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Short Communication | Comunicación Corta

Determination of the antioxidant capacity of two seagrass species according to the extraction method

[Determinación de la capacidad antioxidante de dos pastos marinos de acuerdo al método de extracción]

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Abstract

Context: There is a wide variety of methods for obtaining of plant extracts that enable a good yield of bioactive metabolites. For several years, extractive techniques have been perfected for obtaining natural extracts with powerful pharmacological properties.

Aims: To determine the influence of various extraction methods (infusion, decoction, microwave, maceration with heat and agitation, and constant heat and agitation) on the content of solids, phenolic compounds and antioxidant capacity of marine angiosperms *Thalassia testudinum* Banks ex König (Hyrocharitacea) and *Syringodium filiforme* kützing (Cymodoceaceae).

Methods: The soluble solids content was determined by the gravimetric method; total phenolic content, using Folin-Ciocalteu method, and the antioxidant capacity by 2,2-diphenyl-1 picrylhydrazyl (DPPH) method.

Results: Results showed the effectiveness of extraction by decoction for *T. testudinum* and by microwave for *S. filiforme*, among the methods that use water as the extraction solvent. In the case those that use the hydroalcoholic mixture as solvent extraction, maceration with agitation and heat extraction showed the higher yields of soluble solids and total polyphenols, as well as a higher antioxidant activity for both species.

Conclusions: Results showed the effectiveness of extraction by decoction for *T. testudinum* and by microwave for *S. filiforme*, among the methods that use water as extraction solvent. In the case those that use hydroalcoholic mixture as solvent extraction, maceration with agitation and heat extraction showed the higher yields of soluble solids and total polyphenols, as well as a higher antioxidant activity for both species.

Keywords: Extraction methods; *Syringodium filiforme*; *Thalassia testudinum*; total phenolic compounds.

Resumen

Contexto: Existe una gran variedad de métodos para la obtención de extractos vegetales que posibiliten un buen rendimiento de metabolitos bioactivos. A lo largo de algunos años, se han perfeccionado las técnicas extractivas que han permitido obtener extractos naturales con propiedades farmacológicas potentes.

Objetivos: Determinar la influencia de varios métodos de extracción (infusión, decocción, microondas, maceración con agitación y calor y agitación y calor constante) sobre el contenido de sólidos, polifenoles y actividad antioxidante de las angiospermas marinas *Syringodium filiforme* Kützing (Cymodoceaceae) and *Thalassia testudinum* Banks ex König (Hyrocharitacea).

Métodos: El contenido de sólidos solubles y de fenoles totales fue determinado usando el método de Folin-Ciocalteu. La actividad antioxidante fue determinada por el método 2,2-difenil-1 picrilhidazilo (DPPH).

Resultados: Los resultados muestran la efectividad de la extracción por decocción para *T. testudinum* y de la extracción por microondas para *S. filiforme* entre los métodos que utilizan el agua como disolvente de extracción. En el caso de los métodos que utilizan la mezcla hidroalcohólica como disolvente de extracción, la extracción por maceración con agitación y calor mostró los mayores rendimientos de sólidos solubles y polifenoles totales, así como una mejor actividad antioxidante.

Conclusiones: Para la preparación de extractos de *Thalassia testudinum* y *Syringodium filiforme,* la mezcla etanol/agua es la mejor opción para la extracción de compuestos fenólicos totales. Para la extracción de ambas especies la maceración seguida por agitación y calor muestra ser la opción más interesante con vistas a obtener el mayor contenido de polifenoles y la mejor capacidad antioxidante.

Palabras Clave: Compuestos fenólicos totales; métodos de extracción; *Syringodium filiforme*; *Thalassia testudinum*.

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INTRODUCTION

Basic knowledge on marine organisms and the interactions they establish in their midst is still scarce, being even more limited, the understanding of the potential of the use of marine biodiversity in bioproducts industry. In this regard, the strengthening of scientific and technological communities is essential in understanding and sustainably using the biological diversity of marine ecosystems, since its potential largely depends on correct valuation. Even when it is known of the existence of numerous species containing bioactive compounds with potential for the pharmaceutical, cosmetic, nutraceutical and food industries, this knowledge is still limited. Also being insufficient in some cases because the effects attributed to them, have not been sufficiently validated by bioassays, and even less known are the structures of the principles or molecules responsible for these effects (Heip, 1998).

The genetic and biochemical diversity in the sea is believed to be higher than that on land. Moreover, marine organisms are subject to unique environmental conditions (high pressure, high concentration of salts, and predation, among other), forcing them to synthesize molecules that have no equivalence with the terrestrial organisms (Bhakuri and Rawat, 2005).

Algae are an important marine resource. Their use in food and feed is has been known since 5000 years ago and, scientifically, this knowledge has been validated because of the nutritional values they have (Plaza et al., 2008; 2009). Also, there are reports on their use in obtaining possible therapeutic formulations and the preparation of cosmetic products. In Cuba, antioxidant, anti-inflammatory activity and analgesic effects of extracts obtained from different algae species have been described (Llanio et al., 2003); as well as neuropharmacological properties (Menendez et al., 2014; 2015), and antineoplastic properties (Valdés et al., 2004).

Of particular interest are the actions observed in extracts from plants and marine algae, such as the regeneration of collagen fiber (Regalado et al., 2009), which are potentially usable as cosmetics to delay or prevent the effects of aging (Noel and Se-Kwon, 2013).

A great variety of pure solvents has usually been employed in extraction process during manufacturing of pharmaceuticals. Therefore, the solubility of bioactive compounds has a broad application and great importance in this industry. Some extraction methods have been used by the population to consume the natural products, like infusion and decoction, and some of them have been used by the pharmaceutical industry to prepare formulations like syrups, tinctures and others. However, knowing about the best extraction method to keep the pharmacological properties in the extract is essential (Miranda and Cuellar, 2001).

It is well known that solubility behavior of drugs in solvent mixtures is very significant because solvents mixtures are frequently used in purification methods, pre-formulation studies, among other applications (Acosta et al., 2016).

The wealth of Cuban marine ecosystem represents a promising source for this type of study. Specifically, the coastline of Havana province is subject to the threats arising from natural phenomena and human activity, and constitutes an ecosystem of great interest to know and preserve for both environmental and tourism purposes.

The aim of this study was to determine the influence of various extraction methods on the content of soluble solids, and phenolic compounds, as well as on the antioxidant capacity of extracts from marine angiosperms *Thalassia testudinum* and *Syringodium filiforme* that support the potential of these species for use in the biotechnology industry.

MATERIAL AND METHODS

Plant material

The species *S. filiforme* and *T. testudinum* were collected in September 2015 in "Rincon de Guanabo" beach (23°10'44"N - 82°07'01"W), Havana, Cuba. The species were authenticated by specialist Beatriz Martinez Daranas (Ph.D. in Marine Biology, from the Center of Marine Researches, Havana), and deposited in the collection of the National Aquarium of Cuba as IDO 165 and IDO39 respectively. Plants were washed with distilled water to remove salt and sand, and then dried at room temperature and later in an oven at 60°C until constant weight. Finally, they were grinded to 6 mm approximate particle size using a hammer mill

(Reuther, Germany). This particle size was chosen taking into account the technique described by Miranda and Cuellar (2001).

Preparation of extracts

For all extraction systems it was used the proportion vegetable material/solvent of 1/10 weight/volume, defined by Miranda and Cuellar (2001) is as one of the most commonly used when the drug does not have drastic substances. All extractions were performed in triplicate for further statistical analysis.

Infusion

It was carried out by dipping 10 g of the vegetal material in 100 mL of distilled water at 100°C, and then the Erlenmeyer was covered and was left to rest for 30 min, after which the infusion was cooled at room temperature and filtered by fast qualitative filter paper Whatman No. 1. Finally, the extract was kept frozen at -20°C for further analysis. This procedure was carried out following the description by Miranda and Cuellar (2001) in the book of Pharmacognosy and Natural Products.

Decoction

It was carried out with the aforementioned relation, the vegetal material was added to boiling distilled water, and after 30 minutes it was removed from the heat. Then the decoction was cooled at room temperature and filtered by fast qualitative filter paper Whatman No. 1. Finally, the extract was kept frozen at -20°C for further analysis. This procedure was carried out following the description by Miranda and Cuellar and in the book of Pharmacognosy and Natural Products.

Microwave-assisted extraction

It was carried out for one minute with a 700 W power in a domestic Daewoo microwave oven. As in the previous case, the solvent used for the extraction was distilled water. It was subsequently filtered using fast qualitative filter paper Whatman No. 1, and then it was kept frozen at -20°C for further analysis. In this case, a study conducted at the Cuban Center for Drug Research and Development was taken into account for the preparation of *Aloe vera* extracts, which require a 700 W power and one min of extraction time.

Maceration with heat and agitation

Carried out subjecting plant material to an extraction by maceration during 72 h using ethanol: water (1:1 v/v) as the extraction solvent, after 72 h mixture was placed in a water bath (named "Bain-Marie") at 60°C and stirred at 800 rpm for 2 h. Then the mixture was cooled at room temperature and filtered using fast qualitative filter paper Whatman No.1; the extract was kept in refrigeration at 10°C for further analysis. In this case, the extraction was carried out taking into account the approach by Dai and Mumpher (2010) regarding positive influence on the extraction using agitation and heat. The agitation allowed greater exchange between the drug and solvent extraction, and heat helped release the content of plant cells and therefore favors the extraction.

Agitation with heat

It was carried out by placing the mixture of plant material/solvent (ethanol: water 1: 1 v/v) in a water bath (named "Bain-Marie") at 60°C and stirred at 800 rpm for 2 hours. Then the mixture was cooled at room temperature and filtered by fast qualitative filter paper Whatman No. 1, after which it was kept in refrigeration at 10°C for further analysis. In this case, the extraction is carried out taking into account the already described for the previous method and previous experience in the development of these extracts.

Determination of soluble solids

For every extraction variant, the soluble solids content was determined by the gravimetric method described in British Pharmacopoeia (2010). Three empty capsules were taken and dried at 105°C for 1h, and then the capsules were placed in a desiccator until they were cooled at room temperature. Afterwards, 1 mL of extract was added for to each capsule. Subsequently, the capsules were placed in an oven at 105°C for 1 h. Finally, the capsules were placed in a desiccator until they were cooled and weighed. Each determination was performed in

triplicate. The total solids were calculated as follows:

Total solid content= weight of capsule with dry extract-weight of empty capsule

Determination of total phenolic compounds

The procedure followed, was referred in British Pharmacopeia 2010, using the Folin-Ciocalteu reagent in basic medium, where the concentration of phenolic compounds was detected through formation of tungsten and molybdenum salts, quantifiable by spectrophotometry at 760 nm. The total extract (96 µL) was mixed with 480 µL of distilled water, 48 µL of the acid reactive phosphomolybdic- phosphotungstic (known as the Folin-Ciocalteu reagent) and 576 µL of Na2CO3 at 29%. The mixture was incubated for 30 minutes at room temperature and the absorbance was measured at 760 nm in a spectrophotometer Shimadzu UV 1201, Japan.

The phenolic content was calculated by calibration curve using different concentrations of pyrogallol as standard, and it was expressed as equivalents of pyrogallol in mg per gram of dry weight of the extract. The reference pattern of was prepared at a concentration of 0.5 mg/mL in volumetric of 50 mL amber, protected from light and prepared at the time of use. Each determination was performed in triplicate.

Determination of the antioxidant effect

The used method was a modification to that described by Tabart (2009). Five concentrations of the extract were prepared for the analysis; of which 750 µL were taken and were mixed with 1500 µL of reagent DPPH (0.075 mg/mL). As blank it was used ethanol 95%, and as control it was used 750 µL of ethanol 95% and 1500 µL of reagent DPPH. The reaction was left in the dark for 30 min and, subsequently, samples were read at 517 nm in a spectrophotometer (Shimadzu UV 1201, Japan). Each determination was performed in triplicate. Trolox was used as reference compound. The percent of inhibition was calculated using the following formula:

% inhibition of the DPPH= [(Abs control - Abs sample)/Abs control] x 100

Where:
Abs control: Abs of ethanol +DPPH
Abs sample: Abs of extract +DPPH

Statistical analysis

Data normality was analyzed using the non-parametric test of Komodorov and Smirnov and the homogeneity of the variance of the results for a level of significance ($p \leq 0.05$) using the ANOVA. To enable a real comparison and carry out statistical analysis, the data from the extracts prepared with distilled water and those from the extracts prepared with the mixture ethanol: water (1:1 v/v) as extraction solvent were compared. The comparison of results where there were statistically significant differences between the extracts, it was performed an analysis of Tukey test. All analyses were conducted using the statistical package SPSS 10.0.1. The mean effective concentration (EC_{50}) was determined using the statistical program Origin Version 6.0.

RESULTS

Table 1 shows the calculated values of the contents of soluble solids and phenolic compounds, as well as the antioxidant capacity in extracts obtained by different extraction methods of from marine angiosperms *T. testudinum* and *S. filiforme*, using in some cases distilled water and in others ethanol 50% as the extraction solvent. To enable a real comparison and carry out statistical analysis, the data from the extracts prepared with distilled water and those from the extracts prepared with the mixture ethanol:water (1:1 v/v) as extraction solvent were separated.

For the *T. testudinum* species, as it can be observed in extracts that used water as extraction solvent, no significant differences were found ($p > 0.05$) in the soluble solids content between the extracts obtained using decoction and microwave-assisted extraction; but statistically significant differences were found with respect to extraction prepared by infusion of vegetal material ($p < 0.05$). The greatest amount of soluble solids is present in the extract obtained by infusion. Regarding the content of phenols, statistically significant differ-

ences were found between extracts ($p<0.05$); the highest content of total phenolic compounds was found in the extract obtained by decoction and the lower content, in the extract obtained by infusion of the plant material.

For the aqueous extractions of *S. filiforme*, no significant differences were found ($p>0.05$) in the soluble solids content between the extracts. Also, results revealed that there was no statistically significant differences between infusion and decoction ($p>0.05$) for the phenolic content, but there were statistically significant differences with regard to microwave-assisted extraction ($p<0.05$), which showed the highest value. Concerning the hydroalcoholic extracts from *S. filiforme*, the soluble solids content showed significant differences among the mean values obtained for different extracts ($p<0.05$). Results showed that the highest value of solids content was found in the extract obtained by maceration with heat and agitation. In the case of the polyphenolics, significant differences were found among the mean values ($p<0.05$), the extract prepared by maceration with heat and agitation showed the highest phenolic content.

Antiradical activity given by the effect of entrapment of the DPPH free radical in different extracts of *T. testudinum* and *S. filiformeis* shown in Table 1.

The most significant antioxidant activity occurred when the IC_{50} was lower, which means that lower sample concentrations of are required for achieving the 50% of reduction of the DPPH radical. For aqueous extracts of *T. testudinum*, the greater antioxidant effect was evidenced by the extract prepared by decoction, coinciding with the higher content of polyphenols in the extracts. As for the aqueous extracts of *S. filiforme*, the one obtained through extraction by microwave showed higher antioxidant capacity, also coinciding with higher content of phenolic compounds. These results confirmed the correspondence between the content of phenolic compounds and antioxidant activity of natural extracts.

DISCUSSION

For hydroalcoholic extracts of *T. testudinum*, an increased activity was evidenced by the extract prepared with agitation and heat; while for *S. filiforme* the extract with the highest antioxidant capacity was that obtained through maceration, followed by agitation with heat, coinciding with increased content of phenolic extract. Considering the vast biodiversity of marine species, the use of appropriate methodologies that can rapidly screen different marine sources for bioactive compounds is of great interest. To design this screening methodology, different parameters have to be considered, including the possible nature of the desirable bioactive compounds (regarding solubility, heat resistance, or molecular weight) and the bioactivity that is sought. Initially, a suitable extraction technique should be selected in accordance with the predicted nature of the expected/target bioactive compound(s). However, several extraction techniques could also be used to fully characterize the potential of the different natural sources, introducing different extraction selectivity (Dai and Mumpher, 2010; Ibañez et al., 2012).

Table 1. Content of soluble solids, phenolic compounds and antioxidant capacity in extracts of *T. testudinum* and *S. filiforme*.

Extraction methods	Soluble solids (mg/mL)		Phenolic compounds (mg/g d.w)		DPPH (IC_{50}, mg/mL)		
	T. t	*S. f*	*T. t*	*S. f*	*T. t*	*S. f*	*Trolox*
Infusion	8.73 ± 0.23^{a}	25.93 ± 0.19^{a}	28.52 ± 0.38^{d}	6.43 ± 0.07^{c}	1.5 ± 0.23^{b}	2.5 ± 0.12^{d}	
Decoction	7.73 ± 0.48^{b}	26.53 ± 0.45^{a}	36.12 ± 0.43^{b}	5.56 ± 0.59^{c}	0.8 ± 0.35^{a}	1.2 ± 0.58^{c}	
Microwave	7.41 ± 0.36^{b}	25.87 ± 0.27^{a}	32.31 ± 0.54^{c}	8.92 ± 0.36^{b}	1.7 ± 0.09^{c}	1.05 ± 0.10^{b}	0.004
Mac/Agit/Heat	7.23 ± 0.09^{b}	25.35 ± 0.52^{a}	40.04 ± 29.0^{a}	9.25 ± 0.64^{a}	1.3 ± 0.45^{b}	0.8 ± 0.75^{a}	
Agit/Heat	6.42 ± 0.64^{c}	23.41 ± 0.13^{b}	37.41 ± 21.0^{b}	7.82 ± 0.17^{b}	0.8 ± 0.63^{a}	0.9 ± 0.26^{a}	

T. t: Thalassia. testudinum; S. f: Syringodium filiforme, d.w; dry weight. The values are expressed as mean ± SD (n=3). Different letters in the same column denote statistically significant differences ($p<0.05$).

The use of environmentally clean advanced extraction techniques allows for the attainment of the target compound(s) of interest with more efficient extraction procedures, while, at the same time, minimizing the use of organic toxic solvents. Depending on the selected extraction techniques, different extraction parameters should be tested in order to study the influence of solvents, temperatures, pressures, and other relevant parameters that might have a significant influence on the outcome of the extraction process employed. The different extracts, obtained using diverse conditions, must then be tested for biological bioactivities by performing the appropriate functional activity assay (Ibañez et al., 2012). Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water. Selecting the right solvent affects the amount and rate of polyphenols extracted (Xu and Chang, 2007).

Phenols are among the interesting antioxidant compounds previously isolated from marine resources, including microalgae and seaweed, generally using a mixture of solvents such as ethanol/water. At least 8000 different bioactive compounds are considered polyphenols (Bravo, 1998). In general, phenolic compounds are divided into ten types, based on their structure. These ten groups are simple phenols, phenolic acids, hydroxycinnamic acids, coumarins, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids, and lignins. Among them, flavonoids are the group known to have the greatest number of different structures, and at least 5000 flavonoids have been characterized and referenced in literature to date (Wollgast and Anklam, 2000). For the species in this study the isolation of thalassiolins a, b and c (glycosylated flavonoids) from *T. testudinum* were reported (Regalado et al., 2009, 2012); and the presence of caffeic acid and ferrulic acid in the crude extract of *S. filiforme* was also referred (Nussier et al., 2010).

It is understood that the intensity of the antioxidant activity of these complex polyphenols is related to the degree of polyphenol polymerization. In general, lower degrees of polymerization result in greater antioxidant activities. Nevertheless, the main activity related to phenolic compounds is antioxidant activity (Li et al., 2009).

CONCLUSIONS

The ethanol/water mixture is the most appropriated option for *Thalassia testudinum* and *Syringodium filiforme* to obtain phenolic compounds, under the experimental conditions performed in this study. Maceration, followed by agitation and heat, proved to be the most interesting option to obtain the highest content of phenolic compounds in both species with appropriated antioxidant capacity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Concepts	x				
Design	x	x			
Definition of intellectual content	x	x	x		
Literature search	x			x	
Clinical studies					
Experimental studies	x	x			x
Data acquisition	x	x			x
Data analysis	x	x	x		
Statistical analysis			x	x	x
Manuscript preparation	x				
Manuscript editing	x	x		x	
Manuscript review				x	

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