High Value-Added Biomolecules from Beach Waste of Marine Origin-Screening for Potential Candidates among Seagrass of the Cymodoceaceae Family

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Abstract:

Background: Detrital leaves from seagrass of the Cymodoceaceae family were assayed for biologically active molecules that have commercial as well as societal benefits.

Objective: We focused on L-chiro-inositol, a very rare natural occurring cyclitol, and chicoric acid, a polyphenolic compound, in which both applications were found in the nutraceutical segment.

Method: Six species of seagrass belonging to the genera Syringodium, Cymodocea and Halodule were collected from their native habitat. The L-chiro-inositol content of the crude aqueous extracts prepared from different batches of Cymodocea nodosa flotsam was measured by quantitative ¹H-NMR spectroscopy. High concentrations were found with values ranging from 22.2 to 35.0 mg.g⁻¹ plant dw. The presence of L-chiro-inositol in the tropical species C. rotundata, C. serrulata, Syringodium isoetifolium, and Halodule pinifolia was also characterized by qualitative NMR. The chicoric acid content of crude aqueous methanolic extracts prepared from C. rotundata, C. serrulata, S. isoetifolium, and Halodule pinifolia was determined by quantitative HPLC-DAD. The values found ranged from 0.39 to 6.15 mg.g⁻¹ dry weight. Chicoric acid was unambiguously identified as the major phenolic in S. isoetifolium, and Halodule pinifolia, while it was found in mixture with flavonol derivatives in C. rotundata and C. serrulata. Flavonol derivatives are also of interest for their bioactivity.

Result: Considering the demonstrated therapeutic applications of L-chiro-inositol and chicoric acid, their high value on the nutraceutical market, and their rare occurrence in the plant kingdom, their presence in Syringodium, Cymodocea and Halodule detrital leaves makes this abundant biomass of interest for dietary and pharmaceutical applications.

Conclusion: These results show that there is a real potential for harvesting beachcast Cymodoceaceae.

Keywords: L-Chiro-Inositol, Chicoric Acid, Cymodoceaceae Family, Cymodocea, Syringodium, Halodule, Quantitative NMR and HPLC.

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1. INTRODUCTION

During the last decade, the global nutraceutical market for medicinal herbs has grown at an unprecedented rate, as consumers focus on health maintenance using natural products and health-enhancing foods. As a result, plant secondary metabolites have become economically important in the field of food additives, nutraceuticals and drugs. This is the case of chiro-inositol and chicoric acid.

Inositols constitute an important class of biologically active compounds [1 - 4]. Only five are known to occur in plants in variable amounts. Chiro-inositol exits in two enantiomeric forms, i.e. D-chiro-inositol (DCI) and L-chiro-inositol (LCI). They both exhibit a positive effect on glucose metabolism [3], but are very rare in nature, especially LCI, whose distribution in higher plants is very limited. LCI has been identified, for instance, in Amyema miquelii [5], Chrysanthemum [6], Cremanthodium ellisii [7], Euphorbia resinifera [8], and Euphorbia pilulifera [9]. More recently, LCI has been found in Ageratina petiolaris, a plant traditionally used in Mexico for the treatment of diabetes [10].

Chicoric acid (CA; Fig. (1)) is a rare and valuable natural product of special interest owing to its large spectrum of biological properties including antioxidant activities, anti-obesity effects, anti-HIV, and others [11]. These properties explain its success on the nutraceutical market [12, 13]. CA is the main phenolic compound in Echinacea purpurea, but it does not occur in appreciable amounts in other species such as E. pallida and E. angustifolia [14]. The identification of any novel sources of CA is of economic interest.

Marine biodiversity offers potential resources for a wide variety of non-drug nutritional natural products. Several species of seaweeds are used as human food or as raw material for the production of nutraceuticals and cosmeceuticals [15]. Compared to algae, seagrasses remain less exploited despite they offer promising opportunities to find valuable phytochemicals [16, 17].

Fifty-nine seagrass species are recognised worldwide, distributed in four families [18]. The Cymodoceaceae family (Manatee Grasses) encompasses the greatest variety with 15 species in five genera.

The bioactivity so far reported for seagrass metabolites has been recently reviewed [19]. Examples include cytotoxicity, antioxidant-, antimicrobial-, antiviral-, or anti-inflammatory activity. The ability of Zosterin (a pectin isolated from Zostera asiatica) to decrease toxicity of antitumor drugs and to purge heavy metals from human organisms [20, 21] led to marketed seagrass-derived food and drugs in Russia [see as examples: 22-24]. For our part, we have demonstrated that Z. marina and Z. noltei detrital leaves can compete with Rosmarinus officinalis for the production of rosmarinic acid, which is marketed as a nutraceutical [17].

Seagrass beds generate considerable standing biomass, which could be exploited as raw material. In places dependent on tourism, local governments are under great public pressure to remove seagrass detritus from beaches and shorelines used for recreational purposes. In most cases, the collected biomass is disposed of in waste disposal sites.

The increasing demand for alternative medicine, and the harvesting and collection pressures for the medicinal plants of interest point up the need for alternative resources. Using dead seagrass biomass as a source of phytochemicals offers some particular advantages:

1. Physical events (storm, waves, currents, etc…), and natural seasonal leaf drop result in massive deposit of leaf litter along shorelines. As they originate from large areas of seagrass bed, and sometimes even from different meadows, they offer a more representative metabolite content than isolated samples from a single meadow.
2. Seagrasses are much more resistant to decomposition than are freshwater angiosperms or algae. The rate of decomposition of seagrass detritus is generally low (< 1% of dry wt/day) compared with other sources of detritus.
3. We have previously shown that significant concentrations of cyclitol and/or phenolic compounds remain in the detrital leaves of species of Zosteraeaceae and Cymodoceaceae [16, 17, 25 - 27].
4. In contrast to terrestrial plants, vegetative reproduction is more important in seagrasses than pollination. This limits the plant-to-plant variation and consequent genetic inconsistencies in the type and level of phytochemicals produced by terrestrial plants.
5. Heretofore, there has been no market for Cymodoceaceae flotsam, so that the cost of the same is simply that of harvesting.
Some members of the Cymodoceaceae family have been reported to accumulate cyclitols, including myo- and L-chiro-inositol. However, LCI had not been isolated and its stereochemical assignment was only based on a hypothetical biosynthesis pathway [28].

We have recently reported the isolation of LCI and CA from the dead biomass of the tropical seagrass Syringodium filiforme [25, 26], and CA from the temperate Cymodocea nodosa [27]. Considering the economic potential of these two substances within the pharmaceutical, cosmetic, and food industries, it appears of interest to evaluate the potential of other tropical members of Cymodoceaceae.

The aim of this study was to examine the phytochemical content of detrital leaves, with a view toward exploiting the flotsam. This work reports the quantitative determination of LCI in the temperate C. nodosa, and the first quantitative characterization of polyphenols in C. rotundata, C. serrulata, S. isoetifolium, and Halodule pinifolia, which are found throughout the Indian and Pacific Oceans [29]. In addition, these tropical species were screened for the possible presence of LCI.

2. MATERIAL AND METHODS

2.1. General

All the solvents used were HPLC grade. Trifluoroacetic acid (TFA), anhydrous pyridine, trimethylchlorosilane (TMCS), and hexamethyldisilazane (HMDS) were purchased from Aldrich Chemical Company (Saint-Louis, Missouri, USA), and analytical-grade water from Sodipro Company (Echirolles, France). NMR spectra were recorded on a Bruker AVANCE 400 MHz (Billerica, MA, USA). All the NMR solvents (DMSO, D₂O, or CD₃OD) and trimethylsilyl d₄-propionic acid (TMSP) used as internal standards for the qNMR experiments were purchased from Euriso-Top (Gif-sur-Yvette, France). Ultraviolet (UV) spectra were recorded on a V-630 UV-VIS Jasco spectrophotometer (Easton, USA) in HPLC grade water. Rotatory power was measured on a Perkin Elmer 241 polarimeter. Authentic sample of caftaric- and chicoric acid (also known as cichoric acid and dicaffeoyltartaric acid) was given by Eburon Organics (B-2310 Rijkevorsel, Belgium). All the other standards were purchased from Extrasynthèse (Genay, France). Chlorogenic acid (Extrasynthèse) was used for the HPLC quantification of CA and caftaric acid (CAF).

2.2. Plant Material

The following species were studied: Cymodocea nodosa (Ucria) Ascherson, Cymodocea rotundata, Ehrenberg & Hemprich. Ex Ascherson, Cymodocea serrulata (R. Brown) Ascherson & Magnus, Halodule pinifolia (Miki) den Hartog, and Syringodium isoetifolium (Ascherson) Dandy (Table 1). The temperate seagrass C. nodosa was sampled in Canary Island and throughout the Mediterranean Sea, while the tropical species were collected along the coast of Palk Bay, India (Table 1). Identification of the species was based on morphology and microscopic leaf anatomy of both living tissue and fresh green detrital leaves, and on comparison with identification keys reported in literature [see as
examples: 30-32].

Whatever the species and the sampling site, green freshly detached leaves were collected in the intertidal zone and from large accumulations of detritus along shorelines (Table 1). After collection, the samples were thoroughly rinsed in seawater and then quickly washed in freshwater to remove sand and salt. Associated debris and algae were removed when present, then plant material was air-dried at room temperature in the dark. When shoreline accumulation contain several seagrass species, each of them were separated before drying.

Table 1. Collection data.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Common Name</th>
<th>Geographical Range</th>
<th>Sampling Site</th>
<th>Collection Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringodium</td>
<td>Kützing</td>
<td>S. filiforme</td>
<td>Western tropical Atlantic from Florida (USA) to Venezuela, including the Gulf of</td>
<td>Guadeloupe, French</td>
<td>July to October</td>
</tr>
<tr>
<td></td>
<td>Kützing</td>
<td>Kützing</td>
<td>Mexico and the Caribbean Sea, as well as Bermuda.</td>
<td>West Indies</td>
<td>2007</td>
</tr>
<tr>
<td></td>
<td>Synonyms:</td>
<td>Cymodocea</td>
<td></td>
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<tr>
<td></td>
<td>filiformis</td>
<td>filiformis</td>
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<tr>
<td></td>
<td>Cymodocea manatorum</td>
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<tr>
<td></td>
<td>Phucagrostis manatorum</td>
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<tr>
<td>S. isoetifolium (Ascherson)</td>
<td>Dandy</td>
<td>noodle seagrass</td>
<td>Indo-Pacific region, including Fiji and Samoa.</td>
<td>Palk Bay, Tamil</td>
<td>October to</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nadu, India</td>
<td>December 2012</td>
</tr>
<tr>
<td>Cymodocea</td>
<td>König</td>
<td>C. nodosa</td>
<td>Mediterranean Sea and Atlantic Ocean (Portugal, Mauritania, Senegal, Canary,</td>
<td>Canary island,</td>
<td>14/07/2007 up to</td>
</tr>
<tr>
<td></td>
<td>Ucria (Ascherson)</td>
<td>little Neptune</td>
<td>Madeira and Cape Verde Islands)</td>
<td>Spain</td>
<td>21/05/2008</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>Cadi, Spain</td>
<td>18/07/2008</td>
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<td></td>
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<td>Alfacs Bay, Spain</td>
<td>28/07/2008</td>
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<td>Monastir, Tunisia,</td>
<td></td>
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<td>Zeytineli, Turkey</td>
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<tr>
<td>C. rotundata</td>
<td>Ehrenberg &amp;</td>
<td>Indo-Pacific</td>
<td></td>
<td>Palk Bay, Tamil</td>
<td>October to</td>
</tr>
<tr>
<td></td>
<td>Hemprich ex Ascherson</td>
<td></td>
<td></td>
<td>Nadu, India</td>
<td>December 2012</td>
</tr>
<tr>
<td>C. serrulata (R. Brown)</td>
<td>Ascherson &amp; Magnus</td>
<td>Indo-Pacific</td>
<td></td>
<td>Palk Bay, Tamil</td>
<td>October to</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nadu, India</td>
<td>December 2012</td>
</tr>
<tr>
<td>Halodule</td>
<td>Endlinger</td>
<td>H. pinifolia</td>
<td>Asian Pacific and eastern Indian Oceans</td>
<td>Palk Bay, Tamil</td>
<td>October to</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nadu, India</td>
<td>December 2012</td>
</tr>
</tbody>
</table>

2.3. Extraction of Seagrasses and Determination of their Composition

2.3.1. Typical Procedure for Extraction of LCI

Dried ground leaves (5 g) were extracted at room temperature with water (40 mL, 24 h). The process was twice repeated, and then the extracts were pooled, lyophilized, and analyzed by NMR.

2.3.2. Isolation and Identification of LCI

A pure sample of LCI (72 mg) was isolated from a crude aqueous extract of C. nodosa (12 g), following the silylation-desilylation process we have previously developed [25]. The purity was confirmed by 1H NMR and the rotatory power was measured on a Perkin Elmer 241 polarimeter at 25°C ([α]D 25 = - 67.8, c = 0.5, H2O).

2.3.3. Determination of the LCI Concentrations by 1H NMR

Samples were prepared with 3.00 mg of dried crude extract, 0.4 mL of D2O and 100 µL of a D2O solution of TMSP (internal standard), and transferred to a 5 mm NMR tube. The internal standard solution was prepared by dissolving TMSP of known purity in 5 mL of D2O. One dimensional 1H NMR spectra were acquired with an AVANCE DPX 400 MHz spectrometer (Bruker) in the conditions previously described [25]. For quantitative analysis, manual integrations of the concerned peaks were achieved. LCI was quantified by integrating the area of the d (2 H) at 3.95 ppm and the internal reference signal (s, 9 H) at 0.0 ppm. The amount of the internal standard was calculated to give peaks of similar intensities for both analyte and standard. This similarity helps minimising the error in measurements.

The amount of LCI for each extract was calculated from the resultant analyte to standard peak ratio according to the following equation:

\[
\text{Amount of LCI (extract)} = \frac{MW_{LCI} \times W_s \times I_{LCI} \times N_s \times N_{LCI} \times P_s}{MW_{IS} \times \frac{W_s}{W_{extract} \times I_{IS}} \times N_s \times N_{IS} \times P_s}
\]
Where:

LCI and IS refer to L-chiro inositol and the internal standard respectively, $MW$ are the respective molecular weights, $W$ the amount of substance used, $I$ the integrated peak area, $N$ the number of atoms that gives rise to the measured NMR signals ($N_{IS} = 9, N_{LCI} = 2$), $\rho_{IS}$ the percentage purity of the internal standard (98%).

The amount of LCI in the plant is obtained according to the equation:

$$\text{Amount in the plant} = \text{Amount of LCI (extract)} \times \text{extraction yield}.$$ 

Data are expressed in milligrams per gram (mg.g$^{-1}$ of dry matter of leave tissue, mean ± standard deviation (SD) of three determinations).

### 2.3.4. Typical Procedure for Extraction of Phenolics

Dried ground leaves (10 g) were extracted at room temperature with aqueous methanol (50:50; 120 mL, 24 h). The process was repeated, and then the extracts were pooled, evaporated to dryness, and analyzed by NMR, HPLC and LC-MS.

### 2.3.5. HPLC Analysis

Separation and quantification of phenolics in the crude extracts were performed as previously described [27] using the binary gradient 0.1% (v/v) TFA in water (A) and methanol (B). External standard calibration with chlorogenic acid dissolved in methanol/water (70:30) with the aid of sonication was established on six data points, covering the concentration range 0.0619–0.00619 mg.mL$^{-1}$. Linear regression on the HPLC analyses gave R$^2$ values of 0.9995. Chromatographic peaks were checked for peak purity, and identification was achieved by comparing retention times and UV spectra with those of standards. Quantitative determinations were carried out by peak area measurements at 328 nm, using the calibration curve of chlorogenic acid at the same wavelength and the correction factors, which take into account the differences in the responses of the HPLC detector to CA, CAF, and chlorogenic acid. These factors have been determined by the Institute for Nutraceutical Advancement (INA) in the frame of the INA Methods Validation Program (INA Method 106.000 2000) [33]. The data presented in Table 2 are the average from three experiments, calculated using the following equation and correction factors:

$$\% \text{ W/W individual phenol compound} = \frac{(C \times F \times 100)}{Cs}$$

Where:

- $C$ is the concentration of the tested phenolic compound (mg.mL$^{-1}$) in the analyzed extract, calculated as chlorogenic acid from peak areas and linear regression;
- $F$ is the correction factor of phenolic response against chlorogenic acid (F = 0.888 for CAF and 0.695 for CA); and
- $Cs$ is the concentration of the sample (mg.mL$^{-1}$) diluted in deionized water for analysis.

Data are expressed in milligrams per gram of dry matter of plant material (mg.g$^{-1}$ dw; mean ± standard deviation (SD) of three determinations).

### 2.3.6. LC-MS Analysis

LC-MS was performed using an HP1100 (Hewlett-Packard, Palo-Alto, CA, USA) equipped with an Agilent MSD 1946B simple quad mass spectrometer and HP Chemstation software. Positive mode ESI spectra of the column eluate were recorded in the range of m/z 100–1000 a.m.u. Absorbance was measured at 280 and 320 nm. Compounds were separated using an MN Nucleodur C18 column (Macherey-Nagel, Düren, Germany): 125 mm × 2 mm i.d., 3 μm particle size. The analytes were eluted at a flow rate of 0.3 mL min$^{-1}$ using the binary gradient (v/v) formic acid in water (pH 2.55, A) and methanol (B). The following linear gradient was used: 15% B to 100% B (15 min). Separation of the analytes was carried out at 50°C. The injection volume was 2 μL. For MS analysis, compounds were detected using the following conditions: nebulizing gas pressure, 60 psi; drying gas flow rate, 12 mL.min$^{-1}$; drying gas temperature, 350°C; capillary voltage, 4000 V; temperature source, 350°C. Data were acquired in full scan mode (m/z 100-1000) at a fragmentor voltage of 70 V.
3. RESULTS AND DISCUSSION

3.1. Quantitative Determination of the LCI Content in *C. nodosa*

Quantitative analyses of mixtures of sugars are generally carried out using chromatographic techniques such as HPLC and GC. However, these methods are time consuming and suffer numerous limitations due to the preliminary chemical derivatizations necessary for carbohydrate detection. These problems can be circumvented by using quantitative $^1$H NMR spectroscopy. This fast, non-destructive technique with minimal sample preparation has been proved useful for quantification of individual components in crude extracts without the need for fractionation or isolation procedures [25, 34 - 37].

The LCI content of the crude aqueous extracts prepared from different batches of *C. nodosa* flotsam collected in the Canary Islands and throughout the Mediterranean Sea was measured by quantitative $^1$H-NMR spectroscopy as previously described [25, 37]. The presence of three proton signal at $\delta$ 3.50 (dd), 3.67 (multiplet) and 3.95 (d), and three carbon signals at $\delta$ 70.48, 71.70 and 72.79, allow identification of the major product to free chiro-inositol [38, 39].

The doublet of LCI at 3.95 ppm, and the singlet (9 H) at 0.0 ppm of the TMSP chosen as internal standard were used for all calculations. The values found ranged from 22.2 to 35.0 mg g$^{-1}$ plant dw (Table 2). These values are in the same order as those found in *S. filiforme* [25]. The chiro-inositol contained in *C. nodosa* was isolated from the crude extract to measure its rotatory power. The purification step was achieved by sequential silylation-desilylation with HMDS/TMCS in pyridine, then hydrolysis of the silylated chiro-inositol with TFA in CH$_2$Cl$_2$ led to a white precipitate of chiro-inositol (see Materials and Methods for details). Its purity was confirmed by $^1$H NMR (Fig. 2), and the observed levorotation ($[\alpha]_D^{25} = -67.8, c = 0.5$ (literature data: $[\alpha]_D^{20} = -70, c = 0.55$ in H$_2$O [40])) allowed to unambiguously identify the chiro-inositol contained in *Cymodoceae* to LCI.

To the best of our knowledge, there are few published quantifications of LCI in plants. Epifano *et al* [41]. found 0.8 mg g$^{-1}$ of LCI in *Phagnalon sordidum* while Ichimura *et al* [6] found concentrations ranging from 0.2 to 1.9 mg g$^{-1}$ (fresh weight) in various organs of the ornamental plant Chrysanthemum. In a sample of living *S. filiforme* leaves from Jamaica, Drew [28] estimated the LCI content at 50 mg g$^{-1}$ but mentioned that his method of quantification could lead to values in excess of 100% e.d.wt. Considering the demonstrated hypoglycaemic action of LCI, the high concentrations found in our study (23-25 mg g$^{-1}$ plant dw) offer promise for the exploitation of *C. nodosa* flotsam as a new cheap source for nutraceutical or therapeutic applications.

The presence of LCI in the tropical species *C. rotundata*, *C. serrulata*, *S. isoetifolium*, and *Halodule pinifolia* collected in Palk Bay (India), was also characterized by qualitative NMR. LCI appeared to be present in variable amount in all the samples.

Few authors have studied the biological properties of LCI, while it is well documented in the case of DCI. DCI has found applications as drugs [42]. It has a positive effect on glucose metabolism and can be used for the treatment of symptomatic insulin-resistant type II diabetes without known toxic or deleterious side effects [43] or as a preventive and treatment agent for cataracts [44]. DCI increases the action of insulin in patients with polycystic ovary syndrome (PCOS), which is the most common cause of anovulatory infertility in the US [45, 46]. Comparison of the respective effects of LCI and DCI on diabetic rats have shown that the stereoisomerism differences between the D and L- forms did not affect the hypoglycaemic action of chiro-inositol [47].

Usefulness of DCI as a nutritional supplement in the treatment or prevention of oxidative stress and related deseases has been claimed [48], which resulted in extensive cultivation of *Fagopyrum sagittatum* Gilib (common name: buckwheat) for the production of DCI-based herbal remedies. The concentrations of LCI found in the leaves of *S. filiforme* (23-25 mg g$^{-1}$) and *C. nodosa* (22-35 mg g$^{-1}$) are greater than the concentration of total DCI in buckwheat (14 mg g$^{-1}$ in farinetta) [49]. The ability of LCI to mimic DCI action, especially management of diabetes, opens promising opportunities for the exploitation of *Cymodoceaeae* beach cast.

3.2. Quantitative Determination of the CA Content in Tropical Seagrasses.

High performance liquid chromatography (HPLC) combined with diode array detection (DAD) was used for both qualitative and quantitative analyses of the crude extract composition. The identification of products in a sample was performed by matching both their HPLC retention time (R$_t$) and 220-400 nm on-line UV-spectra with those of standards. Chromatograms were systematically recorded at 280, 328, and 350 nm, which allowed a clear distinction between C6,
C6-C3, and C6-C3-C6 backbone type of phenolic metabolites. HPLC analysis using a C18 column and methanol: aqueous trifluoroacetic acid (0.1 M) as the mobile phase allowed separation of the major and minor phenolics in one chromatographic run. Individual phenolic compounds were identified by comparing the absorption spectra of the unknown metabolite with that of authentic standards.

Examination of Fig. (3) shows that S. filiforme, S. isoetifolium, Halodule pinifolia and C. nodosa share a very similar phenolic profile largely dominated by a compound eluted at R_t ~ 36.9 min with a typical caffeate UV spectra in good agreement with CA (λ_max 330 nm). The identity of CA was confirmed by comparison of the retention time, on line UV and UV-vis spectra, NMR and LC-MS data (ESIMS m/z: 497 [M+23]+; main product ion at m/z = 163), with those of a commercial standard. All these analytical techniques led to perfectly overlaid results. A minor phenolic eluted at R_t ~ 21.7 min (λ_max 328 nm, ESIMS m/z: 335 [M+23]+; main product ion at m/z = 163) was identified as caftaric acid (CAF, Figs. 1 and 3). This substance often co-occurs with CA in others plants and could originate from partial hydrolysis of CA. The concentrations of CA found for S. isoetifolium and Halodule pinifolia (4.06 and 6.15 mg.g⁻¹, Table 2) are similar to those previously found in S. filiforme. In contrast, C. serrulata and C. rotundata exhibit a complex mixture of phenolics, among which CA is still present but less abundant, while a mixture of flavonols was eluted between 39 and 45 min. Their on-line absorbance and spectral shape were characteristic of 3-O-glycosyl flavonols (Fig. 4). On the basis of LC-MS and comparison to standard, some were tentatively assigned to: peak 2: rutin, MW 610; peaks 8 and 10, Kaempferol glucoside isomers, MW 448; peak 11, Kaempferol pentoside, MW 418. A peak at 21.5 min (λ_max 255 nm) was observed in the two species and identified to p-hydroxy benzoic acid. All the other peaks eluted from 2.0 to 20 min exhibit a spectral shape with λ_max ranging from 250 to 280 nm characteristic of aromatic C6 skeleton.

CA is of special interest due to its rarity in the plant kingdom and its biological properties. It has been reported to possess multifunctional effects, namely immunostimulating, anti-hyaluronidase and antioxidant activities, the ability to promote phagocyte activity in vitro and in vivo., the ability to inhibit HIV-1 integrase and replication [50, 51], and a protective effect on the free radical-induced degradation of collagen [52]. CA has been found to be one of the most potent HIV-1 integrase inhibitor [11]. Rutin and flavonols are also of interest. Epidemiological studies suggest that a high dietary intake of flavonols is associated with reduced risk of vascular disease and improvement in a number of parameters associated with this pathology [53]. Rutin and kaempferol have a variety of biological benefits including antiallergic, anti-inflammatory, antimicrobial, antiproliferative, antidiabetic, and anticarcinogenic properties [54 - 56].
Making direct comparisons between our results and the CA amounts reported in the literature for terrestrial plants appears difficult. Indeed, how the calculations have been done is not always clear, some of the results are expressed on a fresh weight basis, and concentrations were often obtained without taking into account the difference in the responses of the HPLC detector to CA and the standards used for quantification. Using the method developed by the Institute for Nutraceutical Advancement for the *Echinacea* [33] solves these problems, and the data obtained represent the real amounts of CA in the dry plant.

**Table 2.** LCI and CA content values. Data are expressed in milligrams per gram (mg.g⁻¹ of dry matter of leave tissue, mean ± standard deviation (SD) of three determinations).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sampling Site</th>
<th>LCI Content</th>
<th>CA Content</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. filiforme</em></td>
<td>Guadeloupe, French West Indies</td>
<td>23-25⁺</td>
<td>0.94–5.26 ⁺</td>
</tr>
<tr>
<td><em>S. isoetifolium</em></td>
<td>Palk Bay, India</td>
<td>Present, nd</td>
<td>4.06</td>
</tr>
<tr>
<td><em>C. nodosa</em></td>
<td>Canary Island, Spain</td>
<td>35.0 ± 0.3</td>
<td>27.44 ± 0.42⁺</td>
</tr>
<tr>
<td></td>
<td>Alfacs Bay, Spain</td>
<td>32.9 ± 0.6</td>
<td>17.94 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Monastir, Tunisia,</td>
<td>22.2 ± 0.1</td>
<td>18.52 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>Zeytineli, Turkey</td>
<td>31.6 ± 0.6</td>
<td>17.66 ± 0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.0 ± 0.2</td>
<td>12.11 ± 0.22</td>
</tr>
<tr>
<td><em>C. rotundata</em></td>
<td>Palk Bay, India</td>
<td>Present, nd</td>
<td>6.15</td>
</tr>
<tr>
<td><em>C. serrulata</em></td>
<td>Palk Bay, India</td>
<td>Present, nd</td>
<td>0.39</td>
</tr>
<tr>
<td><em>Halodule pinifolia</em></td>
<td>Palk Bay, India</td>
<td>Present, nd</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Values from our previous works are given in bold character [24]; [25]; [26]; nd: non quantified.

**Fig. (3).** HPLC profiles of crude extracts obtained from the six Cymodoceaceae species: traces at 280 nm, abscissa in min, ordinate in mAU. Retention times (min), assignment: 21.7 CAF; 36.9 CA; 38.5–45.0 mixture of flavonols.

Noticeable was the fact that CA and LCI were found in all the species. Although other members of Cymodoceaceae should be analyzed, these results already suggest that CA and LCI could be used as taxonomic markers for Cymodoceaceae. Among the four families of seagrasses, the Cymodoceaceae encompasses the highest variety with 15 species in five genera. Except for the six species presented in this work, the full characterization of the phenolic content of the nine others still needs to be established. Considering the high value properties of both LCI and CA, it appears of interest to investigate these other members of Cymodoceaceae growing in tropical seas.
Fig. (4). Close-ups of the *Cymodocea* HPLC traces (280 nm, min, mAU) showing the flavonal compounds and their respective on-line UV spectrum. (a) *C. rotundata*; (b) *C. serrulata*. Retention times (min), assignment: (1) 39.3, rutin derivative; (2) 39.7, rutin; (3) 42.2 and (10) 43.1, kaempferol glucoside isomers; (11) 44.7, kaempferol pentoside.

**CONCLUDING REMARKS**

Considering the demonstrated therapeutic applications of L-chiro-inositol [3, 4, 48], and chicoric acid [12, 13], their high value on the nutraceutical market, and their rare occurrence in the plant kingdom, their presence in *Syringodium*, *Cymodocea* and *Halodule* detrital leaves makes this abundant biomass of interest for dietary and pharmaceutical applications. These results show that there is a real potential for harvesting beachcast Cymodoceacea.

Seagrass meadows form one of the most widespread and productive coastal systems in the world. Huge amounts of leaves are shed and washed ashore, often building important banks of seagrass litter. Recovery of chiro-inositol or chicoric acid from beach waste material could promote opportunities for the economic development of tropical coastal areas. In the West the demand for herbal drugs has reached a new high in recent years. In India, the total market for nutraceuticals is also growing trying to incorporate traditional herbal ingredients into the nutraceutical portfolio. Indian seagrasses were traditionnaly used for thousands of years for a variety of applications from food to medicine and the engagement of the Tamil Nadu community in collecting and sorting seagrasses has been recently reported [57]. All these features could justify significant exploitation of this abundant renewable resource. Harvesting seagrass litter on areas where it is a nuisance to other economic activities, could be of benefit to all.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

Not applicable.

**HUMAN AND ANIMAL RIGHTS**

No Animals/Humans were used for studies that are base of this research.

**CONSENT FOR PUBLICATION**

Not applicable.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest, financial or otherwise.
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REFERENCES


