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ORIGINAL RESEARCH PAPER

ULTRASOUND ASSISTED EXTRACTION OF CAROTENOIDS FROM GREEN AND ORANGE BIOMASSES OF *DUNALIELLA SALINA* DUNADZ1

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Abstract

Microalgae are described as a potential alternative source of bioactive compounds that are environmentally friendly. *Dunaliella* on its own is well known due to its potential to accumulate large amounts of carotenoids, especially β-carotene. Green and orange biomasses from a new *Dunaliella salina* strain DunaDZ1 isolated from an Algerian Salt Lake were evaluated for carotenoids composition and antioxidant activity. Ultrasound-Assisted Extraction (UAE) was employed using different extracting solvents. Furthermore, UAE extracts were then analyzed for their chemical composition by TLC, HPLC-DAD and for the antioxidant activity. Lutein was the main carotenoid in the green biomass, with the highest amount for ethyl acetate extract (393.19 mg/g). However, for the orange biomass, the main carotenoid was *trans*, β-carotene (131.83 mg/g) in the acetonic extract. Moreover, several others carotenoids were detected, belonging to xanthophylls and carotenes. Additionally, ultrasonic-assisted extraction with ethyl acetate produced the extract with the highest antioxidant activity for both *D. salina* biomasses. These extracts could be used as a natural antioxidant and as an ingredient for functional foods formulation.

Keywords: antioxidant activity**,** β-carotene, biomass, ethyl acetate, lutein

Introduction

In recent decades, microalgae have been an interesting biomass source due to their potential to rapidly accumulate important amounts of added value components that

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vary among the different species. Moreover, their high areal productivity that does not require arable lands enables their use as a promising and environmental-friendly source of bioactive compounds. Algae are considered pigment-producing organisms, the function of these compounds in algae is to carry out photosynthesis. They have a great variety of pigments, which can be classified into two large groups: chlorophylls and carotenoids, the latter is divided into xanthophylls and carotenes. Xanthophylls (fucoxanthin, astaxanthin, lutein, zeaxanthin, and cryptoxanthin) are a type of carotenoids with anti-tumor and anti-inflammatory activities, due to their chemical structure rich in double bonds that provide them antioxidant properties (Jaime *et al.,* 2005; Yao *et al.,* 2015; Garoma and Janda 2016; Gong and Bassi 2016; Safi *et al.,* 2017; De Jesus *et al.,* 2018). In this context, xanthophylls can protect other molecules from oxidative stress by turning off singlet oxygen damage through various mechanisms. On the other hand, β-carotene effects have been widely described as human-related health. Carotenoids have an increasing interest in food, pharmaceutical, and chemical industries (Pereira *et al.,* 2021).

There is a growing demand for extracting natural pigments for their application as a food coloring. The combination of advanced extraction techniques with solvents provides shorter extraction times, less solvent use, and decreases energy consumption, making an overall extraction process more environmentally friendly. Alternative extraction solvents, like ethanol or ethyl acetate, are greener processing fluids for pigments extraction. Ultrasound-Assisted Extraction (UAE) has been demonstrated to be a promising alternative to conventional extraction in order to reduce energy consumption and increase the extraction yield for lipids and carotenoids in microalgae. Cavitation is considered as a fundamental mechanism for UAE, where micro-bubbles form and collapse near the cells, creating cellular disruption (Castejón *et al.,* 2018; De Jesus *et al.,* 2018).

Stress factors such as high salt concentration, high light intensity, and nitrogen deprivation, can bring significant changes in the chemical composition of microalgae. Therefore, several high-value metabolites can be synthesized, such as glycerol, pigments, proteins, fats, amino acids, and polysaccharides. Thus, a high variety in microalgae composition occurs depending on cultivation conditions that could be used to module compounds concentration as an advantage (Yen *et al.,* 2013; Gong and Bassi 2016; Jacob-Lopes *et al.,* 2019).

Dunaliella salina which is a green biflagellate microalga has been described to be a carotenoid-producing, and it has increased interest mainly because of the great amount of β-carotene that could accumulate compared to other microalgae (Saini and Keum 2018; Rammuni *et al.,* 2019).

There are numerous reports in the literature that describe the study of carotenoid extraction from *D. salina* strains using different extraction methods. The application of ultrasounds to carotenoids producers strains belonging to the genus *Dunaliella* has been studied by several authors in recent years (Nejadmansouri *et al.,* 2021; Priyanka *et al.,* 2021). These studies indicated clearly that the amount of carotenoids from *Dunaliella* biomass is highly dependent on the strain studied, the

stress factor applied and the extraction method used. Furthermore, UAE has the benefits of higher efficiency, reduced amount of solvent and extraction time, moderate cost, and simple handling (Castejón *et al.,* 2018). Additionally, ultrasonication can usually be carried out at a low temperature, which reduces potential thermal damage to bioactive ingredients such as carotenoids or the loss of volatile components during extraction (Blanco Llamero *et al.,* 2021).

Orange biomass of *D. salina* is the most studied, whereas the green biomass is less known, even though it has the potential to accumulate large amounts of xanthophylls (Rammuni *et al.,* 2019; Di Lena *et al.,* 2019). For this reason, more comprehensive study on this green biomass is required.

Therefore, the main objective of this study was to evaluate carotenoids composition and antioxidant activity of different Ultrasound-Assisted extracts from green and orange biomasses of the new strain *Dunaliella salina* DunaDZ1.

Materials and methods

Chemicals and reagents

All chemicals used in this study were purchased from commercial sources and they are of analytical grade. DPPH (2.2-diphenyl-1-picrylhydrazyl) was from Fluka (Sigma-Aldrich).

Microalgae biomass production

The microalgae *D. salina* used in this study was isolated from Zahrez Chergui salt lake in Algeria (Yaiche Achour *et al.,* 2018). The strain was maintained in solid f/2 medium with 1 M NaCl (Guillard and Ryther 1962). The incubation conditions were 22°C, 50 µmol photons m^{-2} s^{-1} and a photoperiod of 24h. The culture at lab scale was conducted in a 20 L container, using a liquid f/2 medium. For better carotenoids yields a two-stage process was used. A growth phase (green cells) was first conducted then cells were suggested to salt stress and nitrate starvation, it is what we call stress phase (orange cells). The light was provided by fluorescent lamps (120 µmol photons m^{-2} s^{-1}) under 24h photoperiod. Biomasses were harvested by centrifugation and dried in a vacuum freeze dryer (Chaist). Then stored in darkness at -20°C until extraction (Yaiche Achour *et al.,* 2021).

Ultrasound-assisted solvent extraction

In this study, both *D. salina* biomasses (green and orange) were investigated for carotenoids composition. *Dunaliella salina* biomass:solvent ratio of 0.5:5 (w/v) was used in this study. Extraction solvent, time, and temperature were selected based on previous studies that aimed to extract carotenoids from green microalgae (Plaza *et al.,* 2012; Zou *et al.,* 2013). Ultrasound extraction was carried out in an ultrasound bath (Elma brand S 40H, Singen, Germany) at 37 KHz, at a temperature of 50°C for 30 minutes. Five solvents were employed: *n*-hexane:ethanol at a ratio of (3:4), ethyl acetate, isopropanol, acetone, and isobutanol. Extracts were then filtered and the solvent was removed using HeidolphHei-Vap HB/G3 evaporator at 35°C. The samples were saturated with N_2 and stored at -20°C for further analysis. Extractions were carried out in duplicates.

Extraction yield

Extraction yield is expressed as the percentage of dry biomass according to the equation 1:

$$\% \text{ Yield} = (a-b)/m \times 100 \qquad (1)$$

where *a* represents the weight of the vessel containing the sample, *b* represents the weight of the vessel containing the extract after drying and *m* is the weight of the sample.

Thin Liquid Chromatography analysis (TLC)

Silica gel 60 plates (Macherey-Nagel, Germany) were used to perform TLC. Ten microliters of each extract at a 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 mobile phase. The standard used was β-carotene at a concentration of 5 mg/mL.

HPLC-DAD analysis

Extracts analysis were performed with an HPLC-DAD (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 Eclipse XDB C18 column (5 µm. 150 × 4.6 mm). The mobile phase used was methanol 100%. The flow rate was kept at 1.5 mL/min for 50 min. Quantification of carotenoids was done referring to the standard curve. β-carotene was used as standard at the following concentrations: 0.075; 0.125; 0.25; 0.5 and 1 mg/mL. The detection wavelength was set at 450 nm and the injection volume of the sample extract was 20 µL.

DPPH radical-scavenging activity

DPPH radical-scavenging activity of the extracts was measured according to a previously described method (Tepe and Sokmen 2007). 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 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 and then incubated in darkness for 30 min. Absorbance was read at 517 nm using a spectrophotometer (Jenway 6705). Analysis was carried out in triplicates.

The DPPH radical activity was calculated using the following equation 2:

$$\text{Scavenging effect (\%)} = [(A_0 - A_s)/ A_0] \times 100 \qquad (2)$$

where: A_0 - absorbance of DPPH solution (without extract) (DPPH solution:methanol) (v/v); A_s - 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.

Statistical analysis

Results were expressed as mean ± standard deviation (SD), n=3. One-way ANOVA test was applied to evaluate significant differences ($p < 0.05$). Statistical analysis was performed using IBM SPSS Statistics 25.

Results and discussion

Ultrasound-Assisted Extraction yields

The effectiveness of UAE for carotenoids extraction has been widely studied. As green alternatives to traditional ones for microalgae extraction, UAE was performed on green and orange biomasses employing different extracting solvents widely studied for pigment extraction, including acetone, ethyl acetate, isopropanol, isobutanol, and a mixture previously optimized consisting of hexane and ethanol (3:4). Extraction yield values depended greatly on the extracting solvent employed, they varied from 1.91 to 4.41 % in the case of the green biomass, whereas yields obtained for the orange one ranged from 1.16 to 3.37 % (Figure 1). The mixture *n*-hexane:ethanol (3:4) achieved the highest yield results for both biomasses followed by acetone in the case of the orange one and by isopropanol in the green one.

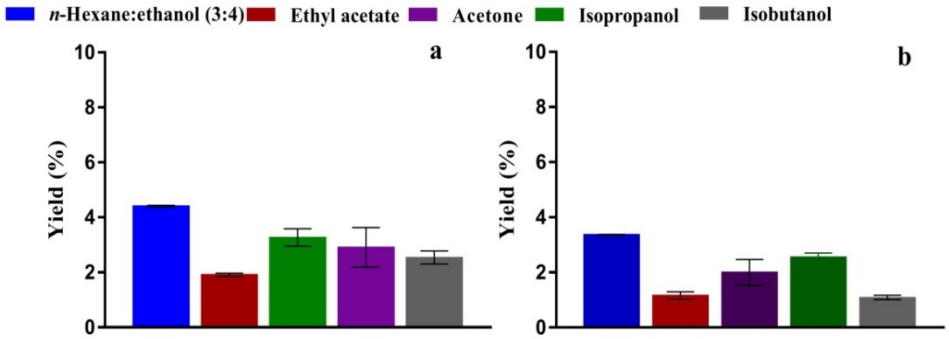

Figure 1. Extraction yield of green biomass (**a**) and orange biomass (**b**) from *D. salina* DunaDZ1. The data are given as mean ± SD of two determinations.

The effectiveness of the mixture of solvents could be explained by the polarity achieved by them, which may be intermediate and have an affinity with a higher range of carotenoids, from the more polar to the more nonpolar as β-carotene, increasing the mass recovery. According to Saini and Keum (2018) a large variety of solvent mixtures have been used to extract carotenoids from different natural sources, which gives a synergistic effect on extraction yield. Rivera and Canela (2012) studied carotenoids extraction from maize. They reported that combinations

of polar solvents with other less polar solvents were more effective than all the solvents tested individually.

Chemical characterization of the UAE extracts by TLC

The UAE extracts obtained from the two biomasses and the different solvents were analyzed by different methods in order to study carotenoids composition and to further compare the different solvents used.

TLC was employed as an easy and fast technique to observe clear differences between extracts. Interesting differences can be seen in Figure 2, where the extracts from the green and orange biomasses are analyzed and the carotenoids are separated at the same concentration of extract. On the one hand, it can be observed how the intensity of the bands highly varied between the different extracts, which is clearly related to the carotenoids concentration in them. The highest spots intensity corresponded to ethyl acetate extracts, in both biomasses, orange and green. However, the extract with the lowest spots intensity was *n*-hexane:ethanol, which is not in agreement with the yield results, meaning that more components apart from carotenoids are extracted in the UAE. That is why it is important to not only measure the yield obtained, but also the biomolecules amounts of the recovered yield. On the other hand, TLC showed the strain *D. salina* DunaDZ1 as rich in a wide range of carotenoids with different polarities. TLC results showed a composition highly different between the two biomasses. Both biomasses are rich in β-carotene, especially the orange one, whereas the green one had a wide range of chlorophylls and xanthophylls

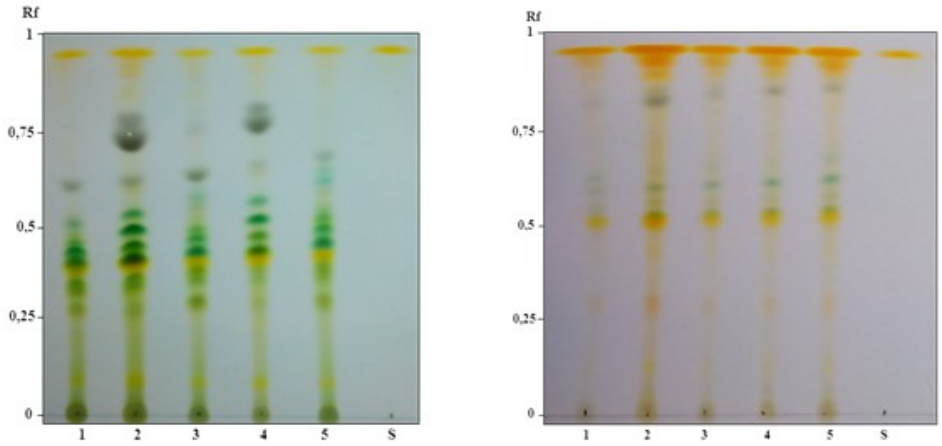

Figure 2. TLC of extracts from green (left) and orange (right) *D. salina* DunaDZ1 biomasses. Rf: retention factor. Extracts: 1: *n*-hexane:ethanol (3:4); 2: ethyl acetate; 3: isopropanol; 4: acetone; 5: isobutanol. Standard (S) is β-carotene at 5 mg/mL.

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Chemical characterization and quantification of the UAE extracts by HPLC-DAD

All UAE extracts obtained from both *D. salina* biomasses were injected into an HPLC-DAD. On the one hand, six carotenoids were detected from the green biomass, among them one was no identified. Astaxanthin, lutein, zeaxanthin, β-carotene and α-carotene were the carotenoids detected and identified from the green *D. salina* biomass. However, from orange biomass, in addition to astaxanthin, lutein and α-carotene, β-carotene isomers were detected. In contrast, great differences were observed between the two-biomasses carotenoids quantification (Tables 1 and 2).

The major carotenoid in the green biomass extracts corresponded to lutein followed by astaxanthin, thus the carotenoid profile of this biomass was mainly polar. Interestingly, great differences were also observed between the different extracts obtained with different solvents. Results showed that the mean values of lutein and astaxanthin to vary significantly ($p < 0.05$) with different extraction solvents. The more polar solvents were the ones that achieved higher amounts of lutein and astaxanthin, as can be seen in the results of ethyl acetate, isopropanol, and acetone. In addition, ethyl acetate extract from the green biomass contained the highest amounts of all detected carotenoids.

In most cases, the mixture *n*-hexane:ethanol which has achieved the highest extraction yield, gave the lowest carotenoids concentration. Jaime *et al.* (2010) have announced that the higher extraction yield was the lower pigment concentration obtained.

An important amount of carotene was also found in the green biomass. The highest amounts of carotene were obtained in the ethyl acetate extract, represented by α-carotene and β-carotene, with concentrations of 62.63 and 72.65±0.03 mg/g, respectively.

On the other hand, the quantification of carotenoids from the orange biomass revealed that it was mainly composed of β-carotene (*Cis*,β-carotene and *Trans*,β-carotene). In this case, solvents achieved different carotenoids amounts, although it must be underlined acetone to achieve the highest amount of *Trans*,β-carotene (131.83 mg/g) and isobutanol to contain the highest amount (84.21 mg/g) of *Cis*,β-carotene, with significant difference with the others solvents tested ($p < 0.05$). In addition, isobutanol was the most efficient for extracting the other carotenoids from *D. salina* orange biomass.

These findings are in agreement with the ones achieved in other works on microalgae carotenoid extraction, in which acetone is described as an effective solvent for this purpose along with isobutanol. Both are defined as promising solvents to avoid hazardous traditional ones such as chloroform or methanol.

Table 1. Carotenoids composition of extracts obtained from *D. salina* DunaDZ1 green biomass. Results are expressed as milligrams per gram of dry extract (mg/g).

Carotenoids	Astaxanthin	Lutein	Zeaxanthin	NI	α-carotene	β-carotene
n-Hexane:Ethanol	66.47±0.32^{e}	161.76±2.49^{e}	37.50±0.33^{d}	36.64±0.44^{d}	47.58±0.72^{b}	43.64±0.43^{c}
Ethyl acetate	131.43±1.69^{a}	393.19±1.69^{a}	39.57±0.26^{b}	45.91±0.26^{b}	62.63±0.43^{a}	72.65±0.03^{a}
Isopropanol	117.55±0.9^{b}	301.43±1.71^{b}	75.20±0.60^{a}	57.31±0.79^{a}	39.49±0.33^{c}	38.53±0.31^{e}
Acetone	106.58±0.48^{c}	291.53±2.45^{c}	38.90±0.67^{c}	42.34±0.09^{c}	61.81±0.41^{a}	60.45±0.37^{b}
Isobutanol	78.56±0.02^{d}	195.78±3.47^{d}	38.80±0.47^{d}	35.92±0.22^{d}	49.21±0.66^{b}	42.18±0.43^{d}

Data are presented as mean ± standard deviation. Within each column, different letters represent significant differences at $p < 0.05$.

NI: no identified carotenoids.

Table 2. Carotenoids composition of extracts obtained from *D. salina* DunaDZ1 orange biomass. Results are expressed as milligrams per gram of dry extract (mg/g).

Carotenoids	Astaxanthin	Lutein	NI 1	NI 2	α-carotene	*Cis*, β-carotene	*Trans*, β-carotene
n-Hexane:Ethanol	42.12±0.83^{a,b}	68.31±0.72^{c}	43.19±0.70^{b}	35.73±0.09^{c}	43±1.34^{a,b}	61.06±0.71^{c}	118.38±0.18^{b}
Ethyl acetate	42.52±1.12^{a}	77.52±0.45^{a}	43.88±0.67^{b,c}	35.73±0.17^{c}	38.37±0.17^{c}	63.01±0.64^{b}	114.04±0.31^{c}
Isopropanol	40.50±0.22^{b}	60.82±0.78^{e}	41.88±0.42^{c,d}	37.40±0.89^{b}	41.22±0.44^{b}	53.39±0.14^{e}	81.30±a0.32^{d}
Acetone	41.01±0.35^{b}	64.10±1.13^{d}	40.74±0.27^{d}	35.73±0.79^{c}	40.82±0.46^{b,c}	58.50±0.12^{d}	131.83±0.10^{a}
Isobutanol	43.62±0.15^{a}	74.38±0.39^{b}	51.58±0.28^{a}	38.13±0.8^{a}	45.77±0.59^{a}	84.21±0.80^{a}	114.54±0.36^{c}

Data are presented as mean ± standard deviation. Within each column, different letters represent significant differences at $p < 0.05$.

NI 1 and 2: no identified carotenoids.

Lin *et al.* (2010) studied by HPLC *D. salina* carotenoids profile, they found that the orange biomass contained 474.82 mg/g of trans,β-carotene, 425.64 mg/g of cis,β-carotene, 22.77 mg/g of lutein, 39.26 mg/g of zeaxanthin and 9.26 mg/g of α-carotene, the extraction was conducted by the mixture *n*-hexane:acetone:ethanol (2:1:1) using a conventional extracting method.

On the other hand, in another study on *D. salina*, lower values were recorded using the pressurized liquid extraction method at different temperatures by *n*-hexane. The carotenoids amounts for the orange biomass ranged from 2.57 to 227.7 mg/g for trans,β-carotene, 4.23 to 22.92 for cis,β-carotene and 0.78 to 24.77 mg/g of α-carotene (Herrero *et al.,* 2006). This difference between the carotenoids composition of *D. salina* strains can be explained by the fact that the accumulation of carotenoids in this microalgae is widely dependent on the culture conditions.

Warkoyo and Saati (2011) studied solvent effectiveness in the extraction process from *Eucheuma cottonii* seaweed and found that acetone tends to produce the highest content of carotenoid in the extract for the green and brown seaweeds compared to the other tested solvents.

Since the β-carotene produced from *D. salina* is proposed for food and nutraceutical applications, acetone is considered the best option for extractions (Kyriakopoulou *et al.,* 2015). According to several studies, the ultrasound-assisted method leads to higher extraction yields due to the cell disruption of the microalgae in shorter extraction times, when compared with supercritical fluid and maceration extraction methods. Ultrasound-assisted method effectiveness has been demonstrated for several food matrices. For example, lutein yield from egg yolk using UAE was four times higher than the yield obtained by conventional extraction using the same solvent (Yue *et al.,* 2006). The extraction of β-carotene from mandarin peel using UAE resulted in a good extraction yields in comparison with conventional extraction (Sun *et al.,* 2011).

Antioxidant capacity of the UAE extracts

The 1,1-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging activities of the different extracts were investigated. The Antioxidant activity was expressed as IC_{50}, low IC_{50} means a high abundance of antioxidant compounds (Table 3).

The results obtained show that the orange biomass has an interesting antioxidant activity in comparison with the green one. This fact could be explained by the richness of the orange biomass by different carotenoids, especially β-carotene. These pigments are known as compounds with strong antioxidant potential (Robman *et al.,* 2007; Saini and Keum 2018). Extracting solvent affect significantly the antioxidant activity ($p < 0.05$). Ethyl acetate extract has the highest antioxidant activity for both biomasses. This is could be explained by the polarity of the solvents used. Apolar carotenoids (e.g., β-carotene) are more soluble in non-polar solvents (*n*-hexane). While polar carotenoids pigments (e.g., lutein) show better solubility in ethanol and acetone.

In a study conducted on the antioxidant activity of different Moroccan microalgae, a better activity (IC_{50} = 283 µg/mL) was recorded in the strain *Dunaliella* sp.

(Maadane *et al.,* 2015). Otherwise, Cakmak *et al.* (2012) recorded IC_{50} varying from 450 to 3460 µg/mL for *D. salina* extracts obtained by different solvents.

Carotenoids are the most responsible for the antioxidant activity in *D. salina* biomasses, but other molecules such as phenolic compounds, polyunsaturated fatty acids, and polysaccharides may also be present and contribute to increase antioxidant activity (Al-Snafi, 2015; Shahidi and Ambigaipalan, 2015). The difference between the antioxidant activities obtained by different solvents is explained by the difference in antioxidant compounds polarity contained in the *D. salina* biomasses.

A previous study on ultrasound-assisted method for bioactive molecules extraction found this method can promote the release of not only carotenoids but also other bioactive compounds that contribute to the increase in antioxidant activity (Um *et al.,* 2017). In addition, the ultrasound-assisted method caused lower thermal degradation of bioactive compounds, which could have a positive effect on antioxidant activity.

Many other studies have also indicated that the application of ultrasound in the extraction of carotenoids can improve the extraction efficiency, enhance the antioxidant activities of extracts and reduce the extraction time compared to the conventional extraction methods (Plaza *et al.,* 2012; Um *et al.,* 2017).

Table 3. Antioxidant activities of extracts from green and orange biomass of *D. salina* DunaDZ1, values are the mean ± SD of three determinations.

Solvents	IC_{50} (µg/mL)	
	Green biomass	**Orange biomass**
n-Hexane:ethanol (3:4)	630.60 ± 0.38^{a}	316.30 ± 0.26^{c}
Ethyl acetate	451.29 ± 0.18^{e}	250.49 ± 0.32^{e}
Isopropanol	533.86 ± 0.20^{d}	432.33 ± 0.62^{b}
Acetone	551.27 ± 0.31^{c}	299.31 ± 0.43^{d}
Isobutanol	605.80 ± 0.52^{b}	480.53 ± 0.23^{a}

Different letters in the same column are significantly different ($p < 0.05$).

Conclusions

A new *Dunaliella salina* Strain DunaDZ1 Isolated from an Algerian Salt Lake was investigated and described in terms of carotenoids composition and antioxidant activity employing the UAE extraction technique and analytical method as HPLC-DAD. *Dunaliella salina* strain DunaDZ1 demonstrated to be a rich source of carotenoids, it is one among the *D. salina* strains existing which are considered as the highest natural source of this pigment. It is important to note that carotenoids from *D. salina* green biomass have never been characterized. Our study demonstrates that not only the orange biomass is a good candidate for carotenoids extraction, but also the green one is as well an important source of carotenoids mainly xanthophylls. Results obtained showed that *D. salina* green biomass has

almost the same carotenoids profile as the orange one with a predominance of lutein extracted with ethyl acetate, this characteristic is not well documented, which is of great interest. In contrast, great differences were observed between the two-biomasses carotenoids quantification. Ethyl acetate extracts were the ones with the highest antioxidant activity. Ultrasound-assisted extraction showed to be a suitable and efficient technique for carotenoids recovery from the microalgae *D. salina*. Furthermore, extract from the strain DunaDZ1 could be used as a functional ingredient in food. However, due to the variability in content and composition of carotenoids between green and orange biomasses, and due to their different polarities, extraction optimization separately for both biomasses is required and the interactive effects of extractions parameters should be studied.

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