

Liverwort *Radula* species from Portugal: chemotaxonomical evaluation of volatiles composition

A. Cristina Figueiredo,^{a*} Manuela Sim-Sim,^{b,e} José G. Barroso,^a Luis G. Pedro,^a M. Glória Esquível,^c Susana Fontinha,^d Leena Luís,^e Soraia Martins,^e Carlos Lobo^f and Michael Stech^g

ABSTRACT: The volatiles isolated by distillation-extraction from 48 samples of seven liverwort *Radula* species collected on Azores and Madeira archipelagos and mainland Portugal, as well as one from Switzerland, were analysed by GC and GC-MS. Cluster analysis of the volatiles composition resulted in two major clusters. The chemically very diverse cluster I included the oil samples of *Radula aquilegia* from Madeira, *R. jonesii*, *R. holtii*, *R. nudicaulis*, *R. lindenberiana* as well as *R. complanata* from Switzerland. Cluster II was characterized by high relative amounts of several sesquiterpenes and included the oil samples of *R. aquilegia* from the Azores as well as *R. carringtonii* and *R. wichurae*. 3-Methoxy bibenzyl was the major oil component from *R. complanata* and *R. lindenberiana*. Except for *R. aquilegia* and the closely related species *R. complanata* and *R. lindenberiana*, the cluster analysis allows distinction of all studied species, indicating the chemotaxonomical value of the volatile oil characters. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: *Radula aquilegia*; *Radula carringtonii*; *Radula complanata*; *Radula jonesii*; *Radula holtii*; *Radula lindenberiana*; *Radula nudicaulis*; *Radula wichurae*; liverworts; bryophytes; volatiles; GC; GC-MS

Introduction

Radula, the only genus of the family Radulaceae (order Radiales, Marchantiophyta) comprises ca. 150–200 species.^[1] Its distribution is mainly tropical, extending to subtropical and temperate areas. In Europe, the highest diversity occurs in Atlantic Europe and the Macaronesian archipelagos. Eight *Radula* species are known to occur in Portugal. Of these, three are referred for the mainland [*R. complanata*, *R. holtii* and *R. lindenberiana* (= *R. lindenberiana*^[2])], seven for Madeira (*R. aquilegia*, *R. carringtonii*, *R. holtii*, *R. lindenberiana*, *R. nudicaulis* and two Macaronesian endemics, *R. jonesii* and *R. wichurae*^[3]), and six for the Azores (*R. aquilegia*, *R. carringtonii*, *R. holtii*, *R. lindenberiana*, *R. nudicaulis* and one Macaronesian endemic, *R. wichurae*^[4]).

The aqueous and organic solvent extracts of about 20 *Radula* species have been evaluated so far (Table 1). *Radula* species are known to produce flavonoids,^[5,6] several linear and cyclic prenyl bibenzyls,^[7–16] cyclopropanochroman derivatives,^[17] sesquiterpenoids^[8,9] and clerodane diterpenoids.^[15,16] Based on these studies, detailed on Table 1, *Radula* is chemically characterized by the presence of particular flavone glycosides,^[5] prenylated bibenzyls and very low levels or an almost total lack of terpenoids.^[10,18–22]

To the best of our knowledge, only two studies have addressed the volatile fraction, obtained by either hydrodistillation or distillation-extraction, of *Radula* species. Suire^[23] extracted the essential oil from *R. complanata* from France (2% w/f.w.) and identified α -pinene, β -pinene, camphene, six sesquiterpenes (of which β -caryophyllene was identified), and 3-methoxybibenzyl. Tesso *et al.*^[24] isolated the essential oil of *Radula perrottetii* from Japan and identified several mono-, sesqui- and diterpenes and

aromatic compounds. No comparative study of the volatiles of *Radula* species from a certain geographic area and evaluation of their chemotaxonomical value has been performed hitherto.

As part of a chemotaxonomical survey on the genus *Radula* in Portugal, we hereby report the comparison of the volatiles isolated from seven of the eight Portuguese *Radula* species:

*Correspondence to: A. C. Figueiredo, Universidade de Lisboa, Faculdade de Ciências de Lisboa, Departamento de Biologia Vegetal, Instituto de Biotecnologia e Bioengenharia, Centro de Biotecnologia Vegetal, C2, Campo Grande, 1749-016 Lisboa, Portugal. E-mail: acsf@fc.ul.pt

^aUniversidade de Lisboa, Faculdade Ciências Lisboa, DBV, Instituto de Biotecnologia e Bioengenharia, Centro Biotecnologia Vegetal, C2, Campo Grande, 1749-016 Lisboa, Portugal

^bUniversidade de Lisboa, Faculdade Ciências Lisboa, DBV, Centro de Biologia Ambiental, C2, Campo Grande, 1749-016 Lisboa, Portugal

^cCentro de Botânica Aplicada à Agricultura, Dep. Botânica e Engenharia Biológica, ISA, UTL, 1349-017 Lisboa, Portugal

^dCentro de Estudos da Macaronésia Universidade da Madeira, Campus da Penteadá, 9000-390 Funchal, Madeira, Portugal

^eUniversidade de Lisboa, Museu Nacional de História Natural, Jardim Botânico/CBA. Rua da Escola Politécnica 58, 1250-102 Lisboa, Portugal

^fJardim Botânico da Madeira, Caminho do Meio, Quinta do Bom Sucesso, 9050-251 Funchal, Madeira, Portugal

^gNationaal Herbarium Nederland, Universiteit Leiden branch, PO Box 9514, 2300 RA Leiden, The Netherlands

Table 1. Survey on the collection site and main type of components isolated by aqueous and organic solvent extracts of the liverwort *Radula* species in previous studies. Studies on the volatile fraction obtained by a distillation procedure are detailed in the Introduction

<i>Radula</i> species	Collection site	Type of solvent	Type of isolated compounds	Reference	
<i>R. appressa</i>	Madagascar	Ether	Bibenzyls, chromenes	15	
	Madagascar	Ether	Bibenzyls, chromenes	28	
<i>R. buccinifera</i>	Australia	Ether + methanol	Bibenzyls and one sesquiterpene	9	
	New Zealand	Methanol	Flavone glycosides	5	
<i>R. brunnea</i>	Japan	Not mentioned	Bibenzyls	18	
<i>R. campanigera</i>	Japan	Not mentioned	Bibenzyls	18	
<i>R. carringtonii</i>	Madeira, Portugal	Aqueous methanol	Flavone glycosides	5	
<i>R. chinensis</i>	Japan	Not mentioned	Bibenzyls	18	
<i>R. complanata</i>	France	Different solvents (not specified)	Bibenzyl	23	
	Japan	Methanol	Bibenzyls	29	
	France	Ether + methanol	Bibenzyls and one sesquiterpene	9	
	Austria, Canada, France, Germany, Italy, Poland, Spain, Switzerland	Aqueous methanol	Flavone glycosides	5	
	Switzerland	Aqueous extracts	Flavone glycoside	6	
	France	Not mentioned	Bibenzyls	18	
	Japan	Methanol	Bibenzyls	11	
	Switzerland	Methanol	Bibenzyl	21	
	<i>R. constricta</i>	Japan	Not mentioned	Bibenzyls	18
	<i>R. grandis</i>	New Zealand	Aqueous methanol	Flavone glycosides	5
<i>R. japonica</i>	Japan	Acetone	Bibenzyls	8	
	Japan	Ether + methanol	Bibenzyls and sesquiterpenes	9	
	Japan	Not mentioned	Bibenzyls	18	
<i>R. javanica</i> (= <i>R. variabilis</i>)	Japan	Methanol	Bibenzyls	7	
	Japan	Methanol	Bibenzyls and a dihydrochalcone	30	
	Japan	Ether	Bibenzyls and a dihydrochalcone	9	
	Japan	Not mentioned	Bibenzyls	18	
	Japan	Methanol	Bibenzyls, cyclopropanochromans and one sesquiterpene	17	
<i>R. kojana</i>	Japan	Not mentioned	Bibenzyls	18	
	Japan	Methanol	Bibenzyls, sesquiterpenes and triterpenes	10	
<i>R. laxiramea</i>	Costa Rica	Dichloromethane	Bibenzyls, chromene, cannabinoid	20	
<i>R. lindenbergiana</i> (= <i>R. lindbergiana</i>)	Austria, Azores, Portugal, Canary Islands, Spain, Crete, Germany, Spain, Yugoslavia	Aqueous methanol	Flavone glycosides	5	
<i>R. marginata</i>	New Zealand	Ether	Bibenzyls, bibenzyl cannabinoids	13	
<i>R. nudicaulis</i>	Azores, Portugal	Aqueous methanol	Flavone glycosides	5	
<i>R. obconica</i>	USA	Ether	Bibenzyls	31	
<i>R. obtusiloba</i>	Japan	Not mentioned	Bibenzyls	18	
<i>R. okamuraana</i>	Japan	Not mentioned	Bibenzyls	18	
<i>R. oyamensis</i>	Japan	Ether + methanol	Bibenzyls and one sesquiterpene	9	
	Japan	Not mentioned	Bibenzyls	18	
<i>R. perrottetii</i>	Japan	Ether + methanol	Bibenzyls, sesquiterpenes and dihydrochalcone	9	
	Japan	Ether + methanol	Bibenzyls	32	
	Japan	Not mentioned	Bibenzyls	18	
	Japan	Methanol	Bibenzyls	11	
	Japan	Ether	Bibenzyl cannabinoid, bibenzyls sesquiterpenes, one diterpene and one terpene quinone	12	

Table 1. Continued

Radula species	Collection site	Type of solvent	Type of isolated compounds	Reference
	Japan	Ether	Bibenzyl cannabinoid, bibenzyls sesquiterpenes	33
<i>R. plicata</i>	New Zealand	Aqueous methanol	Flavone glycosides	5
<i>R. tasmanica</i>	New Zealand	Methanol	Flavone glycosides	5
<i>R. tokiensis</i>	Japan	Acetone	Bibenzyls and sesquiterpenes	8
	Japan	Ether	Bibenzyls and sesquiterpenes	9
<i>R. uvifera</i>	Japan	Not mentioned	Bibenzyls	18
	New Zealand	Aqueous methanol	Flavone glycosides	5
<i>R. variabilis</i> (= <i>R. javanica</i>)				
<i>R. voluta</i>	Ecuador	Chloroform	Bibenzyls, terpenes	34, 14
<i>R. wichuræ</i>	Azores, Portugal	Aqueous methanol	Flavone glycosides	5

R. aquilegia (Hook. f. & Taylor) Gottsche et al., *R. carringtonii* J. B. Jack, *R. holtii* Spruce, *R. jonesii* Bouman et al., *R. lindenberiana* Gottsche ex C. Hartm., *R. nudicaulis* Steph. and *R. wichuræ* Steph. Several populations of *Radula* were collected, in different years, in mainland Portugal and on the Madeira and Azores archipelagos, to evaluate whether volatiles could serve as chemical markers within the genus. *R. complanata* (L.) Dumort., due to its rarity in Portugal, could not be obtained for volatiles analysis. Thus, two samples of *R. complanata* from Switzerland were used for comparison purposes.

Materials and Methods

Plant Material

Radula samples collected on the Azores and Madeira archipelagos, mainland Portugal and Switzerland are listed in Table 2. Voucher specimens (Table 2) have been deposited in the Herbarium of the Botanical Garden of Lisbon (LISU) and in the Herbarium of the Madeira Botanical Garden (MADJ).

Volatiles Extraction

The volatiles were isolated by distillation–extraction for 3 h, using a Likens–Nickerson-type apparatus,^[25] with distilled *n*-pentane (50 ml) as organic solvent. The isolation procedure was run at a distillation rate of 3 ml/min. The oil recovered in pentane was concentrated at room temperature under reduced pressure on a rotary evaporator and collected in a vial and concentrated to a minimum volume, again at room temperature, under nitrogen flux. The volatiles were stored at –20°C in the dark until analysis.

Gas Chromatography

Gas chromatographic analyses were performed using a Perkin-Elmer (Shelton, CT, USA) Autosystem XL gas chromatograph equipped with two flame ionization detectors (FIDs), a data-handling system and a vapourizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-

silica column (30 m × 0.25 mm i.d., film thickness 0.15 µm; J&W Scientific Inc.). Oven temperature was programmed to rise from 45°C to 175°C at 3°C/min, subsequently at 15°C/min to 300°C, and then held isothermal for 10 min; injector and detector temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using a split sampling technique, ratio 1:50. The volume of injection was 0.1 µl of a pentane–oil solution. The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as mean values of two injections from each oil, without using correction factors.

Gas Chromatography–Mass Spectrometry

The GC–MS unit consisted of a Perkin-Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1; Perkin-Elmer). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 µA; scan range, 40–300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C₈–C₂₄ *n*-alkane indices and GC–MS spectra from a laboratory-made library, constructed based on the analyses of reference oils, laboratory-synthesized components and commercially available standards. In addition, some sesquiterpenes could be identified by comparison with those of authentic samples present in reference oils of *Petasites hybridus* (plant material provided by W. Frey, Berlin, Germany) and *Tritomaria polita* (plant material provided by L. Hedenäs, Stockholm, Sweden).

Statistical Analysis

The percentage composition of the isolated essential oils was used to determine the relationship between the different samples by cluster analysis, using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc software, version 2.2, Exeter Software, Setauket, NY, USA).^[26] For cluster analysis, a correlation coefficient was selected as a measure of similarity among all accessions, and the unweighted pair group method with arithmetical averages

Table 2. Geographical origin, collection year, voucher numbers and abbreviation of the different *Radula* species evaluated. With the exception of *Radula complanata*, all other *Radula* species were collected in Portugal (mainland, Azores and Madeira archipelagos)

<i>Radula</i> sp.	Island/mainland	Month/year	Voucher LISU	Voucher MADJ	Abbreviation
<i>R. aquilegia</i>	Madeira	May-06	LISU232855	3084	<i>Raquilegia_M06a</i>
	Madeira	May-06	LISU232859	3085	<i>Raquilegia_M06b</i>
	Madeira	Nov-07	LISU232860	3276	<i>Raquilegia_M07</i>
	Azores, Pico	Sep-07	LISU232878		<i>Raquilegia_AP07a</i>
	Azores, Pico	Sep-07	LISU232877		<i>Raquilegia_AP07b</i>
	Azores, Pico	Sep-07	LISU232876		<i>Raquilegia_AP07c</i>
	Azores, Flores	Sep-08	LISU232879		<i>Raquilegia_AF08</i>
<i>R. carringtonii</i>	Madeira	May-06	LISU232856	3083	<i>Rcarringtonii_M06a</i>
	Madeira	May-06	LISU232857		<i>Rcarringtonii_M06b</i>
	Madeira	Apr-08	LISU232858		<i>Rcarringtonii_M08</i>
	Azores, Pico	Sep-07	LISU232887		<i>Rcarringtonii_AP07a</i>
	Azores, Pico	Sep-07	LISU232886		<i>Rcarringtonii_AP07b</i>
	Azores, Pico	Sep-07	LISU232884		<i>Rcarringtonii_AP07c</i>
	Azores, Pico	Sep-07	LISU232885		<i>Rcarringtonii_AP07d</i>
	Azores, Flores	Sep-08	LISU232880		<i>Rcarringtonii_AF08a</i>
	Azores, Flores	Sep-08	LISU232881		<i>Rcarringtonii_AF08b</i>
	Azores, Flores	Sep-08	LISU232882		<i>Rcarringtonii_AF08c</i>
Azores, Flores	Sep-08	LISU232883		<i>Rcarringtonii_AF08d</i>	
<i>R. complanata</i>	Switzerland, Birmensdorf	Feb-08	LISU232898		<i>Rcomplanata_SW08a</i>
	Switzerland, Schaffhausen	Feb-08	LISU232899		<i>Rcomplanata_SW08b</i>
<i>R. holtii</i>	Madeira	Dec-06	LISU232861	8450	<i>Rholtii_M06a</i>
	Madeira	Aug-06	LISU232862		<i>Rholtii_M06b</i>
	Mainland	Jun-08	LISU232894		<i>Rholtii_ML08a</i>
	Mainland	Jun-08	LISU232895		<i>Rholtii_ML08b</i>
<i>R. jonesii</i>	Madeira	Nov-07	LISU232863	3277	<i>Rjonesii_M07</i>
<i>R. lindenbergiana</i>	Madeira	Aug-05	LISU232864		<i>Rlindenbergiana_M05</i>
	Madeira	May-06	LISU232866	3086	<i>Rlindenbergiana_M06a</i>
	Madeira	May-06	LISU232865	3087	<i>Rlindenbergiana_M06b</i>
	Madeira	Oct-06	LISU232867	3268	<i>Rlindenbergiana_M06c</i>
	Mainland	Apr-99	LISU232897		<i>Rlindenbergiana_ML99</i>
	Mainland	Jun-06	LISU152346		<i>Rlindenbergiana_ML06</i>
	Mainland	Feb-08	LISU232896		<i>Rlindenbergiana_ML08</i>
<i>R. nudicaulis</i>	Madeira	May-06	LISU232870	3081	<i>Rnudicaulis_M06a</i>
	Madeira	May-06	LISU232869	3082	<i>Rnudicaulis_M06b</i>
	Madeira	Oct-06	LISU232868		<i>Rnudicaulis_M06c</i>
	Madeira	Dec-07	LISU232871	3275	<i>Rnudicaulis_M07</i>
	Madeira	Sep-08	LISU232872	3280	<i>Rnudicaulis_M08</i>
<i>R. wichurae</i>	Madeira	May-06	LISU232873	3088	<i>Rwichurae_M06</i>
	Madeira	Nov-07	LISU232874	3278	<i>Rwichurae_M07</i>
	Porto Santo	Feb-08	LISU232875	3279	<i>Rwichurae_PS08</i>
	Azores, Flores	Aug-99	LISU232888		<i>Rwichurae_AF99a</i>
	Azores, Graciosa	Jun-99	LISU232889		<i>Rwichurae_AG99b</i>
	Azores, Terceira	Sep-07	LISU232890		<i>Rwichurae_AT07a</i>
	Azores, Terceira	Sep-07	LISU232891		<i>Rwichurae_AT07b</i>
	Azores, Pico	Sep-07	LISU232892		<i>Rwichurae_AP07</i>
	Azores, Flores	Sep-08	LISU232893		<i>Rwichurae_AF08</i>
	Azores, São Jorge	Sep-08	LISU232901		<i>Rwichurae_ASJ08a</i>
	Azores, São Jorge	Sep-08	LISU232900		<i>Rwichurae_ASJ08b</i>

Table 3. Percentage composition of the volatiles isolated by distillation–extraction from the seven *Radula* species collected on the Azores and Madeira archipelagos and mainland Portugal and the Swiss *R. complanata*

Components	RI	<i>Radula aquilegia</i>			<i>R. carringtonii</i>			<i>R. holtii</i>			<i>R. lindenbergiana</i>			<i>R. nudicaulis</i>			<i>R. wichuraea</i>			<i>R. complanata</i>							
		Madeira Min Max	Azores Min Max	Madeira Min Max	Madeira Min Max	Azores Min Max	Madeira Min Max	Mainland Min Max	Madeira Min Max	Mainland Min Max	Madeira Min Max	Mainland Min Max	Madeira Min Max	Mainland Min Max	Madeira Min Max	Azores Min Max	Madeira Min Max	Mainland Min Max	Madeira Min Max	Azores Min Max	Swiss Min Max						
<i>n</i> -Hexanol	882	t	0.1	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t						
Anisole	921	t	0.2	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t						
Benzaldehyde	927	t	0.2	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t						
α -Pinene	930	t	0.2	t	t	t	t	t	5.3	t	t	t	t	t	t	t	t	t	t	t	t						
6-Methyl-5-hepten-2-one	960	0.1	1.1	t	t	t	t	t	4.3	t	t	t	t	t	t	t	t	t	0.2	t	0.5						
1-Octen-3-ol	961	t	1.1	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	0.2	t	0.6						
1,2,4-Trimethyl benzene	978								t	t	t	t	t	t	t	t	t	t	t	t	t						
α -Phellandrene	995								t	t	5.8	7.5															
<i>n</i> -Decane	1000								t	5.0	t	t															
Limonene	1009			t	t				t	t	t	t									0.1						
2-Methyl decane	1058								t	3.1	t	t									t						
<i>n</i> -Nonanal	1073	t	t	t	0.5	t	0.6	t	t	t	t	t	t	t	t	t	t	t	t	t	t						
Linalool	1074	t	t	t	0.1	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t						
<i>n</i> -Undecane	1100								4.3	6.5	t	t															
UIA	1288	1.5	5.3	3.6	8.1	1.9	11.7	1.4	t	t	t	t	0.5	0.6	2.7	0.7	1.1	t	0.9	t	3.0	t	5.2	t	1.2		
Pentalenene	1332	0.8	3.3	1.5	5.8	2.9	4.0	7.4	11.4	t	t	t	3.0	t	0.9	t	1.2	1.0	4.8	t	5.3	t	8.2	0.2	1.6		
UIS	1334	2.6	4.2	t	0.5	t	0.2	t	1.3	t	t	t	t	t	t	0.2	t	2.4	8.8	t	t	t	t	t	t		
UIB	1353	2.2	3.5	t	1.3	t	1.4	t	t	t	t	t	7.1	0.3	1.3	1.1	3.2	t	t	t	0.7	12.6	t	8.7	0.7	2.0	
UIC	1365				t	t	0.3	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	
α -Funebrene	1375				t	2.4	t	4.9	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	
α -Copaene	1375			t	0.6																						
β -Bourbonene	1379																										
Petasitene	1387	t	1.2	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
β -Elemene	1388																										
7- <i>epi</i> - α -Cedrene	1396	t	t	t	1.5	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
α -Cedrene	1400	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
α -Santalene	1422																										
Sesquisabinene B*	1438	1.6	2.3																								
Acora-3,5-diene*	1414				t	0.5	t	0.5	t	0.5	t	1.5															
β -Caryophyllene	1414	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
β -Cedrene	1414																										
<i>cis</i> - β -Farnesene	1420	2.1	4.6	t	t	t	t	t	t	t	t	t	5.1	t	0.7	t	t	t	t	t	t	t	t	t	t	t	t
γ -Elemene*	1430				t	0.7	t	t	t	t	t	t															
α -Pinguisene	1434																										
<i>trans</i> - α -Bergamotene	1434																										
α -Humulene	1447	t	t	t	0.2	t	0.2	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
Khusimene* (= zizaene*)	1449																										
Eudesma-1,4(15)-11-triene*	1642				t	1.7	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t

Table 3. Continued

Components	RI	<i>Radula aquilegia</i>			<i>R. carringtonii</i>			<i>R. holtii</i>			<i>R. lindenbergiana</i>			<i>R. nudicaulis</i>			<i>R. wichurae</i>			<i>R. complanata</i>						
		Madeira	Azores	Madeira	Madeira	Azores	Madeira	Mainland	Madeira	Mainland	Madeira	Mainland	Madeira	Mainland	Madeira	Azores	Madeira	Azores	Madeira	Azores	Madeira	Azores	Madeira	Azores		
<i>β</i> -Helmiscapene*	1455																									
<i>trans</i> - <i>β</i> -Farnesene	1455	41.9	63.7	t	1.0	t	0.9	t	t																	
<i>β</i> -Santalene	1455																									
<i>allo</i> -Aromadendrene	1456																									
<i>α</i> -Acoradiene*	1456																									
<i>β</i> -Acoradiene*	1463	0.6	1.9	12.2	31.3	t	0.9	t	t																	
Drima-7,9(11)-diene*	1467	t	t	t	11.1	t	0.9	t	t																	
UI D	1471	t	t	t	4.8	t	t	t	t																	
UI F	1471	t	t	t		t	t	t	t																	
UI F'	1475																									
<i>β</i> -Selinene	1476																									
Eremophilene	1480	2.5	7.7	t	14.6	t	3.0	2.8	3.7																	
Valencene	1484																									
<i>α</i> -Helmiscapene*	1489																									
<i>α</i> -Selinene*	1491																									
Cuparene	1491																									
<i>trans,trans</i> - <i>α</i> -Farnesene	1500	t	2.2			t	t	t	t																	
<i>β</i> -Bisabolene	1500	t	1.5	t	t	t	t	t	12.4	15.3	9.4	9.9														
<i>β</i> -Curcumene*	1500																									
7- <i>epi</i> - <i>α</i> -Selinene	1500																									
<i>cis</i> - <i>γ</i> -Bisabolene*	1501	t	t			t	t	t	t	t	22.0	22.1														
<i>δ</i> -7(14)- <i>α</i> -Himachalene*	1501					t	1.5	t	t																	
<i>δ</i> -Cadinene	1505	t	t			t	2.0	1.2	4.2																	
Zonarene*	1506					t	2.0	t	4.2																	
Bisabola-1,3,5,7(14),10-pentaene*	1517					t	2.0	t	4.2																	
Myli-4(15)-ene*	1538																									
<i>trans</i> -Nerolidol	1549																									
UI Q	1585	t	t			t	4.9	t	0.2																	
UI L	1672																									
3-Methoxy bibenzyl	1717					t	1.1	t	t																	
UI M	1718					t	2.2	t	2.2																	
UI N	1726					t	5.2	t	t																	
Xanthorizol*	1712																									

Table 3. Continued

Components	RI	<i>Radula aquilegia</i>			<i>R. carringtonii</i>			<i>R. holtii</i>			<i>R. jonesii</i>			<i>R. lindenbergiana</i>			<i>R. nudicaulis</i>			<i>R. wichurae</i>			<i>R. complanata</i>																														
		Madeira	Azores	Min	Madeira	Azores	Min	Madeira	Mainland	Min	Madeira	Mainland	Min	Madeira	Mainland	Min	Madeira	Azores	Min	Madeira	Azores	Min	Madeira	Azores	Min	Madeira	Azores	Min	Madeira	Azores	Min	Madeira	Azores	Min	Madeira	Azores	Min	Madeira	Azores	Min	Madeira	Azores	Min										
6,11-Epoxy-15-nor-3,4-dioxo-5,10-pinguisadien-12-acetate*	1760	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t													
UII	1771				t	t																																															
UIJ	1781				t	t																																															
ent-2,3-Dihydroxycuparene*	1802	t	t	t	2.4	t	2.8																																														
Hexadecanoic acid (= palmitic acid)	1908	t	0.3		t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t						
UIO	1922	1.0	1.7		t	1.2	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t						
3,4'-Dimethoxybibenzyl	1952	t	1.6	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t				
ent-2,3-Dihydroxycuparene*	1975				t	0.2	t																																														
Linoleic acid	2125				t	7.3	2.0	10.4	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t			
2,2-Dimethyl-5-hydroxy-7-(2-phenylethyl)-chromene*	2248				t	7.3	2.0	10.4	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	
Radulanin H*	2345				t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t

The minimum and maximum values represent the lowest and highest percentage, respectively, of each component as found for each species. For samples studied from each species, see Table 2

RI, retention index relative to C₈–C₂₄ n-alkanes on the DB-1 column; t, trace (<0.05%); UI, unidentified compounds.

*Identification based on mass spectra only.

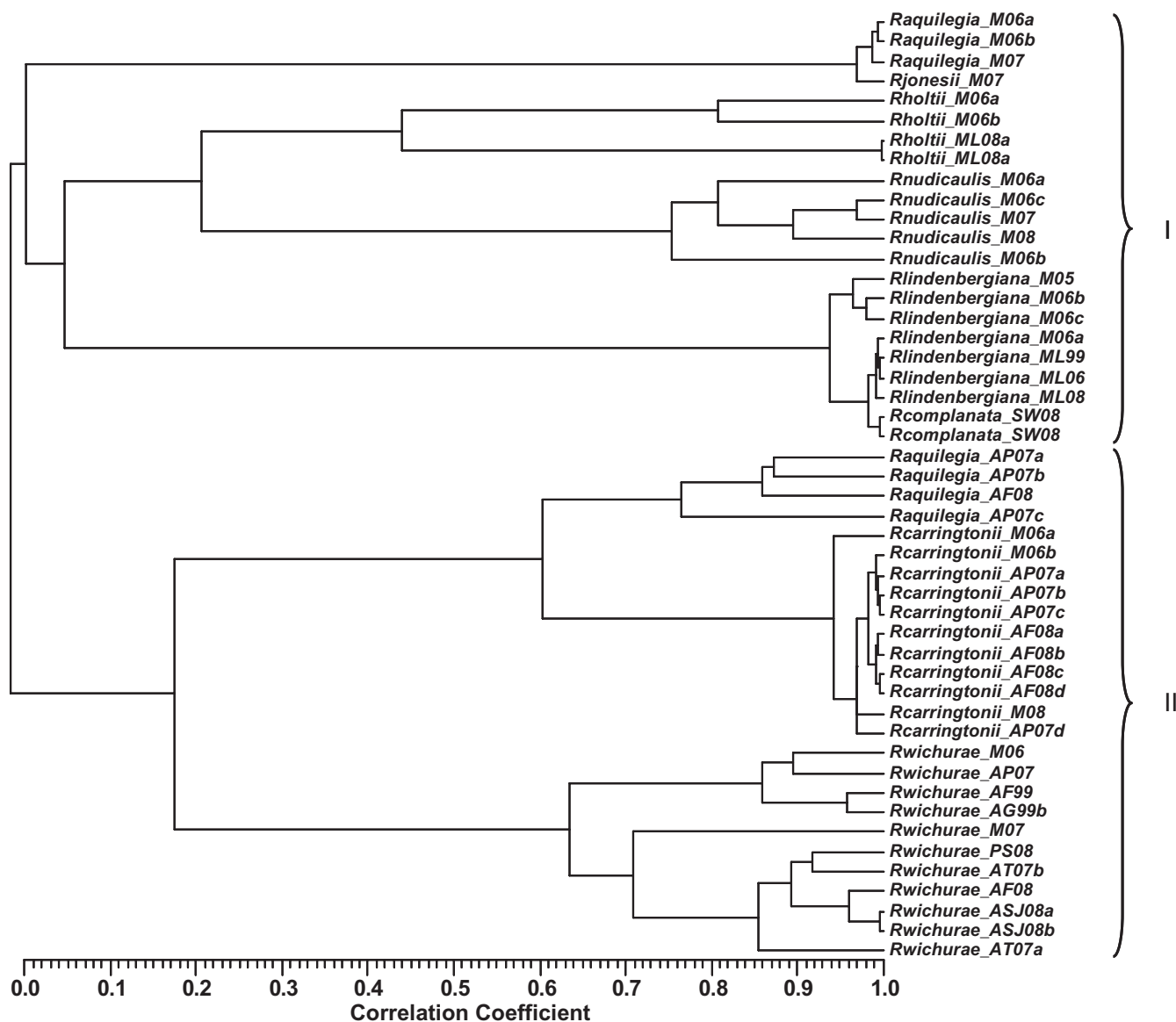


Figure 1. Dendrogram obtained by cluster analysis of the percentage composition of the volatiles isolated from the *Radula* species studied, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA). For abbreviations, see Table 2

(UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro^[27] and classified as very high (0.9–1.0), high (0.7–0.89), moderate (0.4–0.69), low (0.2–0.39) and very low (<0.2).

Results and Discussion

All studied *Radula* samples afforded light yellow oils. The identified oil components are listed in Table 3 in order of their elution on the DB-1 column. Due to the limited amounts of fresh material available, some components could not yet be identified, although their presence was detected in the volatile oils isolated from several of the studied *Radula* species. These unidentified compounds are marked 'U' in Table 3.

A major grouping of the studied samples into two main clusters could be observed based on the volatiles composition,

which was confirmed by cluster analysis (Figure 1, cluster I), which was in itself chemically very diverse (Figure 2), was formed by 22 of the 48 oil samples studied, including the oil samples of *R. aquilegia* from Madeira, *R. jonesii*, *R. holtii*, *R. nudicaulis*, *R. lindenbergiana* as well as the Swiss *R. complanata* used for comparison (Figure 1). The oil samples of *R. complanata* showed a high chemical correlation with those of the Portuguese *R. lindenbergiana*, due to the high amounts of 3-methoxy bibenzyl that occurred in both species. Cluster II was composed of 26 oil samples, comprising *R. aquilegia* from the Azores as well as *R. carringtonii* and *R. wichurae* (Figure 1). All oil samples of cluster II showed high relative amounts of several sesquiterpenes (cf. Table 3, Figure 2).

Except for *R. aquilegia* and the distinction between *R. complanata* and *R. lindenbergiana*, the cluster analysis allows distinction of all the species studied (Figure 1), indicating the

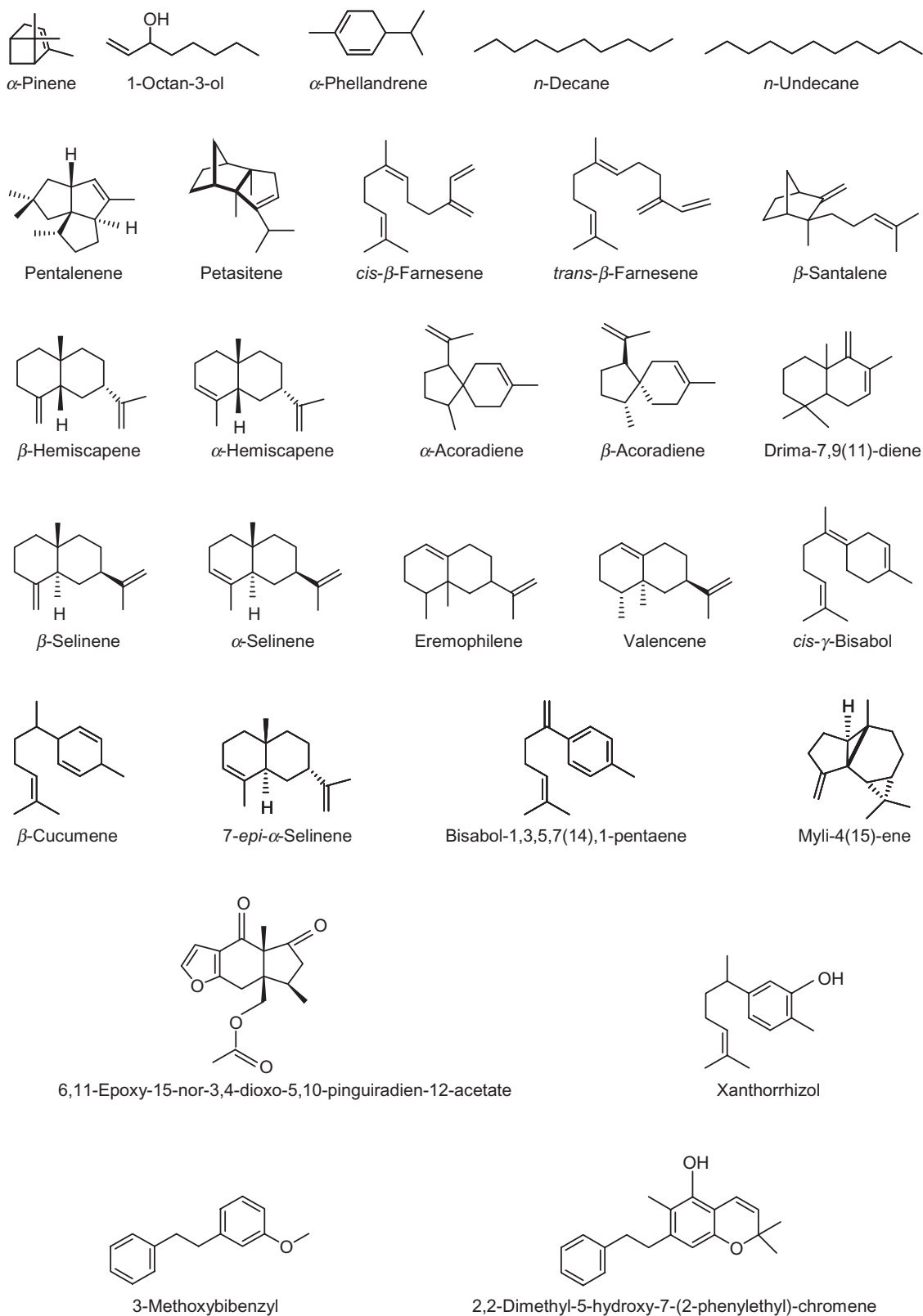


Figure 2. Chemical structures of the compounds identified in a relative amount of $\geq 5\%$, in the volatiles isolated from the *Radula* species studied

chemotaxonomical value of the essential oil characters. The volatile analyses of *Radula* species may thus constitute a helpful tool for *Radula* species identification and characterization. Further geographic differentiation based on intraspecific chemical variability is indicated in *R. holtii* (Madeira vs. mainland Portugal), whereas in *R. carringtonii*, *R. wichurae* and *R. lindenbergiana* no geographic differentiation of the analysed samples is possible. Noteworthy is the major chemical heterogeneity of *R. aquilegia* oils from samples collected on Madeira and the Azores, which resulted in two separate subclusters of this species within cluster I (Madeiran samples) and cluster II (Azorean samples), respectively (Figure 1). These patterns may indicate that the morphological species *R. aquilegia* comprises different taxa that may have originated from independent evolution on both archipelagos. Detailed morphological and molecular phylogenetic analyses of non-coding DNA markers are in progress to evaluate the present chemotaxonomical results.

Based on the aqueous and organic solvent extract studies performed so far (Table 1), *Radula* is a genus chemically categorized by the presence of particular flavone glycosides, prenylated bibenzyls and the almost total lack of terpenoids. In the present study and by volatiles analysis, the presence of bibenzyl compounds was frequent in the volatiles isolated from some Portuguese *Radula* species, such as in *R. complanata* and *R. lindenbergiana* (Table 3), whereas other species showed high relative amounts of sesquiterpenes, such as *R. aquilegia*, *R. carringtonii*, *R. nudicaulis* and *R. wichurae*. In contrast to earlier analyses based on organic solvent extracts, the present study thus shows that the genus *Radula* is not particularly poor in terpenes.

Acknowledgements

This study was partially funded by the Fundação para a Ciência e Tecnologia (FCT) under research contract POCI/AGR/57487/2004. The authors gratefully acknowledge Professor Dr Wolfgang Frey from Freie Universität, Institut für Biologie, Systematische Botanik und Pflanzengeographie, Berlin, Germany, for kindly providing a sample of *Petasites hybridus*, and Dr Lars Hedenäs from the Swedish Museum of Natural History, Department of Cryptogamic Botany, Stockholm, Sweden, for samples of *Tritomaria polita*, both species used for isolation of reference compounds. We are also indebted to Dr Ariel Bergamini from WSL, Swiss Research Institute, Zürich, Switzerland, for support in the sampling of *R. complanata*.

References

1. W. Frey, M. Stech. In *Adolf Engler's Syllabus of Plant Families*, 13th edn, part 3, Frey W. (ed.). Gebr. Borntraeger: Berlin, Stuttgart, **2009**, (in press).
2. P. Isoviita. *Ann. Bot. Fennici* **1977**, *14*, 95.
3. L. Luís, D. Draper, M. Sim-Sim. *Lindbergia* **2005**, *30*, 3.
4. L. Söderström, E. Urmi, J. Vána. *Lindbergia* **2002**, *27*, 21.
5. R. Mues. Proceedings of the Third Meeting of Bryologists from Central and East Europe, **1984**, 37.
6. K. R. Markham, R. Mues. *Z. Naturforsch.* **1984**, *39c*, 309.
7. Y. Asakawa, M. Toyota, T. Takemoto. *Experientia* **1978a**, *34*, 971.
8. Y. Asakawa, R. Takeda, M. Toyota, T. Takemoto. *Phytochemistry* **1981**, *20*, 858.
9. Y. Asakawa, K. Takikawa, M. Toyota, T. Takemoto. *Phytochemistry* **1982**, *21*, 2481.
10. Y. Asakawa, K. Kondo, M. Tori, T. Hashimoto, S. Ogawa. *Phytochemistry* **1991a**, *30*, 219.
11. Y. Asakawa, T. Hashimoto, K. Takikawa, M. Tori, S. Ogawa. *Phytochemistry* **1991b**, *30*, 235.
12. M. Toyota, T. Kinugawa, Y. Asakawa. *Phytochemistry* **1994**, *37*, 859.
13. M. Toyota, T. Shimamura, H. Ishii, M. Renner, J. Braggins, Y. Asakawa. *Chem Pharm Bull* **2002**, *50*, 1390.
14. L. Kraut, R. Mues, H. D. Zinsmeister. *Corrigendum in Phytochemistry* **2006**, *67*, 1297.
15. L. Harinantenaina, Y. Takahara, T. Nishizawa, C. Kohchi, G.-I. Soma, Y. Asakawa. *Chem. Pharm. Bull.* **2006**, *54*, 1046.
16. F. Nagashima, F. Kuba, Y. Asakawa. *Chem. Pharm. Bull.* **2006**, *54*, 902.
17. Y. Asakawa, K. Kondo, M. Tori. *Phytochemistry* **1991c**, *30*, 325.
18. K. Takikawa, M. Tori, Y. Asakawa. *J. Hattori Bot. Lab.* **1989**, *67*, 365.
19. Y. Asakawa. In *Progress in the Chemistry of Organic Natural Products*, vol. 65, W. Hertz, G. W. Kirby, R. E. Moore, W. Steglich, C. Tamm (eds). Springer-Verlag: Vienna, **1995**.
20. F. Cullmann, H. Z. Becker. *Z. Naturforsch.* **1999**, *54c*, 147.
21. M. Flegel, H. Becker. *Plant Biol.* **2000**, *2*, 208.
22. Y. Asakawa. *Phytochemistry* **2004**, *65*, 623.
23. C. Suire. *Le Botaniste* **1970**, *53*, 125.
24. H. Tesso, W. A. König, Y. Asakawa. *Phytochemistry* **2005**, *66*, 941.
25. S. T. Likens, G. B. Nickerson. Proceedings of the American Society of Brewing Chemists, **1964**, 5.
26. J. F. Rohlf. *NTSYS-pc, Numerical Taxonomy and Multivariate System*. Applied Biostatistics: New York, **2000**.
27. M. H. Pestana, J. N. Gageiro. *Análise de Dados para Ciências Sociais. A Complementaridade do SPSS*. Edições Sílabo: Lisbon, **2000**.
28. L. Harinantenaina, Y. Asakawa. *Nat. Prod. Comm.* **2008**, *2*, 701.
29. Y. Asakawa, E. Kusube, T. Takemoto, C. Suire. *Phytochemistry* **1978c**, *17*, 2115.
30. Y. Asakawa, M. Toyota, T. Takemoto. *Phytochemistry* **1978b**, *17*, 2005.
31. K. D. L. Millar, B. J. Crandall-Stotler, J. F. S. Ferreira, K. V. Wood. *Cryptogam. Bryol.* **2007**, *28*, 197.
32. M. Toyota, M. Tori, K. Takikawa, Y. Shiobara, M. Kodama, Y. Asakawa. *Tetrahedr. Lett.* **1985**, *26*, 6097.
33. A. Ludwiczuk, F. Nagashima, R. S. Gradstein, Y. Asakawa. *Nat. Prod. Comm.* **2008**, *3*, 133.
34. L. Kraut, R. Mues, H. D. Zinsmeister. *Phytochemistry* **1997**, *45*, 1249.