Algerian Journal of Environmental Science and Technology
December edition. Vol.8. N°4. (2022)
ISSN : 2437-1114
www.aljest.org

Seasonal variation in polyphenol content and antioxidant activity of the Brown Alga *Cystoseira amentacea* var. *stricta* (*Sargassaceae*) from Northwest coast of Algeria

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ARTICLE INFO

Article History :

Received ***: 11/03/2020***
Accepted ***: 28/02/2021***

***Key Words*:**

Cystoseira amentacea var. stricta; Seasonal variation; Phenols;Antioxidant activity; TLC analysis.

ABSTRACT/RESUME

Abstract: *In this study, we evaluated the seasonal variation in phenol and antioxidant activity of Cystoseira amentacea var. stricta. Algal material was harvested monthly and extracted by four solvents (methanol, acetone, hexane and diethyl ether), separately. Total phenol, flavonoid contents, as well total condensed tannins were determined in crude extracts. Antioxidant activity was evaluated using several in vitro assays: Total Antioxidant Capacity, Ferric Reducing Antioxidant Power and diphenyl picrylhydrazyl radical scavenging activity. The results of TLC analysis obtained by Vanillin-sulfuric acid and those obtained by bio-autographic screening of active antioxidant compound show the presence of common molecules between the highly active extracts studied.*

I. Introduction

Marine world represents the greatest unexploited source of important pharmaceutical potential. A great interest is actually given to the extraction of natural and safe antioxidants from marine algae. Marine algae produce a wide range of secondary metabolites and contain interesting antioxidant molecules that possess novel chemical structures, which can be used to improve human health. Several reports revealed that seaweeds represent a rich source of antioxidant compounds [1, 2, 3]. Recently, the potential antioxidant compounds were identified in some pigments (e.g., carotenoid and fucoxanthine), phenolic compounds, polyphenols (e.g., catechin, gallate, flavonoids and phlorotannins), sulphated polysaccharides and vitamins (e.g., ascorbate) [4, 5, 6, 7, 8]. The presence of antioxidant substances in seaweeds is found to be a protection mechanism against oxidative stress, in response to extreme environmental conditions, such as high doses of ultraviolet and high oxygen concentrations that lead to the formation of free radicals and other strong oxidizing agents [9] Previous studies revealed that *Phaeophyceae* shows comparatively higher antioxidant activity and contains the highest levels of phenols than green and red algae [10]. Phenol contents in brown seaweed show seasonal [11] and geographical variations [12], since the level of phenolic content is regulated by seasonal fluctuations of light intensity [13], temperature ([14] salinity [15] and emersion [16]. Seasonal variations of polyphenols in brown algae have been investigated by many authors [17, 18]. However, a few studies describe the seasonal antioxidant activity variation in brown algae [19]. *Cystiseira amentaceae* var. *stricta* is a brown alga belonging to Sargassaceae family, growing abundantly and widely distributed in Mediterranean Sea. Variation in biochemical composition of this alga was studied by Pellegrini et al. [20] who found that this fluctuation reflects the several physiological activities of alga. Moreover, many terpenoids, alkaloids and steroids have been isolated from *C. amentacea* var. *stricta*. Ruberto et al. [21] reported that the antioxidant activity of the lipid fractions of *C. amentacea* var. *stricta* extract was the most active among eight marine algae belonging to the *Cystoseira* genus. Algeria with more than 1200 Km of coast lines along the Mediterranean Sea, housing

high marine biodiversity and a large variety of marine organisms and seaweeds could present an intersting source for marine pharmacology. However, data and studies are still very much in short supply in Algeria about algae valuation. Reagarding their phytochemical profile, an insufficient attention is dedicated to evaluate the possible temporal variation in antioxidant activities of *C. amentacea* var. *stricta.* To date, there is no published report concerning the effects of solvent extraction and seasonal variation on total phenol, flavonoid, condensed tannins contents and antioxidant activity of this coast alga. That is why the objectives of this study are focused on the determination of solvent extraction effects on total phenol, flavonoid and condensed tannins contents the antioxidant activity of *C. amentacea* var. *stricta* as well as the evaluation of the seasonal variation on polyphenol contents and antioxidant activity.

II. Materials and methods

II.1. Algal material

The alga was hand-collected at a depth range between 0 to 1 m from sea water surface, between March 2013 to February 2014, from Madrid beach at 33 km from Ain-Temouchent (lat 35°18' N, long 1°28' O), in the northwest coast of Algeria. After collection, the seaweed was rinsed with sea water, cleaned of epiphytes and then transported to the laboratory in polyethylene bags. In laboratory, alga samples were washed again with tap water and thereafter with distilled water to remove salt and associated debris. The cleaned material was then dried at room temperature in the shade. The dried samples were stored in paper bag until use.

II.2. Preparation of algal crude extracts

The dry material was crushed into powder in an electric mixer and then macerated with solvents of decreasing polarity: methanol, acetone, diethyl ether and hexane. In a typical procedure, the dried powder (1 g) was added to 20 ml of solvent, and gently stirred for 48 h. After filtration through Whatman N°1 filter paper, the solvent was eliminated under reduced pressure in a rotary evaporator at 40 °C. The residue (crude extract) was dissolved in methanol for analysis. The same procedure was applied using methanol, acetone, hexane and diethyl ether.

II.3. Determination of total phenol (TP)

The TP of prepared crude alga extracts was determined by the spectrophotometric method using Folin-Ciocalteu phenol reagent [22]. 1mL of Folin-Ciocalteu reagent diluted 10 times with water was added to 200 µL of the extract and 0.8 mL of a 7.5% sodium carbonate solution in a test tube. The mixture was left to stand for 30 min in the dark. The absorbance was measured at 765 nm. Gallic acid was used to prepare the standard curve. Phenol estimation was carried out in triplicate and results were expressed as mg of gallic acid equivalents per gram of dry matter (mg GAE/g DM).

II.4. Total flavonoids content

Aluminium chloride colorimetric technique was used for flavonoids content estimation [23]. 500µL of methanolic extracts were mixed with 1500 µL of distilled water in a test tube, followed by addition of 150 µL of sodium nitrite (5%) solution, at time zero. After 5 min, 150 µL of aluminum trichloride (10%, w/v) were added. After 6 min of incubation, at room temperature, 500 µL of NaOH (4%) were added. The absorbance of the solution was measured at 510 nm against the blank. The mean ± SD results for triplicate analyses were expressed as milligram catechin equivalents of dry matter (mg CE/ g DM).

II.5. Determination of condensed tannins

Proanthocyanidins were measured using the vanillin assay as described by Julkunen-Titto [24]. A volume of 50 µL of extract was mixed with 1500 µL of vanillin/methanol solution (4%, w/v). Then, 750 µL of concentrated hydrochloric acid were added. After 20 min of incubation, the absorbance was measured at 550 nm against the blank. The amount of condensed tannins was expressed as milligrams of catechin equivalents per gram of dry matter (mg CE/ g DM) from the calibration curve.

II.6. Determination of antioxidant activity

II.6.1. Total antioxidant capacity

Total antioxidant capacity of seaweed crude extracts was determined according to the method of Prieto et al. [25]. Briefly, 0.3 mL of sample was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Then the reaction mixture was incubated at 95° C for 90 min. The absorbance of all of the samples was measured at 695 nm. Total antioxidant capacity is expressed as the number of milligrams of ascorbic acid equivalents per gram of dry matter (mg AAE/ g DM).

II.6.2. DPPH radical - scavenging activity

The free radical scavenging activity of the seaweed extracts was measured by 2,2-diphenyl-1-picryl-hydrazil (DPPH) following the method of Sanchez-Moreno et al. [26]. This method is based on the reduction of stable DPPH radical in a methanolic solution. Briefly, 50µL of various concentrations of methanolic extracts were added to 1.950 mL of DPPH methanolic solution (0.025 g/L). The mixture was vortexed and then left to stand at room temperature for 30 min in the dark. The absorbance was measured against a blank at 515 nm. The radical scavenging activity (RSA) was calculated as

a percentage of DPPH discoloration using the following equation:

RSA % = [(Acontrol –Asample) / Acontrol] ×100

Where: Acontrol is the absorbance of the DPPH solution (without sample); Asample is the absorbance of the test sample with DPPH solution.The EC50, which is the concentration of sample (mg/mL) required to scavenge 50% of the DPPHradical in the reaction mixture was calculated from the plotted graph of inhibition percentage against extract concentrations. Ascorbic acid methanolic solution was used as positive control.

II.6.3. Reducing power

Reducing power of crude extracts was determined following the method of Oyaizu [27]. 1 mL of extracts at various concentrations was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL potassium ferricyanide (1%). The reaction mixture was incubated for 20 min at 50°C. After incubation, 2.5 mL of trichloroacetic acid (10%) was added and the mixture was centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%) solution freshly prepared. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increasing reducing power. Gallic acid was used as a reference.

II.7. Thin-layer chromatography (TLC) of algae extract

II.7.1. Analytical TLC

The highly active extracts were submitted to TLC analysis (Silica gel GF254, Germany) using the following mobile phase system: Toluene/ ethyl acetate/ methanol (10: 2: 0.5mL). In this experiment, two TLC plates were developed in parallel and will be used for staining revelation methods. The developed plates were dried under normal air and the spots were visualized under visible light and under UV light (λ = 366 nm).

II.7.2. TLC with revelation

II.7.2.1. Vanillin-sulfuric acid reagent

Vanillin sulfuric acid reagent was prepared by dissolving 2 g of vanillin in 100 mL of ethanol, with 5 mL of concentrated sulfuric acid. The developed TLC plate was sprayed with Vanillin-sulfuric acid regent. Sprayed plate was heated at 110°C until spots turned brown, grey or black. This method is used to detect terpenes, sterols and phenols.

II.7.2.2. Thin-layer chromatography (TLC) bioautography analysis

TLC bioautographic method, which combines chromatographic separation and biological activity determination, gives direct localization of antioxidant compounds on a TLC chromatogram. The most active extracts were deposited as spots onto the TLC plate. The developed TLC plate was sprayed with 2.54 mM DPPH methanol solution. Bands with the antioxidant capacity were observed as yellow bands on purple background.

II.8. Statistical analysis

All the reported experimental data were expressed as means ± standard deviation (S.D) from triplicate measurements using Excel program.

The data obtained were submitted to a multivariate statistical analysis using IBM SPSS Statistics, V.19 (© Copyright 1989, 2010). The significance of the seasonal variation was checked by ANOVA analysis at (α=0.01).

III. Results and discussion

III.1. Total phenol (TP)

The effect of the seasons and the solvents used for the extraction on TP of *C. amentacea* var. *stricta* are presented in figure.1 and table 1.

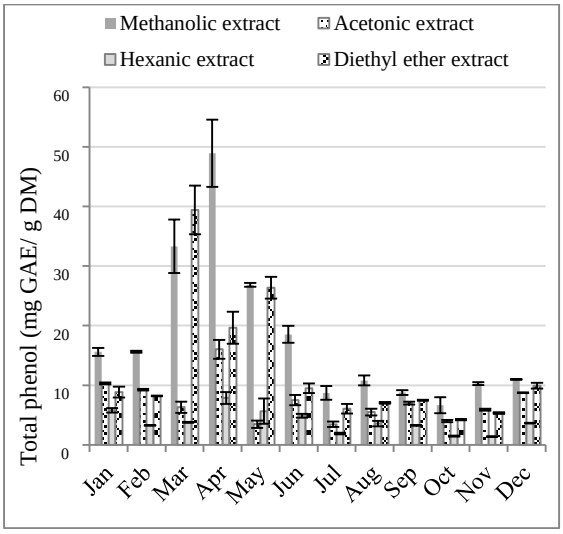

Figure 1. *Seasonal variation in total phenol of C. stricta*

Table 1. *Variance analysis for total phenol, flavonoids and condensed tannins content, total antioxidant capacity, DPPH free radical scavenging assay and ferric-reducing power (α=0.01).*

Source of variations	Total phenol		Total flavonoids content		condensed tannins		CAT		DPPH		FRAP	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Between solvents	10.21	6.6058E-05***	17.60	5.3132E-07***	3.76	0.02 (NS)	47.34	4.7052E-12***	6.55	0.00135089*	20.43	1.1523E-07***
Between months	3.41	0.00303619*	3.54	0.00234085*	1.44	0.2 (NS)	15.40	5.4079E-10***	1.65	00.13 (NS)	2.8	0.01 (NS)

***very high significant difference; * high significant difference; NS no significant difference

In all crude extracts, TP increased significantly in spring and decreased in dry season, it stayed at a low level in summer and in the rest of the year as well. The highest TP was recorded in April for methanolic extract (48.92 ± 5.64 mg GAE/g DM) and in March for diethyl ether extract (39.42 ± 4.09 mg GAE/g DM). The lowest concentrations of TP were observed for the hexanic and acetonic crude extracts, highest values were found in April (16.03 ± 1.01 and 7.86 ± 1.59 mg GAE/g DM, respectively). ANOVA analysis (at α= 0.01) applied on the TP showed highly significant difference between solvents (F=10.21, P=0.000) and significant difference between months (F=3.41, P=0.003). These results indicated that phenolic compounds in *C. amentacea* var. *stricta* are more soluble in polar solvent such as methanol. This finding is in agreement with the study of Trigui et al. [28] who found that TP depends on the type and polarity of the extracting solvents. Commonly phenolic compounds are found in polar extracts such as methanol [29]. Distribution of phenolic compounds through different solvents can also depend on their structures and amphipathic properties [29]. Sadati et al. [30] reported that TP of methanolic and hexanic extracts of *C. myrica*, collected in June from the coast of Libya, was 10.08 ± 1.13 and 3.01 ± 0.00 mg GAE/g DM, respectively. In our study, the levels of TP observed in the same month for methanolic (18.53 ± 1.41 GAE/g DM) and hexanic (4.88±0.30 GAE/g DM) extracts were relatively higher. The differences in the TP between our study and that of Sadati et al. [30] may be the consequences of different conditions at collection site. Variations in TP can also be attributed to the different protocols used both in sample preparation and phenol extraction [31]. According to the study performed by Connan et al. [17] on seasonal variation of phenol in several brown macroalgae from Brittany coast, the highest levels of phenol were found in summer but observed in spring which supports our finding. It has been reported that the level of phenol in algae is usually increasing with excessive exposure to solar radiation and higher temperature in the dry season but peak phenol is often not found in summer [32]. In a recent study, phenol in *C. tamariscifolia* from the southern coast of Spain were higher in winter and spring than in summer and autumn [32]. Mannino et al. [14] reported that the highest TP of *C. amentacea*, collected in the north-western coast of Sicily, was observed in winter and in spring. The declining of phenol in summer in *Cystoseira* genus can be attributed to light damage when UV radiation peaks [33]. Seasonal variations in TP in brown algae is a response to a combination of several factors such as temperature, irradiance levels and salinity but also to the reproductive state of the algae [13, 15, 17, 34].

III.2. Total flavonoids content

The determination of flavonoids was conducted using the aluminium chloride method with catechin as standard. The seasonal variation of total flavonoids contents of *C. amentacea* var. *stricta* obtained in this study was showed in figure.2 and table 1. Total flavonoids content was solvent and season dependent. Higher flavonoids content was found in the extraction with acetone whereas hexanic extract reflected small quantity of flavonoids. During the year, the highest flavonoids content occurred in autumn (September), in winter (December and February) and spring (April) for most crude extracts, whereas the lowest level was found in summer. The highest flavonoids concentration was observed in December for the acetonic extract (6.40 ± 0.19 mg CE /g DM) and in September for diethyl ether extract (6.35 ± 0.35 mg CE/g DM).

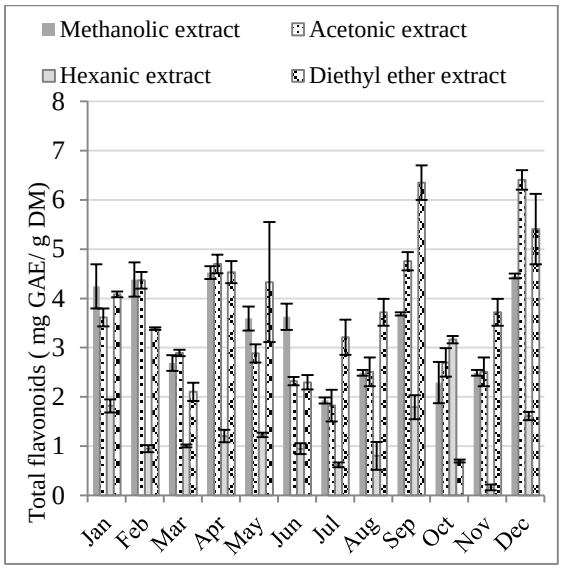

Figure 2. *Seasonal variation in total flavonoids content of C. stricta*

Different stress factors, temperature, UV radiations, salinity variation and the reproductive state of the algae may influence the level of flavonoids content. The same factors contribute also to phenolic variations [13, 15 and 17]. It was reported that high flavonoid content was obtained for acetonic extract for some algae [35, 36]. To our knowledge, there are no reports on the flavonoids contents of *C. amentacea* var. *stricta*. Compared to other species of brown alga, Meenakshi et al. [37] found that *Sargassum wightii* was rich in flavonoids (2.02±0.07 mg GAE/g) compared to other species of green algae. Kosanić et al. [38] found that acetonic extract of *C. amentacea*, collected in June from the Adriatic Sea, was rich in flavonoids (64.58 ± 1.099 µg RE/mg) compared to *C. barbata* and *C. compressa*. ANOVA analysis (at α= 0.01) applied on the total flavonoids content showed a highly significant difference between solvents (F=17.6, P=0.000) and a significant difference between months (F=3.54, P=0.002).

III.3. Condensed tannins

Our results showed that the total condensed tannins in *C. amentacea* var. *stricta* fluctuated throughout the year as seen in figure 3 and table 1. In the first half of the year, the quantity of condensed tannins in *C. amentacea* var. *stricta* crude extracts increased in February. In this month methanolic extract showed the highest content (23.51±1.15 mg CE/g DM) among other extracts.

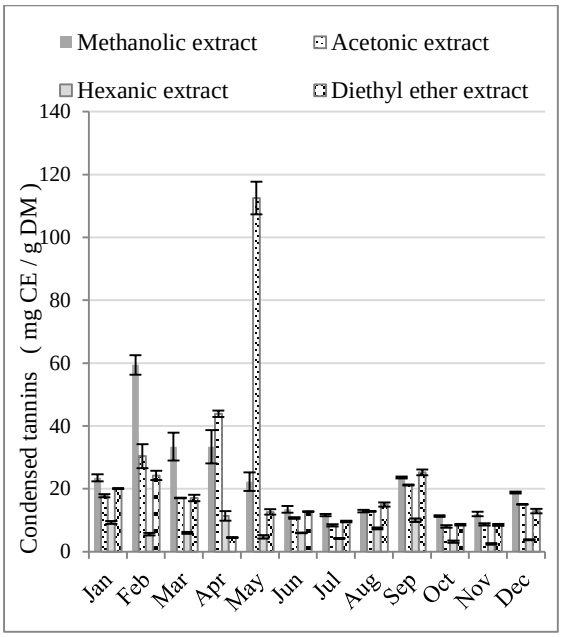

Figure 3. *Seasonal variation in condensed tannins of C. stricta*

For the acetonic crude extract, the condensed tannin increased in April and reached its peak in May (112.51±5.20 mg CE /g DM) and then decreased in summer. In contrast, in the others crude extracts, the contents decrease from March to July where the lowest level was found. In the second half of the year, we observed a slight increase during September and in December. No significant difference was observed between neither the solvents (F=3.76, P=0.02; at α= 0.01) nor between the months (F=1.44, P=0.2; at α= 0.01) for the condensed tannin contents. Chkhikvishvili and Ramazanov [39] found that the contents of phlorotannins, similarly to condensed tannins in *C. compressia* were higher compared to other species of brown algae. Custódio et al. [40] reported that *C. tamariscifolia* had a higher content of condensed tannins (64.2±1.0 mg CE/g DW) than *C. nodicaulis* (4.23±0.78 mg CE/g DW) [2].

III.4. Antioxidant activities

III.4.1. Total antioxidant capacity

The total antioxidant capacity of *C. amentacea* var. *stricta* crude extracts was determined by phosphomolybdate assay varied as seen in figure 4 and table 1.

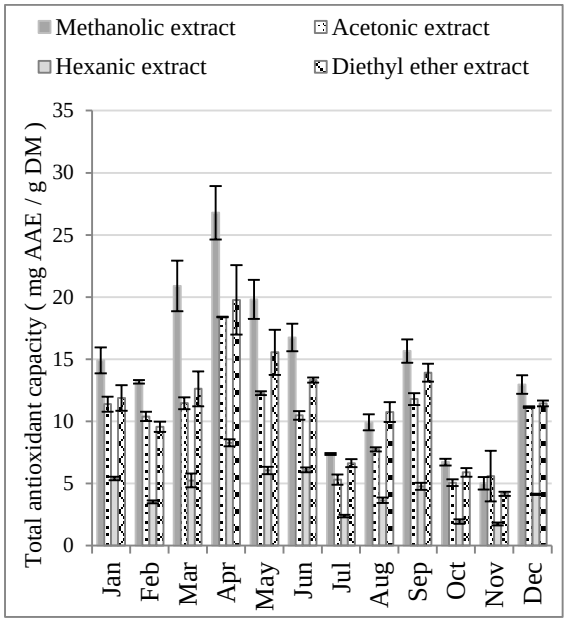

Figure 4. *Seasonal variation in total antioxidant capacity of C. stricta crude extracts*

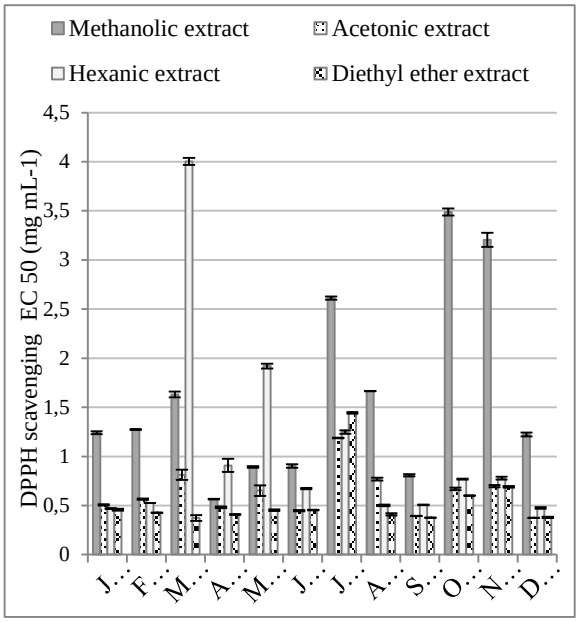

Figure 5. *Seasonal variation in DPPH radical scavenging effect of C. stricta crude extracts*

During the year, all samples displayed the same variation. The methanolic extract exhibit the highest antioxidant capacity followed by the diethyl ether and the acetonic extracts. The highest value was reached in April for the methanolic extract (26.78±2.14 mg AAE/g DM) followed by the diethyl ether extract (19.76±2.79 mg AAE/g DM). In September and December, we observed other maxima, but not as prominent as the ones in April, while the lowest total antioxidant capacity was found in July and November. Several studies have reported that the antioxidant activity of extracts from various types of seaweed may be correlated with the total phenol of the extract [1]. In the present study the total antioxidant activity of *C. amentacea* var. *stricta* extracts could be due to these compounds. ANOVA analysis (at α= 0.01) applied on the total antioxidant capacity showed a highly significant difference between solvents (F=47.34, P=0.000) and between months (F=15.40, P=0.000).

III.4.2. DPPH radical - scavenging activity

When tested with the DPPH free radical scavenging assay, all extracts of *C. amentacea* var. *stricta* showed antioxidant activities (Figure.5 and table 1). Diethyl ether and acetonic extracts proved to have powerful antioxidant activity. The diethyl ether extract displayed the highest DPPH radical-scavenging activity with an EC_{50}value of 0.37 mg/mL observed in March, September and December. In acetonic extract, this activity was observed to be higher in December (EC_{50} of 0.37 mg/mL) and in September (EC_{50} of 0.39 mg/mL).

The hexanic extract had the highest antioxidant activity in January (EC_{50} of 0.46 mg/mL) and in December (EC_{50} of 0.47 mg/mL). However, the antioxidant activity to scavenge DPPH free radical of *C. amentacea* var. *stricta* extracts was lower than that of ascorbic acid ($EC_{50}$of 0.090±0.002 mg/mL). In the present work, some extracts were found to have allowed total phenol and also showed good antioxidative effects. The amount of polyphenols doesn't seem to determine the antioxidant activity, the difference in activities would probably be the result of qualitative differences of these phenolic compounds nature. Some other co-extracted active compounds such as pigments and tocopherols may contribute to the antioxidant properties of the extracts, which require further research. ANOVA analysis (at α= 0.01) applied on the DPPH free radical scavenging assay showed a significant difference between solvents (F=6.55, P=0.001) but no significant difference was recorded between months (F=1.65, P=0.13). In comparison with another study, Zubia et al.[41] found that the methanolic crude extract of *C. tamariscifolia*, collected in April 2007 from Brittany coast, displayed a high antioxidant activity with an EC_{50} of 0.49 ± 0.01 mg/mL, which is stronger than our result for the same month and the same extract (0.56±0.004 mg/mL). Celis-Plá et al. [32] found higher DPPH scavenging in spring in *C. tamariscifolia* from southern Spain. In our study, *C. amentacea* var. *stricta* showed a strong antioxidant activity during most of the year, which can be related to higher irradiance levels occurring in Algerian coast compared to other areas in Mediterranean Sea.

III.4.3. Reducing power

The ferric-reducing power was variable among the 4 crude extracts of *C. amentacea* var. *stricta* (Figure 6 and table 1). The acetonic extract exhibited the highest ability to reduce Fe^{3+} (EC_{50} of 0.25± 0.00 mg/mL, in June), followed by the diethyl ether extract (EC_{50} of 0.26± 0.00 mg/mL, in March and June) and hexanic extract (EC_{50} of 28±0.00 mg/mL, in December). Despite its high phenol, methanolic extract showed the lowest activity when compared to other extracts, the highest reducing power value was observed in June with an EC_{50} of 0.54±0.01 mg/mL. The lower correlation between ferric-reducing power and the phenolic contents indicated that not only the phenolic substances were involved in the antioxidant activity but there could be some effects involving other active compounds.

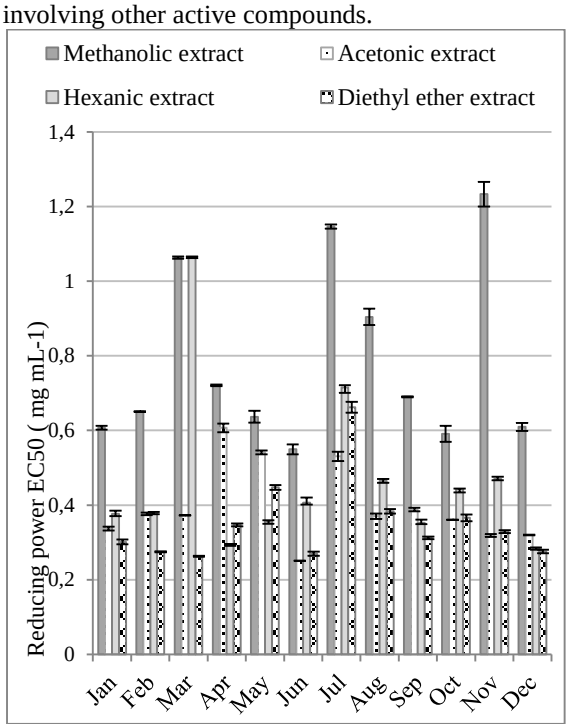

Figure 6. *Seasonal variation in reducing power of C. stricta crude extracts*

ANOVA analysis (at α= 0.01) applied on the ferric-reducing power showed a highly significant difference between solvents (F=20.43, P=0.000) but no significant difference was recorded between months (F=2.8, P= 0.01). Compared to the other species, methanolic extracts of *C. myrica* (14.80 ± 0.9 mmol FeII /100 g DM) and *C. tamariscifolia* (91.83 ± 0.32%) showed a good reducing activity, reported by Sadati et al. [30] and Zubiaet al. [41] respectively, but still lower than that of the ascorbic acid (EC_{50} of 0.063 ± 0.002 mg/mL).

III.5. Thin-layer chromatography (TLC) of algae extract

TLC technique is the first step of the analysis and presents the most convenient purification method of individual biomolecules in mixtures [42]. The crude extracts selected for TLC analysis were the highly active. The TLC crude extracts plate, visualized under visible light, indicated the presence of colourful bands that could possibly correspond to chlorophyll and carotenoids pigments. The TLC plate was also inspected under UV light (λ=366 nm) and many fluorescent compounds were detected (Figure.7). The results obtained by Vanillin-sulfuric acid and those obtained by bio-autographic screening of active antioxidant compound show the presence of common molecules between the different extracts studied corresponding to Rf-values 0.39 (methanolic extract of April, diethyl ether extract of March and of September), 0.78 (diethyl ether extract of March and of September) and 0.79 (diethyl ether of march and of December, Acetonic extract of June). It should be noted that these molecules were also fluorescent.

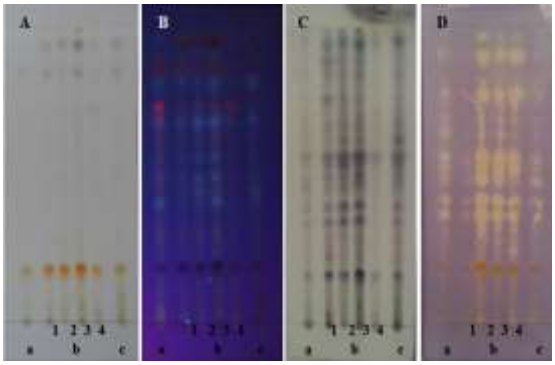

Figure 7. *TLC visualized: under visible light (A), under UV light (366nm) (B), using vanillin-sulfuric acid reagent (C), bio-autographic screening of active antioxidant compound stained with 2.54 mM DPPH· (D)*

IV. Conclusion

This study was the first report about the effects of solvent extraction and seasonal variation on total phenol, flavonoids and condensed tannins contents; and antioxidant activity of *C. amentacea* var. *stricta* from Algerian coast. Results indicate that the phenolic substances and the antioxidant activity of *C. amentacea* var. *stricta* were depending on the solvent used in the extraction as well as on the season's variation. Acetonic and diethyl ether extracts showed the highest antioxidant activities. A strong antioxidant activity was observed in March,

June, September and December. Further studies are needed to isolate and identify the bioactive compounds present in most active extracts of this alga.

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Please cite this Article as:
Benmahdjoub M., Belyagoubi-Benhammou N., Belyagoubi L., Aissaoui Gh., El Zerey-Belaskri A., Kerzabi-Kanoun Kh., Benguedda-Rahal W., Atik-Bekkara F., Seasonal variation in polyphenol content and antioxidant activity of the Brown Alga *Cystoseira amentacea* var. *stricta* (*Sargassaceae*) from Northwest coast of Algeria, ***Algerian J. Env. Sc. Technology**, **8:4 (2022)** **2782-2790***