipids and the risk of cancer [12]. Of the two major classes of n-6 and n-3 PUFAs, n-3 remained the dominant one in brown algae in both analysed lipid fraction. The opposite trends were found for red seaweed. In this case, the n-6 PUFA content was higher in TL lipid fraction (10.26 mg.g^{-1} lipid), whereas n-3 content was more in PL fractions (0.47 mg.g^{-1} lipid). The observed n-6/n-3 ratios for red species showed significant differences between two lipid fractions. The highest calculated ratio was found in TL (1.64), whereas it is 6 times lower (0.26) in PL fraction. In brown algae, the calculated ratio is similar for both lipid fractions (0.68). All the examined species exhibited a favourable n-6/n-3 ratio below 10. Kumari et al., (2010) reported significant high values for the n-6/n-3 ratio for Rhodophyta (up to 27.7) and Phaeophyta (up to 5.15) species from the coast of India [8]. In this study, the PUFA/SFA ratio was found lower than 4.0 (Table 1), which is within the recommendations of the Department of Health (1994) [13].

Table 1. Fatty acid composition of TL and PL of *Gelidium crinale* and Cystoseira *barbata* from the Black Sea coast

	Gelidium crinale (red algae)				*Cystoseira barbata* (brown algae)			
	Phospholipids		Total lipids		Phospholipids		Total lipids	
	% of TPL	mg.g^{-1} lipid	% of FA	mg.g^{-1} lipid	% of TPL	mg.g^{-1} lipid	% of FA	mg.g^{-1} lipid
SFA	78.09	4.22	57.00	29.7	67.09	4.10	70.63	23.3
MUFA	9.09	0.49	10.62	5.42	12.79	0.78	13.08	4.32
PUFA	12.82	0.70	32.38	16.51	20.12	1.23	16.29	5.38
PUFA/SFA	***0.16***		***0.57***		***0.30***		***0.23***	
C_{18} PUFA	1.88	0.10	9.20	4.69	Nd		6.42	2.12
C_{20} PUFA	8.55	0.46	20.29	10.35	20.12	1.23	9.22	3.04
n-6	2.25	0.12	20.12	10.25	8.12	0.49	6.53	2.15
n-3	8.63	0.47	12.27	6.26	12.01	0.73	9.76	3.23
n-6/n-3	***0.26***		***1.64***		***0.68***		***0.67***	

Fat soluble vitamins and carotenoids

Among the compounds found in seaweeds, those with antioxidant activity have an excellent potential for application in cosmetics and pharmacology industry. In this study, the analysed fat soluble components with antioxidant activity are vitamin E (α-tocopherol) and carotenoids (β-carotene and astaxanthin). Observed results are presented in Table 2. Alpha-tocopherol, the most bioactive form of vitamin E, was determined in both algae, in the range 1.55 (red) to 4.70 mg.g^{-1} lipid (brown). These results present a promising value since they are significantly higher than the values reported for another seaweed as *U. Pinnatifida* (1.32 mg.g^{-1} lipid) [14]. According to Ortiz et al., (2006) brown algae contain more a-tocopherol than red seaweeds [15]. Our results fully confirm his claim. Algae do not contain intrinsic vitamin A, but its pro-vitamin β-carotene. In this study, 2.5 times higher amount of β-carotene was found in brown species (8.35 mg.g^{-1} lipid) compared to red algae (3.43 mg.g^{-1} lipid). The vitamin A activity, calculated on the basis of β-carotene content in 100 g DW as retinol equivalent (RE) was in the range from 2.91 mg RE/100 g DW (red algae) to 4.6 mg RE/100 g DW (red algae). According to Norziah and Ching, (2000) classification of RE values, both Black Sea algae can be classified as very high sources (> 1000 μg RE) of pro-vitamin A [16]. Thus *Cystoseira barbata* is an interesting potential source having a high vitamin A activity despite the low TL amount. Astaxanthin has high antioxidant activity - 10 times more than β-carotene and 100 times more that α-tocopherol. Currently, there is a wide variety of astaxanthin – containing supplements, and most of them are manufactured from seaweed extracts. Due to their high antioxidant properties, they have potential properties against many diseases; anti-inflammatory activity, anticataract prevention activity, etc. The recommended dose is 5 mg per day astaxanthin [17]. In present study astaxanthin values were in the range: 0.19mg (red algae) to 0.45 mg.100g^{-1} lipid (brown algae). In our case, 300 g dry mass of *Cystoseira barbata* and 520 g dry mass of *Gelidium crinale* from the Black Sea, contain 5.1 mg and 5.0 mg astaxanthin, respectively.

Table 2. Fat soluble vitamin and carotenoids content of *Gelidium crinale* and Cystoseira *barbata* from the Black Sea coast (in mg.g^{-1} lipid)

	Gelidium crinale (red algae)	*Cystoseira barbata* (brown algae)
α- tocopherol	1.55 ± 0.09	4.70 ± 0.23
Astaxanthin	0.19 ± 0.01	0.45 ± 0.04
β- carotene	3.43 ± 0.09	8.35 ± 0.30

CONCLUSION

Marine algae are generating a lot of interest because of their wide distribution and valuable chemical composition. Phospholipids, fatty acids composition, α-tocopherol, β-carotene and astaxanthin content in two of the most widespread Black Sea algae were evaluated in the present study. The FA profile of both lipid fractions presented a similar distribution for analysed algae species: SFA>PUFA>MUFA. The two examined species exhibited a favourable n-6/n-3 ratio below 10. The study reveals that lipids from Black Sea algae have a high potential as natural sources of biologically active ingredients. High concentrations of α-tocopherol, β-carotene, polyunsaturated fatty acids and the presence of the powerful antioxidant astaxanthin demonstrate possible application of these macroalgae as supplements for use in pharmaceutical, cosmetic industry and aquaculture.

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