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An Analysis of Volatile Components of the Liverworts *Dumortiera hirsuta subsp. hirsuta and Dumortiera hirsuta subsp. nepalensis* (Dumortieraceae) from Panama and Taxonomic Observations on the Species

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We report for the first time the chemical composition of volatile components (VOCs) of two subspecies, *D. hirsuta* subsp. *hirsuta* and *D. hirsuta* subsp. *nepalensis*, of the liverwort *Dumortiera hirsuta* from Panama by using headspace-solid phase microextraction-gas chromatography-mass spectrometry in order to assess distinguishing markers between the two species. Forty VOCs were identified in total for both subspecies. Of these, 34 are reported for the first time in *D. hirsuta*. Furthermore, both subspecies showed clear differences in the type and amount of VOCs. The major compounds in *D. hirsuta* subsp. *hirsuta* were α-gurjunene, β-selinene, α-guaiene, α-humulene and β-caryophyllene; while in *D. hirsuta* subsp. *nepalensis* were ledene, α-gurjunene, β-caryophyllene and α-guaiene, respectively. Two oxygenated sesquiterpenes, globulol and nerolidol, could be considered as possible distinguishing chemical markers between these two subspecies. We conclude that both morphotypes of *D.* hirsuta are chemically different.

Keywords: Natural products, Terpenoids, *D. hirsuta* subsp. *hirsuta*, *D. hirsuta* subsp. *nepalensis*, HS-SPME-GC-MS.

Dumortiera hirsuta (Sw.) Nees is a thallose liverwort widely distributed in the tropical and subtropical regions of the world, with infrequent presence in the more warmer and humid areas of the temperate zones [1-2]. This species is not characterized by the presence of internally differentiated thalli into distinct layers such as other liverworts of the order Marchiantiales [2]. *Dumortiera hirsuta* had previously been assigned to the Marchantiaceae family, but based on molecular evidence, it has now been placed in its own family Dumortieraceae [3]. Most taxonomist consider *D. hirsuta* as a single species. This species is frequently divided into two subspecies, *D. hirsuta* subsp. *hirsuta* (Sw.) Nees and *D. hirsuta* subsp. *nepalensis* (Tayl.) R.M. Schust., because of differences in the thallus morphology [4]. The occurrence of both subspecies has been reported Asia, Europe, in the Northern and Southern countries of the Americas, but also in Central America and Panama [2, 5]. Previous reports on the chemical composition of *D. hirsuta* species identified the presence of flavonoids, steroids, bibenzyls, aromatic compounds, monoterpenoids, diterpenoids, sesquiterpenoids and acetogenins in samples collected from Asia, Europe and the New World [6]. In general, sesquiterpenoids are the primary secondary metabolites identified so far for *D. hirsuta* species investigated. In the American continent, these studies have been performed mainly on plants from South America (Argentina, Brazil, and Ecuador), and Mexico [6]. No studies have been reported so far on the chemical composition of *D. hirsuta* from Central America and Panama. Previous chemical studies on the chemical composition of *D. hirsuta* have been carried out, without evaluating the differences between the two subspecies, even though this is valuable for supporting the taxonomic knowledge of the species. It is worth noting that this species is the only one included within its own family Dumortieraceae [2,6].

Liverworts, such as *D. hirsuta*, possess oil bodies in their cells, which are organelles containing lipophilic globules, which vary in number and distribution and that exhibit chemical and morphological variations [7]. These unique structures host an array of secondary metabolites such as mono-, sesqui-, and diterpenes, alcohols, phenolic compounds and polyketides [7-8]. Thus, the characteristic composition of these oil bodies make them valuable for chemosystematic studies. Indeed, it is remarkable that the volatile composition of liverworts has favored the differentiation of liverworts species, and resolved relevant taxonomic questions [9]. Furthermore, VOcs are secondary metabolites which are essential for the defense of plants against predators and for protection against environmental pathogens [10]. Headspace solid phase microextraction (HS-SPME) is a simple, fast, and sensitive method that allows for extraction of volatile compounds present in plants by using a minimal amount of sample [11]. This technique coupled with gas chromatography-mass spectrometry (GC-MS) has been widely used for the analysis of VOcs and for determination of chemical markers in plants [11-15], as well as in a vast diversity of bryophytes [6].

Notwithstanding that, there are numerous reports on the chemical composition of *D. hirsuta*, no studies have been reported on the chemistry of the subspecies of the plant, i.e. *D. hirsuta* subsp. *hirsuta* and *D. hirsuta* subsp. *nepalensis*. Therefore, we undertook this study using, HS-SPME-GC-MS analysis for determining the

Table 1: Volatile compounds identified in the Panamanian *D. hirsuta* subsp *hirsuta (Dhh)* and *D. hirsuta* subsp *nepalensis (Dhn)* by HS-SPME-GC-MS.

Peak No.	KRI	KRI*	Compounds	Relative content (%)	
				Dhh	*Dhn*
1	874	865	1-Hexanol	0.2	0.1
2	981	986	1-Octen-3-ol	0.3	5.2
3	990	975	3-Octanone	0.4	0.2
4	1000	985	Mesitylene	-	Trace
5	1055	995	Benzeneacetaldehyde	0.1	0.2
6	1070	1049	Acetophenone	-	Trace
7	1103	1065	β-Linalool	0.9	0.5
8	1119	1104	Phenylethyl alcohol	-	0.1
9	1159	1114	Veratrole	-	Trace
10	1148	1143	Camphor	Trace	-
11	1161	1162	2-Nonenal	0.2	-
12	1176	1147	Menthol	0.2	0.2
13	1204	1173	Decanal	-	0.1
14	1205	1195	*trans*-Dihydrocarvone	Trace	-
15	1206	1200	E-dihydrocarvone	-	Trace
16	1221	1200	Octyl acetate	0.2	0.1
17	1227	1215	β-Cyclocitral	0.1	Trace
18	1250	1222	Carvone	0.1	0.1
19	1303	1242	Methylnaphthalene	-	Trace
20	1300	1294	Menthyl acetate	0.1	-
21	1306	1298	Anethole	0.1	Trace
22	1357	1301	α-Cubebene	0.2	0.2
23	1387	1354	α-Gurjunene	37.3	18.1
24	1401	1396	Sativene	0.3	-
25	1407	1408	Isolongifolene	-	0.5
26	1414	1402	α-Cedrene	1.2	0.5
27	1421	1408	β-Caryophyllene	5.5	7.6
28	1432	1417	Aristolene	-	4.4
29	1438	1447	β-Gurjurene	-	3.3
30	1448	1440	α-Guaiene	9.3	6.4
31	1467	1443	Alloaromadendrene	0.2	2.7
32	1457	1452	α-Humulene	5.6	-
33	1441	1456	Aromandendrene	0.4	-
34	1474	1457	γ-Gurjunene	0.8	3.0
35	1485	1479	Valencene	0.5	6.0
36	1492	1484	δ-Cadinene	2.3	3.1
37	1498	1519	Ledene	-	35.5
38	1515	1493	β-Selinene	33.7	1.0
39	1570	1489	Nerolidol	-	0.4
40	1609	1566	Globulol	-	0.3

KRI: Kovat's retention index; KRI*: reported Kovats retention index from reference databases. Trace: less than 0.05 %.

chemical composition of VOCs from *D. hirsuta* subsp. *hirsuta* and *D. hirsuta* subsp. *nepalensis* from Panama. This study could be useful in the chemical differentiation of these *Dumortiera hirsuta* subspecies.

Both subspecies of *Dumortiera* that occur in Panama are morphologically distinct in thallus color and aspect, the presence of vestigial air chambers, and the density of dorsal papillae. *Dumortiera hirsuta* subsp. *hirsuta* includes plants, with a shiny dark green thallus, smooth dorsal surface with absence of vestigial air chambers or these restricted to the most apical section of the plant and with few dorsal papillae [2]. The second subspecies, *D. hirsuta* subsp. *nepalensis* has a light green thallus, with a velvety appearance, and remnants of air chambers with numerous papillae. These occur in most of the thallus length [2]. For this subspecies, Gudiño (unpublished) in a study of *Dumortiera* of Panama reported a density of ca. 4,000-5,800 papillae/mm^2.

Extraction of the volatile and semivolatile compounds of the whole plants of *Dumortiera hirsuta* subsp. *hirsuta* and *D. hirsuta* subsp. *nepalensis* were obtained by HS-SPME and then separated and identified by GC-MS. Headspace-solid phase microextraction (HS-SPME) is a known analytical technique that favors the detection of compounds present in the sample in small quantities, permitting identification of compounds otherwise undetectable by using other methods. The main VOCs identified in each subspecies of *D. hirsuta* with their Kovat's retention indices and percentages are presented in Table 1.

In total, the HS-SPME-GC-MS analyses allowed the identification of 40 volatile compounds for both subspecies of *D. hirsuta.* Twenty-eight compounds were identified in *Dumortiera hirsuta* subsp. *hirsuta*, while 37 compounds in *D. hirsuta* subsp. *nepalensis*. When considering only *D. hirsuta* species from Panama, 34 of the most compounds identified have not been reported for the *D. hirsuta* species of Argentina, Brazil, China, Ecuador, Indonesia, Japan, Portugal and Spain analyzed before [6]. Sesquiterpenes were the main components identified in *D. hirsuta* subsp. *hirsuta* and *D. hirsuta* subsp. *nepalensis*, representing 97.5% and 93.1% of the detected compounds, respectively. In *D.hirsuta* subsp. *hirsuta* monoterpenes (1.3%) were the second main compounds identified, followed by alcohols (0.4%), ketones (0.4%), aldehydes (0.2%), carboxylic acids (0.2%) and aromatic compounds (0.1%). On the other hand, alcohols (5.4%), monoterpenes (0.8%), aldehydes (0.3%), ketones (0.2%), aromatic compounds (0.1%) and carboxylic acids (0.1%) were identified in *D. hirsuta* subsp. *nepalensis*. Our results revealed that the main volatile compounds of *D.hirsuta* subsp. *hirsuta* were α-gurjunene (37.3%) and β-selinene (33.7%), followed by α-guaiene (9.3%), α-humulene (5.6%) and β-caryophyllene (5.5%); while the major constituents of *D. hirsuta* subsp. *nepalensis* were ledene (35.5%) as the main compound, followed by α-gurjunene (18.1%), β-caryophyllene (7.6%) and α-guaiene (6.4%) (Table 1). Earlier studies have shown that terpenes, which are found in the oil bodies of liverworts, are important markers for establishing chemical taxonomic differences between species [7-9]. Sesquiterpenes were the prevalent VOCs of both Panamanian subspecies of *D. hirsuta* analyzed in this study, while monoterpenes are present in smaller quantities. Overall, there were important differences in the type and percentage of compounds found between the two subspecies. Exclusive compounds detected in *D. hirsuta* subsp. *hirsuta* were 2-nonenal, camphor, *trans*-dihydrocarvone, menthyl acetate, sativene, aromadendrene and humelene, while, the particular compounds for the *D. hirsuta* subsp *nepalensis* were mesitylene, acetophenone, phenylethyl alcohol, veratrole, *E*-dihydrocarvone, decanal, methylnaphthalene, isolongifolene, aristolene, ledene, nerolidol, globulol, and β-gurjurene. Most of the sesquiterpenes identified for both subspecies were sesquiterpenes hydrocarbons. It is worth noting that oxygenated sesquiterpenes, nerolidol and globulol, were present only in *D. hirsuta* subsp *nepalensis*.

Sesquiterpenoids are the most characteristic compounds of *D. hirsuta* species identified so far, which have been reported mainly for samples from Asia and the American continent [6]. Saritas et al. [16], by using gas chromatography, identified β-caryophyllene, α-cubebene, valencene, α-guaiene, γ-gurjunene, in samples of *D. hirsuta* from Brazil. These components were also found in *D. hirsuta* subsp. *hirsuta* and *D. hirsuta* subsp. *nepalensis* from Panama surveyed. The volatile composition of *D. hirsuta* from Indonesia was investigated by Komala et al. [17]. They identified neophytadiene, nootkatene, guaia-6,9-dien-4β-ol, α-humulene, a dumortane derivative and α-humulene epoxide. This compound was found only in samples of *D. hirsuta* subsp. *hirsuta*. This might suggest that the subspecies of *D. hirsuta* from Indonesia could be the subspecies *hirsuta*. By using GC-MS an unknown sesquiterpenoid, germacrene D, ent-1(10) E,5Egermacradien-11-ol and 4β-hydroxygermacra-1(10),5-diene were found in the Japanese *D. hirsuta* [18]. These compounds were not found in the Panamanian subspecies of *D. hirsuta*.

Our results suggest a chemical difference between the *D. hirsuta subspecies* studied. The oxygenated sesquiterpenes globulol and nerolidol were found only in *D. hirsuta* subsp. *nepalensis*, which could probably be useful as chemical markers for differentiation

between this subspecies and *D. hirsuta*. To the best of our knowledge, this is the first report of the chemical composition of *D. hirsuta* from Central America, and that a chemical differentiation based on the volatile composition of subspecies of this liverwort is established.

HS-SPME analysis of *D. hirsuta* subsp. *hirsuta* and *D. hirsuta* subsp. *nepalensis* collected in Panama indicated that both subspecies produce characteristic volatile compounds that could be used as chemical markers for taxonomic identification. Nevertheless, this needs to be confirmed by study of samples from other geographical locations. On the other hand, when the chemical constituents of *D. hirsuta* species reported so far in the literature is compared with the species of Panama, the chemical composition of VOCs seems to be quite different from other *D. hirsuta* studied earlier, which might suggest a geographical difference in the VOCs observed within the same species.

Experimental

Plant Material: Fresh samples of *D. hirsuta* subsp. *hirsuta* and *D. hirsuta* subsp. *nepalensis* were collected at Monumento Natural Cerro Gaital, El Valle de Anton, Province of Coclé (8°37'34.6" N, 80°08'13.7" O) and in Santa Fe National Park, Province of Veraguas (08°31'37" N, 81°09'00" O) , between August and November. Samples were first identified by Jose Gudiño, and identification was 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 *D. hirsuta* subsp. *hirsuta* (JGL VA04) and *D. hirsuta* subsp. *nepalensis* (JGL VA03) were deposited at the Herbarium of the University of Panama (PMA), Panama, Panama. Both subspecies of *D. hirsuta* were classified according to Schuster (1992) [4]. Two samples of each liverwort were analyzed in duplicate and mean reported in Table 1 as percent relative concentration of each compound.

Isolation of volatile compounds by Headspace Solid-Phase Microextraction (HSPME): The divinylbenzene-carboxen-polydimethylsiloxane StableFlex SPME fiber (DVB/CAR/PDMS; 50/30 μm) and the fiber holder were purchased from Supelco (Bellefonte, PA, USA). The fiber was conditioned in the gas chromatography (GC) following the manufacturer's recommendation. A blank test was done before each analysis to verify for possible carry-over effect. For SPME analysis of the samples of *D. hirsuta* subsp *hirsuta* and *D. hirsuta* subsp *nepalensis*, 1g of each sample and 3 mL of NaCl (5%) were added in a 10 mL hermetically sealed amber vial. The DVB/CAR/PDMS fiber was then exposed to the headspace of the sample and maintained there for 15 min in a thermostatic bath at 50° C. All experiments were done under constant stirring of 500 rpm. After extraction of the volatile compounds, the SPME fiber was removed and inserted in the GC injection port for desorption of the compounds.

Analysis of Volatile Compounds by Gas Chromatography-Quadrupole Mass Spectrometry (GC-QMS): GC-QMS analyses were performed in a GC 6890 N coupled to a mass spectrometer 5975C (Agilent Technologies, Palo Alto, CA, USA). The injection port was set at 240°C and operated in splitless mode for 2 min. Helium was used as carrier gas at 1 mL/min. The 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). The oven temperature was programmed at 50°C for 2 min, then increased to 240°C at 6°C min^{-1} and held for 5 min. The mass spectrometry 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 – 550 m/z. All experiments were performed in duplicate and the results obtained expressed as average. Identification of volatile compounds was achieved by comparing the mass spectra with those provided by the National Institute of Standards and Technology library (NIST/11) and the Registry of Mass Spectral Data with Structures library (Wiley 7th edition, USA); and by using authentic standards when available. Moreover, a 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, 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 the Table for each subspecies are means of two samples of each subspecies and each analysis carried out in duplicate.

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