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Chemical Composition of the Brown Alga *Padina pavonia* (L.) Gaill. from the Adriatic Sea

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The chemical composition of the brown alga *Padina pavonia* (L.) Gaill. from the southern Adriatic Sea was investigated. Twelve sterols were identified in the sterol fraction, the main ones being cholesterol and fucosterol. The main fatty acids in the lipids were also identified. The most abundant fatty acid was palmitic acid, followed by oleic and myristic acids. The concentration of polyunsaturated fatty acids was unusually low for a marine alga. By GC/MS analysis of the volatile and polar fractions, 40 compounds were identified. Some of them probably possess defensive functions. In the volatile fraction free fatty acids, aromatic esters, benzyl alcohol and benzaldehyde predominated. Low concentrations of terpenoids, phenols and sulfur containing compounds were also identified. The n-butanol extract contained mainly fatty acids and polyols. Some of the extracts had an antibacterial activity.

Introduction

The brown alga *Padina pavonia* (L.) Gaill. is widespread in the Adriatic Sea. It belongs to the family Dictyotaceae, order Dictyotales, subclass Isogeneratae, class Phaeophyceae. There are limited data on the chemical composition of the algae from the genus *Padina.* Fatty acids, containing 14–22 carbon atoms have been identified in different *Padina* species (Qasim 1986, Karawya *et al.* 1987, Kanias *et al.* 1992, Wahbeh 1997). Significant differences in the sterol composition were found within the different *Padina* species. Fucosterol predominated in *Padina gymnospora* (Kütz.) Sond. and no cholesterol was found (Al Easa *et al.* 1995). Fucosterol was also the main sterol in *Padina crassa* Yamada (Enoloh *et al.* 1981). In *Padina vickersiae* (Kütz.) Sond. (Combaut *et al.* 1985, Aknin *et al.* 1992*)* from Senegal waters and in *Padina pavonia* from the Aegean Sea (Kanias *et al.* 1992) the main sterol was cholesterol. The biogenetic precursor of fucosterol, 24-methylene cholesterol, was found in significant amounts in *Padina vickersiae* (Aknin *et al.* 1992) and in *Padina gymnospora* from the Qatar coast (Al Easa *et al.* 1995). In the Mediterranean *Padina pavonia* (Iatrides *et al.* 1983) the main sterol appeared to be fucosterol instead of cholesterol.

Several oxidised sterol derivatives have been found in some *Padina* species, 24-hydroperoxy-24-vinyl-cholesterol in *Padina pavonia* (Ktari and Guyot 1999), 7-ketocholesterol in *Padina tetrastromatica* Hauck. (Parameswaran *et al.* 1994) and 7α-hydroxyfucosterol in *Padina crassa* (Tan *et al.* 1992).

Few terpenoids have been found in *Padina* species. Halogenated terpenoids were identified in *Padina tetrastromatica* (Parameswaran *et al.* 1994, 1996). Loliolide was identified in *P. tetrastromatica* (Rao and Pullaiah 1982) and in *P. crassa* (Tan *et al.* 1992). Hexahydrofarnesylacetone was found in *P. tetrastromatica* (Rao and Pullaiah 1982).

Dimethylsulfide and dimethyl-β-propiothetin were identified in *Padina arborescens* Holmes (Iida *et al.* 1985). Pigments were investigated in *Padina pavonia* by Hegazi *et al.* (1998). The characteristic brown algal polysaccharides (alginates and laminarans) were found in *Padina pavonia* (Khafaji 1986) and *P. tetrastromatica* (Rao *et al.* 1984). In *P. tetrastromatica* two new sulphated heteropolysaccharides, containing sugar and protein residues were also found (Rao *et al.* 1984). Galactol was identified in *P. tetrastromatica* (Parameswaran *et al.* 1996).

We concentrated our efforts on the analysis of fatty acids from lipids, sterols, volatile compounds and the polar fraction from *P. pavonia.* Sterols and lipids are important constituents of the cell membranes and are responsible for many of the cell functions. Due to their significant concentrations in living organisms they might have a practical application. Volatile compounds often contain biologically active compounds, some of them with allopathic activity. Polar fractions, extracted with n-butanol, are very complex and there are limited data on their composition. They often contain biologically active compounds and their investigation with modern methods

like gas chromatography/mass spectroscopy (GC/MS) might produce interesting results.

All the conflicting information about the chemical composition of the different *Padina* species justifies our investigation of the Adriatic *Padina pavonia* (L.) Gaill.

Material and Methods

Collection of the samples

The alga was collected in the Bay of Kotor, southern Adriatic Sea in June 1999 (water temperature 23 °C, salinity 3.3 %). The alga was rinsed with water, dried carefully in the shade in a stream of air and immediately subjected to extraction.

Extraction

The dried alga was extracted with toluene in a Soxhlet apparatus and the residue was extracted with ethanol. The lipid extract was obtained by extraction with a chloroform – methanol mixture (1:1) of another sample of the fresh alga. The extracts were further purified and analysed as described below.

Isolation and analysis of sterols

The chloroform-methanol extract was evaporated under reduced pressure at a temperature of 40 °C. Part of the dry residue (100 mg) was subjected to column chromatography on 10 g of silica gel. Petroleum ether, followed by petroleum ether-acetone mixtures, chloroform and chloroform-methanol mixtures in ascending polarity were used as eluents. Fractions containing sterols were eluted with petroleum ether-acetone (10:1), characterised by thin layer chromatography (TLC) with petroleum ether-acetone (8:1) and combined. The total sterol mixture was investigated by GC and GC/MS.

A GC Pye Unicam 304 (Scientific Instrument Company of Philips, Cambridge, England) equipped with a flame ionisation detector (FID) and a capillary column SPB-1 (30 m × 0.32 mm, 0.25 μm film thickness, Supelco Park, Bellefonte, PA, USA) was used. The temperature programme was 230 °C–300 °C at 4 °C min^{-1} and a 10-min hold. The injector temperature was 300 °C and the detector temperature 320 °C. The carrier gas was N_2.

A GC/MS Hewlett Packard 6890 + MS 5973 (Hewlett Packard, Palo Alto, CA, USA) with a capillary column SPB-50 (30 m × 0.32 mm, 0.25 μm film thickness, Supelco Park, Bellefonte, PA, USA) was used. The carrier gas was helium and a temperature programme of 270 °C–290 °C at 4 °C min^{-1} and a 20-min hold was used. The ion source was set at 250 °C and the ionisation voltage was 70 eV.

Isolation and analysis of volatile compounds by GC/MS

The toluene extract (200 mg) was subjected to a four-hour distillation-extraction in a Lickens-Nickerson apparatus (Hendriks *et al.* 1981). The volatile compounds were extracted from the distillate with diethyl ether (yield: 37 mg, 18.5 % of the extract). They were investigated by analytical GC/MS on the same apparatus as described above but a HP5-MS capillary column was used (30 m × 0.25 mm, 0.25 μm film thickness, Agilent Technologies, Wilmington, DE, USA). The temperature was programmed from 40 °C to 280 °C at a rate of 6 °C min^{-1}. Helium was used as the carrier gas.

Isolation and analysis of polar compounds

After the extraction of the alga with toluene and ethanol, the ethanol extract was evaporated to dryness and subjected to silylation. Part of the dry residue (5 mg) was dissolved in 50 μL pyridine and 75 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added. The mixture was heated at 80 °C for 30 min and analysed by GC/MS on the same apparatus equipped with a capillary column HP5-MS (23 m × 0.2 mm, 0.5 μm film, Agilent Technologies, Wilmington, DE, USA). Helium was used as the carrier gas and the temperature programme was 100 °C–315 °C at 5 °C min^{-1} and a 10-min hold.

Identification of compounds by GC/MS

The GC/MS investigation was based on the interpretation of the mass spectral fragmentation followed by comparisons of the spectra obtained with those of authentic samples. Computer searches in a HP Mass Spectral Library NIST98 (Hewlett Packard, Palo Alto, CA, USA) were also applied. In the cases when the spectra of some isomers were very similar and these compounds could not be identified unambiguously, comparisons of the GC retention times, obtained under the same conditions, were used. When there were no suitable authentic samples and spectra for comparison, no identification was made. Only the unambiguously identified compounds were reported in the Tables.

Investigation of lipids

The fresh alga was extracted twice with methanol-chloroform (1:1). After the addition of an equal volume of water, the lower layer containing the total lipophylic substances was evaporated. Part of it was used for the transesterification of the fatty acids from the total lipids with 5 mL 15 % acetyl chloride in methanol. After four hours at 60 °C the sample was diluted with water and extracted with petroleum ether. The methyl esters produced were purified by

TLC on silica gel with petroleum ether-acetone 95:5. They were analysed by a Hewlett Packard 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA), equipped with FID and 30 m × 0.25 mm i.d. fused silica capillary column SP – 2340 (Supelco Park, Bellefonte, PA, USA). The temperature was programmed from 150 °C to 210 °C at a rate of 4 °C min^{-1}, and a 10-min hold at 210 °C.

Biological activity

Antibacterial tests

Staphylococcus aureus Rosenbach 209 (from the Bulgarian Type Culture Collection) and *Escherichia coli* Castellani and Chalmers WF+ (from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences) were used as test microorganisms. Antibacterial activity was elucidated by the agar cup method described by Spooner and Sykes (1979). Briefly, 0.2 mL of bacterial suspension (1.0×10^5 cfu/mL) was plated on a meat peptone agar layer into Petri dishes (10 cm d). The samples were dissolved in 96 % ethanol at a concentration of 5mg/mL. After preparing 5 wells with a diameter of 10 mm per dish, 0.1 mL of each sample was dropped into each well. For pre-diffusion the Petri dishes were placed for 2 h at 4 °C. The antibacterial activity was measured by the diameter of inhibitory zones in the agar layer after a 48-h incubation at 37 °C. An inhibitory zone with a diameter less than 10 mm indicated a lack of activity. Control experiments with solvent showed that it has no activity.

Antifungal tests

The agar cup method of Spooner and Sykes (1979) was used, too. As a test microorganism *Candida albicans* Berkhaut 562 from the collection of the Institute of Microbiology (Bulgarian Academy of Sciences) was used. The yeast was cultured on Sabouraud glucose agar. Two hundred microliters of a *C. albicans* suspension (1.0×10^7 cells/mL) were plated following the same experimental approach as it has been previously described.

Results and Discussion

Analysis of sterols

The results obtained from the analysis of the sterol fraction are summarised in Table I.

As indicated in the Introduction, different *Padina* species possess different sterol compositions. One of the main sterols we identified in *Padina pavonia* was fucosterol, which is characteristic of most of the brown algae. Its concentration was relatively low (24.3 %) while the concentration of cholesterol appeared to be high (34 %). It is known that most of the brown algae contain over 70 % of the total sterol mixture as fucosterol (Goad 1978, Elyakov and Stonic 1988), but cholesterol also occurs and the proportions of both sterols vary during the life cycle of the algae (Patterson 1990, Aknin *et al.* 1992). In our sample the ratio of fucosterol to cholesterol is 0.7:1. This is higher than the 0.3:1 previously found in the Aegean Sea *Padina pavonia* (Kanias *et al.* 1992), but much lower than in the French Mediterranean Sea *Padina pavonia*, where the fucosterol to cholesterol ratio was found to be 16:1 (Iatrides *et al.* 1983). The differences found in the sterol composition could be due to differences in the life cycle of the algae (Patterson 1990) and to differences in the ecological conditions (Petkov *et al.* 1992).

Table I. Sterol composition (% of the total sterol fraction*).

Sterols	%
27-nor-24-Methyl-cholesta-5,22-dien-3β-ol (occelasterol) or (22*Z*)-Cholesta-5,22-dien-3β-ol	1.5
(22*E*)-Cholesta-5,22-dien-3β-ol	2.7
Cholesterol	34.0
5α-Cholestan-3β-ol (cholestanol)	2.2
Cholest-4-en-3-one	2.5
24-Ethyl-cholesta-5,22-dien-3β-ol	5.5
Cholesta-4,6-dien-3-one	3.6
(24*E*)-Ethyl-cholesta-5,24(28)-dien-3β-ol (Fucosterol)	24.3
24-Ethyl-cholest-5-en-3β-ol	8.0

* Values obtained from three parallel measurements. The standard deviations (related to peak proportion on the chromatogram) are as follows: ± 0.2 for cholesterol and fucosterol and ± 0.1 for the others.

In a sample of *Padina pavonia* from the Aegean Sea (Kanias *et al.* 1992) only four sterols were detected (cholesterol, fucosterol, stigmasterol and campesterol), comprising 98 % of the total sterol mixture (Kanias *et al.* 1992). In our sample, 12 sterols were identified, the four former sterols comprising 72 % of the total sterol mixture.

In *Padina pavonia* we found, in low concentrations, some sterols rarely found in macroalgae, 27-nor-24-methylcholesta-5,22-dien-3β-ol or (22Z)-cholesta-5,22-dien-3β-ol (these two sterols cannot be separated by GC and possess identical mass spectra) and 5α-cholestan-3β-ol. The latter was found in some red algae (Goad 1978, Elyakov and Stonic 1988). Saturated sterols are not characteristic of brown algae.

Two steroidal ketones have also been identified (Table I). Such compounds are in some cases found in sterol mixtures isolated from marine organisms. In such cases, there is always a question about their origin, because they can be artefacts, produced by oxidation through the isolation process. We have never found steroidal ketones in the Black Sea algae. Since the isolation procedure for the *Padina pavonia* sterols was the same, it is likely that the production of

the two steroidal ketones identified is due to oxidation processes within the organism and they are not artefacts. We also did not find saringasterol and other oxidised derivatives of fucosterol, which confirms the absence of oxidation in the sample investigated. It must be mentioned that cholest-4-en-3-one is a precursor in the biogenesis of the saturated sterols in animals (Goad 1978) and possibly 5α-cholestan-3β-ol can be produced from it.

Analysis of the volatile compounds

An increasing number of investigations of volatile compounds from non-essential oil plants and marine algae have been published in recent years (Gally *et al.* 1993, Mahran *et al.* 1993, Kamenarska *et al.* 2000). Hundreds of compounds were identified by GC/MS and other techniques, but there are no investigations on the volatile compounds from *Padina pavonia*. Some of the volatile compounds identified have distinctive chemical structures characteristic of the corresponding organism, and more information on the composition of volatile compounds from related organisms could be useful for taxonomic conclusions. These biomarker compounds can also be valuable for the identification of the food chains in the sea. Some of them can act as allelochemicals, defensive compounds, attractants, alarming pheromones, etc.

The volatile compounds isolated were investigated by GC/MS and the results obtained are summarised in Table II. According to their structures the volatile compounds identified belong to few different groups.

Most of the fatty acids identified are common for marine organisms, and are normally found as esters in different groups of lipids. However, in the volatile compounds of *Padina pavonia* the fatty acids identified were shown to be free. The presence of free acids is often accepted as a result of hydrolysis during the isolation procedure. The distillation-extraction is a relatively mild process and we did not expect a degradation of the lipids, so the fatty acids identified might exist in a free state in the algae investigated. This is in agreement with their identification in the polar fraction, because during their isolation and silylation there was no possibility of hydrolysis. Free fatty acids were previously found in *Padina gymnospora* (Parekh *et al.* 1984) and in *Padina australis* Hauck (Chen 1991). It is also reported in the literature that the antibiotic activity of some algae species could be attributed to their content of a mixture of organic acids such as: capric, lauric, linoleic, myristic, oleic, palmitic, stearic (Kanias *et al.* 1992).

The rare 2-formylbenzoic acid was found at a low concentration. It probably possesses some defensive functions, at least against certain bacteria and fungi.

All of the esters identified contain an aromatic residue. The main components of the ester fraction were benzyl esters of acetic and formic acids. Such compounds are rare natural products (Rzama *et al.* 1995, Jongaramruong and Blackman 2000, Kamenarska *et al.* 2000) and a further investigation into volatile compounds from other brown algae can show if these compounds can serve as biomarkers for these algae. Three esters of benzoic acid were also identified, among them ethyl benzoate, which is rare in nature.

Table II. Composition of the volatile fraction (% of the total volatiles*).

Compounds	%
Acids	9.98
Caprylic acid	0.23
Pelargonic acid	0.60
Myristic acid	2.50
Palmitic acid	6.24
Stearic acid	0.41
2-Formylbenzoic acid	<0.10
Esters	35.57
Benzyl acetate	16.54
Benzyl format	17.50
Ethyl benzoate	0.82
o-Tolyl benzoate	0.31
Benzyl benzoate	0.40
Phenols	3.14
o-Cresol	1.85
p-Cresol	1.29
Alcohols	18.22
Benzyl alcohol	18.22
Aldehydes	4.21
Benzaldehyde	4.21
Aromatic hydrocarbons	0.40
n-Butylbenzenel	0.40
Terpenes	1.51
Dihydroactinidiolide	0.97
β-Ionone	0.54
Others	2.62
Methylethyl disulfide	1.11
Diethyl disulfide	0.68
Benzothiazole	0.83

* The ion current generated depends on the characteristics of the compound and is not a true quantification.

Besides esters of benzoic acid we found in significant concentrations the biogenetic precursors benzaldehyde and benzyl alcohol.

Two methyl phenols were detected. Such compounds are unambiguously defensive (Cowan 1999) due to their antibacterial and antifungal activity.

Low concentrations of the common marine algal terpenoids, dihydroactinidiolide and β-ionone, were found. It is known that β-ionone has a strong deterring action against some Arthropods (Wang *et al.* 1999).

Three sulfur-containing compounds were also identified. Their functions in the organism are not

clear. Two of them are disulfides, which are rarely found in algae. Disulfides were earlier found in *Padina arborescens* (Iida *et al.* 1985).

We can conclude that the volatile compounds of *Padina pavonia* are a complex mixture, containing a number of interesting compounds, particularly aromatics and some of them might possess defensive functions.

Analysis of the polar compounds

The information regarding polar compounds in marine algae is scarce. Due to the difficult separation and purification of polar, water-soluble compounds, there are limited investigations on them. Investigation by GC/MS is one of the most suitable methods. The results obtained are summarised in Table III.

The main part of the polar compounds was free fatty acids (a small part of them was found also in the volatile fraction). Their composition was typical of marine organisms, with palmitic acid being the main fatty acid, followed by oleic acid. As we discussed above, the presence of free fatty acids is probably not a consequence of hydrolysis of lipids during the isolation and separation procedures. The differences in the composition of the free and esterified fatty acids (see Tables III and IV) are in accordance with our suggestion.

Table III. Composition of the polar fraction (% of the total silylated fraction*).

Compounds	%
Acids	59.77
3-Hydroxybutanoic acid	0.36
Lauric acid	0.16
Myristic acid	5.57
Pentadecanoic acid	0.50
Palmitic acid	33.00
Palmitoleic acid	4.57
Stearic acid	1.79
Oleic acid	10.12
Linoleic acid	1.53
Arachidic acid	0.21
Arachidonic acid	1.30
Benzoic acid	0.27
Phenylacetic acid	0.27
4-Hydroxybenzoic acid	0.12
N-containing compounds	0.37
Uracil	0.17
Thymine	0.20
Polyols	10.61
Glycerol	1.14
Mannitol	9.47
Esters	0.30
Propyl ester of 2,3-dihydroxyhexadecanoic acid	0.30

* The ion current generated depends on the characteristics of the compound and is not a true quantification.

The appearance of benzoic and 4-hydroxybenzoic acids identified together with benzoic acid derivatives in the volatile fraction is an indication that the chemical defence of the algae investigated is based partially on benzoic acid and its derivatives.

In our sample we detected significant amounts of mannitol, similarly to what was found in *Padina australis* by Chen (1991).

Analysis of the fatty acids from the total lipids

The fatty acids of the total lipids were converted to methyl esters and analysed by GC. The results obtained are summarised in Table IV.

Padina pavonia contained fatty acids with 12–22 carbon atoms, a profile similar to other brown algae (Kanias *et al.* 1992). The main acid in *Padina pavonia* was palmitic acid followed by oleic and myristic, a composition similar to that of *Padina vickersiae* (Aknin *et al.* 1992). Palmitic acid appears to be the most abundant fatty acid in algae no matter their systematic position. The C_{18} and C_{20} polyunsaturated fatty acids (PUFA) are characteristic of brown algae (Wagner and Pohl 1965, El-Naggar 1998), while high contents of 20:5 acid are characteristic of red algae. The green algae are found to have high proportions of 16:4 acid (El-Naggar 1998). Contrary to *Padina boryana* Thivy from the Saudi Arabian coast, in which high contents of 20:4 were found (El-Naggar 1998), the concentrations of PUFA in our *Padina pavonia* are unusually low. Such low concentrations were found earlier in *Padina vickersiae* from the Senegalese coast (Aknin *et al.* 1992).

Table IV. Fatty acid composition of the total lipids (% of the total lipids*).

Compounds	Total fatty acids
12:0	0.4
14:0	13.0
15:0	1.2
16:0	49.0
16:1	6.4
16:2	4.2
16:3	<0.1
16:4	0.5
18:0	2.3
18:1	15.9
18:2	4.8
18:3	1.3
18:4	<0.1
20:4 /n-6/	0.4
20:4 /n-3/	0.5
22:5	<0.1

* Values obtained from three parallel measurements. The standard deviations (related to peak proportions on the chromatogram) are as follows: ± 0.3 for 14:0, 16:0 and 18:0, ± 0.2 for 16:1, 16:2, 18:0 and 18:2, and ± 0.1 for the others.

Table V. Antibacterial and antifungal activity of extracts from *Padina pavonia*.

Extract	Diameter of the inhibitory zone (mm)*		
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*
Toluene	18.0 ± 0.5	0	0
Ethanol (after the toluene extraction)	16 ± 1	0	15 ± 0
Methanol:Chloroform (1:1)	15.7 ± 1.2	0	0

* Diameter of the inhibition zone less than 10 mm means absence of activity.

The fatty acid composition of our material differs a lot from the fatty acid composition of *Padina pavonia* from the Aegean Sea, in which oleic acid was found to be the main fatty acid, and palmitic acid appears in low concentrations (Kanias *et al.* 1992). It is more similar to the composition of the Red Sea *Padina pavonia* in which palmitic, myristic and stearic acids comprised 80 % of the fatty acids (Karawya *et al.* 1987), compared to 66 % in our sample.

Biological activity

As shown above, we identified a significant number of compounds in the volatile and polar fractions, which might possess an antibacterial and antifungal activity. In order to confirm this assumption, we investigated the antibacterial and antifungal activity of the different extracts from *Padina pavonia*. The toluene (non-polar compounds, including volatile compounds) and methanol:chloroform (1:1) extracts (compounds with average polarity) showed a moderate activity only against the gram-positive bacteria *Staphylococcus aureus* (18.0 ± 0.5 mm and 15.7 ± 1.2 mm inhibition zones, respectively). The ethanol extract (more polar compounds) showed not only a moderate activity against *S. aureus* (16 ± 1 mm) but also a moderate antifungal activity (Table V) against *Candida albicans* (15 ± 0 mm). Similarly to most marine organisms, no activity against Gram (-) bacteria *Escherichia coli* was detected.

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