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Pressurized Liquid Extraction for the Recovery of Carotenoids and Functional Compounds from Green and Orange *Dunaliella salina* Biomasses

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Abstract

In recent years, intensive research has been conducted on natural carotenoids extraction using several processes. Conventional extraction methods require high amounts of solvents and a long extraction time. However, pressurized liquid extraction demonstrated to be an interesting method. The extraction efficiencies of pressurized liquid for the recovery of carotenoids, from the green and the orange biomasses of the microalga *Dunaliella salina* DunaDZ1, are described. Organic solvents were tested including ethanol, *n*-hexane, ethyl acetate and a mixture of *n*-hexane:ethanol (3:4). Moreover, three extraction temperatures were used (90, 120 and 150 °C) at constant pressure. Extraction efficiency and extracts characterization were conducted. Results have shown that temperature has a positive effect on extraction yield. HPLC characterization showed that *β*-carotene is the main carotenoid in the orange biomass, and lutein in the green biomass, with the presence of other minor carotenoids in both biomasses. The highest carotenoid amounts were found in the *n*-hexane orange biomass extract, with *β*-carotene isomers as the main carotenoid (138.54 and 357.10 mg/g of dry extract, for *cis* and *trans* isomers, respectively). Otherwise, extracts obtained at the lowest tested temperature provided the best carotenoid yields. The best results for the antioxidant activity were obtained at 120 °C for orange biomass ethyl acetate extract.

Keywords

carotenoids, *Dunaliella salina*, pressurized liquid extraction, temperature, antioxidant activity

1 Introduction

Carotenoids are a group of pigments that are gaining more attention due to their potential health benefits and their essential role in preventing human diseases (cardiovascular diseases, macular degeneration and cancers). They are considered as natural antioxidants, which can help maintain the stability and coloring of foods [1–4]. One of the major interesting carotenoid is *β*-carotene, which can be found in several natural sources (e.g. carrots, sweet potatoes, apricots, etc.), with the microalgae *Dunaliella salina* as the main source of this compound (up to 16% of dry matter) [5–7].

Some microalgae species are cultivated on a large-scale for their ability to produce bioactive compounds with multiple industrial, biotechnological and biorefinery applications [8–10]. Numerous species of microalgae are reported to produce different types of carotenoids, e.g. *Dunaliella salina*, *Porphyridium cruentum*, *Isochrysis galbana*, *Haematococcus pluvialis*, etc. Astaxanthin, *β*-carotene, lutein, lycopene and canthaxanthin are the main carotenoids found in microalgae, which are receiving increased attention thanks to their biological activity and their potential health benefits [1, 11–13].

The green biflagellate microalgae *D. salina* has pear-shape cells and thin elastic plasma membranes. The main characteristic of this species is the lack of a rigid cell wall.

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It is considered as good resource for commercial production of *β*-carotene. *D. salina* carotenoids are synthesized in response to stress factors such as salinity, light and temperature [14, 15]. In the cells, *β*-carotene is usually present with other carotenoids such as lutein, neoxanthin, zeaxanthin, *α*-carotene and violaxanthin [5, 16].

The choice of extraction method for obtaining carotenoids from microalgae is crucial and should take into consideration several factors, such as the oxidative characteristics of carotenoids which limit the exposure to excess heat and long extraction times [17]. Nowadays, several carotenoids extraction methods have been investigated, which can be classified as conventional: Soxhlet, maceration, etc., and non-conventional methods: pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), pulsed electric field (PEF), etc. [11, 18–20]. Most of these methods were tested by several authors on different strains of *D. salina*, but were only evaluated for their application to the orange biomass [21–24].

PLE technique uses solvents at high temperatures and pressures to extract various analytes mainly from solid samples. These conditions cause changes in solvent proprieties, enhancing mass transfer rates and decrease solvent surface tension and viscosity and hence the solubility of analytes increases. This technique has the advantage on being fast and requires smaller solvent volumes compared to conventional extraction techniques [25, 26]. Therefore, PLE has been used to extract several compounds from different microalgae species, for example, pigments (mainly carotenoids), omega-3 acylglycerols and polyphenols [27–30].

The aim of this work was to study carotenoids composition and antioxidant activity of different pressurized liquid extracts of green and orange biomass from *D. salina* strain DunaDZ1 isolated from Zahrez Chergui salt lake in Algeria. PLE carotenoids extraction from *D. salina* green biomass is, to the best of our knowledge, investigated for the first time.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals used in this study were purchased from commercial sources and they are of analytical grade, acetone, ethyl acetate, methanol, and petroleum ether were from LabScan (Australia), *n*-haxane from Macron (Göteborg, Sweden), and absolute ethanol from AppliChem (Darmstadt, Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl) and carotenoids standard (*β*-carotene, violaxanthin, lutein, *α*-carotene, zeaxanthin) were obtained from Sigma-Aldrich.

2.2 Microalgae biomass

Dunaliella salina strain DunaDZ1 was isolated from Zahrez Chergui salt lake in Djelfa province, Algeria. It was identified using molecular tools in a previous study [31]. *D. salina* was cultivated in *f*/2 medium using one-step method for the green biomass [32]. Cultures were incubated at 22 ± 2 °C with continuous illumination provided by white lamps (120 µmol photon m^{-2} s^{-1}). However, for the orange biomass (carotenogenesis) a two-step method was used.

The first step (growth phase) consisted of the accumulation of a high biomass level by culturing *D. salina* under optimal conditions. When the culture had produced a high level of biomass, it was used for the second step (stress phase for carotenoids production). Cultures at the end of the exponential phase were transferred into another medium to induce cells for carotenogenesis (free nitrate medium, at 2 M NaCl). Biomasses were harvested by centrifugation and dried in a vacuum freeze dryer (Chaist). These were then stored in darkness at –20 °C until extraction.

2.3 Pressurized liquid extraction of carotenoids

Extractions were carried out with an accelerated solvent extractor Dunix ASE 350 (Sunnyvale, California). Green or orange freeze-dried biomass was mixed in a 1:9 ratio with sea sand (300 to 350 µm of diameter, from Scharlau, Spain). This mixture was added to 10 mL cells. A filter paper was placed at the bottom of the extraction cell and then was automatically filled with solvents. The solvents used were ethanol, *n*-hexane, ethyl acetate and a mixture of *n*-hexane:ethanol (3:4). Extractions were performed at three different temperatures: 90, 120 and 150 °C, with a static extraction time of 15 min and pressure of 110 bar. These conditions were selected based on published [11] and unpublished data that aimed to extract carotenoids from green microalgae using PLE. Solvents were removed using HeidolphHei-Vap HB/G3 evaporator at 35 °C. The samples were saturated with N_2 and were stored in darkness at 4 °C until their analysis. Extractions were carried out in duplicate.

2.4 Thin Layer Chromatography (TLC) analysis

Silica gel 60 plate (Macherey-Nagel. Germany) was used to perform TLC. 10 µL of each extract at concentration of 20 mg/mL were spotted on the Silica plate. A mixture of petroleum ether and acetone 3:1 (v/v) was employed as the

mobile phase. The standard used was *β*-carotene at a concentration of 5 mg/mL.

2.5 HPLC analysis

Analyses of the extracts were performed with an HPLC (Varian ProStar 218) equipped with a diode array detector. Twenty microliters of each extract (2 mg/mL) were injected into the HPLC. This HPLC is equipped with an Eclipse XDB-C18 column (5 µm, 150 × 4.6 mm). The mobile phase used was 100% methanol. The flow rate was kept at 1.5 mL/min for 50 min. Quantification of carotenoids was done by referring to a standard curve, using carotenoid standards (*β*-carotene, violaxanthin, lutein, α-carotene, zeaxanthin, by Sigma-Aldrich). The detection wavelength was set at 450 nm.

2.6 DPPH radical-scavenging activity

DPPH radical-scavenging activity of PLE extracts was measured according to a previously described method of Tepe et al. [33]. A sample of 1 mL (extracts at different concentrations: 300 to 1000 µg/mL) was added to 1 mL 0.004% DPPH solution in methanol. Since our PLE extracts from green and orange biomass absorb at 517 nm, it was necessary to prepare a control. This control contains 1 mL of each sample and 1 mL of methanol. Whereas the blank contains only methanol. The mixture was shaken then incubated in darkness for 30 min. Absorbance was read at 517 nm using a spectrophotometer (Jenway 6705). Analysis was carried out in triplicate.

The DPPH radical activity was calculated using the Eq. (1):

$$\text{Scarvenging effect } \% = \left[\left(\frac{A_0 - A_s}{A_0}\right) \times 100\right], \quad (1)$$

where A_0 represents absorbance of DPPH solution (without extract) (DPPH solution:methanol) (v/v), A_s represents absorbance of DPPH solution mixed with extracts dilutions.

The results were expressed as IC_{50} (concentration providing 50% inhibition). IC_{50} values were calculated from the plotted graph of scavenging activity against the concentrations of the samples.

2.7 Statistical analysis

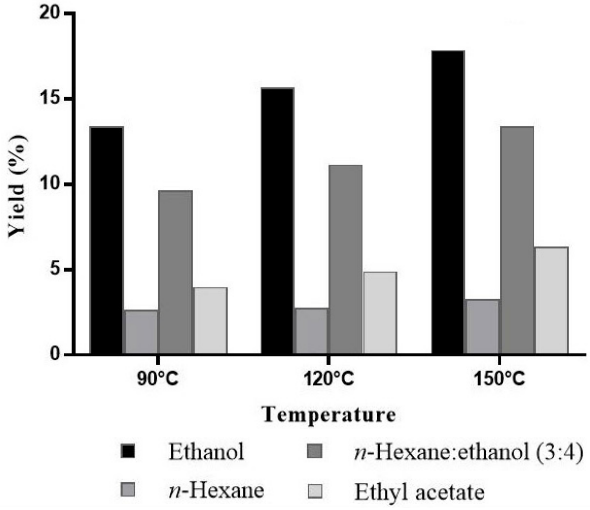
Statistical analyses were performed using analysis of variance (ANOVA) and Tukey's test. The software used was SPSS 21.0 (SPSS Inc., Chicago, USA). Pearson's correlation coefficient correlation was calculated to determine the relationship between parameters.

3 Results and discussion

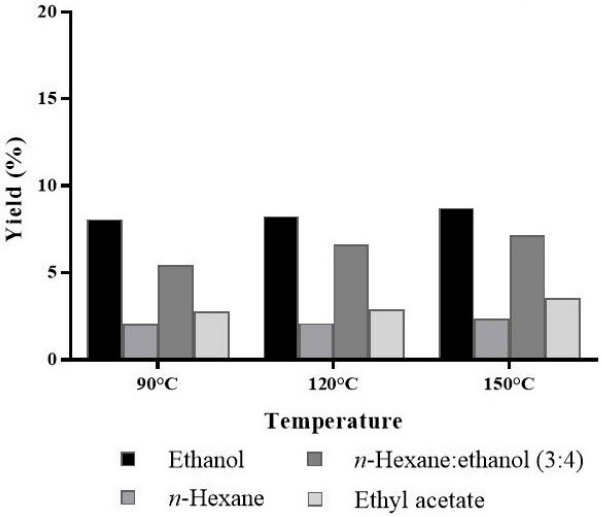
3.1 PLE yields

PLE extraction yields from green and orange *D. salina* biomass are presented in Fig. 1. Extraction yields values are closely related to temperature. Fig. 1 demonstrates that temperature enhances the extraction yields for both biomasses. The highest yield was obtained for ethanol extracts at 150 °C and this is for both *D. salina* biomasses, green (17.83%) and orange (8.68%). It is clear that the temperature increased the solubility of solutes in the solvent. The extraction yield of *D. salina* biomass also improved in the presence of polar solvent (ethanol).

Previous works have shown the effect of temperature to enhance yields, it improves mass transfer rates, promoting



(a)



(b)

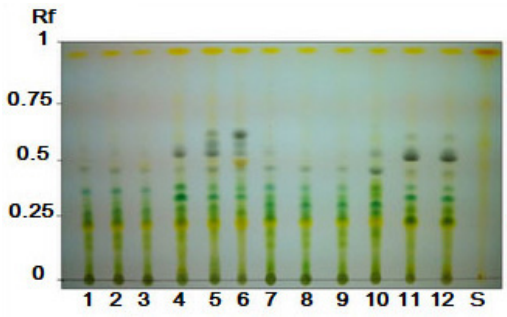
Fig. 1 PLE extraction yield of green biomass (a) and orange biomass (b) from *D. salina* DunaDZ1

solutes extraction from biomass to the solvent. This is explained by the increase of the solute solubility and diffusion coefficients; it also reduces solvent surface tension and viscosity [11, 17, 34].

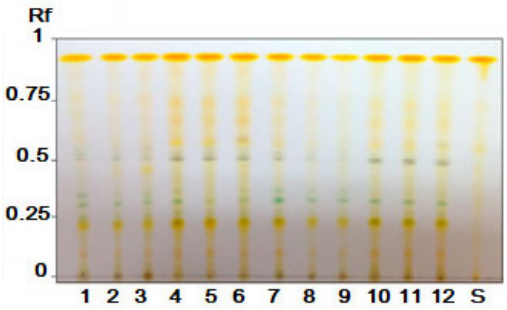
3.2 PLE extracts analysis

3.2.1 TLC analysis

Thin layer chromatograms are shown in Fig. 2. The retention factor (*Rf*) value of each spot was calculated and then compared with the scientific literature. The TLC analysis of green biomass revealed several green spots, which are characteristic of chlorophylls. Grey spots are also detected at different *Rf*, with a high intensity for *n*-hexane and ethyl acetate extracts. Spots intensity is higher with the highest temperature tested 150 °C. These spots are pheophytins, which are chlorophylls degradation products. Pheophytin is a chlorophyll molecule lacking a central Mg^{2+} ion after heating or at low pH [35]. The same remarks have been announced by Jaime et al. [11], for *n*-hexane PLE extracts from the microalgae *Haematococcus pluvialis*. Carotenoids are also revealed in *D. salina* green biomass at different *Rf*, with lutein (*Rf* ~ 0.21) as the dominant carotenoids.



(a)



(b)

Fig. 2 Thin layer chromatogram of PLE extracts from green (a) and orange (b) *D. salina* DunaDZ1 biomasses. *Rf*: retention factor, Extracts: 1: *n*-hexane:ethanol (3:4), 2: ethyl acetate, 3: isopropanol, 4: acetone, 5: isobutanol, Standard (*S*) is *β*-carotene at 5 mg/mL

However, for *D. salina* orange biomass, yellow to orange spots are revealed, with *β*-carotene as the main pigment (*Rf* ~ 0.9). Furthermore, spots intensity depends on the solvent used. As shown in Fig. 2, almost all chlorophyll spots have disappeared. Pheophytins are also present but with less intensity compared with the green biomass. It is well known that *D. salina* cells turn to orange aplanospores when subjected to stress.

3.2.2 Analysis and quantification of carotenoids

Carotenoid identification was determined by the retention time and the UV-visible spectral produced, and they were compared with scientific literature. In this study only carotenoids are quantified.

Carotenoid concentration and temperature effects are shown in Table 1, lutein is the main carotenoid in the green biomass, with the higher amount (279.7 mg/g dry extract) was obtained for the *n*-hexane extract at 90 °C. However, to our knowledge, there are no studies on carotenoids from the green biomass of *D. salina*, which is characterized by the presence of a large amount of lutein. In addition to lutein, other xanthophylls (violaxanthin and zeaxanthin) were found in *D. salina* green extracts, with an amount between 36 and 91.7 mg/g dry extract. Furthermore, *α*-carotene and *β*-carotene were also detected and presented amounts ranging from 37.1 to 62.6 mg/g dry extract. As shown, and among all solvents tested, temperature has a positive effect on carotenes extraction (α and *β*), except *n*-hexane,

Table 1 Carotenoid composition of PLE extracts obtained from *D. salina* DunaDZ1 green biomass. Results are expressed in mg/g of dry extract

	Ethanol			*n*-Hexane			*n*-Hexane:ethanol			Ethyl acetate		
	90 °C	120 °C	150 °C	90 °C	120 °C	150 °C	90 °C	120 °C	150 °C	90 °C	120 °C	150 °C
Violaxanthin	52.3	36.2	46.5	91.7	76.4	78.1	58.8	50.5	79.8	73.4	68.2	66.7
Lutein	157.7	107.5	49.5	279.7	105.8	115.4	127.2	95.5	207.1	182.8	161.2	157.7
Zeaxanthin	45.3	36	46.7	39.1	51.3	52	56.1	53.1	66	39.7	39.7	38.4
NI	35.8	35.7	40.3	43.8	45.6	43.5	43.5	43.4	60.2	40.8	37.6	42.1
α-carotene	41.4	40.8	44.1	62.6	37.3	37.1	47.8	40	53.1	49.7	47.8	53.3
β-carotene	37.7	37.6	46	54.4	38.1	38.1	39	38.7	48.6	44.5	48.4	56.4
TC	370.2	293.8	273	571.4	354.5	364,2	372.4	321.2	514.8	431	403	414.6

NI: no identified carotenoid; TC: Total carotenoids

in which the highest amount is obtained at the lowest temperature tested (90 °C). Otherwise, temperature has a negative effect on the other carotenoids (xanthophylls), and the best temperature is the lowest tested (90 °C). Carotenoids concentration order in the green biomass is as follows: lutein > violaxanthin > *α*-carotene > *β*-carotene.

Nonetheless, HPLC results of *D. salina* orange biomass show *trans β*-carotene as the main carotenoid. The highest amount of this pigment (357.1 mg/g dry extract) is revealed in *n*-hexane extract at 90 °C. *Cis β*-carotene is also present in the orange biomass at lower levels than *trans β*-carotene, except for ethanolic extracts, where the amount of *cis β*-carotene exceeds *trans β*-carotene when extraction was done at 120 and 150 °C.

Carotenoids profiles and concentration from the *D. salina* orange biomass are summarized in Table 2. In general, the highest carotenoid amounts are obtained at the lowest temperature tested (90 °C). The *α*-carotene is also detected in *D. salina* orange biomass, with the higher level (57 mg/g dry extract) in the *n*-hexane extract at 90 °C. Lutein is also present in the orange biomass with the higher level (102.3 mg/g dry extract) for *n*-hexane at 120 °C, this level is lower than the obtained for the green biomass. Violaxanthin is also identified in the orange biomass with the higher level (48 mg/g dry extract) in hexanic extract at 90 °C. Moreover, two other carotenoids are detected in the orange biomass but not identified. Quantities of these pigments vary from 36.5 to 51.2 mg/g dry extract.

Therefore, for the orange biomass, carotenoids concentration order is *trans β*-carotene > *cis β*-carotene > lutein > *α*-carotene > violaxanthin.

The microalga *D. salina* DunaDZ1 produces not only *β*-carotene, but is considered also as a good source of lutein which can help to prevent degenerative diseases as for example age-related macular degeneration [36].

Lin et al. [37] have investigated the *D. salina* orange biomass, and they found 474.8 mg/g *trans β*-carotene, 425.6 mg/g *cis β*-carotene, 22.8 mg/g lutein, 39.3 mg/g zeaxanthin and 9.3 mg/g *α*-carotene, using a solvent mixture of *n*-hexane:acetone:ethanol (2:1:1) and conventional extraction techniques. However, lower carotenoid amount has been reported by Herrero et al. [38] using PLE at different temperatures (40, 100 and 160 °C) and *n*-hexane as solvent. Amounts were 2.6–227.7 mg/g for *trans β*-carotene, 4.2–22.9 mg/g for *cis β*-carotene and 0.8 to 24.8 mg/g for *α*-carotene. Carotenoids are characterized by a highly unsaturated structure which makes them susceptible to degradation mainly under high temperatures [39].

It is important to note that, amounts of carotenoids found depend greatly on not only the microalgae species or strain studied but also on the culture conditions (whether the one or two step approach were used, the stress factor used, etc.). Salinity, light intensity, nitrate concentration, photoperiod are the main factors determining biomass composition [40, 41].

3.2.3 Antioxidant activity

The antioxidant potential of the PLE extracts was estimated and results are summarized in Table 3. PLE extracts obtained from orange and green *D. salina* biomass were analyzed to evaluate antioxidant activity and the results are expressed as IC_{50} values (μg of extract per mL). Results showed that the orange biomass is the most active. Temperature affects the IC_{50} values, the best activity was found at 120 °C.

The lowest IC_{50} (important antioxidant activity) is obtained in the ethyl acetate extract, with 194 ± 0.22 and 336.2 ± 0.59 μg/mL, for orange and green biomasses, respectively.

The highest IC_{50} was obtained at 90 °C for the orange biomass and at 150 °C for the green one. The difference

Table 2 Carotenoid composition of PLE extracts obtained from *D. salina* DunaDZ1 Orange biomass. Results are expressed in mg/g of dry extract

	Ethanol			*n*-Hexane			*n*-Hexane:ethanol			Ethyl acetate		
	90 °C	120 °C	150 °C	90 °C	120 °C	150 °C	90 °C	120 °C	150 °C	90 °C	120 °C	150 °C
Violaxanthin	39.7	38.4	39.4	48	45	43.6	42.5	38	38	47.1	45.4	42.2
Lutein	58.3	51.3	42.7	87.2	102.3	52	81.1	53.5	69.4	101.7	95.5	78
NI n°1	43.3	40	36.6	49.7	51.2	39.8	43.5	39.1	43.3	47.7	44	41.1
NI n°2	40.6	37	38	47.7	44.6	37.6	39.7	37.1	36.5	44	43.6	40.6
α-carotene	41.3	41.2	38.1	57	50	38.3	48	43.3	36.8	50.7	51.3	45
Cis β-carotene	68.1	147.5	72.8	138.5	127.8	49.7	107.6	102.7	39	92.6	77.8	74.2
Trans β-carotene	177.6	105.4	66.5	357.1	234.8	52	295.2	142	43	289.7	254.4	170.8
TC	469	460.8	334.2	785.2	655.7	313	657	455.	306	673.5	612	492

NI: no identified carotenoid; TC: total carotenoids

Table 3 Antioxidant activities of PLE extracts from DunaDZ1green and orange biomass of *D. salina* DunaDZ1. Values are the mean ± SD of three separate experiments. IC_{50}: concentration providing 50% inhibition, *T*: temperature

Solvent	*T* (°C)	IC_{50} (μg/mL)	
		Green biomass	Orange biomass
Ethanol	90	674.7 ± 0.28	568.6 ± 0.48
	120	604.8 ± 0.34	456.5 ± 0.22
	150	692.2 ± 0.38	545.4 ± 0.32
n-Hexane	90	758.5 ± 0.13	496.8 ± 0.25
	120	650.4 ± 0.26	359.1 ± 0.32
	150	774.8 ± 0.44	423.8 ± 0.21
n-Hexane:ethanol (3:4)	90	605.7 ± 0.52	494.4 ± 0.35
	120	522.8 ± 0.60	371.1 ± 0.24
	150	707.6 ± 0.49	402 ± 0.32
Ethyl acetate	90	427.4 ± 0.38	279.2 ± 0.63
	120	336.2 ± 0.59	194 ± 0.22
	150	446.7 ± 0.41	344.3 ± 0.33

between the antioxidant activities obtained by different solvents is explained by the difference in polarity of the antioxidant compounds contained in *D. salina* biomasses.

The IC_{50} obtained are comparable with *β*-carotene, a reference compound, which present an IC_{50} of 257.3 μg/mL as it was reported by Coulombier et al. [42]. Otherwise, the IC_{50} of *D. salina* extracts were highest then IC_{50} of other reference compounds with 6.3 μg/mL and 2.5 μg/mL, vitamin C and butylated hydroxytoluene (BHT), respectively [43].

Statistical analysis showed non-significant positive correlation between IC_{50} and total carotenoids from the green biomass ($r = 0.107$; $p = 0.741$; $p > 0.05$). However, for the orange biomass a non-significant negative correlation was revealed ($r = –0.236$; $p = 0.461$; $p > 0.05$). Carotenoids are known as an antioxidant agent. Protecting cells and tissues against damaging reactive oxygen species (ROS). They have a high free-radical scavenging proprieties thanks to the elongation in the conjugated double bond system. Carotenoid pigments are responsible for a major part of antioxidant activity in *D. salina*. They have a high free-radical scavenging proprieties thanks to the elongation in the conjugated double bond system. But other molecules such as phenolic compounds, polyunsaturated fatty acids and polysaccharides may also be present and consequently contribute to the increase of antioxidant activity [44, 45].

Otherwise, the non-significant correlation between total carotenoids and DPPH methods could be explained by the efficiency weakness of the method used to estimate the antioxidant activity (DPPH), as it is reported by several authors [46, 47].

In another study conducted on the antioxidant activity of Moroccan microalgae, the best result ($IC_{50} = 283$ μg/mL) was found in a *Dunaliella* sp. strain [43]. However, Cakmak et al. [48] reported IC_{50} values ranging from 450 to 3460 μg/mL for extracts obtained by different solvents from a strain of *D. salina*.

Moreover, Hu et al. [49] have demonstrated that the antioxidant activity of carotenoids extracted from *D. salina* is higher than the antioxidant activity of synthetic carotenoids, which opens numerous ways to utilize and commercialize these compounds.

4 Conclusions

PLE extracts of *D. salina* DunaDZ1 were found to contain major carotenoids, *trans β*-carotene for the orange and lutein for the green biomass, as well as other minor carotenoids. PLE hexanic extracts has shown the highest *trans β*-carotene yield (357.10 mg/g) in the orange biomass, and the highest lutein yield (279.67 mg/g) in the green biomass. The best temperature for PLE extraction from both biomasses is the lowest tested (90 °C). However, better antioxidant activity was obtained in the orange biomass extracted with ethyl acetate in comparison with the green one. The non-significant correlation between carotenoid amount and antioxidant activity could be explained by whether the weak efficiency of the method used and/or by the presence of other compounds in the biomass which contribute to improve this activity. In this study we have shown that both biomasses, green and orange, from *D. salina* DunaDZ1 are of great interest considering the industrial and commercial applications of *β*-carotene and lutein. Furthermore, PLE has advantages compared to traditional methods for being shorter in time and using small quantities of solvents.

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