International Journal of Phytocosmetics and Natural Ingredients 2015;2:8

doi 10.15171/ijpni.2015.08

Original Article

Rapid Screening of Chemical Compositions of *Gracilaria dura* and *Hypnea musciformis* (Rhodophyta) From Corsican Lagoon

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Received 2 June 2015
Accepted 29 July 2015
ePublished 6 August 2015

Abstract

Marine macroalgae are important ecological and commercial biomass resources to many regions of the world. They are valuable food resources and are most promising sources of bioactive compounds for the cosmetic industries. Phytochemical off-line investigation by the separation and identification of secondary metabolites are time, material and human resource consuming. Thus, in our studies, a rapid gas chromatography-mass spectrometry (GC-MS) on-line analysis with pretreatment (chemical derivatisations) was adapted for chemical profiling of two wild growing algae: *Gracilaria dura* and *Hypnea musciformis* from Corsica (France). Two GC-MS derivatisation methods were used: transmethylation for the fatty acids (FAs), and trimethylsilylation for compounds which bear -OH/-NH function. The application of the method on *Gracilaria dura*, and *Hypnea musciformis* leads to an identification of FAs, phytosterols, esters, simple phenolic compounds, and sugars from *n*-pentane, ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) extracts. This method is quick and low-cost for chemical profiling of marine biomass. It could be applied not only by the fundamental but also cosmetic industrial research. In addition, this study showed that all algae extracts had modest DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activities and the *n*-BuOH extract of *Hypnea musciformis* had a high total phenol content.
Keywords: GC-MS, Chemical derivatisations, Chemical profiling, Corsican, Red algae

Introduction

Algae has been one of the richest and most promising sources of bioactive primary and secondary metabolites[1-3] and their discoveries have significantly expanded in the past three decades.[4,5] According to Capo et al,[6] the genus *Gracilaria* was the most attractive candidate because of its ability to achieve high yields while producing commercially valuable extracts. Numerous bioactive primary and secondary metabolites identified within the genus *Gracilaria* and *Hypnea* have been reported in literature.[7-9] In the light of these results and of worldwide great interest in marine biomass as a source of bioactive compounds (for the nutraceutical, pharmaceutical and cosmetic industry), the aim of this work was to screen rapidly the chemical compositions of two of the most abundant algae growing in Corsican lagoons (Its' ecosystem was formerly studied by Pasqualini et al[10]) and to take into account the algae biomass as a potential multi product source for the cosmetic industry.

Materials and Methods

Biomass Collection and Preparation

Fresh algae was collected from the surface to the depth (maximum depth 3 m) in Urbinu lagoon, located on the eastern coast of Corsica, France (Mediterranean Sea; Figure 1) on March 2013.The samples were afterwards carefully washed with artificial sea and tap water; cleaned algae was next dried by a lyophilizer. Five hundred grams of each dried and grounded alga was soaked in 500 mL methanol (MeOH) at room temperature for 3 × 72 hours then filtered. The extracts were concentrated in vacuum at 35°C, suspended in ultra pure water (250 mL) and further partitioned between *n*-pentane (3×250 mL), ethyl acetate (EtOAc) (3×250 mL) and *n*-BuOH (3×250 mL). For algae *G. dura*: 1000.2 mg *n*-pentane extract, 502.7 mg EtOAc extract and 1544.6 mg *n*-BuOH extract were obtained. For algae *H. musciformis*, 313.0 mg *n*-pentane extract, 97.1 mg EtOAc extract and 1000.0 mg *n*-BuOH extract were obtained. All organic phases were concentrated to dryness

Please cite this paper as: Xu T, Sutour S, Casabianca H, et al. Rapid screening of chemical compositions of Gracilaria dura and Hypnea musciformis (Rhodophyta) from Corsican lagoon. *International Journal of Phytocosmetics and Natural Ingredients*. 2015;2:8. doi:10.15171/ijpni.2015.08.

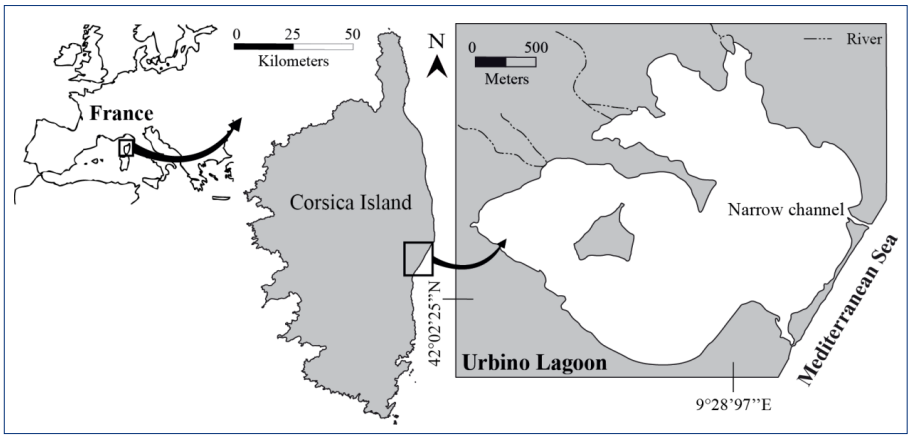

Figure 1. Location of Urbinu lagoon (Western Mediterranean Sea, Corsica, France).

under reduced pressure and stored at 4°C prior to analysis.

Preparation of Fatty Acids Methyl Esters and Trimethylsilyl Derivatisation

In a 25 mL round-bottomed flask, *n*-pentane extract of algae were converted to methyl esters by heating at 80°C for 4 hours after adding NaOH- methanol solution (4M) and various quantities of tridecanoic acid (C13) as external references. Until the mixture cooled at room temperature, 5 mL BF_3-methanol solution (1.3M) was added and followed by 10 minutes reaction. Liquid-liquid extraction was performed twice between the mixture and 10 mL *n*-pentane, *n*-pentane extract was then washed with saturated $NaHCO_3$ and NaCl solutions. After concentrated in vacuum at 30°C, 1.5 mL *n*-pentane was added and subjected GC-MS analysis. 10-20 mg of EtOAc and *n*-BuOH extracts of algae was dissolved in 1 mL of pyridine for trimethylsilyl (TMSi) derivatisation, 0.2 mL hexamethyldisilane and 0.1 mL chlorotrimethylsilane. The mixture was then put under stirring for 2 hours. After 12 hours precipitation, the upper layer of the solution were sampled and subjected to gas chromatography-mass spectrometry (GC-MS) analysis.

Gas Chromatography Coupled With Mass Spectrometry in Electron Impact Mode

Chemical derived algae extracts were analyzed on an Agilent 6890N-5975 inert masse selective detector (quadrupole) system equipped with a splitless-split injector and a HP-1 (methyl siloxane) column (50 m × 0.32 mm I.D., 0.52 µm film thickness), the initial oven temperature was programmed from 100°C for 1 minute, then from 100°C to 350°C (10°C/min) and isothermal for 44 minutes. The injection (1 µL) was performed in split mode with helium as carrier gas (3 mL/min, constant rate). The ion source temperature was set at 230°C. The mass spectrometer was used in electron impact (EI) mode (70 eV) and operated from 35 to 550 Da.

Nuclear Magnetic Resonance

All nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 400 (Wissembourg, France) in deuterated solvents ($CDCl_3$, MeOD, DMSO), with all shifts referred to internal tetramethylsilane (TMS). The ^{13}C-NMR spectra were recorded with the following parameters: pulse width, 4 µs (flip angle 45°); acquisition time, 2.7 seconds for 128 K data table with a spectral width of 24000 Hz (240 ppm); composite pulse decoupling (CPD) mode decoupling; digital resolution, 0.183 Hz/pt.

Identifications of Individual Components

Identification of compounds was based on: (1) Comparison of their GC retention times with commercial standards. (2) Computer search using digital libraries of mass spectral data (Wiley 275.L, NIST05.L and aeocnrs_13.2.1). (3) ^{13}C-NMR spectroscopy, following a computerized method developed in our laboratory.[11-13]

Quantification of Fatty Acids Methyl Esters and Sterols

Quantification of fatty acids methyl esters (FAMEs) were performed on an Agilent 6890N chromatograph system equipped with FID and a HP-INNOWax (polyethylene glycol) fused-silica capillary columns (60 m × 0.32 mm, I.D., 0.5 µm film thickness). The oven temperature was programmed from 60°C-425°C at 2°C/min and then held isothermal at 245°C for 35 minutes, injector temperature: 250°C; detector temperature: 250°C; carrier gas: helium (1 mL/min); split: 1/120. Quantification of sterols were performed on the same system using a HP-5 (5% phenyl)-methylpolysiloxane) fused-silica capillary columns (30 m × 0.25 mm, I.D., film thickness 0.25 µm). The oven temperature was programmed from 260°C to 325°C at 2°C/min held isothermal at 260°C for 60 minutes then held isothermal at 325°C for 2 minutes; the injector temperature was: 250°C; detector temperature was: 280°C; carrier gas was: helium (1 ml/min); and split: 1/35.

Antioxidant Activity and Total Phenolic Contents

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging potentials of different algae extracts were measured based on the method written by Blois.[14] The results were expressed in terms of % DPPH-scavenging activity rela-

tive to the control sample. Meanwhile, butylated hydroxytoluene (BHT) was used as the positive control. Total phenolic content was estimated by the Folin-Ciocalteu method.[15] Gallic acid (0-500 mg/mL) was used for the standard calibration curve. The results were expressed as gallic acid equivalent GAE g^{-1} weight of algae extracts. All experiments were carried out in triplicate.

Results and Discussion

Fatty Acids Methyl Esters and Sterols

In the present study, FAs from *n*-pentane extract of algae had been identified by GC-MS in their FAMEs forms. Palmitic acid is the predominant FA in *G. dura* (28.4%) and *Hypnea musciformis* (10.7%). Saturated fatty acids (SFAs) are at 34.0% in *G. dura* and at 19.8% in *Hypnea musciformis.* Monounsaturated fatty acids (MUFAs) extend 15.0% in *H. musciformis* but solely 6.0% in *G. dura*. Polyunsaturated fatty acids (PUFAs) take up 2.7% and 1.0% of *n*-pentane extract of *G. dura* and *H. musciformis* respectively. Four derivatives of sterols had been identified by GC-MS (Table 1). Among them, cholesterol **14** and β-sitosterol **16** had been confirmed further by ^{13}C-NMR spectroscopy (comparison with home-made libraries). Percentage of MUFAs of *H. musciformis* and *G. dura* corresponds with reported data.[16] However, the SFAs and PUFAs contents are much lower than which were reported.[16]

Saturated Dicarboxylic Acids

Low molecular weight dicarboxylic acids C4-C9 were for the first time identified in EtOAc extract of *G. dura* in their bi-TMSi esters form by GC-MS (Table 2). This series of molecules are recognized as ubiquitous aerosol constituents.[16] They were for the first time reported from marine biomass.

Phenolic Compounds

Simple phenolic compounds **24**-**29** were identified in their TMSi esters form from EtOAc extracts of algae by GC-MS. Only *p*-hydroxybenzoic acid **28** was identified in both algae (Table 2). All phenolic compounds in our study were reported for the first time from *G. dura* and *H. musciformis.* Although, **25** and **29** were previously reported in microalgae *Spirulina maxima*.[18] However, no phenolic compounds had been identified in the *n*-BuOH extract with GC-MS. Firstly, the GC-MS with derivatisation are limited to the "small molecules" that is to say, this method is not adapted to the identification of polyphenols. Secondly, *n*-BuOH is a high polar organic solvent, so the simple phenolic compounds will be extracted by EtOAc instead of *n*-BuOH.

Others

Glycerol **30** was identified in EtOAc extracts of two algae. Diactetin **31**, nucleoside derivatives **32** and **33** were identified only in EtOAc extract of *G. dura* by GC-MS and NMR. Simple saccharides were identified in *n*-BuOH extract of two algae in their multi-TMSi esters forms by GC-MS (Table 2). One vitamin **44** was identified in *n*-BuOH extract of *G.dura*. Compounds **32**-**44** were for the first time reported in *G. dura* and *H. musciformis.*

DPPH Scavenging activity and total phenol content

The DPPH scavenging activity of algae extracts were presented in the Table 3. The activity of *n*-pentane extract of *G. dura* (EC_{50} = 5.6 mg/mL) and *n*-BuOH extract of *Hypnea musciformis* (EC_{50} = 5.2 mg/mL) are the most active ones; the activity of this extract of *G. dura* may came from the non-identified unsaponifiable compounds of *n*-pentane extract (carotenoid etc.). Moderate scavenging activity of *H. musciformis n*-BuOH extract (EC_{50} = 5.2 mg/mL) is perhaps due to its modest phenol contents (157 GAE g^{-1}). The highest concentration of algae extracts' total phenol content was found in the *n*-BuOH extract of *H. musciformis* (157 GAE g^{-1}). Interestingly, this concentration of total phenol contents was much higher than the value reported for some of the red algae: *G. gracilis* (solely 65 mg GAE g^{-1}).[19]

Conclusions

The chemical compositions of different polarity extracts

Table 1. Percentage of Apolar Constituents From *n*-Pentane Extracts of *Gracilaria dura* and *Hypnea musciformis* by GC-MS After Transmethylation

		G. dura (%)	*H. musciformis* (%)
	Saturated Fatty Acids		
1	Lauric (C12:0)	0.2	0.1
2	Myristic (C14:0)	2.2	5.1
3	Palmitic (C16:0)	28.4	10.7
4	Stearic (C18:0)	0.7	0.5
5	Arachidic (C20:0)	2.5	2.5
6	Behenic (C22:0)	nd[a]	0.5
	Monounsaturated Fatty Acids		
7	Palmiteladic (C16:1 ω7*t*)	nd	0.3
8	Palmitoleic (C16:1 ω7*c*)	2.8	1.0
9	Elaidic (C18:1 ω9*t*)	1.5	6.8
10	Oleic (C18:1 ω9*c*)	1.6	7.3
	Polyunsaturated Fatty Acids		
11	Dihomo-γ-linolenic (C20:3 ω6)	1.3	0.1
12	Arachidanoic (C20:4 ω6)	1.5	0.8
	Sterols		
13	Cholesta-4,6-dien-3-ol	tr	nd
14	Cholesterol[b]	1.0	tr
15	Cholesta-3,5-dien-7-one	tr[c]	nd
16	β-Sitosterol[b]	nd	0.3
	MUFAs	5.9	15.4
	PUFAs	2.8	0.9
	Sterols	1.0	0.3

[a] Not detected, [b] Structure of compounds confirmed by ^{13}C-NMR, [c] Percentage ≤0.05%.

Tables 2. Chemical Compositions of EtOAc and *n*-BuOH Extracts of *Gracilaria dura* and *Hypnea musciformis* by GC-MS After Trimethylsilylation

No.			*G. dura*	*H. musciformis*
		EtOAc Extracts		
	Rt[a]	**Compounds[b]**		
		Saturated Dicarboxylic Acids		
17	14.38	Succinic acid	X[c]	nd[d]
18	15.59	2-Hydroxypentanedioic acid	X	nd
19	16.92	Adipic acid	X	nd
20	19.22	Suberic acid	X	nd
21	20.30	Azelaic acid	X	nd
22	21.31	Sebacic acid	X	nd
23	22.30	Uundecanedioic acid	X	nd
		Phenolic Compounds		
24	13.56	Benzoic acid	nd	X
25	15.87	*p*-Hydroxybenzaldehyde	nd	X
26	17.89	Tyrosol	X	nd
27	17.95	*p*-Hydroxyphenylacetic acid methyl ester	nd	X
28	18.51	*p*-Hydroxybenzoic acid	X	X
29	19.97	Vanillic acid	X	nd
		Ester/Alcohol		
30	22.32	Diacetin[e]	X	nd
31	14.30	Glycerol[e]	X	X
		Nucleoside Derivatives		
32	15.91	Thymine[e]	X	nd
33		3-Bromo-uracile[e,f]	X	nd
		n-BuOH Extracts		
		Saccharides		
34	18.47	α-D-xylopyranose	nd	X
35	22.24	α-D-mannopyranose	nd	X
36	23.61	Inositol	X	X
37	24.91	α-D-glucopyranose	X	nd
38	25.08	Floridoside	X	nd
39	25.43	D-glactose	X	nd
40	25.47	Talose	X	nd
41	32.23	Maltose	X	nd
42	35.06	Melibiose	X	nd
43	18.47	α-D-xylopyranose	X	nd
		Other		
44		D-Pantothenic acid (vitamine B5)	nd	X

Abbreviations: EtOAc, ethyl acetate; *n*-BuOH, *n*-butanol; GC-MS, gas chromatography-mass spectrometry.

[a] Retention times in minute, [b] Compounds in their TMSi derivatives, [c] Percentage of compounds stay undetermined, [d] Not detected, [e] Structure of compounds confirmed by ^{13}C-NMR, [f] Purified compound.

Table 3. DPPH Radical Scavenging Activity (EC_{50} Value) and GAE in Extracts of *Gracilaria dura* and *Hypnea musciformis*

Algae	Solvent Extracts	GAE (mg/g)	EC_{50}[a] (mg/mL)
	n-Pentane	nd[b]	5.6 ± 0.18
G. dura	EtOAc	60.2 ± 0.01[c]	8.7 ± 0.10
	n-BuOH	4.8 ± 0.04	7.1 ± 0.03
	n-Pentane	nd	>10.0
H. musciformis	EtOAc	102.2 ± 0.03	7.1 ± 0.15
	n-BuOH	157.0 ± 0.02	5.2 ± 0.01
	BHT		13.2×10^{-3} ± 0.01

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; GAE, gallic acid equivalent; EtOAc, ethyl acetate; *n*-BuOH, *n*-butanol; BHT, butylated hydroxytoluene.

[a] Half maximal effective concentration; [b] Not detected; [c] All values are mean ± Standard Deviation.

from *G. dura* and *H. musciformis* were screened by GC-MS with chemical derivatisations. Forty-four compounds including lipids, esters, simple phenol compounds, and sugars were identified. This is the first application of this analytical method for two Corsican lagoon algae. The method is less solvent and time consuming compared to the off-line analysis, and could be a possible way to do the rapid screening of marine biomass before advanced researches. In addition, from the results of total phenol content, *n*-BuOH extract of *H. musciformis* may have the potential to be developed as anti-aging cosmetic product.[20.]

Acknowledgements

We are grateful to the technical team of Stella-Mare for their assistance in the field and indebted to the CTC (Collectivité Territoriale de Corse) for the financial support

through FEDER funding.

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