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Chemical Composition and Antimycobacterial Activity of the Essential Oil from *Anemia tomentosa* var. *anthriscifolia*

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The essential oil from *Anemia tomentosa* var. *anthriscifolia* showed *in vitro* activity against *Mycobacterium tuberculosis* (MIC 100 μg/ml) and therefore was characterized by gas chromatography (GC) and by gas chromatography coupled with mass spectrometry (GC-MS). The major constituents of this essential oil were triquinane sesquiterpenes: silphiperfol-6-ene (14.7%), (–)-*epi*-presilphiperfolan-1-ol (30.6%), presilphiperfol-7-ene (3.9%), cameroonan-7α-ol (4.4%), prenopsan-8-ol (1.9%) and presilphiperfolan-8-ol (8.3%), suggesting the existence of different chemotypes for this species. The essential oil was fractionated by column chromatography and its major constituent and fractions were assayed against *Mycobacterium tuberculosis* and *M. smegmatis*. (–)-*epi*-Presilphiperfolan-1-ol exhibited an MIC of 120 μg/ml against *M. tuberculosis* H37Rv.

Keywords: *Anemia tomentosa*, triquinane sesquiterpenes, *Mycobacterium tuberculosis,* Pteridophyta, tuberculosis.

Ferns are abundant in the fossil record and today include about 11,000 species, placing this group as one of the largest, after flowering plants. They are the most diverse in form and habit, and their diversity is greatest in the tropics, where approximately 75% of its population is distributed [1]. The genus *Anemia* occurs mainly in the Americas [2]. In Brazil, species from this genus are found in the central and southeast regions, where many of them are endemic [3a]. *Anemia tomentosa* (Savigny) Swartz var. *anthriscifolia* (Schrader) Mickel occurs in rocky regions and has very aromatic leaves. This variety is predominant over the four other known varieties [3b]. The leaves of *A. tomentosa* var. *tomentosa* are used as a digestive aid, expectorant and antigripal [4]. In previous studies, the essential oil of *A. tomentosa* var. *anthriscifolia* showed antimicrobial activity and mosquito repellent activity [5a,5b]. The present investigation deals with the chemical and antimycobacterial studies on essential oil of *A. tomentosa* var. *anthriscifolia*.

The essential oil was characterized by gas chromatography (GC) and by gas chromatography coupled with mass spectrometry (GC-MS) analysis. Sixty compounds were detected in the chromatogram. Thirty substances were identified by mass spectrometry and retention indices (Table 1), of which, the major components were triquinane sesquiterpenes such as silphiperfol-6-ene (14.7%), (–)-*epi*-presilphiperfolan-1-ol (30.6%), presilphiperfol -7-ene (3.9%), cameroonan-7α-ol (4.4%), prenopsan-8-ol (1.9%) and presilphiperfolan-8-ol (8.3%), which accounted for a total of 63.8 % of the total substances in the oil.

The biosynthetic origin of the triquinane sesquiterpenes from the caryophyllenyl ion was originally proposed by Bohlmann [6a,6b] based on the co-occurrence of the triquinane sesquiterpenes isocomene, modhephene, silphiperfolenes and silphinenes along with caryophyllene in *Silphium perfoliatum* L. (Asteraceae) [6a]. The co-occurrence of isocomene, modhephene, and caryophyllene in *Isocoma wrightii* (Asteraceae) was first noted by Zalkow *et al*. [6c,6d]. Additional evidence came from the report of the co-occurrence of silphiperfolenes and silphinenes with presilphiperfolanol sesquiterpenes in *Flourensia heterolepis* (Asteraceae) [6b]. Subsequently, Weyerstahl *et al*. described the presence of sesquiterpene skeletons of the type silphiperfolane, presilphiperfolane, isocomene, modhephene and caryophyllene in the essential oil of *Echinops giganteus* var. *lelyi* C. D. Adams (Asteraceae). Three new types of triquinane skeletons - cameroonane, prenopsane and nopsane - were elucidated. Based on these data, Weyerstahl proposed a biosynthetic route that covered all of those constituents, indicating a possible interrelationship between them [7]. The similarity between the compositions of essential oils from *Echinops giganteus* var. *lelyi* and from *A. tomentosa* var. *anthriscifolia* gives additional support to the biosynthetic correlation proposed by Weyerstahl. The essential oil from *A. tomentosa* var. *anthriscifolia* (Anemiaceae) is the second one that presents a large amount of triquinane sesquiterpenes in its composition biosynthetically correlated between them.

The essential oils from the plants of *A. tomentosa* have already been described in the literature. Studies from Juliani *et al*. with plants collected in Argentina described α-bisabolol as its major constituent [3b], while Santos *et al*. described isoafricanol as the main sesquiterpene from the oil obtained from a specimen collected in Rio de Janeiro, Brazil [8a]. In both studies, the presence of triquinane sesquiterpenes was not described. These data suggest the existence of different chemotypes for this species.

The essential of *A. tomentosa* var. *anthriscifolia* oil was fractionated by liquid column chromatography to access the antimycobacterial activity of its major constituent, (–)-*epi*-presilphiperfolan-1-ol, and fractions. By this procedure, 18 fractions were generated (A1 to A18) and characterized by GC-MS (Table 2). The triquinane sesquiterpene (–)-*epi*-presilphiperfolan-1-ol [16] was isolated in fraction A9 with 99% purity (by GC-FID) (Table 2) which showed lower antimycobacterial activity (MIC of 120 μg/mL) than the essential oil. The antimycobacterial activities of fractions A1 to A-18 are shown in Table 2. Fraction A1, consisting of unsaturated hydrocarbon compounds, mainly α-guaiene and silphiperfol-6-ene, showed an MIC of 25 μg/mL.

Table 1: Chemical composition of the essential oil from *A. tomentosa var. anthriscifolia*.

Compound	$RI_{calc.}$	$RI_{lit.}$	%	IM[#]
α-Pinene	938	939	0.1	1, 2
trans-Sabinol	1142	1142	0.6	1, 2
trans-2-Caren-4-ol	1149	-	0.2	2
Pinocarvone	1166	1165	0.2	1, 2
p-Menta-1,5-dien-8-ol	1170	1170	0.1	1, 2
cis-Pinocamphone	1178	1175	0.1	1, 2
Thymol	1293	1290	0.1	1, 2
Silphiperfol-5-ene	1327	1329	0.6	1, 2
Presilphiperfol-7-ene	1334	1337	3.9	1, 2
7-*epi*-Silphiperfol-5-ene	1346	1348	1.6	1, 2
α-Cubebene	1348	1351	0.1	1, 2
Silphiperfol-4,7(14)-diene	1359	1361	0.1	1, 2
Longicyclene	1370	1374	1.0	1, 2
Silphiperfol-6-ene	1378	1379	14.7	1, 2
α-Isocomene	1388	1388	0.1	1, 2
β-Elemene	1393	1391	0.2	1, 2
(*E*)-Caryophyllene	1421	1419	0.6	1, 2
α-Guaiene	1437	1440	5.2	1, 2
α-Muurolene	1482	1480	0.1	1, 2
Cameroonan-7-α-ol	1509	1512	4.4	1, 2
(–)-*epi*-Presilphiperfolan-1-ol	1518	-	30.6	3
Silphiperfolan-7-β-ol	1523	1521	0.7	1, 2
Nopsan-4-ol	1529	1531	1.0	1, 2
Silphiperfolan-6-β-ol	1546	1548	1.1	1, 2
Prenopsan-8-ol	1575	1576	1.9	1, 2
Presilphiperfolan-8-ol	1584	1586	8.3	1,2
di-*epi*-α-Cedrene epoxide	1591	-	0.4	2*
β-Atlantol	1610	1608	0.7	1, 2
Caryophylla-4(14),8(15)-dien-5-α-ol	1638	1641	1.0	1, 2
Ishwarone	1681	1682	0.5	1, 2
α-Bisabolol	1687	1686	0.8	1, 2
Monoterpene hydrocarbons			0.1	
Oxygen containing monoterpenes			1.2	
Sesquiterpene hydrocarbons			28.2	
Oxygen containing sesquiterpenes			51.4	
Total identified			81.0	

[#]Identification methods: 1- retention indices; 2- Wiley library; 3- ^{1}H and ^{13}C-NMR [8]. *tentative identification.

Haermers *et al*. [9a] reported the high lipophilicity of substances as an important feature for antimycobacterial activity. Since the cell wall of mycobacteria contain lipophilic substances such as mycolic acid, more lipophilic substances are likely to penetrate more easily into the cell [9b]. This may partially explain why the fraction containing the unsaturated sesquiterpenes (A1) showed higher activity against the *M. tuberculosis*. However, lipophilicity is not the only requirement for antimycobacterial activity, since A3, also containing unsaturated sesquiterpenes (but also unidentified oxygenated sesquiterpenes), displayed a higher MIC than A1. β-bisabolene and caryophyllene oxide, which were identified in fraction A3, were not

Table 2: Composition (GC-MS/GC-FID) of the fractions from the essential oil of *A. tomentosa* var. *anthriscifolia* and their minimum inhibitory concentration (MIC, in μm/mL) against *Mycobacterium tuberculosis* (H37Rv) and *M. smegmatis* (mc^2155).

Fraction	Identified Constituents (%)	MIC	
		H37Rv	mc^2 155
A1	α-Guaiene (17.3), Silphiperfol-6-ene (39.6)	50	200
A3	Silphiperfol-5-ene (1.8), Presilphiperfol-7-ene (8.5), Silphiperfol-6-ene (48.0), α-Isocomene (1.0), β-Elemene (0.5), *E*-Caryophyllene (2.3), α-Guaiene (17.0), α-Muurolene (0.4), β-Bisabolene (5.0), Caryophyllene oxide (0.7)	100	200
A5	Pinocarvone (0.5), Presilphiperfol-7-ene (0.3), Silphiperfol-6-eno (0.1), Cameroonan-7-α-ol (18.9), Silphiperfolan-7-β-ol (2.1), Prenopsan-8-ol (7.3), Presilphiperfolan-8-ol (31.3)	100	200
A7	(–)-*epi*-Presilphiperfolan-1-ol (89.0), Prenopsan-8-ol (2.0), di-*epi*-α-Cedrene epoxide (t), Cameroonan-7-α-ol (t), Ishwarone (t)	100	200
A8	(–)-*epi*-Presilphiperfolan-1-ol (91.0)	100	200
A9	(–)-*epi*-Presilphiperfolan-1-ol (99.0)	120	-
A18	*trans*-Sabinol (1.6), (–)-*epi*-Presilphiperfolan-1-ol (0.9), Silphiperfolan-7-β-ol (1.4), Silphiperfolan-6-β-ol (31.2)	R	-

t < 0.1%; R – resistant; - not tested

detected in the crude oil and may be artifacts that formed during essential oil fractionation procedures [10a,10b]. Fractions A5 to A8, containing sesquiterpene alcohols, showed the same MIC as the crude oil. Fractions A10 to A17 showed similar chromatographic profiles by TLC and GC, consisting basically of (–)-*epi*-presilphiperfolan-1-ol in different degrees of purity (always above 80%, data not shown) and therefore were not screened for antimycobacterial activity nor fully characterized by GC-MS. The major compound of fraction A18 is silphiperfolan-6β-ol (31.2%, Table 2), which is an isomer of silphiperfolan-6α-ol, a triquinane sesquiterpene isolated from the red algae *Laurencia majuscula* [11]. The latter showed an MIC of 120 μg/mL, corroborating our results for this fraction, which was inactive at 100 μg/mL towards *M. tuberculosis* H37RV (Table 2). In addition to the susceptible strain of *M. tuberculosis* (H37Rv), the essential oil fractions from *A. tomentosa* var. *anthriscifolia* were assayed against *M. smegmatis* (mc^2155), one environment mycobacterial species (Table 2). The assayed fractions, as well as (–)-*epi*-presilphiperfolan-1-ol, were active against this strain only at 200 μg/mL. In fact, environmental species show higher resistance profiles than classical pathogens due to lower permeability or increased drug efflux.

To the best of our knowledge, this is the first report of the antimycobacterial activity of the essential oil from *A. tomentosa* var. *anthriscifolia*. From comparison with chemical data published for this variety, the occurrence of chemotypes is being suggested.

Experimental

General procedures: Gas chromatography analyses were performed with a HP 5890 Series II gas chromatograph equipped with a FID detector and an HP-5 (5% phenyl/95% polydimethylsiloxane) fused silica capillary column (25 m x 0.2 mm, film thickness 0.33 μm) using hydrogen as carrier gas (1.0 mL min^{-1}). The injector temperature was 250ºC and the column oven programmed was 60–240ºC at 3ºC min^{-1}. The detector (FID) was operated at 280ºC. The GC/MS was performed with an Agilent 5973MSD coupled to an Agilent 6890 gas chromatograph, using helium as carrier gas, and the same column and oven conditions as above. Transfer line temperature was 240ºC, ion source was at 230ºC, EIMS, 70 eV. Constituents of the oil were identified by comparing the experimental gas chromatographic retention indices RI and MS fragmentation pattern with corresponding reference data [12a,12b]. A standard solution of *n*-alkanes (C_7–C_{26}) was used to obtain the retention indices.

Plant material and extraction: Aerial parts of *A. tomentosa* var. *anthriscifolia* were collected on a rocky hillside of Vila Velha, Espírito Santo State, Brazil. The plant was identified by Dr. Claudine Mynssen from the Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, and a voucher specimen (RB438912) is deposited at the herbarium.. The essential oils from fresh aerial parts were obtained by hydrodistillation in a Clevenger-type apparatus for 2 hours, yielding 0.3 % of a light yellow essential oil.

CC separations: The column (h=54cm; Ø=1.5cm) was packed with silica gel 60 (230-400 Mesh ASTM) as the stationary phase. Mixtures of hexane/ethyl acetate (100:0 to 95:5, v/v) of increasing polarity were used as eluent. The sample of essential oil (1.5 g) to be separated into components was dissolved in a small amount of hexane. This solution is loaded onto the column.

Antimycobacterial tests: Samples were screened against *Mycobaterium tuberculosis* strain H37Rv (ATCC - 27294), *Mycobacterium smegmatis* (mc^2 155), using the resazurin (redox) bioassay [13-15]. The final concentration of the essential oil, substances and fractions was either 200 µg/mL or 100 µg/mL. Media plus bacteria with and without rifampicin were used as controls. In brief, the assay is accomplished in microplates (96 wells) using resazurin as indicator of cellular viability. The minimal inhibitory concentration (MIC) was determined (starting from 200 µg/mL in 1:2 serial dilutions).

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