Original Article

Natural Product Communications
August 2019: 1–4

DOI: 10.1177/1934578X19868875
journals.sagepub.com/home/npx

Chemical Profiling of Volatile Components of the Gametophyte and Sporophyte Stages of the Hornwort *Leiosporoceros dussii* (Leiosporocerotaceae) From Panama by HS-SPME-GC-MS

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Abstract

We report for the first time the chemical profiling of volatile organic compounds (VOCs) of gametophyte and sporophyte life stages of *Leiosporoceros dussii,* from Panama by using headspace-solid phase microextraction-gas chromatography-mass spectrometry in order to assess distinguishing chemical markers between the male and female gametophytes, and sporophytes of this hornwort. A total of 27 VOCs were identified in *L. dussii*. Furthermore, the gametophyte and sporophyte showed clear differences in the type and amount of VOCs. The main constituents of *L. dussii* female thalli were menthacamphor (17.8%), hexanol (12.3%), and menthyl acetate (12.3%), while the major compounds of the male thalli were hexanol (25.3%), β-ionone (21.1%), benzeneacetaldehyde (17.6%), and β-cyclocitral (14.0%). The main VOCs of the sporophytes were hexanal (19.3%), β-cyclocitral (17.6%), 2-nonenal (15.8%), hexanol (12.5%), and β-ionone (10.2%). Unique compounds found in the female thalli were 3-pentanone, 3-octenol, nonanol, estragole, and menthyl acetate, and in the male thalli were methyl heptenone, nonanal, neoisomenthol, and bornyl acetate. Isomenthol, thymol, isomenthol acetate, and β-methylnaphthalene were only found in the sporophyte. The characteristic VOCs identified in *L. dussii* suggest a difference between the chemical constituents of *L. dussii* and other hornworts species. The presence of simple VOCs when compared with compounds previously characterized in another hornwort genera may support the distinct genetic nature of this species.

Keywords

natural products, hornwort, anthocerotophyta, *Leiosporoceros dussii*, gametophyte, sporophyte, chemosystematics, HS-SPME-GC-MS

Received: March 8th, 2019; Accepted: June 17th, 2019.

Bryophytes (mosses, liverworts, and hornworts) are a group of plants, which are taxonomically placed between the pteridophytes and the algae. More than 20,000 species have been reported worldwide, among which the phylum Anthoceratophyta (named hornworts) contains around 300 species.[1] A haploid-dominant life cycle characterizes all bryophytes with an alternance of a multicellular gametophyte (*n*) and a multicellular sporophyte (2*n*). Bryophytes are known for being resilient organisms and resistant to the attack of bacteria, fungi, insects, or snails.[1] In contrast to flowering plants, the chemical characterization and pharmacological potential of these plants have been underexplored, although many bryophytes have been used as medicinal plants to cure diseases such as tuberculosis, snake bite, neurasthenia, pneumonia, convulsions and uropathy.[2]

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The hornwort *Leiosporoceros dussii* (Steph.) Hässel is a dioicous species (separate male and female plants) native to the Americas. The species is found in Panama, Colombia, Costa Rica, the Greater Antilles, Ecuador, and Mexico.[3] It is worth noting that *L. dussii* is the only species included within its own family Leiosporocerotaceae. Only 1 study has reported so far the chemical composition of *L. dussii* species from the Neotropics.[4] In this study, the chemical differences between the different stages of the plant (gametophyte and sporophyte) were not evaluated, although this is valuable for supporting the biochemical knowledge, and potential pharmacological use of the species.

The study of the volatile composition of bryophytes has facilitated the differentiation between species, and resolved important taxonomic questions.[5,6] Volatile organic compounds (VOCs) are remarkable secondary metabolites for protection of plants against environmental pathogens and predators.[7] Solid phase microextraction (SPME) has been widely used for research and identification of volatile compounds present in plants, including bryophytes.[6,8-10] SPME is a more sensitive and effective method, when compared with solvent extraction techniques for the analysis of VOCs in plants and fruits.[6,11] SPME is an analytical method that favors the isolation of volatile and semivolatile compounds present in small amounts in the sample, allowing for the identification of compounds otherwise undetectable by using other methods. In this paper, isolation and identification of chemical compounds from the gametophyte and sporophyte stages of *L. dussii* from Panama by headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) are reported for the first time. Furthermore, 27 compounds not previously reported in *L. dussii* were also identified.

In general, the chemical compositions of hornworts species have not been investigated thoroughly. Among VOCs, only a small number of volatile mono-, phytane-type di-, and sesquiterpenoids have been identified in few hornworts.[1] Regarding the chemical composition of *L. dussii* the only study reported so far was done in species from Panama, by using solvent extraction technique. Two compounds were detected, 16-kaurene and 4-hydroxy-3,3',4-trimethoxystilbene.[4] To undertake a more in-depth study on the chemical constituents of *L. dussii*, both the gametophyte (female and male thalli) and the sporophyte stages were investigated for the identification of the VOCs present in this plant, by using SPME. The isolated compounds were separated by gas chromatography (GC) and detected by mass spectrometry (MS). The main VOCs identified in the female and male thalli, and sporophyte of *L. dussii*, and their Kovat's retention indices and percentages are presented in Table 1. In total, HS-SPME-GC-MS permitted the identification of 27 VOCs in *L. dussii*. Eighteen compounds were identified in the female thalli and in the sporophyte, and 14 compounds in the male thalli. Alcohols (42.3%) were the main components identified in the female thalli, while aldehydes were the most abundant both in male thalli (41.2%) and sporophyte (56.9%). In the female thalli, aldehydes (25.7%) were the second main compounds followed by monoterpenoids (18.9%), ketones (6.7%), esters (3.2%), and aromatic compounds (2.9%). On the other hand, alcohols (30.0%), ketones (22.0%), aromatic compounds (4.6%), and monoterpenoids (2.2%) were identified in the male thallus, while alcohols (16.4%), ketones and monoterpenoids (10.2%), esters (4.1%), and aromatic compounds (2.3%) were found in the sporophytes. None of the compounds identified in this study have been reported for *L. dussii*. Our results revealed that the main constituents of *L. dussii* female thalli were menthacamphor (17.8%), hexanol (12.3%), and menthyl acetate (12.3%) (Table 1). Furthermore, the major compounds of the male thalli were hexanol (25.3%), β-ionone (21.1%), benzeneacetaldehyde (17.6%), and β-cyclocitral (14.0%), while the main VOCs of the sporophytes were hexanal (19.3%), β-cyclocitral (17.6%), 2-nonenal (15.8%), hexanol (12.5%), and β-ionone (10.2%). The presence of the diterpenoid 16-kaurene was reported recently in *L. dussii* from Panama;[4] however, this compound was not detected in this study.

Anthocerotophyta phylum is known to produce small amounts of terpenoids due to the absence of oil bodies in their biological structure.[12] Furthermore, *L. dussii* is the most simple of the Anthocerotophyta.[3] These 2 facts might explain why terpenoids such as di- and sesquiterpenoids were not identified in this hornwort in our study, and why there was a huge presence of alcohols and aldehydes in the plant. Diterpenoids and sesquiterpenoids have been found in more biologically evolved hornwort species such as *Anthoceros caucasicus* and *Megaceros flagellaris*.[13,14] To the best of our knowledge none of the VOCs found in this investigation for *L. dussii* have been identified before in other Anthocerotophyta.

HS-SPME-GC-MS allowed for chemical differentiation between gametophyte and sporophyte of *L. dussii*. Overall, there were important differences in the type and percentage of compounds found between gametophyte and sporophyte. Unique compounds found in the female thallus were 3-pentanone, 3-octenol, nonanol, estragole, and menthyl acetate, and in the male thallus were methyl heptenone, nonanal, neoisomenthol, and bornyl acetate. Isomenthol, thymol, isomenthol acetate and β-methylnaphthalene were only found in the sporophyte.

Our results suggest a difference between the chemical constituents of *L. dussii* and other hornworts species. The presence of simple VOCs in *L. dussii* when compared with compounds previously characterized in other Anthocerotophyta confirmed the simple nature of this species. Due to the fact that *L. dussii* do not possess oil bodies, is morphologically very small, and that it is difficult to collect enough plant material, the investigation on the volatile composition has been limited. Nevertheless, HS-SPME-GC-MS allowed for the profiling of the VOCs from *L. dussii* with success, and also in

Table 1. Volatile Compounds Identified in the Female Thallus, Male Thallus, and Sporophyte of the Panamanian *Leiosporoceros Dussii* by Headspace-Solid Phase Microextraction-Gas Chromatography-Mass spectrometry.

Peak No.	KRI	KRI*	Compounds	Relative content (%)		
				Ft	***Mt***	***S***
1	652	647	3-Pentanone	0.4	-	-
2	808	800	Hexanal	7.7	5.1	19.3
3	871	865	Hexanol	12.3	25.3	12.5
4	1003	987	Methyl heptenone	-	0.9	-
5	1107	1102	Nonanal	-	3.4	-
6	1042	1036	3-Octenol	3.4	-	-
7	1057	1049	Benzeneacetaldehyde	2.6	17.6	4.0
8	1151	1147	2-Nonenal	5.1	-	15.8
9	1180	1173	Nonanol	5.6	-	-
10	1146	1141	Menthacamphor	17.8	1.6	3.9
11	1150	1146	Camphor	0.7	1.4	0.4
12	1189	1182	Isomenthol	-	-	3.1
13	1195	1188	Neoisomenthol	-	3.1	-
14	1197	1190	α-Terpineol	0.2	-	0.5
15	1200	1192	Dihydrocarveol	5.7	-	1.5
16	1205	1196	Estragole	3.5	-	-
17	1208	1200	Decanal	0.5	1.0	0.2
18	1227	1222	β-Cyclocitral	9.9	14.0	17.6
19	1285	1281	β-Methylnaphthalene	-	-	1.0
20	1288	1283	Bornyl acetate	-	0.8	-
21	1298	1294	Menthyl acetate	12.3	-	
22	1301	1297	Thymol	-	-	1.6
23	1307	1301	Anethole	2.9	4.2	1.2
24	1311	1305	Isomenthol acetate	-	-	3.0
25	1452	1446	Citronellyl propanoate	3.2	-	4.1
26	1494	1490	β-Ionone	6.3	21.1	10.2
27	1537	1532	Dihydroactinidiolide	-	0.3	0.1

Ft, female thallus; KRI: Kovat's retention index;KRI*, reported Kovats retention index from reference databases; Mt, male thallus; S, sporophyte.

differentiating between the chemical nature of the different stages of the plant

Experimental

Plant Material

Fresh samples of *L. dussii*, female and male thallus, were collected at Monumento Natural Cerro Gaital, El Valle de Anton, Province of Coclé (8°37'34.6" N, 80°08'13.7" O), between August and December. Samples were initially identified by Jose Gudiño, and identification was finally confirmed by the bryologist Noris Salazar Allen. The plants were cleaned of debris, protected from light and humidity, and stored at a temperature of −20°C until analysis. Voucher specimens of *L. dussii* were deposited at the Herbarium of the University of Panama, Panama, Panama. Sporophytes were separated from the thallus for further analyses. Two samples of each type of thalli and sporophytes were analyzed in duplicate and mean reported in Table 1 as percent relative concentration of each compound.

Analysis of the Volatile Chemical Composition of L. Dussii by HS-SPME-GC-MS

HS-SPME-GC-MS analysis of the female thallus, male thallus and sporophytes was done following the procedure described by Durant et al,[8] with some modifications. Briefly, divinylbenzene-carboxen-polydimethylsiloxane StableFlex SPME fiber (50/30 µm) (Supelco, Bellefonte, PA, USA) was conditioned in the GC following the manufacturer's recommendation. A blank test was done before each analysis to verify for possible carry-over effect. For SPME analysis 1 g of each sample and 1 mL of NaCl solution were added in a 10 mL hermetically sealed vial. The fiber was then exposed to the headspace of the sample and maintained there for 15 minutes in a thermostatic bath at 50°C. All experiments were done under constant stirring of 400 rpm. GC quadrupole MS analyses were performed

in a GC 6890N coupled to a mass spectrometer 5975C (Agilent Technologies, Palo Alto, CA, USA). After extraction of the volatile compounds the SPME fiber was inserted in the GC injection port. Desorption of the compounds was performed at 240°C in splitless mode for 2 minutes. Separation of the extracted compounds was achieved on a HP-5MS capillary column (30 m length, 0.25 mm id, 0.25 μm film thickness). Helium was used as a carrier gas at 1 mL/min. The oven temperature was programmed at 50°C for 2 minutes, then increased to 240°C at 6 °C/min and held for 5 minutes. The MS detector was operated in electron impact mode (EV = 70 eV); with an ion source temperature of 250°C; and operated in scan mode from 30 to 550 m/z. All experiments were performed in duplicate and the results obtained expressed as average. Identification of VOCs was achieved by comparing the mass spectra with those provided by the Registry of Mass Spectral Data with Structures library (Wiley 7th edition, USA), National Institute of Standards and Technology library (NIST/11), and by using authentic standards when available. Further identification was performed by comparison of the calculated Kovat's retention index (KI) with those reported in the literature. KI was determined by using an alkane standard solution C8-C20 (Sigma-Aldrich, Saint Louis, MO, USA). The relative quantities of the volatile compounds are expressed as percent peak areas relative to the total peak area of identified compounds. The percentages reported in Table 1 for each stage of the plant are means of 2 samples of each stage and each analysis carried out in duplicate.

Acknowledgments

Mahabir P. Gupta and Armando Durant-Archibold gratefully acknowledge the National Secretariat for Science, Technology, and Innovation of Panama (SENACYT) for support through its National System of Research Awards (SNI). Armando Durant-Archibold wishes to acknowledge Dr. Jagannatha Rao, Director of the Institute for Scientific Research and Technology Services (INDICASAT AIP) for support with the GC-MS. Noris Salazar Allen and José Gudiño acknowledge support provided by research funds from the Smithsonian Tropical Research Institute (STRI).

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by National Secretariat for Science, Technology, and Innovation of Panama (SENACYT) through its National System of Research Awards (SNI).

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