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Bioactive agents from beach waste: *Syringodium* flotsam evaluation as a new source of L-chiro-inositol

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Received 2 November 2007; accepted 9 December 2007

Editor proof receive date 18 January 2008

Abstract

Flotsam from the seagrass *Syringodium filiforme* were assayed for inositols, a class of cyclitol well known for their biological activities and applications. Free L-chiro-inositol (LCI), a very rare natural occurring cyclitol, was isolated from aqueous extracts of dried detrital leaves. The structure was unambiguously established by NMR and polarimetry. The LCI content of the crude aqueous extracts prepared from different batches of *Syringodium* flotsam was measured by quantitative ¹H-NMR spectroscopy. The high concentrations found (2.3–2.5% dry weight) offer promise for the exploitation of *Syringodium* flotsam as a new cheap source for nutraceutical or therapeutic applications, considering the demonstrated hypoglycaemic action of LCI.

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Keywords: L-chiro-inositol; *Syringodium filiforme* flotsam; Hypoglycaemic agent; Nutraceutical; Quantitative NMR

Industrial relevance: In the West the demand for herbal drugs has reached a new high in recent years. As the demand for alternative medicine has grown, so have the harvesting and collection pressures for numerous ecologies that produce the medicinal plants of interest. There is evidence in literature that chiro-inositol can be used in managing diabetes. Flotsam of the seagrass *Syringodium filiforme*, which accumulate in huge quantities on the beaches of the Caribbean Sea, could become a new and interesting source to obtain extracts rich in chiro-inositol. Heretofore, there has been no market for *Syringodium flotsam*, so that the cost of the same is simply that of harvesting. Recovery of inositol from this waste material could offer very interesting economic possibilities to tropical coastal areas suffering from increased rates of unemployment.

1. Introduction

Plant secondary metabolites are economically important in the field of food additives, nutraceutical and drugs. During the last decade, market for medicinal herbs has grown at an unprecedented rate. This growth is consumer-driven as people focus on health maintenance using natural products. The oceans are 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 compounds of nutritional interest (Cardozo et al., 2007). Compared to algae,

seagrasses remain less exploited despite the fact that they offer tremendous opportunities to find new commercially valuable phytochemicals. One of the rare real applications seems to be zosterin, a bioactive pectin from the eelgrass *Zostera asiatica*, which decreases toxicity of antitumor drugs and purges heavy metals from human organisms (Dolgi et al., 1999; Loenko, Artyukov, Kozlovskaya, Miroshnichenko, & Elyakov, 1997). These properties led to marketed drug and food in Russia (see as examples: Bobylin & Vozhdaeva, 1999; Bobylin & Ostroumova, 1999; Gavriljuk & Gavriljuk, 2004).

Seagrass meadows form the most widespread and productive coastal systems in the world. They produce large amounts of leaf material that is shed and washed ashore, often building important banks of seagrass litter. These deposits represent a source of concern for the manager, whenever they accumulate

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Table 1
Extraction yields and concentrations of LCI in the three batches

Samples	Dates and collection sites	Extraction yields (% /dried plant)			Concentrations of LCI (mean±SD)		
		Extracts a	Extracts b	Global yields	Extracts a	Extracts b	Global concentrations (% /dried plant)
Batch 1	2005-06-15, Gosier	22.5	9.8	32.8	8.48±0.05	6.15±0.06	2.51±0.01
Batch 2	2005-07-27, Sainte-Anne	20.6	7.2	27.8	8.73±0.10	6.65±0.13	2.29±0.02
Batch 3	2005-08-04, Gosier	23.9	7.1	31.0	8.34±0.10	5.17±0.16	2.36±0.03

on beaches and shorelines used for recreational purposes. The negative repercussions on the tourist industry of the affected areas lead to expensive beach cleaning and elimination processes. In most cases, the collected biomass is disposed of in waste disposal sites.

French West Indies are particularly concerned about this problem. They are skirted by highly productive *Syringodium filiforme* meadows (Cymodoceaceae, common name: Manatee Grass), and large amount of seagrass litter are washed ashore. In Guadeloupe, beaches are cleaned and flotsam are currently stored in garbage dumps. However, according to the new European Community guidelines on environmental protection, land filling is no longer possible without former treatment and it has become necessary to recycle the seagrass detritus.

As seagrasses are very resistant to decomposition, answers may come from the recycling of the flotsam as a new renewable resource for the production of bioactive substances.

The aim of this study was to describe the phytochemical content of *Syringodium* detrital leaves, with a view toward exploiting the flotsam otherwise deposited in landfill site. We focused on cyclitols, which constitute an important class of biologically active compounds (Takahashi, Kittaka, & Ikegami, 2001). Myo-inositol and its derivatives are well known for their antitumor properties (Somasundar et al., 2005) and their antidepressant activity in humans (Einat, Clenet, Shaldubina, Belmaker, & Bourrin, 2001). Myo-inositol is also marketed as nutraceutical. Another naturally occurring form of inositol is chiro-inositol. This molecule has a twofold axis of symmetry leading to the existence of two enantiomers, i.e. D-chiro-inositol (DCI) and L-chiro-inositol (LCI). Both are bioactive products. Compared to myo-inositol, DCI and especially LCI are very rare in nature. Seagrasses of the Zannichelliaceae have been reported to accumulate large amounts and a great range of cyclitols, and in particular LCI in the case of *Syringodium* (Drew, 1983). However, LCI had not been isolated by this author and the structural assignment was only based on a hypothetical biosynthesis pathway. This work reports the isolation of LCI from aqueous extract of *Syringodium* flotsam, its unambiguous structural assignment and quantification by polarimetry and NMR.

2. Materials and methods

2.1. General

Anhydrous pyridine, sodium acetate, trimethylchlorosilane (TMCS), and hexamethyldisilazane (HMDS) were purchased from Aldrich Chemical Company, plastic-backed TLC plates

pre-coated with silica UV₂₅₄ from Macherey Nagel. Qualitative NMR spectra were recorded on an AVANCE 300 MHz (Bruker). For the determination of the LCI concentration in the crude aqueous extract, spectra were recorded on a 400 MHz spectrometer (Bruker). Rotatory power was measured on a Perkin Elmer 241 polarimeter.

2.2. Materials

Dead leaves of *Syringodium filiforme* Kütz. (Cymodoceaceae) were collected from piles in the intertidal zone at Gosier and Sainte-Anne, Guadeloupe (French West Indies). Only the more recent green deposits were sampled from June to August 2005. Dates and collection sites are listed in Table 1. After collection, they were quickly washed in freshwater for 1 to 2 min to remove sand and salt, then air-dried at room temperature.

2.3. Extractions

Dried finely crushed leaves (20 g) were extracted for 24 h in 140 mL of de-ionised water at room temperature. The extraction was twice repeated, leading to extracts a and b (Fig. 1), which

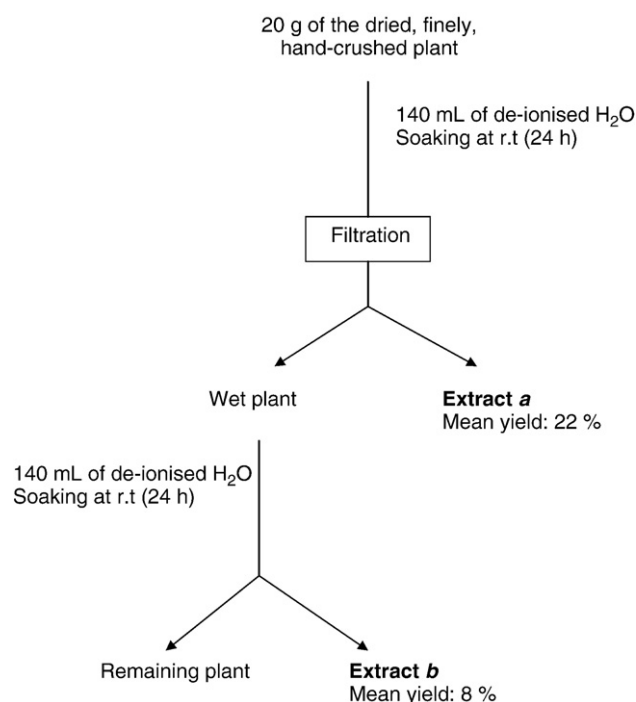


Fig. 1. Methods for the extractions of dried leaves.

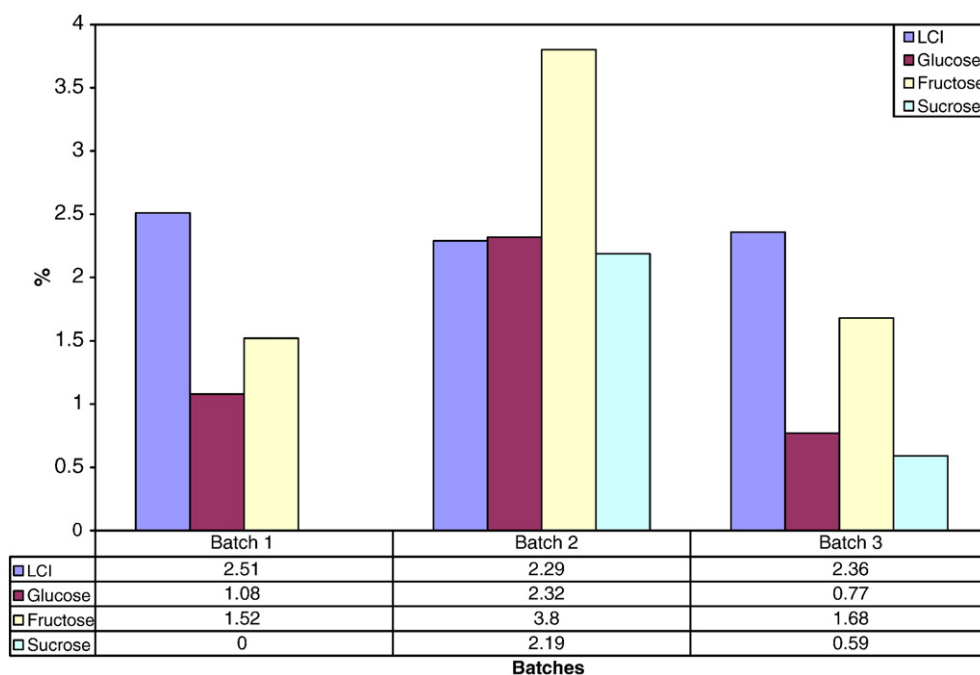


Fig. 2. LCI and sugar contents in the three tested batches (quantification by $^1\text{H-NMR}$).

were filtered through a glass funnel then freeze-dried. Yields are reported in Table 1. The crude extracts were systematically analysed by TLC and NMR.

2.4. Isolation and identification of LCI

HMDS (10 mL) and TMCS (10 mL) were added to a stirred solution of crude aqueous extract (12 g) in pyridine (100 mL), and the mixture was heated at reflux for 2 h. Reaction progress was monitored by TLC. After total evaporation of the solvent, the residual solid was partitioned between water (90 mL) and CH_2Cl_2 (90 mL). An aliquot of the organic layer was evaporated to dryness, and analysed by NMR in order to check the efficiency of the silylation step. The fully silylated chiro-inositol was hydrolysed by slowly adding 1 mL of trifluoroacetic acid (TFA) under stirring. A white solid precipitated very gradually and was removed by filtration, then washed in 10 mL of MeOH, yielding 90 mg of pure L-chiro-inositol as shown by $^1\text{H-NMR}$ and polarimetry.

2.5. Determination of the LCI concentrations by $^1\text{H NMR}$

Samples were prepared with 6.0 mg of dried extract, 0.5 mL of D_2O (Euriso-Top, Gif-Sur-Yvette) and 100 μL of a D_2O solution of sodium acetate (internal standard), and transferred to 5 mm NMR tubes. The internal standard solution was prepared by dissolving 50 mg of pure sodium acetate (Aldrich) in 5 mL of D_2O . $^1\text{H-NMR}$ spectra were recorded on an AVANCE DPX 400 MHz spectrometer (Bruker). For each sample, 128 scans were recorded with the following parameters: 0.134 Hz/point; sweep width, 4401 Hz; 90° pulse width, 5.0 μs ; relaxation delay, 1 s; acquisition time, 3.72 s; temperature, 27 $^\circ\text{C}$. Each sample was recorded in triplicate. Phase adjustments and baseline corrections

were applied prior to integrations. For quantitative analysis, manual integrations of the concerned peaks were achieved.

3. Results and discussion

Three collections of dried *Syringodium* leaves were extracted by two successive soakings with de-ionised water, at room temperature (see Materials and methods section). Extraction yields of all the extracts *a* (mean: 22%) are always noticeably higher than for extracts *b* (mean: 8%), indicating the efficacy of the first soaking. Global yields are of the same order, about 30%/ dried plant (Table 1).

The ^1H and ^{13}C NMR spectra of the different aqueous extracts were recorded in D_2O . They showed one predominant cyclitol, along with traces of myo-inositol and the common carbohydrates glucose, fructose and sucrose, which were identified from spectra of authentic samples recorded in the same conditions (Fig. 2). The presence of three protons at δ 3.50 (dd), 3.67 (multiplet) and 3.95 (d), and three carbons signals at δ 70.48, 71.70 and 72.79 (Table 2, Fig. 3), allow identification of the major product to free chiro-inositol by comparison with

Table 2
 ^1H and ^{13}C NMR chemical shifts of free LCI, isolated from *Syringodium* leaves (D_2O)

Position (no.)	Chemical shifts (ppm)	
	δ_{H}	δ_{C}
1	3.95	71.70
2	3.67	70.48
3	3.50	72.79
4	3.50	72.79
5	3.67	70.48
6	3.95	71.70

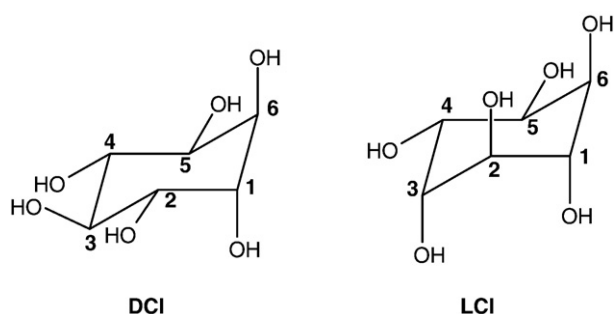


Fig. 3. Structures of LCI and DCI.

literature data (Abraham, Byrne, Griffiths, & Konioutou, 2005; Angyal & Odier, 1982). Of the nine possible inositols, only five are known to occur in plants, among which myo-inositol is the most abundant, while chiro-inositol is relatively rare (Kawa, Taylor, & Przybylski, 2003). Drew assumed that the chiro-inositol contained in seagrasses would originate from an enzymatic cyclization of D-glucose and as a result should be levorotatory (Drew, 1983; Larkum, Drew, & Ralph 2006). However, no physical chemistry data were presented to confirm this hypothesis. In order to unambiguously assign the stereochemistry of the chiro-inositol contained in *Syringodium*, we decided to isolate a pure sample to measure its rotatory power. Purification of the crude aqueous extract was achieved by sequential silylation with HMDS/TMCS in pyridine, and hydrolysis of the silylated chiro-inositol with TFA in CH_2Cl_2 led to a white precipitate of pure chiro-inositol, as confirmed by $^1\text{H-NMR}$ (Table 2). The rotatory power was measured in water: $[\alpha]_{\text{D}}^{25} = -62$, $c=0.5$ (literature data: $[\alpha]_{\text{D}}^{20} = -70$, $c=0.55$ in H_2O , Podeschwa et al., 2003) and confirmed the L-stereochemistry, in agreement with Drew's hypothesis.

3.1. Quantification of LCI

In general, quantitative analyses of mixtures of sugars are carried out using chromatographic techniques such as high performance liquid chromatography (HPLC) and gas chromatography. However, these methods are time consuming and suffer numerous limitations. Chromatographic analysis of sugars requires chemical derivatizations for the conversion into suitably volatile derivatives (GC) or to introduce chromophore (HPLC with UV detection). The fact that these reactions are not always specific or quantitative introduces errors in the quantification of the substances studied. In addition, calibration curves must be constructed from a series of appropriate standards across a range of concentrations near the expected unknown concentration. These processes may use significant quantities of expensive chemicals and result in an increased amount of waste for disposal. All these problems can be circumvented by using quantitative NMR spectroscopy on the crude extract. Indeed, NMR spectroscopy has proved to be useful for quantification of individual components in crude extracts without the need for fractionation or isolation procedures (Rivero-Cruz, Rivero-Cruz, Rodriguez, Cerda-Garcia-Rojas, & Mata, 2006). Moreover, NMR is a fast, non-destructive technique with minimal sample preparation (Al-

Deen, Hibbert, Hook, & Wells, 2004), and can compete with or even surpass chromatographic validation based on molecular analysis (Pauli, 2001). The internal standard suitable for quantification should be a non-volatile and stable compound, preferably with a singlet well separated from signals of the target compound (Choi, Choi, Peltenburg-Leoman, Lefeber, & Verpoorte, 2004). For this purpose, anhydrous sodium acetate, soluble in D_2O as LCI and with a singlet at 1.83 ppm, has been chosen. The doublet of LCI at 3.95 ppm, well defined and recognizable in the spectrum was used for all calculations.

The contents of LCI, calculated as established by Martin and Martin (1971), and the standard deviations are given in Table 1 for the three tested batches. The contents of LCI in all the extracts *a* are of the same order, as well as for extracts *b*. The total concentrations of LCI in dried leaves of *Syringodium* were found to be in the range of 2.29–2.51% (standard deviations ≤ 0.16).

Distribution of LCI in higher plants is very limited and they are few references in the literature with real quantifications. In a sample of living *Syringodium* leaves collected in Jamaica, Drew (1983) estimated the content of LCI at 5.1%, but mentioned that in some cases his method of quantification led to values in excess of 100% e.d.wt. Epifano, Marcotullio and Menghini (2002) found 0.08% (dry weight) of LCI in *Phagnalon sordidum*. Ichimura et al., (2000) reported concentrations ranging from 0.02 to 0.19% (fresh weight) in various organs of *Chrysanthemum*, an ornamental plant. LCI has also been identified without quantification in *Cremanthodium ellisii* (Wang, Zhang, & Jia, 2004), in *Euphorbia resinifera* (Bøe, Winsnes, Nordal, & Bernatek, 1969), in *Euphorbia pilulifera* (El-Naggar, Beal, Parks, Salman, & Patil, 1978), and in mistletoe (*Amyema miquelii*, Loranthaceae) (Hensens, Lewis, & Mulquiney, 1971).

To the best of our knowledge, few authors have studied the biological properties and activities of LCI, while it is well documented in the case of DCI. Several animal and human studies have shown that DCI has a positive effect on glucose metabolism (Kawa et al., 2003), and can be used for the treatment of symptomatic insulin-resistant type II diabetes without known toxic or deleterious side effects (Lamer & Kennington, 1992). DCI increases the action of insulin in patients with the polycystic ovary syndrome (PCOS), and thereby improving ovulatory function and decreasing serum androgen concentration (Nestler, Jakubowicz, Reamer, Gunn, & Allan, 1999 and ref. therein). PCOS is the most common cause of anovulatory infertility in the US (Baillargeon et al., 2006). Comparisons of the effects of LCI and DCI on diabetic rats have shown that the stereoisomerism differences did not affect the hypoglycaemic action of the chiro-inositol (Musalmah, Elkkairee, Lau & Wan Ngah, 2001).

Buckwheat is a broad-leaved crop produced extensively for the production of DCI-based herbal remedies from the seeds (luorno & Nestler, 2001; Wijngaard & Arendt, 2006). The highest values were found to be 1.37% (dry weight) and 0.16% (dry weight), respectively for total and free DCI (Steadman et al., 2000). The concentrations of LCI found in *Syringodium* leaves are greater than the concentration of total DCI in buckwheat (1.4% in farinetta).

The ability of LCI to mimic the hypoglycaemic effect of DCI opens interesting opportunities for the exploitation of *Syringodium* flotsam.

4. Conclusion

Free L-chiro-inositol (LCI) was isolated from dried detrital leaves and its structure was unambiguously assigned by NMR and polarimetry. Extraction was done by water, with no chemical reagents. Water as a solvent is not only abundant and inexpensive, but also environmentally benign and easy to recycle. For the first time, LCI was quantitatively assessed in crude extract with good accuracy using $^1\text{H-NMR}$ spectroscopy. Based on the NMR results, *Syringodium filiforme* flotsam contain higher amounts of LCI than all other previously reported terrestrial resources. Noteworthy, the LCI content remains almost constant in all the batches, in contrast to the concentration in carbohydrate. The high concentrations found, the rare natural occurrence, the potential biological applications and the environmentally-friendly extraction process constitute the main points of interest. They could justify significant exploitation of this low cost, very abundant and renewable but heretofore unused marine resource for food and pharmaceutical industry.

Acknowledgments

We are thankful to Claude Bouchon (Laboratoire de Biologie Marine, Université des Antilles et de la Guyane) for helpful advices and identification of plant material, Jean-Claude Lartigue (CESAMO) who recorded quantitative NMR spectra. One of the authors (G. N.) is grateful to the Conseil Général de Guadeloupe (French West Indies) for providing a post-doctoral grant.

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