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Analytical Methods

The effects of solvents on the phenolic contents and antioxidant activity of *Stypocaulon scoparium* algae extracts

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ABSTRACT

Water, water/methanol (1/1), methanol and ethanol crude extracts from a brown alga *Stypocaulon scoparium* were examined for total phenolic contents (TPC) using Folin–Ciocalteu method. DPPH scavenging assay was performed to measure the radical scavenging activities (RSA) of the extracts. Results showed a significant association between the antioxidant potency and the TPC. The aqueous extract showed both, the highest antioxidant activity and highest phenolic contents. The identification and quantification of phenolic antioxidants were carried out with a rapid and simple method of reverse phase high performance liquid chromatography (RP-HPLC). This method was developed for the simultaneous analysis of 14 polyphenols, namely gallic acid, catechin, epicatechin, rutin, *p*-coumaric acid, myricetin, quercetin and protocatechuic, vanillic, caffeic, ferulic, chlorogenic, syringic and gentisic acids. The chromatographic separation of 14 polyphenols was achieved in less than 40 min by RP-HPLC (Varian, Pursuit XRs C18 column, 5 μm, 250 mm × 4.6 mm) using linear gradient elution of methanol and water (0.1% formic acid) with a flow rate of 1 ml/min. Gallic acid was by far the predominant polyphenol.

1. Introduction

Recently phytochemicals in herbal plants have attracted a great deal of attention mainly concentrated on their role in preventing diseases caused as a result of oxidative stress (Southon, 2000). Dietary antioxidants from plants are believed to help prevent ageing and many degenerative diseases such as cardiovascular diseases and cancers through radical scavenging activity (Dillard & German, 2000; Prior & Cao, 2000; Steinmetz & Potter, 1996; Virgili & Scaccini, 2003; Wargovich, 2000; Yang, Landau, Huang, & Newmark, 2001). There is considerable interest in preventive medicine in the development of natural antioxidants obtained from botanical sources (Kaur & Kapoor, 2002). Seaweeds are considered to be a rich source of antioxidants (Cahyana, Shuto, & Kinoshita, 1992; Lim, Cheung, Ooi, & Ang, 2002), and different types of antioxidants from various species of seaweeds have been reported (Yan, Chuda, Suzuki, & Nagata, 1999).

The high level of solar radiation and the high temperature prevailing in the regions of Canary Islands (African Northwestern Coast) forces plants to develop defence mechanisms against ultraviolet radiation and excessive production of free radicals through the accumulation of antioxidant substances. This prompted us to evaluate total phenolic content of the crude extracts derived from *Stypocaulon scoparium* collected from Canary Islands.

The effects of different extracting solvents have been tested for the extraction of polyphenols from plant material (Pinelo, Rubilar, Sineiro, & Nunez, 2004). Extraction yield is dependent on the solvent and the method of extraction (Hayouni, Abedrabba, Bouix, & Hamdi, 2007). Khokhar and Magnusdottír (2002) reported that aqueous ethanol was superior to methanol and acetone for extracting flavonoids from tea. Thus, the objective of this study was to investigate the effect of several extracting solvents on the total phenolic compounds of the crude extracts derived from *S. scoparium*. It is obvious that total phenolics measured by Folin–Ciocalteu procedure do not give a full picture of the quantity or quality of the phenolic constituents in the extracts. Several methods have been developed to identify polyphenols from plants, fruits and vegetables using RP-HPLC analysis (Andre et al., 2007; Keyhanian & Stahl-Biskup, 2007; Khokhar & Magnusdottír, 2002). We report here the rapid determination of 14 polyphenols in crude extracts derived from a brown alga *S. scoparium*, to compare the ability of each solvent to dissolve a selected group of polyphenols. In addition, we will investigate the correlation between TPC and RSA of the extracts. Several reports have convincingly shown a close relationship between antioxidant activity and

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total phenolic content (Duan, Wu, & Jiang, 2007; Zhao, Yang, Wang, Li, & Jiang, 2006). However, other reports indicated that this correlation doesn't exist and it was concluded that phenolic compounds are not responsible of the antioxidant activity (Kähkönen et al., 1999; Patthamakanokporn, Puwastien, Nitithamyong, & Sirichakwal, 2008; Rapisarda, Lo Bianco, Pannuzzo, & Timpanaro, 2008). Although valid in some cases, this interpretation does not consider factors such as differences in the phenolic profiles between samples (Jacobo-Velazquez & Cisneros-Zevallos, 2009).

2. Materials and methods

2.1. Chemicals

Methanol (Panreac, Barcelona) and ethanol (Panreac, Barcelona) were of HPLC grade. 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–Ciocalteu's phenol reagent and sodium carbonate were from Sigma-Aldrich Chemie (Steinheim, Germany).

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

2.2. Plant material

The brown alga *S. scoparium* used for this study was freshly collected from Canary Island, Spain, at 0–0.1 m depth between March and April 2008. The algae were rinsed carefully in fresh seawater and then frozen. The frozen samples were lyophilised, pulverised into powder by a blender (Moulinex, 600 W, France) and were kept in the dark at −20 °C under nitrogen.

2.3. Preparation of seaweed extracts for both, DPPH assays and TPC determinations

Dried powders (2.0 g) were separately extracted for 2 h at room temperature in 30 ml of one of these solvents: absolute methanol, absolute ethanol, water and a mixture water/methanol (50%) by mixing using a magnetic stirrer.

Each extract was filtered for removal of alga particles. After centrifugation at 2000*g* for 20 min, the supernatant was collected and filtered through 0.45 μm filter paper and stored (10 ml) at 4 °C.

Extraction solutions (20 ml) were dried by vacuum-evaporator. The dried residues were weighed and the yield for extractable substances was calculated based on the weight of dry alga powder.

2.4. Preparation of seaweed extracts prior to HPLC injection

Approximately 50 mg of powdered freeze-dried material was mixed separately with 1.0 ml of each solvent (methanol, ethanol, water and methanol/water 50%). The mixture was homogenised using a vortex for 30 s and shaken for 60 min at room temperature in the darkness. After centrifugation at 2000*g* for 20 min at 4 °C, the supernatant was collected. Supernatants were evaporated to dryness and residues were suspended in 500 μl of water and filtered through a 45 μm nylon syringe filter prior to injection.

2.5. Determination of total phenolics

The amount of total phenolics in extracts was determined according to the Folin–Ciocalteu assay (Julkunen-Tiitto, 1985). Samples (100 μl) were introduced into test tubes containing 8.4 ml of water; 0.5 ml of Folin–Ciocalteu's reagent and 1 ml of sodium carbonate (20%) were added. The tube were mixed and allowed to stand for 1 h in the 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 were averaged. A calibration curve of gallic acid (ranging from 0.050 to 0.9 mg/ml) was prepared (in methanol), and the results, determined by the regression equation of the calibration curve ($y = 0.00029x - 0.00025$); correlation coefficient $r = 0.9992$), were expressed as gallic acid (GA) mg equivalents/100 g dry alga powder.

2.6. DPPH scavenging activity

The free radical scavenging activity was measured using the method of Chu, Chang, and Hsu (2000) with some modification. One ml of 0.1 mM DPPH (1,1-diphenyl-2-pikryl-hydrazin) solution in methanol was added to 100 μl of the sample solution. The decline in absorbance was recorded at 515 nm against a methanol blank over a period of 20 min. The scavenging activity (%) (RSA) on DPPH radicals was calculated as follows: RSA (%) = (1 − absorbance of sample/absorbance of control) × 100.

2.7. Analytical data

Chromatographic analysis was performed on a Varian ProStar 210 system, equipped with a vacuum degasser, a binary pump, a thermostatted column compartment and a diode array detector (DAD), connected to a ChemStation software. The separation was performed with 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). 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 27 °C. The elution conditions applied were: 0–5 min, 20% B isocratic; 5–30 min, linear gradient from 20% to 60% B; 30–35 min, 60% B isocratic; 35–40, 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, coumaric acid and ferulic acid) and 373 nm (rutin, myricetin, and quercetin) for quantification.

2.7.1. Calibration curves

Stock solution containing standards was prepared and diluted with methanol to appropriate concentration in the range of 1.0–200 μg/ml for establishing calibration curves. For quantitative analysis, five different concentrations of fourteen analytes were injected in triplicate. The calibration curves were constructed by plotting the peak areas versus the concentration of each analyte.

2.7.2. Selectivity

The selectivity of the method was determined by analysis of standard compounds and samples. The peaks of polyphenols were identified by comparing their retention times and overlaying of UV spectra with those of standard compounds.

2.7.3. Linearity

Previous experiments showed us the correct ranges for concentrations to construct calibration curves. Stock solution was prepared containing 200, 100, 50, 10, 2.0 μg/ml for gallic acid; 75, 40, 20, 10 and 5.0 μg/ml for catechin; 50, 25, 10, 5.0 and 1.0 μg/ml for rutin; 25.0, 10.0, 5.0, 3.0 and 1.0 μg/ml for caffeic acid; 75,