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Chapter 7

PHENOLIC PROFILE OF CRUDE EXTRACTS DERIVED FROM A RED ALGA, *CORALLINAELONGATA* ELLIS AND SOLANDER

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ABSTRACT

In the present chapter, we have evaluated the total phenolic contents and antioxidant activities of crude extracts from a red alga, *Corallinaelongata*. The phenolic compounds in the extracts have been identified and quantified by reverse-phase high-performance liquid chromatography. Phloroglucinol was by far the predominant polyphenol found in the extracts, followed by catechin and epicatechin.

Extracting solvents such as water, methanol and water/methanol (1/1) significantly affected the total phenolic amounts determined using the Folin-Ciocalteu method. The total phenolic contents ranged from 443.0 to 487.4 mg gallic acid equivalents per 100 g of dry plant material. The antioxidant efficiency of the extracts was evaluated by measuring the ability to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl, finding that it ranged from 37.0 % to 66.4% (inhibition percentage). The high correlation between the yield of extraction, the total phenolic content and the radical scavenging activity was found, allowing us to conclude that phenolic constituents are mainly responsible for the observed antioxidant activity in the extracts. Because of the wide range of biological activities of phloroglucinol and the other polyphenols detected in the extracts, *Corallinaelongate* appears to have many potential industrial uses.

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INTRODUCTION

Terrestrial and marine plants contain a diverse group of phenolic compounds with the structural requirements of free radical scavengers due to their high reactivity as hydrogen or electron donors and to the ability of polyphenol-derived radicals to stabilize and delocalize unpaired electron (Pietta, 2000, Quideau, Deffieux, Douat-Casassus, Pouysegu, 2011). These compounds have been reported to prevent diseases caused as a result of oxidative stress including cancers, cardiovascular malfunction, cataracts, rheumatism and many other auto-immune diseases besides ageing (Steinmetz and Potter, 1996; Dillar and German, 2000). Therefore, crude extracts of plant materials are increasingly of interest in food and pharmaceutical industries (Southon, 2000).

Seaweeds are considered to be a rich source of phenolic compounds with a number of important biological activities such as phlorotannins, which are polymers of phloroglucinol and constitute an extremely heterogeneous group of molecules (Target and Arnold, 1998; Shibata *et al*., 2002; Nagayama*et al*., 2003; Anh*et al*., 2004; Kang *et al*., 2004; Yu, Hu, Wu and Liu, 2009). The presence of phloroglucinol is associated to algae containing phlorotannins (Koch and Gregson, 1984; Targett, Boettcher, Targett and Vrolijk, 1995; Shibata *et al*., 2004). Phloroglucinol was found to inhibit cell damage induced by radiation through scavenging of reactive oxygen species (Kang *et al*., 2006). Moreover, phloroglucinol showed anti-fouling activity (Lau and Qian, 1997) and therapeutic potential for prevention of chronic inflammation (Kim and Kim, 2010).

Several studies have focused on *Corallinaelongata* algae: with articles on carotenoid composition and xanthophyll regulation in*Corallinaelongata* already published (Esteban *et al*., 2009); proteins such as R-Phycoerythrin (a protein acting as an accessory photosynthetic pigment in red algae and highly important in many biotechnological applications) also having been isolated from *Corallinaelongata* (Rossano*et al*., 2003) and with reported metal concentration and structural changes in *Corallinaelongata* from hydrothermal vents (Couto, Neto and Rodríguez, 2010). A study has also been made to determine the quality (as food) of *Corallinaelongata* and some 11 other macrophytes by recording ingestion, absorption and total growth rates of *Paracentrotuslividus* fed unlimited rations of the alga over a 6-month period (Frantzisl and Gremaree, 1992). Despite of low absorption rates, the echinoids fed *C. elongata* showed high growth rates (an index of food quality). However, no information of the phenolic profile of *C. elongata* has been reported to date.

The present chapter focuses on identifying the phenolic profile of *C. elongata* and the relationships between antioxidant activities and the phenolic profile of several extracts derived from *C. elongate*. Differences in the phenolic profiles of the extracts were determined by simultaneous identification and quantification of 14 polyphenols (gallic acid, catechin, epicatechin, rutin, p-coumaric acid, myricetin, quercetin and protocatechuic, vanillic, caffeic, ferulic, chlorogenic, syringic and gentisic acids) using reverse-phase high-performance liquid chromatography with a diode array detector (Lopez, Rico, Rivero and Suarez de Tangil, 2011). Given the importance of phloroglucinol and its widely demonstrated activity and presence in algae, we also determined the amounts of phloroglucinol in the extracts.

MATERIALS AND METHODS

Chemicals

Methanol (of HPLC grade) was obtained from Panreac (Barcelona, Spain). Water was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Formic acid provided by Merck (Darmstadt, Germany) was of analytical quality. Folin-Cicolteau`s phenol reagent, sodium carbonate and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were from Sigma-Aldrich Chemie (Steinheim, Germany).

Polyphenol standards were purchased as follows: gallic acid, protocatechuic acid, chlorogenic acid, (-) epicatechin, quercetin, myricetin, ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, (+) catechin, phloroglucinol, from Sigma-Aldrich Chemie (Steinheim, Germany); rutin and gentisic and caffeic acids from Merck (Darmstadt, Germany).

Plant Material

The red alga *C. elongata* was freshly collected from the Canary Islands, Spain, at 0-0.1 m depth between February and March 2009. Soon after collection, the algae were rinsed carefully in fresh seawater and then frozen. The frozen samples were lyophilized, pulverized using a blender (Moulinex, 600 W, France) and were kept in darkness at -20ºC under nitrogen.

Preparation of the Seaweed Extracts

Dry powders (5.0 g) were extracted by stirring at room temperature for 3 h using 1.5 ml acetic acid and 48.5 ml of one of the following solvents: absolute methanol, water, water/methanol (1/1).Each extract was filtered for removal of the alga particles. After centrifugation at 7600 g for 10 min, the supernatant was collected and filtered through 0.45 μm filter paper and stored at 4 ºC.

Yield Determinations

Extraction solutions (5 ml) were evaporated, the residues weighed and the amount of extractable substances of each extract expressed as a percentage by weight of the dry alga powder.

Determination of Total Phenolic Contents

The total phenolic content of each extract was estimated using the Folin-Ciocalteu reagent where values were expressed as mg of gallic acid equivalent to 100 gram dry alga powder (Julkunen-Tiitto, 1985). Sample solutions (100 μl) were mixed with 8.4 mL of H_2O

and 0.5 ml of Folin-Ciocalteu reagent. One ml of 20 % Na_2CO_3 was added. After 1 h incubation in darkness at room temperature, the absorbance was measured at 765 nm using a SHIMADZU 1700 UV-Vis spectrophotometer. The estimation of phenolic compounds was carried out in triplicate, and the results averaged. A calibration curve of gallic acid (ranging from 50 to 900 μg/ml) was prepared and the results were determined using the regression equation of the calibration curve ($y = 0.00029x – 0.00025$; $r^2 = 0.9992$).

Free Radical Scavenging Activity on DPPH

The antioxidant activity was measured in terms of radical scavenging capacity, using the DPPH assay (Chu, Chang and Hsu, 2000). One ml of a methanol solution of 0.1 mM DPPH (1,1-diphenyl-2-pikryl-hydrazin) was mixed rapidly into the sample solution (100 μl). The decline in absorbance was recorded at 515 nm against a methanol blank over a period of 16 min. The scavenging activity (%) (RSA) on DPPH radicals was calculated using the equation: RSA = 100 (1-Abs in the presence of sample/Abs in the absence of sample).

Determination of the Phenolic Profile Using HPLC

The dried algae powder (100 mg) was mixed and homogenized using a vortex for 30 s with 0.6 ml of the following: methanol, water and water/methanol (1/1). The mixture was stirred in a rotary shaker for 60 min at room temperature in darkness. After centrifugation at 7000 g for 20 min at 4ºC, the supernatant was colleted and filtered through a 45 μm nylon syringe filter prior to injection. Chromatographic analysis was performed using a Varian ProStar 210 system, equipped with a vacuum de-gasser, binary pump, thermostat column compartment, diode array detector (DAD), a reverse phase Pursuit XRs C18 (250 mm×4.6 mm, 5 μm) column and a Pursuit XRs C18 (10 mm×4.6 mm, 5 μm) guard column (Varian, Barcelona). The separation of gallic acid, (+) catechin, chlorogenic acid, protocatechuic, gentisic and vanillic acids, (-) epicatechin, ferulic acid, myricetin, caffeic acid, rutin, syringic acid, *p*-coumaric acid and quercetin was carried out using a method previously reported by us (Lopez *et al*., 2011). A gradient system was used involving two mobile phases. Eluent A was water with 0.1% formic acid and eluent B methanol. The flow rate was 1.0 ml/min, and the injection volume was 60 μl of crude extracts. The system operated at 27ºC. The elution conditions applied were: 0-5 min, 20%B isocratic; 5-30 min, with a linear gradient from 20% to 60% B; 30-35 min, 60%B isocratic; 35-40, with a linear gradient from 60% to 20% B and finally, washing and reconditioning of the column. Simultaneous monitoring was set at 270 nm (gallic acid, protocatechuic acid, (+) catechin, vanillic acid, (-) epicatechin and syringic acid), 324 nm (chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid and ferulic acid) and 373 nm (rutin, myricetin, and quercetin) for quantification. The retention times (RT) were as follows: gallic acid (RT: 5.3 min), protocatechuic (RT: 10.0 min), catechin (RT: 12.7 min), chlorogenic acid (RT: 14.9 min), gentisic acid (RT: 17.1 min), vanillic acid (RT: 17.7 min), epicatechin (RT: 17.9 min), caffeic acid (17.9 min), syringic acid (RT: 18.9 min), coumaric acid (RT: 23.4 min), rutin (RT: 28.1 min), ferulic acid (RT: 24.3 min), myricetin (RT: 30.6 min) and quercetin (RT: 34.6 min). To quantify phloroglucinol in the extracts, five different

solutions containing phloroglucinol with concentrations in the range of 1.0-200 μg/ml were injected in triplicate establishing the calibration curve by plotting the peak areas versus the concentration of each solution (y = 7149.1x + 28800). The gradient elution profile was as follow: 0-5 min, 20% B isocratic; 5-10 min, with a linear gradient from 20% to 60% B; 10-17 min, linear gradient from 60% to 20% B and finally, washing and re-conditioning of the column. Monitoring was set at 266 nm and the retention time was 7.05 min. The limit of detection (LOD) was found to stand at 0.0014 μg/ml, with the limit of quantification (LOQ) at 0.0046 μg/ml and the recovery 98.42 ± 6.92 (%). The injections were performed in duplicate.

RESULTS

Yield

Solvents such as methanol, ethanol, acetone, chloroform and water have been commonly used for the extraction of phenolics from brown and red algae (Duan, Zhang, Li and Wang, 2006; Yuan and Walsh, 2006). Yields of several extracts derived from *C. elongata* were evaluated as presented in Table 1. The highest yield was obtained using water as solvent (7.95 %) whereas the methanol and aqueous methanol gave similar yields. The results fully align previous studies where extraction yield was found to be strongly dependent on the solvent polarity and extracts prepared with polar solvents were found to give the highest percentages of extractable substances (Hayouni, Abedrabba, Bouix and Hamdi, 2007). As compared to our results, the results of Ganesan, Chandini and Bhaskar (2008) offer lower yields of crude methanol extracts of three red seaweeds (5.01 %, 3.98 % and 2.85 %).

Table 1. The effects of different solvents on the yield, the total phenolic content (TPC) and the radical scavenging activities (RSA) in *Corallinaelongata* algae extracts

Solvent	Yield[a]	TPC[a,b]	RSA[a,c]
Water	7.95 ± 0.21	487.4 ± 0.87	66.4 ± 0.73
Water methanol	6,52 ± 0,22	464.2 ± 1.52	57.5 ± 0.82
Methanol	6,29 ± 0,25	443.0 ± 4.01	37.0 ± 0.47

[a] Means ± standard deviation of three measurements.
[b] Values are expressed as mg gallic acid equivalents per 100 gram of dry alga power.
[c] Values are expressed as % inhibition.

Total Phenolic Contents

From the results shown in Table 1, it is evident that the recovery of phenolic compounds was dependent on the solvent used and its polarity. The order of TPC from high to low was: water (487.4) > water/methanol (464.2) > methanol (443.0). Similar findings were also reported by Kuda, Tsunekawa, Goto and Araki(2005). Our results in Table 1 show high correlation between the total extractable content and the total phenolic content, in line with previously published results (Chew, Lim, Omar, Khoo, 2008).

Earlier research investigations with respect to the crude methanol extracts of three different brown seaweeds gave a total phenolic content of under 290 mg gallic acid equivalents per 100 g seaweed on a dry weight basis (Chandini, Ganesan and Bhaskar, 2008), values inferior to those found here.

The DPPH Radical-Scavenging Activity

The order of the radical-scavenging activity in the extracts from high to low was: water > water/methanol > methanol (Table 1). The higher extraction yield corresponds to the higher antioxidant activity. The water extract showed the highest amount of total phenolic compounds (487.4) and the strongest DPPH radical scavenging activity (66.4%), whereas the methanol extract gave the lowest activity (37.0). A high correlation was found between TPC and RSA. The present findings corroborate well with earlier studies on algae extracts where it was concluded that high levels of total phenolic contents gave high RSA values indicating that phenolic compounds were the major constituents contributing to the DPPH scavenging activities of the extracts (Duan*et al*., 2006; Chew *et al*., 2008; Yangthong, Hutadilok-Towatana, Phromkunthong, 2009).

Table 2. Polyphenol amounts in extracts derived from *Corallinaelongata*algae determined by HPLC

Compounds	Solvent		
	Methanol[a] [b]µg / 100 g	Water/methanol[a] [b]µg / 100 g	Water[a] [b]µg / 100 g
Gallic acid	643.5 ± 16.94	426.4 ± 36.96	1700 ± 119.1
Protocatechuic	573.5 ± 11.68	752.4 ± 70.37	704.8 ± 21.6
Catechin	3518 ± 5.208	3699 ± 246.2	4444 ± 125.6
Vanillic acid	630.3 ± 37.59	558.2 ± 29.22	1121 ± 12.19
Epicatechin	1794 ± 99.21	1474 ± 82.69	7349 ± 697.8
Syringic acid	809.9 ± 70.83	769.3 ± 17.02	987.2 ± 98.82
Chlorogenic acid	1102 ± 43.78	1068 ± 35.91	1058 ± 17.19
Gentisic acid	1069 ± 80.32	1110 ± 54.91	1003 ± 21.08
Caffeic acid	374.3 ± 40.77	387.5 ± 35.40	409.0 ± 37.38
Coumaric acid	435.3 ± 42.34	412.9 ± 11.01	471.6 ± 28.07
Ferulic acid	638.5 ± 7.663	623.1 ± 16.64	696.8 ± 4.70
Rutin	501.4 ± 46.66	440.7 ± 26.29	566.3 ± 33.83
Quercetin	nd[c]	nd[c]	nd[c]
Phloroglucinol	nd[c]	17,64 ± 110.0	24,82 ± 70.04
Total amount of phenolics (mg per 100g of dry alga power)			
Sum	13.18	30.50	46.46
Sum - phloroglucinol	13.18	12.89	21.64

[a] Means ± standard deviation of three measurements.

[b] µg / 100 g indicates per 100 gram of dry algae powder.

[c] nd indicates not detected.

The Determination of Phenolic Profile by HPLC

Each extract showed the presence of all the standards with the exceptions of quercetin, which was not to be detected (Table 2). In this analysis, phloroglucinol was by far the predominant polyphenol in the water and water/methanol extracts (24.8 and 17.6 mg per 100 gram dry alga respectively), although it was not to be detected in the methanol extract. Significant amounts of catechin and epicatechin were detected in all the extracts. As compared to our results, Onofrejová*et al*. (2010) reported lower amounts of protocatechuic, *p*-coumaric, vanillic, caffeic and chlorogenic acids extracted from *in vitro* culture of two freshwater algae and from food products of marine macroalgae. High correlation was found between the total phenolic content calculated as the sums of the quantified by HPLC polyphenols (Table 2) and the radical scavenging activity. It must be noted that this high correlation depends strongly on the amount of phloroglucinol. As a general rule, the water gave higher contents of each polyphenol than the other solvents, thereby explaining why the water extract showed higher RSA.

CONCLUSION

Several reports have convincingly shown a close relationship between antioxidant activity and total phenolic content in algae extracts and in other plant materials, concluding that phenolic compounds are responsible for antioxidant activity (Duan*et al*., 2006; Chew *et al.*, 2008; Alothman, Bhat and Karim, 2009).

Chemical assays based on the removal of stable DPPH free radicals have been shown to indicate the presence of reductive compounds in terms of hydrogen donation capacity under test conditions. The radical scavenging activity of the extracts increased when the total phenolic content increased. These findings indicated that phenolic compounds are the main microconstituents contributing to the antioxidant activity of the algal extracts.

The results of the present work indicate that extracts of *Corallina elongate* algae can be used as a source of phloroglucinol. Phloroglucinol has been reported to be an inhibitory of *Hydroideselegans* larval settlement and the growth of certain marine bacteria, the two mechanisms that have been suggested to explain the antifouling effects of natural compounds (Lau and Qian, 1997). Therefore, aqueous extracts of *Corallinaelongata* can be used as an easy and accessible antifouling agent in coastal areas. In addition, phloroglucinol was found to have many biotechnological applications in food science and therapy (Kang *et al*., 2004; Kang *et al*., 2006; Kim and Kim, 2010).

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