Antioxidant and anti-cancer activities of brown and red seaweed extracts from Chilean coasts

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ABSTRACT. This study evaluates the content of polyphenols, flavonoids and anthraquinones from sequential extracts from four algae species from along the Chilean coastline: Desmarestia ligulata, Dictyota kunthii, Laurencia chilensis and Chondracanthus chamissoi. The antioxidant capacity of these extracts was evaluated through three complementary assays: the TRAP, FRAP, and DPPH assays. Additionally, the cytotoxic activity of these extracts was determined through sulfrohodamine B assays on two cancer cell lines, one colon (HT-29) and one breast (MCF-7), and one non-tumor control group of epithelial colon cells (CoN). The greatest antioxidant activity was detected in the ethyl acetate and dichloromethane extracts from L. chilensis in its TRAP potential, ethyl acetate of D. kunthii in its FRAP potential, and finally D. ligulata in its DPPH radical scavenging activity. The activities of this D. ligulata and L. chilensis extracts were significantly correlated with their flavonoid contents. In addition, the dichloromethane extracts from D. kunthii and C. chamissoi showed strong cytotoxic activity against HT-29 and MCF-7; however, the activity was not selective. Future research is necessary to purify the bioactive compounds of interest and improve their selectivity for eventual therapeutic use.

Keywords: algae, antioxidant activity, cytotoxicity, cancer, polyphenols, flavonoids, Chilean coast.

INTRODUCTION

Reactive oxygen species (ROS) are constantly generated in aerobic organisms under normal conditions, as part of metabolic processes. These species react with biological molecules in order to contribute to processes related to cellular signaling (Naqui et al., 1996; Apel & Hirt, 2004; Kalyanaraman, 2013; Mailloux, 2015). However, under abnormal metabolic conditions, there is an imbalance in the production of ROS and the biological system’s inherent antioxidant defense mechanisms; this phenomenon is known as oxidative stress (OS) (Storey, 1996; Valavanidis et al., 2006). The main cellular components affected by OS include membrane lipids, proteins, and nucleic acids (Sies, 1993), leading to the development of pathologies such as arteriosclerosis, cardiovascular and neurodegenerative diseases, diabetes, rheumatism and cancer (Rao et al., 2007). The latter can be attributed to the mutagenic effects of ROS on DNA and is one of the main causes of death in populations worldwide (Halliwell, 2007; WHO, 2016). According to the World Health Organization (WHO), the types of cancer with the greatest prevalence around the globe are lung, liver, stomach, colorectal, mammmary, and esophageal cancer (WHO, 2016).

In order to prevent or mitigate the deleterious effects produced by OS, different antioxidant treatments have
been generated; favoring the use of natural compounds over those of synthetic origin, due to the latter’s paradoxical carcinogenic behavior (Sindhi et al., 2013). Algae are known to be a good source of natural antioxidant compounds, since they have efficient defense systems aimed to tolerate a wide array of stress-inducing factors (Lobban & Harrison, 1997; Collén & Davison, 1999; Stengel et al., 2011; Balboa et al., 2013; Sordet et al., 2014; Gaete et al., 2016).

Given these characteristics, marine algae have been widely studied for potential therapeutic uses, especially due to their ability to biosynthesize a vast diversity of metabolites of biological and ecological importance (e.g., terpenoids, polyphenolic compounds, sterols, liposoluble and hydrosoluble vitamins, polysaccharides, and polyunsaturated fatty acids) (Batista et al., 2009).

Numerous studies have reported antioxidant and anti-carcinogenic properties in Ochrophyta (brown algae) and Rhodophyta (red algae) species (Zubia et al., 2009a, 2009b; Jiménez-Escrig et al., 2012; Murphy et al., 2014). This is the case of the genus Desmarestia and Dictyota (Ochrophyta), which biosynthesize a great variety of compounds, e.g., phlorotannins and diterpenes, which are known to possess antioxidant and anti-carcinogenic activity (Balboa et al., 2013). In addition, Zubia et al. (2009b) reported antioxidant activity and a strong cytotoxic effect against leukemia cell lines from crude extracts of Desmarestia ligulata and Dictyota dichotoma. The genus Laurencia (Rhodophyta) has been widely studied due to its extensive distribution in marine systems and because it is an abundant source of secondary metabolites with bioactive properties (Mao & Guo, 2010). Some compounds isolated from Laurencia undulata (Chondrophycus undulatus (Yamada) Garbary & Harper), Laurencia catarinensis, and Laurencia microcladia have been shown to possess antioxidant and anti-carcinogenic activity against skin, lung, prostate, and breast cancer lines (Li et al., 2009; Lhullier et al., 2010; Campos et al., 2012). In the case, a species of Rhodophyta, Piao et al. (2012) and Lee & Kim (2015) evidenced photoprotective and antioxidant activities on human keratinocytes exposed to UV-B radiation and antioxidant activity in extracts from Chondracanthus tenellus. However, there has been little evidence relating this genus to compounds with antioxidant capacity or anti-carcinogenic properties.

In the Chilean coast, Desmarestia ligulata is present from central to southern Chile (Valparaíso to Cape Horn). Dictyota kunthii can be found from Arica in the north, to the Chacao Channel in Chiloé Island, southern Chile (Santelices, 1989). As a defense against herbivores both species biosynthesizes compounds (Pereira et al., 2000; Pelletreau & Muller-Parker, 2002). Chondracanthus chamissoi is present along the coast from Iquique (northern Chile) to Chiloé Island in the south, whereas the endemic species Laurencia chilensis (Santelices, 1989; Hoffmann & Santelices, 1997) can be found only from central Chile to the Magellan Strait in the south. Chondracanthus chamissoi has defense mechanisms that help to prevent its interaction with herbivores and surface fouling, which explains the production of secondary metabolites (Rothäusler et al., 2005; Bulboa et al., 2007). Studies by Henríquez et al. (1979) and Valdebenito et al. (1982) have found antimicrobial activity in extracts and compounds obtained from L. chilensis, but studies showing antioxidant and anti-tumor activities in this species are scarce or non-existent. The present study is the first to evaluate phytochemical content and antioxidant and anti-carcinogenic capacities of differing-polarity extracts obtained from D. ligulata, D. kunthii, C. chamissoi, and L. chilensis.

MATERIALS AND METHODS

Collection algal material

The algae were collected in January 2013, at Caleta Cocholgüe (36°36’30”S, 72°57’52”W), Biobío region in Chile. Species were identified in situ. Individuals of each species were randomly collected in the rocky intertidal zone, during low tide until completing approximately 1 kg of wet weight collected per species (Table 1).

Preparation of algal extracts

About 1 kg of wet algae was dried to 40°C for one week. Once dried, seaweed compounds were extracted using sequential extraction with increasing polarity methods (dichloromethane, ethyl acetate, and ethanol) at room temperature, for 72 h. This procedure was performed twice. These steps were followed by filtration and concentration at reduced pressure. Finally, every extract was re-suspended in ethanol as a stock solution to 10 mg mL⁻¹ of concentration for all the analyses.

The concentration of polyphenols directly depends on the polarity of the solvents used in extraction, as well as their solubility. It is possible to obtain high yields of polyphenols when using mixtures of water and methanol, ethanol, or acetone (Waterman & Mole, 1994; Khlifi et al., 2013).

Phytochemical estimation

Estimation of total polyphenols content

The extracts’ total phenol contents were determined by following a slightly modified version of Waterman and Mole’s protocol (Waterman & Mole, 1994). In short, a
diluted solution of each extract (0.5 mL, 1.0 mg mL$^{-1}$) was mixed with Folin-Ciocalteu reagent (0.2 N, 2.5 mL). This mixture was allowed to stand at room temperature for 5 min and then a sodium carbonate solution (7.5% in water, 2.0 mL) was added. After 2 h of incubation in the dark at room temperature, absorbance was measured at 700 nm against the corresponding solvent as blank. A standard calibration curve was plotted using gallic acid (0-200 mg L$^{-1}$) and results were expressed as μg of gallic acid equivalents per g of dry extract (μg GAE/g d.e.). All measurements were performed in triplicate, and the average were expressed as mean ± standard error (SE).

**Estimation of total flavonoid content**

The total flavonoid content was measured by the Dowd method, adapted by (Arvouet-Grand et al., 1994) with slight modifications. Here, a diluted solution (5 mL, 1.0 mg mL$^{-1}$) of each extract was mixed with a solution (5 mL) of aluminum trichloride (AlCl$_3$) 2% in ethanol. The absorbance was read at 415 nm after 10 min, against a blank consisting of ethanol (5 mL) and extract (5 mL) without AlCl$_3$. Quercetin was used as a reference, to produce the standard curve, and the results were expressed as μg of quercetin equivalents per g of dry extract (μg QE/g d.e.). All measurements were performed in triplicate.

**Estimation of total anthraquinone content**

Total anthraquinone content was measured according to Mellado (2012). For this, 5 mL of 2% aluminum trichloride (AlCl$_3$) in ethanol was mixed with the same volume of extract solution in ethanol (1.0 mg mL$^{-1}$). Absorbance to 485 nm was measured after 10 min against a blank sample consisting of a 5 mL extract solution with 5 mL of ethanol without AlCl$_3$. The total anthraquinone content was determined using a standard curve using emodin (0-70 mg L$^{-1}$) as standard. Three readings were averaged and expressed as μg of emodin equivalents per g dry extract (μg EE/g d.e.). All measurements were replicated three times.

**Antioxidant activity**

**TRAP assay (Total Reactive Antioxidant Potential)**

The method developed by (Romay et al., 1996) was slightly modified in this experiment. A 10 mM solution of ABAP (2,2′-azo-bis (2-amidino propane)) was mixed with 150 μM solution of ABTS$^{+}$ (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) in 100 mM solution of PBS (phosphate buffered saline) at pH 7.4. The mixture was incubated at 45°C for 30 min. 10 μL of extract solution (1.0 mg mL$^{-1}$) was added to 990 μL of the resulting blue-green ABTS radical solution. The absorbance reduction (734 nm) was recorded after 50 s at room temperature against a blank solution without extract. The total antioxidant capacity TRAP of extracts was expressed in μM of Trolox$^\text{TM}$ equivalents antioxidant capacity (TEAC μM), using a standard curve of Trolox$^\text{TM}$ (0-120 mg L$^{-1}$). All measurements were replicated three times. The percentage of radical inhibition (RI) was calculated, using the following equation:

$$RI \% = \left( \frac{A_0 - A_{50}}{A_0} \right) \times 100$$

where $A_0$ and $A_{50}$ correspond to the absorbance registered at 0 and 50 seconds, respectively.

**FRAP assay (Ferric Reducing Antioxidant Potential)**

The ferric reducing power of plant extracts was determined using the FRAP assay (Dudonné et al., 2009) with minor modifications. The FRAP reagent consisted of 10 volumes of 300 mM acetate buffer, 1 volume of 20 mM FeCl3 and 1 volume of 10 mM 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) solution. 100 μL of extract (1.0 mg mL$^{-1}$) in distilled water (300 μL) was added to the FRAP reagent and shook for 15 s. After incubating for 30 min at 37°C in a water bath, the absorbance was measured at 593 nm. The FRAP of each extract was expressed in μM of Trolox$^\text{TM}$ equivalents antioxidant capacity (TEAC μM), using a Trolox$^\text{TM}$ (0-120 mg L$^{-1}$) standard curve. All measurements were replicated three times.

**DPPH assay**

Antioxidant scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) following a slightly modified version of (Brand-Williams et al., 1995). In short, 0.1 mL of extract (0.0625-1.0 mg mL$^{-1}$) was mixed with 2.9 mL of an ethanolic 50 μM DPPH solution. After incubating for 15 min at room tempera-

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**Table 1. Taxonomic information on species in the study.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Phylum</th>
<th>Family</th>
<th>Order</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Desmarestia ligulata</em> (Stackhouse) Lamouroux</td>
<td>Ochrophyta</td>
<td>Desmarestiaceae</td>
<td>Desmarestiales</td>
</tr>
<tr>
<td><em>Dictyota kunthii</em> (C. Agardh) Greville</td>
<td>Ochrophyta</td>
<td>Dictyotaceae</td>
<td>Dictyotales</td>
</tr>
<tr>
<td><em>Chondracanthus chamissoi</em> (C. Agardh) Kützing</td>
<td>Rhodophyta</td>
<td>Gigartinae</td>
<td>Gigartinales</td>
</tr>
<tr>
<td><em>Laurencia chilensis</em> De Toni, Forte &amp; Howe</td>
<td>Rhodophyta</td>
<td>Rhodomelaceae</td>
<td>Ceramiales</td>
</tr>
</tbody>
</table>
nature, the absorbance at 517 nm and the wavelength of maximum absorbance of DPPH\(^*\), was recorded \((A_{\text{sample}})\). A control experiment applying the same procedure to a solution without the test material and the absorbance was recorded \((A_{\text{control}})\). The percent radical scavenging capacity (RSC) was calculated by the following equation:

\[
\text{RSC}(\%) = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where \(A_{\text{control}}\) corresponds to the absorbance of the control solution, and \(A_{\text{sample}}\) represents extract’s absorbance. The IC\(_{50}\) values corresponded to the %RSC representing the concentration of extract that caused 50% neutralization, which was calculated by linear regression analysis. All measurements were performed in triplicate.

**Anti-cancer activity**

**Cell lines**

The experimental cell cultures were obtained from the American Type Culture Collection (Rockville, MD, USA) HT-29 (human colorectal adenocarcinoma), MCF-7 (human breast adenocarcinoma), and the control group CoN (normal human epithelial colon cells). All cancer cell lines were grown in DMEM-F12 medium containing 10% FCS, 100 U mL\(^{-1}\) penicillin, 100 µg mL\(^{-1}\) streptomycin and 1 mM glutamine. Cells were seeded into 96 well microtiter plates in 100 µL at a plating density of 3×10\(^3\) cells/well. After 24 h of incubation at 37°C (under a 5% humidified carbon dioxide to allow cell attachment) the cells were treated with different concentrations of extracts and incubated for 72 h under the same conditions. A stock solution of compounds was prepared in ethanol keeping the solvent’s final concentration constant at 1%. Control cultures received only 1% ethanol.

**In vitro growth inhibition assay**

This assay was performed following Skehan et al. (1990) methods, that allow measuring cellular density after the produced cytotoxic effect (in this case, from algal extracts) based on measurements of cellular protein content. The extracts (1 mg mL\(^{-1}\)) were inoculated onto a 96-well plate for each cell line. For this, each microplate had three wells assigned as negative controls (untreated cells), three positive controls (inoculated with 10 µL ethanol, 10%), and three wells with 10 µL of each algal extract. Afterward, the cultures were incubated at 37°C and 5% CO\(_2\) for 72 h. After this period, the cells were placed into each well with 25 µL trichloroacetic acid (TCA) at 50% and maintained at 4°C for 1 h. Following this, the TCA was removed by rinsing each microplate with distilled water and drying it with paper towels. Then the cells were dyed with 50 µL of 0.1% sulforhodamine B (SRB) solution in 1% acetic acid. To homogeneously dye protein cells, samples were incubated for 30 min at room temperature, after which the SRB was eliminated by rinsing the sample with 1% acetic acid, three times. Once each microplate was dried with paper towels, 100 µL of 10 mM Tris-base was added to each well to suspend the cultured cells. The absorbance of the dyed cells was measured using a BioTek EL 808 reader microplate at 540 nm. The percentage of cellular viability, an indicator of cellular survival after treatment with the extracts, was calculated using the following equation:

\[
\text{Cellular Viability (\%) = } \left( \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where \(A_{\text{sample}}\) corresponds to the absorbance of each well per extract, and \(A_{\text{control}}\) represents the average absorbance of the readings for the positive controls (with 1% ethanol). The IC\(_{50}\) (half inhibitory concentration) was calculated using different extract concentrations (0.5-1 mg mL\(^{-1}\)), in order to determine the specificity of the extracts on the cancer cell lines (Table 2). The selectivity index (SI) was calculated with the following equation:

\[
\text{SI} = \frac{\text{IC}_{50} \text{CoN}}{\text{IC}_{50} \text{cancer cell line}}
\]

An SI > 2 is the threshold for an extract that is selective for cancer cell lines, and SI < 2 for a non-selective extract (i.e., cytotoxic to both cancer and healthy cell lines) (Koch et al., 2005).

**Statistical analysis**

After tests of normality and homoscedasticity (Kolmogorov-Smirnov and Cochran tests, respectively), a two-way ANOVA or Kruskal-Wallis ANOVA test was applied, using a significance level of 5%. To test for associations between linear variables, we used Spearman’s Rank correlation coefficients (\(r_s\)). All tests were performed using Statistica 7.

**RESULTS**

**Phytochemical content**

The three studied phytoconstituents were found in most the extracts tested. Only in a few cases, they were below the detection level threshold. Estimations of polyphenols, flavonoid, and anthraquinone contents of *D. ligulata*, *D. kunthii*, *C. chamissoi* and *L. chilensis* extracts are showed in Figure 1. These species evidenced the presence of polyphenols, however, *L. chilensis* had the highest average polyphenols content.
in the dichloromethane and ethyl acetate extracts (221.67 ± 7.50 and 154.55 ± 18.26 mg GAE g⁻¹ d.e., respectively) (Fig. 1a). These values were significantly higher than those obtained in the other species (P < 0.05). On the other hand, for the Ochrophyta, the content of polyphenolic compounds was higher in ethyl acetate extracts (Fig. 1a), where D. ligulata showed the highest concentrations (92.6 ± 2.01 mg GAE g⁻¹ d.e.), however this extract did not differ with that of D. kunthii (89.8 ± 3.30 mg GAE g⁻¹ d.e.) (P > 0.05).

In relation to the total flavonoid content, low concentrations were found for all species studied (Fig. 1b). The highest concentrations were registered in dichloromethane and ethanol extracts of D. ligulata (3.38 ± 1.23 and 3.78 ± 1.39 mg QE g⁻¹ d.e., respectively). Similar results were obtained in the ethyl acetate extract of de L. chilensis, which registered a concentration of 3.13 ± 1.47 mg QE g⁻¹ d.e. All species showed high concentrations of anthraquinones in the ethanol extracts (Fig. 1c). This type of extract had its largest average concentrations among Ochrophyta (1.68 ± 0.38 mg EE g⁻¹ d.e. for D. ligulata, and 1.55 ± 0.07 mg EE g⁻¹ d.e. for D. kunthii). Nevertheless, the greatest concentrations were detected in dichloromethane and ethyl acetate extracts of de L. chilensis (2.15 ± 0.14 and 2.27 ± 0.35 mg EE g⁻¹ d.e., respectively) (Fig. 1c).

**Antioxidant activity**

The TRAP assay (Fig. 2a) showed a trend similar to the one described above for Polyphenol concentration (Fig. 1a): ethyl acetate extracts have a higher antioxidant activity, except for D. ligulata (Fig. 2a), which had a higher antioxidant activity in the ethanol extract (20.33 ± 0.70 mM TEAC, P < 0.05). Nonetheless, the highest antioxidant activities were detected in L. chilensis, specifically in the ethyl acetate (25.93 ± 0.44 mM TEAC) and dichloromethane extracts (22.93 ± 1.59 mM Trolox). The L. chilensis extracts also showed a positive correlation between its total antioxidant activity (TRAP) and flavonoid and anthraquinone contents (r_s = 0.81 and r_P = 0.78, respectively, P < 0.05).

The FRAP assay showed that the D. kunthii ethyl acetate extract had the most active results (Fig. 2b), with 61.77 ± 5.75 mM Trolox, followed by the D. ligulata ethanol extract, with 46.21 ± 1.14 mM Trolox. In comparison, both species of brown algae showed a higher Fe³⁺ reducing activity (Fig. 2b), compared to C. chamissoi dichloromethane extract (18.53 ± 2.42 mM TEAC). For these three species, there were no significant correlations found between the FRAP activity and the phytochemical content; however, the L. chilensis extracts showed a positive association between FRAP activity and flavonoid content (r_s = 0.68, P < 0.05).

Regarding the DPPH assay, all the extracts tested exhibited DPPH* scavenging activity; the ethyl acetate extracts had the lowest IC₅₀ and consequently, the highest activity. The D. ligulata dichloromethane extract had the most efficient scavenging activity, with an IC₅₀ of 3.01 ± 0.12 mg mL⁻¹, followed by the L. chilensis ethyl acetate extract with an IC₅₀ of 4.31 ± 0.04 mg mL⁻¹. This type of activity showed a negative relationship with flavonoid concentration in D. ligulata and L. chilensis (r_s = -0.73 and r_P = -0.84, respectively, P < 0.05). A similar relationship was observed between the scavenging activity of C. chamissoi extracts and polyphenols content (r_s = -0.70, P < 0.05).

**Cytotoxic activity**

The dichloromethane extracts were more cytotoxic than any of the other extracts (P < 0.05). Dictyota kunthii

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Table 2. Half inhibitory concentration (IC₅₀) and selectivity index (SI) of the algae extracts under study. ND: not determined.

<table>
<thead>
<tr>
<th>Species</th>
<th>Extracts</th>
<th>IC₅₀ (mg mL⁻¹)</th>
<th>SI</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HT-29</td>
<td>MCF-7</td>
</tr>
<tr>
<td>D. ligulata</td>
<td>Dichloromethane</td>
<td>1.49</td>
<td>2.67</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>1.47</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>2.43</td>
<td>3.23</td>
</tr>
<tr>
<td>D. kunthii</td>
<td>Dichloromethane</td>
<td>ND</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>1.13</td>
<td>2.34</td>
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<tr>
<td></td>
<td>Ethanol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C. chamissoi</td>
<td>Dichloromethane</td>
<td>ND</td>
<td>2.49</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
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</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>12.20</td>
<td>115.26</td>
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<tr>
<td>L. chilensis</td>
<td>Dichloromethane</td>
<td>1.03</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>2.92</td>
<td>4.83</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>22.31</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 1. Phytochemical estimation assay. a) Total polyphenols (gallic acid equivalents mg g⁻¹ d.e.), b) flavonoids (quercetin equivalents mg g⁻¹ d.e.), and c) anthraquinones content (emodinequivalents mg g⁻¹ d.e.) for the extracts in this study (n = 3, average ± SE). < LD represents the results that are less than the detection limit. *Indicates significant differences (P < 0.05) among species and among extracts (designated by brackets).

was the species with strongest effects on cancer cell lines (Figs. 3a-3b) showing the lowest percentage of cellular viability for HT-29 (19.81 ± 2.46%) and MCF-7 (28.41 ± 2.37%). The dichloromethane extract from C. chamissoi also showed high activity, with a cellular viability of 27.10 ± 1.54% for HT-29 and 52.23 ± 10.12% for MCF-7 (Figs. 3a-3b).
Figure 2. Antioxidant activities of the extracts studied (n = 3, average ± S.E.). a) Total reactive antioxidant potential (TRAP assay, Trolox equivalent antioxidant capacity mM mg⁻¹ d.e.), b) ferric reducing antioxidant potential (FRAP assay, Trolox equivalent antioxidant capacity mM mg⁻¹ d.e.), and c) DPPH● scavenging activity (IC₅₀ mg mL⁻¹). <LD represents the results that are less than the detection limit. *Indicates significant differences (P < 0.05) among species and among extracts (designated by brackets).
Figure 3. Percentage of cellular viability, with respects to negative and positive controls (EtOH 10% vehicle), of the different algae extracts studied (n = 9, average ± SE), using a treatment with a concentration of 1 mg mL^{-1} per extract, on cell lines HT-29 a) colon adenocarcinoma grade II; b) MCF-7 breast adenocarcinoma), and c) CoN one non-tumor cell line). *Indicates significant differences (P < 0.05) among species and among extracts (designated by brackets).
The D. ligulata extracts, evidenced positive associations between cytotoxicity on HT-29 and polyphenols content ($r_s = 0.81, P < 0.05$) and also with TRAP ($r_s = 0.78, P < 0.05$).

Regarding the CoN cell line, the activity of the extracts followed a similar trend to what was observed for the HT-29 and MCF-7 lines (Fig. 3c), where the dichloromethane extracts from all species were the most cytotoxic, with D. kunthii and C. chamissoi being the most active (23.81 ± 1.98% and 29.28 ± 2.60% cellular viability, respectively). These results show that the most active extracts were not selective, which was corroborated through the Selective Index of the evaluated extracts (Table 1).

**DISCUSSION**

The search for new natural compounds that could benefit human health has been the focus of many investigations during the past decades. Marine organisms, in particular, have had an important role as sources of bioactive chemical compounds with therapeutic uses (Balboa et al., 2013). This study is the first to show the antioxidant and cytotoxic properties of D. kunthii, C. chamissoi and L. chilensis against cancer cell lines, the first to use sequential extraction with increasing polarity in algae and the first one to provide additional information regarding these properties for D. ligulata after Zubia et al. (2009b). Given the high diversity and complexity of the biochemical compounds present in algae (such as polysaccharide sulfates, carotenoids, tocopherols, alkaloids, sterols, terpenoids, and polyphenols), they have great potential as sources of natural bioactive compounds (Gupta & Abu-Ghannam, 2011; Stengel et al., 2011; Murphy et al., 2014).

Usually, the efficiency of a solvent increases with its polarity (Koivikko et al., 2005); therefore, we expected higher polyphenols contents in ethanol extracts. However, the ethanol and dichloromethane extracts had fewer polyphenols than ethyl acetate extracts (specifically D. ligulata, D. kunthii, and C. chamissoi). Ethyl acetate usually extracts polyphenolic compounds with low-molecular-weight (Seidel, 2005), however, the highest content in this study, was registered in the L. chilensis dichloromethane extract (going above 200 mg GAE g⁻¹). These results differ from other findings, where lower concentrations of polyphenols were found in red algae in comparison to brown algae (Jiménez-Escrig et al., 2001, 2012). Given that the Folin-Ciocalteu's reagent can also detect oxidizable (i.e., compounds with high reducing potential) other than polyphenols (Singleton et al., 1999), it is possible that we detected other phyto-constituents in the *L. chilensis* dichloromethane extract (e.g., sesquiterpenes have also been described for the genus Laurencia) (Masuda et al., 2002; Kladi et al., 2005).

The methods used to obtain the increasing polarity extracts, allowed to identify which extracts have greater potential for nutraceutical applications, based on polyphenol concentration (Mellado et al., 2012b; Leyton et al., 2015; Jara et al., 2017). In this context, our extracts have a higher polyphenol concentration than what has been reported for species in the same genus (Zubia et al., 2007, 2009a, 2009b; Matanjun et al., 2008; Jiménez-Escrig et al., 2012).

Our results show that flavonoids and anthraquinones were present in all extracts even though their concentration was low in all the species tested. The presence of both compounds in the dichloromethane extract is unusual given that this solvent extracts apolar compounds such as terpenoids (Seidel, 2005). We attribute the presence of polyphenols on dichloromethane extracts to a possible susceptibility of the measurement to false positives (Mellado, 2012a). It is important to mention that there has been no previous research quantifying the flavonoid or anthraquinone contents in any of the species presented in this study. Precisely identifying these bioactive compounds is a complex task from an analytical point of view. Marine organisms produce secondary metabolites that are very different, in terms of structural diversity, from terrestrial plants and that include a mix of biosynthetic routes. Consequently, the only suitable alternative to process such extracts is by previously purifying them via column chromatography and then analyze them using complementary spectroscopic techniques such as Nuclear Magnetic Resonance (NMR), Mass spectrometry (MS) or infrared spectroscopy (IR) (Mellado, 2012a).

Using a wide array of methods to evaluate antioxidant capabilities, allowed our study to obtain a complete perspective of the mechanisms behind a given extract or compound. We analyzed the antioxidant activity of the extracts using three complementary methods: the TRAP assay (total antioxidant activity), which corresponds to a hydrogen-atoms transfer model; the FRAP (ferric-reducing capacity potential), which is based on electron transfer; and the DPPH assay (DPPH• scavenging activity), where both mechanisms can intervene (Huang et al., 2005; Prior et al., 2005). The TRAP activity in the algae extracts had a similar tendency to that of the DPPH• scavenging activity; in both cases, the one with the highest antioxidant activity was the ethyl acetate extract of *L. chilensis*. Ethanol extracts of *D. ligulata* had a high TRAP activity. The highest DPPH• activity was obtained with the
dichloromethane extracts being the most efficient compared to other extracts. The extracts’ FRAP activities showed a different trend to those observed for the TRAP and DPPH assays, where the highest reducing capacity was detected in ethyl acetate extracts of _D. kunthii_.

The differences in the extracts’ antioxidant capacity depend on the complexity of their composition, which influences their bioactivities, were synergistic effects among the present compounds, can also occur leading to an increase in antioxidant properties (Stengel _et al._, 2011). A wide array of methods have been used to evaluate total antioxidant capacity, however, their lack of correlation with the number of phytoconstituents usually makes it necessary to perform multiple tests in order to get a precise evaluation. In general, our results showed a high association between DPPH* scavenging, FRAP and TRAP activity of the extracts from _D. ligulata_, _D. kunthii_, _C. chamissoi_ and _L. chilensis_ and their respective phytoconstituents. Together, these results suggest that these compounds are responsible for the antioxidant activity of these algal species, validating the battery of test used in this study.

Regarding the TRAP assay, there is no previous information on antioxidant activity measured with this method, for the genus _Desmarestia_. On the other hand, for the genus _Dictyota_, studies by Matarjun _et al._ (2008) in _D. dichotoma_, show that its antioxidant activity is 3.2 to 11.2 times lower than what we found for _D. kunthii_. For Rhodophyta species, there are some studies about the antioxidant activity but comparable only to the order level. In this context, and for species in the order Gigartinales, the methanol extracts from _E. cottonii_ and _E. spinosum_ has a similar TRAP activity, to what we found in the ethanol extract, and lower than the diconmethane and ethyl acetate extracts from _C. chamissoi_ (Matarjun _et al._, 2008). The extracts obtained in this study showed a higher activity in comparison to reference antioxidants used in a previous study (BHT and gallic acid; Jara _et al._, 2017).

The antioxidant activity, based on the reduction potential of Fe³⁺ to Fe²⁺, that was measured with the FRAP assay, showed that all the extracts obtained here, had a higher activity (ranging from 2.5 to 28.1 times higher) than those from _Laurencia_, _Dictyota_ and _Desmarestia_ reported elsewhere (Matarjun _et al._, 2008; Zubia _et al._, 2009a, 2009b; Kelman _et al._, 2012). Additionally, all our extracts had a higher reducing potential than the reference antioxidants used in previous studies (BHT and gallic acid; Jara _et al._, 2017).

The DPPH assay results showed that values estimated for _D. ligulata_ are similar to those reported by Zubia _et al._ for the same species (Zubia _et al._, 2009a, 2009b). For _D. kunthii_ the values for the PPH have a similar behavior to those from _D. dichotoma_ and _D. cervicomis_ (IC₅₀ = 4.73 y 6.42 mg mL⁻¹, respectively) found superior to _D. ciliolata_ y _D. crenulata_ (IC₅₀ = 12.4 and 34.88 mg mL⁻¹, respectively) (Zubia _et al._, 2007, 2009a, 2009b). For the red algae in this study, there are only reports for Laurencia, and the values we found for _L. chilensis_ show at least nine times more activity than _L. intricata_ and _L. obtusa_ (Zubia _et al._, 2007). However, all the extracts showed less activity than the positive control treatments used in previous studies (Trolox™ and gallic acid), (Jara _et al._, 2017).

Regarding anticarcinogenic activity, the dichloromethane extracts from all four species were the most cytotoxic for both cellular models. _Dictyota kunthii_ showed the strongest behavior, followed by _C. chamissoi_. These results suggest that the dichloromethane extracts (i.e., non-polar) from these two species, have liposoluble compounds that were dissolved in the final ethanol 10% solution. This would increase their cytotoxic effectiveness, given that cellular membrane is more permeable to these compounds (Moo-Puc _et al._, 2009). Additionally, in the case of _D. ligulata_, the correlations found between the extracts’ polyphenols contents and their cytotoxicity on HT-29, suggests that this compound has anticarcarinogenic properties as it has been reported by Gomes _et al._ (2003) and Zubia _et al._ (2009a). Our findings regarding the toxicity of the Dictyota extract breast and colon cancer lines are in addition to what was reported by Zubia _et al._ (2009b) on leukemia lines.

The cytotoxic activity of the extracts could be caused by different mechanisms. Cancer cells show uncontrolled proliferation and are able to suppress cell death (e.g., through ROS that acts as secondary messengers in signal cascades to propitiate cancer, Korsmeyer, 1995; Rao _et al._, 2007). In this scenario, anticancer compounds may show cytotoxic effects either through the activation of an apoptotic route or through a cytostatic effect that stops the cellular cycle (Lundberg & Weinberg, 1999; Houghton _et al._, 2007). The identification of the mechanisms involved in the cytotoxicity generated by our extracts requires further research.

The cytotoxicity of the extracts was also evaluated on CoN normal cell lines to account for selectivity. Our results showed that extracts were not selective since they obtained selectivity indices <2 (Koch _et al._, 2005). Given that the extracts also contain other non-purified compounds (Murphy _et al._, 2014), it is possible that some of these caused a damaging effect on the CoN cell line, e.g., some bromophenols present in brown and red algae have this behavior against healthy cells, and have low selectivity (Han _et al._, 2005; Liu _et al._, 2011).
Nevertheless, the most active extracts evaluated in this study remain as possible candidates for eventual therapeutic use, for which we propose further purification and chemical modification to improve selectivity.

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Cytotoxic activities of brown and red seaweed


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