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Original Article

Chemical Composition of Extracts From *Chaetomorpha linum* (Miller) Kütz. A Potential Use in the Cosmetic Industry

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Abstract

The chemical composition of extracts from the green alga *Chaetomorpha linum* (Miller) Kütz., growing wild in Corsican pond, were investigated by GC-MS (derivatisation) and ^{13}C NMR spectroscopy. Eighteen compounds have been identified from both pentane and ethyl acetate extract. Fatty acids, mainly saturated (SFA) are the main compounds from the pentane extract, whereas components of the sterols family are major compounds from the ethyl acetate extract. Besides usual fatty acids founds in green alga extract, we report herein the identification of unusual compounds. Indeed 12 compounds (3, 7-18) were identified for the first time in a *Chaetomorpha* species. Among them three diterpenes bearing the abietane skeleton (abietic acid 12, dehydroabietic acid 13 and methyl dehydroabietate 14) were identified for the first time from a marine source. This chemical composition could be a chemotaxonomic characteristic from the linum species found in the Corsican area.

Keywords: ^{13}C NMR, Abietane, *Chlorophyta*, Corsica, Mediterranean Sea

Introduction

Mediterranean Sea is recognized to be a global biodiversity hotspot. Its surface represents only a small percentage (0.8%) of World Ocean surface; but the biodiversity is paradoxically rather high. More than 25% of Mediterranean marine plants are endemic (26.6%).[1] The *Chaetomorpha* genus belongs to the *Chlorophyta* division. *Chaetomorpha* means stiff hairs. Around 76 species are known from the *Chaetomorpha* genus. Fatty acids composition has been reported from *C. linum* from California (USA),[2] from *C. minima* from Japan[3] and steroids from *C. basiretorsa* from China.[4] However, the *linum* (Miller) Kütz. species, a green alga growing wild in the Mediterranean sea and pond has never been subjected to a phytochemical investigation.

In the course of our ongoing work on the potential valorisation of Mediterranean resources in the cosmetic industry, we investigate the chemical composition and biological activities of various macroalgae from the three divisions: Chlorophyta, Rhodophyta and Chromophyta (Dinophytes and Pheophytes). We report here on the chemical composition of *C. linum* apolar (pentane) and medium polar (ethyl acetate) extracts.

Materials and Methods

Material and Isolation

Chaetomorpha linum (Miller) Kütz. was collected at 20 cm deep in Urbino pond in the East coast of Corsica (France), on September 2013 and has been authenticated by Pr. Vanina Pasqualini (Université de Corse). Alga were dried and then extracted with solvent extracts of increasing polarities (pentane and ethyl acetate) using a Soxhlet apparatus during 4 hours. Solvent was evaporated under reduced pressure to yield a dark yellow (pentane extract) and dark green (ethyl acetate) extract with a low yield (respectively 0.37 and 0.50%). Each extract were chromatographed using a Grace Reveleris® flash chromatography system using a gradient pentane/chloroform/chloroform/ethyl acetate and finally ethyl acetate-methanol. Thirty fractions were collected from the pentane extract and 41 from the ethyl acetate extract.

Saponification

Ethyl Acetate extract (90 mg) was heated to 85°C in a 25 ml round-bottomed flask for 2 hours after adding NaOH (5 ml) water solutions. After the reaction, the mixture was

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suspended in 10 ml of ultra pure water following 10 mn agitation and extracted twice with 10ml pentane. The extract was concentrated under vacuum at 30°C and subjected to Trimethylsilylation (TMSi)-derivatization procedures.

Preparation of Fatty Acids Methyl Esters (FAMEs) and TMSi-Derivatization

In a 25 ml round-bottomed flask, pentane extract of algae were converted to methyl esters by heating to 80°C for 4 hours after adding NaOH- methanol solution (4M) and various quantities of tridecanoic acid. Until the mixture cooled to room temperature, 5ml BF_3 methanol solution (1.3M) was added and followed by a 10 minutes reaction. Liquid-liquid extraction was performed with the mixture and pentane (10 ml). The organic layer was then washed with saturated $NaHCO_3$ and NaCl solutions. After concentrated in vacuum at 30°C, pentane (1.5 ml) was added and submitted to GC-MS analysis. Ten to 20 ml of ethyl acetate extract of algae was dissolved in pyridine (5 ml), hexamethyldisilane (0.2 ml) and chlorotrimethylsilane (0.1 ml). The mixture was stirred for 2 hours. After 12 hours precipitation, upper layer of the solution were sampled and subjected to GC-MS analysis.

GC-MS (EI)

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 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 EI mode (70 eV) and operated from 35-550 Da.

Quantification of Fatty Acids Methyl Esters

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-245°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-325°C at 2°C/min held isothermal at 260°C for 60mn then held isothermal at 325°C for 2 minutes; injector temperature: 250°C; detector temperature: 280°C; carrier gas: helium (1 ml/min); split: 1/35.

^{13}C NMR

NMR spectra were recorded on a Bruker AVANCE 400 Fourier Transform spectrometer operating at 100.13 MHz for ^{13}C, equipped with a 5 mm probe, in deuterated chloroform ($CDCl_3$), with all shifts referred to internal tetramethylsilane (TMS). ^{13}C NMR spectra were recorded with the following parameters: pulse width (PW), 4 µs (flip angle 45°); acquisition time, 2.7 seconds for 128 K data table with a spectral width (SW) of 24 000 Hz (240 ppm); CPD mode decoupling; digital resolution 0.183 Hz/pt. The number of accumulated scans was 3000 for each sample (50-60 mg in 0.5 ml of $CDCl_3$).

Identification of the Components

Identification of the individual components was based on a dereplication method (*a*) on computer search using digital libraries of mass spectral data,[5,6] (*b*) comparison with published data[7,8] and (*c*) by ^{13}C NMR spectroscopy, following the methodology developed and computerized in our laboratory, using home-made software, by comparison with spectral data of reference compounds compiled in a laboratory-built library.[9-11]

Cytotoxic Assay

Dried and powdered *Chaetomorpha linum* was extracted (m/m) during 2 weeks with a mixture of deionised water and propan-1,2-diol (1/1). Cytotoxic assay were performed by the society "Laboratoire Shadeline" using an in vitro test on human keratinocytes. The extract at various concentrations (10, 50, 100, 500 and 1000 µg/mL) was tested in triplicate during 24 hours using the MTT (3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromide) test.[12,13] The extract is considered cytotoxic when the ratio of the absorbance between the positive control (phenol) and the tested extract is inferior to 0.3 (30%). A statistical analysis of the results was done with the *t* test. Significant results occurred when $P < 0.05$.

Results and Discussion

Eighteen compounds (Table 1) have been identified in total from the 2 extracts (pentane and ethyl acetate), by combination of chromatographic and spectroscopic techniques (GC-FID), GC-MS derivatization and ^{13}C NMR, following a computerized method developed in our laboratory, without separation of the individual components. In order to optimize the efficiency of this latter method, a literature data bank were created and compiled with ^{13}C NMR chemical shifts of selected compounds previously described in the literature isolated from a marine source.

All the compounds excepted FAMEs were identified by comparison of ^{13}C NMR chemical shift from the literature and/or recorded at our laboratory compiled in spectral data library. The whole extracts were submitted to GC(FID), GC-MS allowing the identification and quantification of FAME and 2 steroids.

Table 1: Compounds indentified from both Pentane (PE) and Ethyl Acetate Extracts (EAE)

No.	Compounds	PE	EAE	Identification
	Fatty Acid Methyl Esters			
1	Myristic acid ME (C14:0)	×		MS, Rt
2	Palmitic acid ME(C16:0)	×	×	MS, Rt
3	Palmitoleic acid ME (C16:1)	×		MS, Rt
4	Stearic acid ME (C18:0)	×	×	MS, Rt
5	Oleic acid ME (C18:1)	×		MS, Rt
6	Linolelaidic acid ME (C18:2)	×		MS, Rt
	Sterols			
7	Cholesterol	×	×	^{13}C NMR, MS, Rt
8	β-Sitosterol	×	×	^{13}C NMR, MS, Rt
9	24-Methylene-cholesterol		×	^{13}C NMR
10	(*E*,*E*)-Cholesta-5,22-dien-3-ol		×	^{13}C NMR
	Terpenes			
11	Phytol and phytyls derivatives	×		^{13}C NMR
12	Abietic acid	×	×	^{13}C NMR
13	Dehydroabietic acid	×	×	^{13}C NMR
14	Methyl dehydroabietate	×	×	^{13}C NMR
	Phenols derivatives			
15	*p*-Hydroxybenzaldehyde		×	^{13}C NMR, MS
16	*p*-Hydroxybenzoic acid		×	^{13}C NMR, MS
	Others			
17	(*E*)-2-tridecyl-2-heptadecenal	×		^{13}C NMR
18	Thymine			^{13}C NMR, MS

Table 2. Cytotoxic Assay Against Human Keratinocytes

Concentration (µg/mL)	10		50		100		500		1000		negative control		positive control	
	MTT (AU)	%	MTT (AU)	%	MTT (AU)	%	MTT (AU)	%	MTT (AU)	%	MTT (AU)	%	MTT (AU)	%
Chaetomorpha linum extract	0.771 ±0.142	115 NS	0.683 ±0.039	102 NS	0.717 ±0.078	107 NS	0.647 ±0.023	97 NS	0.589 ±0.042	88 P<0.05	0.668 ±0.041	100	0.213 ±0.041	32 P<0.001

NS = Not significant

Fatty Acids Methyl Esters

The pentane extract contains mainly FAMEs, the more abundant being palmitic (10.2%), oleic (5.5%), myristic (5.3%), linolelaidic (2.1%), palmitoleic (0.9%) and stearic methyl ester (0.5%). The ethyl acetate extract contains less FAMEs, qualitatively and quantitatively. Indeed only palmitic and stearic acid methyl ester were identified.

Sterols

Among the sterol derivatives, we were able to identify β-sitosterol and cholesterol by analysis of both crude extracts by ^{13}C NMR (Figure 1). These sterol derivatives are the main compounds of the ethyl acetate extract. The ^{13}C NMR spectrum of this extract exhibits more chemical shifts characteristics of the sterol family than the pentane extract. In order to identify minor compounds, ethyl acetate extract was submitted to a low pressure automated flash liquid chromatography using a silica cartridge. From the ethyl acetate fractions of chromatography we were able to confirm the occurrence of cholesterol and β-sitosterol and identify 2 other sterol derivatives: 24-methylen-cholesterol and (*E*,*E*)-cholesta-5,22-dien-3-ol (Figure 1). These sterols were identified by ^{13}C NMR by comparison with literature data.

Terpenes

After fractionation of the pentane extract we were able to identify additional compounds by ^{13}C NMR (FAMEs excepted). Three of them belong to diterpenes: abietic acid, dehydroabietic acid, methyl dehydroabietate (Figure 2) and exhibit the abietane skeleton. They were previously isolated and identified in our laboratory from the oleoresin of *Pinus nigra* ssp. *Laricio* from Corsica.[14]

The ^{13}C NMR spectra of 3 different fractions of chromatography from the pentane extract give information about the occurrence of phytol derivatives. Indeed, ^{13}C NMR chemical shifts are characteristic of the phytol moiety and of a saturated fatty acid. These observations led us to the occurrence of a phytyl fatty ester. HMBC correlation between the carbon of the carbonyl and the proton in α position of the oxygen from the phytyl moiety lead to the occurrence of three different phytyl fatty esters with dif-

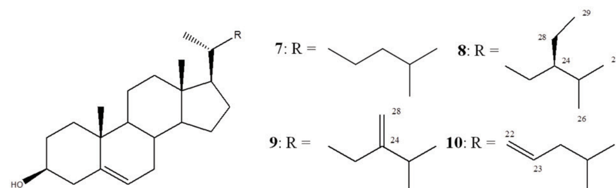

Figure 1. Sterols identified in *C. linum* extracts.

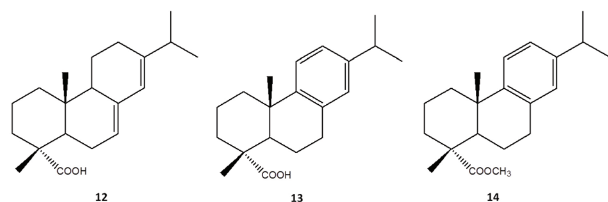

Figure 2. Abietanes identified in *C. linum* extracts.

ferent saturated fatty chain.

Others

From the fractions of chromatography of both pentane and ethyl acetate extracts we were able to identify two phenol derivatives (*p*-hydroxybenzaldehyde **15** and *p*-hydroxybenzoic acid **16**), one aldehyde ((*E*)-2-tridecyl-2-heptadecenal **17**) and a nucleobase (thymine **18**).

Cytotoxic assay

In parallel, *Chaetomorpha linum* was extracted with a mixture water/propan-1,2-diol (v/v) and tested in vitro for its cytotoxicity. Even at the higher concentration tested (1000 µg/mL), the extract exhibits no cytotoxicity against human keratinocytes (Table 2). Indeed the ratio is 0.36 > 0.30.

Conclusions

Red, brown and green algae have different fatty acid and sterol profiles, which have a chemotaxonomic significance for seaweeds.[15-17] Indeed, C20 PUFAs (C20:4n-6 and C20:5n-3) were characteristic profile of red algae; the association of C16:0 and C18:1n-9 and PUFAs with a C18 and C20 chain length were dominant compounds from brown algae.[2] Green algae have high concentrations of C16 and C18 fatty acids and various unsaturation patterns depending on the species. Indeed Pereira et al[18] reported a higher percentage of saturated fatty acids (SFAs) than usual.

C. linum FAMEs composition from Corsica is characteristic of Chlorophyta by the occurrence of C14 and C16 fatty acids as major compounds. Especially, FAMEs composition is close to those reported by Pereira et al[18] for the *Chaetomorpha* sp., *C. linum* from America[2] and *C. minima* from Japan[3] and some other green algae by the abundance of SFAs. However, even from the same species investigated, difference occurs. Indeed, neither stearic nor linolelaidic acid were identified in American extracts of *C. linum*. Otherwise it's noticeable that linolelaidic methyl ester is identified for the first time in this study in the *Chaetomorpha* genus.

Steroids composition is different from those described from *C. basiretorsa* from China. Cholesterol and β-sitosterol are common compounds to alga, however 24-methylen-cholesterol and (*E*,*E*)-cholesta-5,22-dien-3-ol are less usual. Indeed, both sterols were identified from brown and red alga whereas (*E*,*E*)-cholesta-5,22-dien-3-ol was never identified from a green alga.[2,19-21] Phytyl fatty acids were previously described in a *C. gracilis* extract.[22] However; diterpenes bearing an abietane skeleton (Figure 2) are reported for the first time from a marine source.

The combination of complementary analytical methods (GC-MS and ^{13}C NMR) without separation of the individual compounds allowed a quick and efficient screening of major metabolites from pentane and ethyl acetate extracts from *C. linum*.

Moreover *C. linum* extract shows no cytotoxicity against human keratinocytes. The wide biological activity already described of compounds bearing an abietane skeleton,[23] mainly acids let appear a potential use of *C. linum* extract in the cosmetic industry.

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