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Essential Oils from Plants and in Vitro Shoots of *Hypericum androsaemum* L.

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The essential oil yields obtained by hydrodistillation of the aerial parts of *Hypericum androsaemum* cultivated plants varied from 0.94 to 4.09 mg/g of biomass dry weight, depending of the harvest time. The respective analyses performed by gas chromatography and gas chromatography−mass spectrometry revealed more than 80 compounds, 72 of which were identified. Most of the compounds were sesquiterpene hydrocarbons, which, depending of the harvest time, corresponded to 43−78% of the total essential oil. The other compounds were distributed as monoterpene hydrocarbons, oxygen-containing sesquiterpenes, *n*-alkanes, 1-alkenes, and oxygen-containing monoterpenes, these being a minor group. In *H. androsaemum* in vitro shoots, sesquiterpene hydrocarbons represented >80% of the respective essential oil. Differences in the essential oil composition were found depending on the harvest time and origin, in vivo versus in vitro, of the plant material. The essential oil sampled in November was characterized by the highest levels of sesquiterpene hydrocarbons and a high number of *n*-alkanes and 1-alkenes, from C_{18} to C_{28}, whereas that sampled in June of the following year showed the highest levels of *n*-nonane and 1-octene as well as monoterpene hydrocarbons, the second most representative group.

KEYWORDS: ***Hypericum androsaemum*; in vitro shoots; essential oils; sesquiterpenes**

INTRODUCTION

Hypericum androsaemum L. grows wild in shadowy sites, namely, in the northern region of Portugal, where it is widely used as a medicinal herb. According to some authors this species is used in popular medicinal preparations as a cholagogue, hepatoprotector, and diuretic and in kidney failure (*1*, *2*). Usually, in northern Portugal, wild-growing *H. androsaemum* plants are harvested, air-dried, and sold by the local people to tourists, who use it to prepare the tea called "hipericão do Gerês". However, due to intensive harvesting, the local wild populations of this species are in risk of disappearing.

To meet the needs of the consumers, we established four small *H. androsaemum* experimental fields in northern Portugal where the species is being propagated. The respective chemical characterization is also one of the objectives of an integrated research and development program, which has as its goal to restock the local *H. androsaemum* populations and increase the overall knowledge on this species as well as to support its commercial and industrial exploitation.

Most of the few recent reports on the chemical characterization of this species concern the composition of phenolic extracts. Phenolic acids and flavonoids (*2*−*5*) as well as xanthones (*6*) and xanthone *C*-glucosides (*7*) have been identified in *H. androsaemum* plants. The essential oil of this species has been less studied. Nonane, α-pinene, *β*-pinene, myrcene, limonene, and undecane (*8*) as well as geraniol and α-terpineol (*9*) were identified as constituents of the *H. androsaemum* essential oil. The presence of α-terpineol and hydrocarbon waxes ($C_{19}H_{40}$, $C_{21}H_{44}$, and $C_{23}H_{48}$), in the *H. androsaemum* unripened seed capsules, was also reported (*10*). Five monoterpene and eight sesquiterpene compounds as well as the *n*-alkanes nonane and undecane were identified in the essential oils of this species (*11*). However, the available data for chemical characterization of the *H. androsaemum* essential oil are scanty. Less is known on metabolites produced by in vitro cultures of this species. Phenolic acids and flavonoid compounds (*5*) and xanthones (*12*) were identified in in vitro cultures of calli and suspended cells of this species. Up to now, however, we have found no studies on essential oils produced by in vitro cultures of *H. androsaemum*.

In recent years we have performed the micropropagation of *H. androsaemum* with the aim of helping to restock this species. Parallelly with the characterization of the essential oils of the *H. androsaemum* in vivo plants we consider that the capacity

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of the in vitro shoot cultures of this species in producing essential oils would deserve to be evaluated and the respective composition determined. In this paper we report the yields and composition of essential oils accumulated in the aerial part of in vivo *H. androsaemum* plants, cultivated at Arcos de Valdevez, as well as the yield and composition of the essential oil accumulated in the respective in vitro shoots.

MATERIALS AND METHODS

In Vivo Cultivated Plants and Establishment of in Vitro Shoot Cultures. *H. androsaemum* L. plant cultures were established in March 1998 at a DRAEDM experimental farm located at Arcos de Valdevez (northern Portugal). The cultivation was performed by planting ±15 cm long slips obtained from wild *H. androsaemum* plants growing near Ponte de Lima (Facha). A voucher specimen (herbarium voucher ref. H.a.-AV1998) is maintained in ERCA/DRAEDM.

In vitro shoot cultures of *H. androsaemum* were established on chemically defined medium containing the macronutrients of Margara N30K medium (*13*) and the micronutrients and organic constituents of the Murashige and Skoog (MS) medium (*14*) with the exception for thiamin, which was used at 0.8 mg/L. Ascorbic acid at 3 mg/L, with 30 g/L sucrose, 0.5 mg/L benzylaminopurine (BAP), 0.05 mg/L α-naphthaleneacetic acid (NAA), and 0.05 mg/L gibberellin (GA_3), was added to the medium before pH adjustment at 5.7. Agar at 8 g/L was added to solidify the medium before autoclaving at 120 °C for 20 min. Apical buds from wild plants growing in Facha were excised, surface sterilized with a solution of 2% formaldehyde in 70% ethanol, during 10 min, and used as primary explants in the establishment of the cultures. The MS medium supplemented with 0.8 mg/L indol-3-acetic acid (IAA) and 0.5 mg/L kinetin (KIN) was used in multiplication of the shoot cultures. Cultures were maintained in a growth room at 24 ± 2 °C and 60−65% humidity with a photoperiod of 16 h of light/8 h of darkness. Illumination was supplied by cool white fluorescent tubes with a light intensity of 52 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The cultures were subcultivated on the multiplication MS medium with the interval of 2 months.

Hydrodistillation and Analysis of the Essential Oils. For the study of the essential oil composition, some cultivated plants were randomly pruned in July and November 1999 and in June 2000, and subsamples of ∼10 g of fresh biomass of the pruned branches were subjected to hydrodistillation in a Clevenger-type apparatus over 1 h, using volumes of 1.0 mL of *n*-hexane, containing 5α-cholestane (1 mg/mL), for retention of the hydrodistillate components. The humidity percentage from fresh biomass of the samples submitted to hydrodistillation and harvested in July and November 1999 and June 2000 were 79.6, 81.6, and 74.4%, respectively. The dry weight of the plant material was determined after the respective drying at 60 °C in a drying stove during 72 h. The same procedure was followed in the hydrodistillation of 6-month-old in vitro fresh shoots (91.9% of humidity) maintained by subculturing, on the multiplication MS medium, during ∼2 years.

The hydrodistillates from all samples were analyzed by gas chromatography (GC) and gas chromatography−mass spectrometry (GC-MS). GC analyses were performed using a Perkin-Elmer Autosystem gas chromatograph equipped with a fused silica DB-5 column (30 m long × 0.25 i.d., 0.25 μm film thickness composed by 5% phenyl methylpolysiloxane, J&W Scientific). The temperature program was as follows: 60−285 °C at 3 °C min^{-1} for the column, 300 °C for the injector, and 300 °C for the flame ionization detector (FID). H_2 was used as carrier gas with a flow rate of 1.49 mL/min under a column head pressure of 12.5 psi. Injections were performed in a split/splitless injector with the splitter opened at a 1:13 split ratio. Three replicates of each sample were processed in the same way. Percentage values from the listed compounds correspond to the values given in the GC report without correction factors.

Following the procedure reported before for *Salvia officinalis* (*15*), 5α-cholestane was used as an internal standard for estimation of the specific content of each essential oil compound. This internal standard accounted for the differential responses of the FID and for the column inlet discrimination of the essential oil compounds due to the injector split ratio. Considering that the determination of individual correction factors is impractical, due to either the high number of compounds or their absence in the market, compounds of a given group (monoterpene hydrocarbons, oxygen-containing monoterpenes, sesquiterpene hydrocarbons, and oxygen-containing sesquiterpenes) were assumed to have the same GC response factor. Three replicates of mixtures at equal amounts of 5α-cholestane and limonene (monoterpene hydrocarbon), camphor (oxygen-containing monoterpene), (*E*)-caryophyllene (sesquiterpene hydrocarbon), and (*E*,*E*)-farnesol (oxygen-containing sesquiterpene) were prepared and injected three times each. The respective average correction values, corrected for the purity grade of each reference compound, were 0.741 (limonene), 1.014 (camphor), 0.747 [(*E*)-caryophyllene], and 1.018 [(*E*,*E*)-farnesol]. These values were used as GC response factors of the compounds of the corresponding group from *H. androsaemum* essential oil. A correction factor of 1 was assumed for compounds that did not belong to any of these groups, as they are the cases of *n*-alkanes and 1-alkenes. The sum of the specific contents of all individual essential oil compounds was assumed as a parameter for the determination of the total specific essential oil yield. Given the generalization of each response factor to all compounds from the same group, all of the quantitative data expressed in micrograms of the compound per gram of biomass dry weight may be considered as a tentative of approximation to the absolute quantification.

GC-MS analyses were performed with a Perkin-Elmer 8500 gas chromatograph equipped with a fused silica DB-5 column, as that of GC, connected with a Finnigan MAT ion trap detector (ITD; software version 4.1) operating in EI mode at 70 eV. Injector, interface, and ion source temperature were 300, 260, and 220 °C, respectively. The oven temperature program and injection conditions were as above-described for GC. Helium was used as carrier gas with a column head pressure of 12.5 psi. The identification of the compounds was performed with the help of mass spectral libraries. The compounds were considered to be identified when the respective identity was confirmed by at least two methods according to recommendations of the International Organization of the Flavor Industry (*16*). The identification of some compounds (those not denoted in **Table 1**) was confirmed by using authentic standards (all of them from the Sigma-Aldrich group). In the confirmation of the identity of compounds, not available in the market, a terpene library incorporated in the computer database by the GC-MS supplier was used, which allows the comparison of mass spectra from ITD and retention times on DB-5. The coherence of the retention times of the analyzed compounds with the retention times obtained in similar conditions with a DB-5 column and published in the literature, namely, in refs *17* and *18*, constituted an additional criterion in the confirmation of the respective identity.

RESULTS AND DISCUSSION

Multiplication and Maintenance of in Vitro Shoot Cultures. The new shoots formed from the apical buds cultivated on the establishment medium were visible 2−3 weeks after the beginning of the culture. The subculture of nodal shoot segments to the MS medium supplemented with 0.8 mg/L IAA and 0.5 mg/L KIN allowed the multiplication of the cultures by a factor of ∼5, with intervals of 2 months. However, due to slow growth, shoots could be maintained in the same vessel, without subculture, during several (6−8) months. Two years after the establishment of the *H. androsaemum* in vitro cultures, either the shape or the multiplication rate of the shoots was maintained without apparent change. Micropropagation was accomplished by transferring the shoots to half-strength MS medium without growth regulators, for their elongation, followed by auxin shock for rhizogenesis induction (results not shown).

Yields and Composition of Essential Oils Produced by in Vivo Cultivated Plants. The essential oil yields obtained in the hydrodistillation of aerial parts of *H. androsaemum* cultivated plants ranged from 0.9 to 4.1 mg/g of biomass dry weight depending of the harvest time (**Figure 1**). Variations in the essential oil content of *H. androsaemum*, from 0.6 to 1.4 mg/g, had already been reported by other authors (*19*). Drastic

Table 1. Specific Compound Contents and Percentage Composition of the Essential Oils from in Vivo Plants Cultivated at Arcos de Valdevez, Northern Portugal, and from in Vitro Shoots of *H. androsaemum* L.[a]

		in vivo cultivated plants						in vitro shoots	
		July 99		Nov 99		June 00			
compound	retention time (s)	μg/g of dw	%	μg/g of dw	%	μg/g of dw	%	μg/g of dw	%
1-octene	142	10.7	0.9	81.4	1.6	249.0	7.3	1.0	0.2
(*E*)-2-hexenal	176	31.1	2.4	275.1	5.3	271.9	7.9	2.3	0.3
n-hexanol	185	1.4	tr	5.2	0.1	4.5	0.1	1.0	0.2
n-nonane	214	36.5	2.9	69.8	1.3	121.8	3.6	3.8	0.3
α-thujene	249	1.6	tr	4.3	0.1	3.3	0.1	0.5	tr
α-pinene	258	5.6	0.5	13.4	0.3	81.3	3.2		
2,6-dimethyloctane (MS)	303	5.2	0.5	8.2	0.2	7.2	0.2	3.1	0.3
β-pinene	314	38.2	4.2	85.2	2.2	163.5	6.4	14.8	2.1
myrcene	334	9.2	0.9	10.9	0.3	34.4	1.3	1.2	0.2
limonene	397	52.0	5.9	82.7	2.1	393.1	15.4	9.2	1.3
1,8-cineole	401	1.6	tr	3.8	0.1				
(*E*)-β-ocimene	433	12.7	1.4	24.6	0.7	16.0	0.6	1.0	0.2
γ-terpinene	453	3.0	0.5	3.8	0.1	4.7	0.2	0.5	tr
terpinolene	512	36.3	3.9	55.0	1.4	39.0	1.5	4.2	0.6
undecane	536	7.2	0.6	5.8	0.1	28.6	0.9	36.0	3.8
α-thujone	549	5.8	0.5	14.1	0.3	14.3	0.5		
β-thujone	574	1.0	tr	0.6	tr	1.6	tr		
camphor	633	1.5	tr	0.7	tr				
δ-elemene*	1116	3.9	0.6	15.5	0.4	5.1	0.2	0.4	tr
α-terpinyl acetate*	1152	3.0	0.5	6.1	0.1	6.1	0.2	1.4	0.2
α-ylangene*	1201	0.6	tr	0.8	tr	0.8	tr	2.3	0.3
α-copaene	1213	0.8	tr	3.5	0.1	2.0	0.1		
β-bourbonene*	1234	0.8	tr	8.5	0.2	2.0	0.1	0.8	0.2
$C_{15}H_{24}$	1241	1.1	tr	9.1	0.2	2.7	0.1	0.4	tr
β-elemene*	1257	9.8	1.1	104.2	2.7	30.1	1.2	6.8	1.0
(E)-caryophyllene	1325	115.1	12.5	585.0	15.1	243.1	9.4	36.1	5.0
cis-thujopsene	1344	1.7	tr	20.0	0.5	5.9	0.2	2.2	0.3
β-gurjunene	1348	54.9	6.1	601.1	15.5	192.3	7.6	61.2	8.6
α-*trans*-bergamotene*	1360	1.0	tr	2.6	0.1	1.0	tr	1.1	0.2
α-guaiene* + β-humulene	1370	12.4	3.4	2.7	0.1	2.1	0.1	12.5	1.8
$C_{15}H_{24}$	1390	18.7	3.4	127.6	4.9	64.5	2.5	8.3	1.2
α-himachalene*	1399	0.6	tr						
α-humulene	1411	0.3	tr	5.0	0.1	1.2	0.1	4.3	0.7
allo-aromadendrene	1427			2.1	0.1	0.8	tr	0.8	0.2
$C_{15}H_{24}$ (germacrene D isomer[b])	1435	6.5	0.5						
unknown	1464	5.0	0.5	21.9	0.4	14.0	0.4	21.4	2.2
β-chamigrene	1468	tr	tr						
$C_{15}H_{24}$ (germacrene D isomer[b])	1470	21.9	1.9						
γ-muurolene*	1479	36.1	3.8	170.1	4.4	54.4	2.1	108.8	15.3
germacrene D* + γ-curcumene*	1487	43.0	5.0	298.6	7.7	149.3	5.8	30.7	4.3
β-selinene*	1493	5.6	0.8	36.6	0.9	17.0	0.6		
α-selinene*	1514	4.3	0.5	4.9	0.1	1.6	0.1	1.5	0.2
germacrene B[b]	1517	2.9	0.5	40.7	1.1	11.7	0.4	28.6	3.9
α-zingiberene*	1524	2.0	0.5	28.5	0.7	9.1	0.3	6.0	0.8
$C_{15}H_{24}$	1527	1.0	tr	14.0	0.4	1.9	0.1		
α-muurolene*	1540	2.4	0.5	4.6	0.1	1.7	0.1	1.4	0.2
α-(*E*,*E*)-farnesene*	1554	4.2	0.7	4.0	0.1	2.9	0.1	10.9	1.5
(*Z*)-γ-bisabolene*	1573	2.6	0.5	19.5	0.5	8.5	0.4	11.5	1.6
δ-cadinene	1585	5.9	0.6	15.6	0.4	8.5	0.3	7.7	1.1
(*E*)-γ-bisabolene*	1610	8.0	0.9	65.1	1.7	36.1	1.4	77.1	10.8
$C_{15}H_{24}$	1620	6.8	0.6	19.7	0.5	14.4	0.5	13.3	1.9
$C_{15}H_{24}$	1626	54.5	5.2	72.3	1.9	38.3	1.5	78.2	11.0
γ-elemene[b]	1662	73.5	8.5	699.1	17.9	212.1	8.0	69.4	9.8
caryophyllene oxide	1722	3.3	0.5	9.5	0.2	8.2	0.2	1.0	0.2
$C_{15}H_{24}O$	1773	3.6	0.5	7.0	0.1	3.8	0.1		
$C_{15}H_{24}O$	1816	6.9	0.5	38.9	0.7	22.4	0.6	4.8	0.5
epi-α-cadinol*	1869	8.4	0.6	17.9	0.3	15.0	0.4	2.6	0.3
epi-α-muurolol*	1876	14.2	1.1	20.0	0.4	24.6	0.7		
δ-cadinol*	1881	tr	tr	12.2	0.2			8.5	0.9
$C_{15}H_{24}O$	1889	16.3	1.4	41.6	0.8	47.5	0.9		
α-eudesmol*	1896	8.7	0.5			1.7	tr		
α-cadinol*	1904	5.4	0.5	28.4	0.5	22.3	0.6	10.6	1.2
unknown	1918	39.4	2.9	6.9	0.1	3.8	0.1	2.4	0.2
unknown	1922	52.5	6.9	75.0	1.4	74.4	2.1	26.5	2.7
epi-α-bisabolol*	1989	1.7	0.5	3.8	0.1	2.5	0.1	2.3	0.2
8-cedrane-13-al[b] (MS)	2021	2.8	0.5	8.2	0.2	4.4	0.1		
n-octadecane	2631			tr	tr				
1-nonadecene	2811			tr	tr				
n-nonadecane	2822			1.3	tr	0.9	tr		
1-heneicosene	2995			0.7	tr				

Table 1. (Continued)

compound	retention time (s)	in vivo cultivated plants						in vitro shoots	
		July 99		Nov 99		June 00			
		μg/g of dw	%	μg/g of dw	%	μg/g of dw	%	μg/g of dw	%
n-heneicosane	3007			2.1	0.1	1.3	tr		
1-docosene	3173	1.5	tr						
n-docosane	3183			5.6	0.1	3.3	0.1		
1-tricosene	3344			4.7	0.1	0.3	tr		
n-tricosane	3353			3.7	0.1	3.3	0.1		
1-tetracosene	3507			tr	tr				
n-tetracosane	3517	0.6	tr	18.5	0.4	6.4	0.3		
1-pentacosene	3665			2.3	0.1				
n-pentacosane	3673			2.3	0.1	2.7	0.1		
unknown	3724			2.0	0.1				
n-hexacosane	3829			17.1	0.3	3.2	0.2	1.1	0.2
unknown	3882	1.5	tr	0.9	tr				
1-heptacosene	3973			0.9	tr				
n-heptacosane	3979			0.9	tr				
n-octacosane	4116			5.2	0.1	1.8	0.2		
unknown	4315			0.8	tr	0.9	0.1		

[a] Identification of the compounds was confirmed with authentic standards. Both mass spectra and retention times of the compounds marked with an asterisk match with those of the corresponding compounds from the terpene library of the computer database, and the respective retention times match with the corresponding compounds described in the literature (*17*, *18*). dw, biomass dry weight; tr, trace amounts; (MS), tentative identification based on the mass spectra. [b] Both mass spectra and retention times match with the corresponding compounds from the terpene library of the computer database, and the respective retention times match with the corresponding compounds described in ref *17*. However, according to ref *18*, the retention times on DB-5 would be higher for germacrene B and lower for γ-elemene.

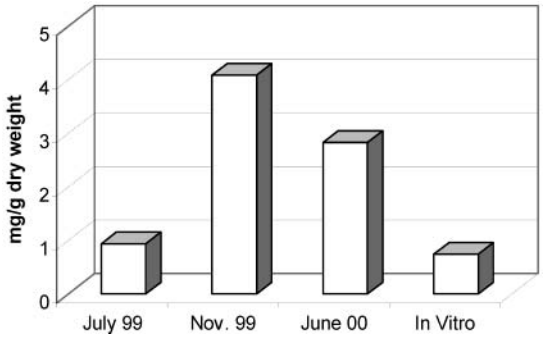

Figure 1. Essential oil contents in in vitro shoots of *H. androsaemum* and in the respective in vivo plants, cultivated at Arcos de Valdevez and harvested in the months of July and November 1999 and June 2000.

variations in the essential oil contents of plants from another species (*S. officinalis*), cultivated at the same site during the same time period, had already been reported (*15*).

More than 80 compounds were detected in the hydrodistillates from *H. androsaemum* in vivo plants, 72 of which were identified, with 4 of them remaining doubtful (**Table 1**). Depending on the harvest time, the identified compounds correspond to 80−90% of the total essential oil specific amount. With the exceptions of linalool, *trans*-β-farnesene, and bicyclogermacrene, the compounds previously reported by Nogueira et al. (*11*) were found in our samples as well as all six of the ones identified by other authors (*8*). Geraniol and α-terpineol, previously reported as constituents of the essential oil of this species (*9*), were not found in our samples. The unidentified compounds include 7 sesquiterpene hydrocarbons and 3 oxygen-containing sesquiterpenes.

For plants harvested in July 1999 and June 2000, 69 and 71 compounds, respectively, were detected in the essential oils (**Table 1**). The highest number of compounds (82) was found in the essential oil from plants harvested in November 1999. The excess compounds detected in essential oils from samples harvested in November 1999 were mainly due to the presence of an almost complete series of *n*-alkanes and 1-alkenes, from C_{18} to C_{28} (**Table 1**). To our knowledge, the presence of these *n*-alkanes and 1-alkenes in the essential oils of *H. androsaemum* has not have yet been reported. However, *n*-nonadecane, *n*-heneicosane, and *n*-tricosane had already been identified in unripened seed capsules of *H. androsaemum* (*10*). The presence of a complete series of *n*-alkanes, from $C_{16}H_{34}$ to $C_{29}H_{60}$, in dried leaf material of *H. perforatum* had also been reported (*20*).

Notwithstanding the high number of constituents, almost 50% of the total essential oil from *H. androsaemum* plants harvested in November 1999 was composed by three sesquiterpenes: (E)-caryophyllene (15.1%), β-gurjunene (15.5%), and the putative γ-elemene (17.9%). These compounds were among the five major constituents of the essential oils of plants harvested in July and June (**Table 1**). However, instead of a sesquiterpene, the major compound in the essential oil sample of June 2000 was a monoterpene (limonene, 15.4%). Undoubtedly, either the content or the composition of the essential oil from *H. androsaemum* plants changes with the time of harvest. Variations in essential oil composition could be induced by different physiological or environmental factors, the variation of which during the vegetative cycle may influence compound turnover. The effects of physiological factors, such as the relative development and maturation of the plant organs, and environmental factors, such as soil mineral fertilization, ligth intensity, climate conditions, and season, on the composition of essential oils of other plant species are well documented (*15* and references cited therein). The attack of the cultivated plants by some organisms constitutes another type of factor that contingently can influence the essential oil composition (*21* and references cited therein). Frequently, *H. androsaemum* plants growing wild in several regions of northern Portugal appear to be contaminated by a rust fungus. On the studied cultivated plants, we identified two contaminant organisms: a rust fungus—*Uromyces* sp.—and an aphis—*Aphis gossypii* Glover. The populations of these contaminants appeared in April 1999 and rose during the following spring and summer months. However, any interpretation inherent to the respective effects on the essential oil composition is speculative, because no type of control of either physiological or environmental factors was made.

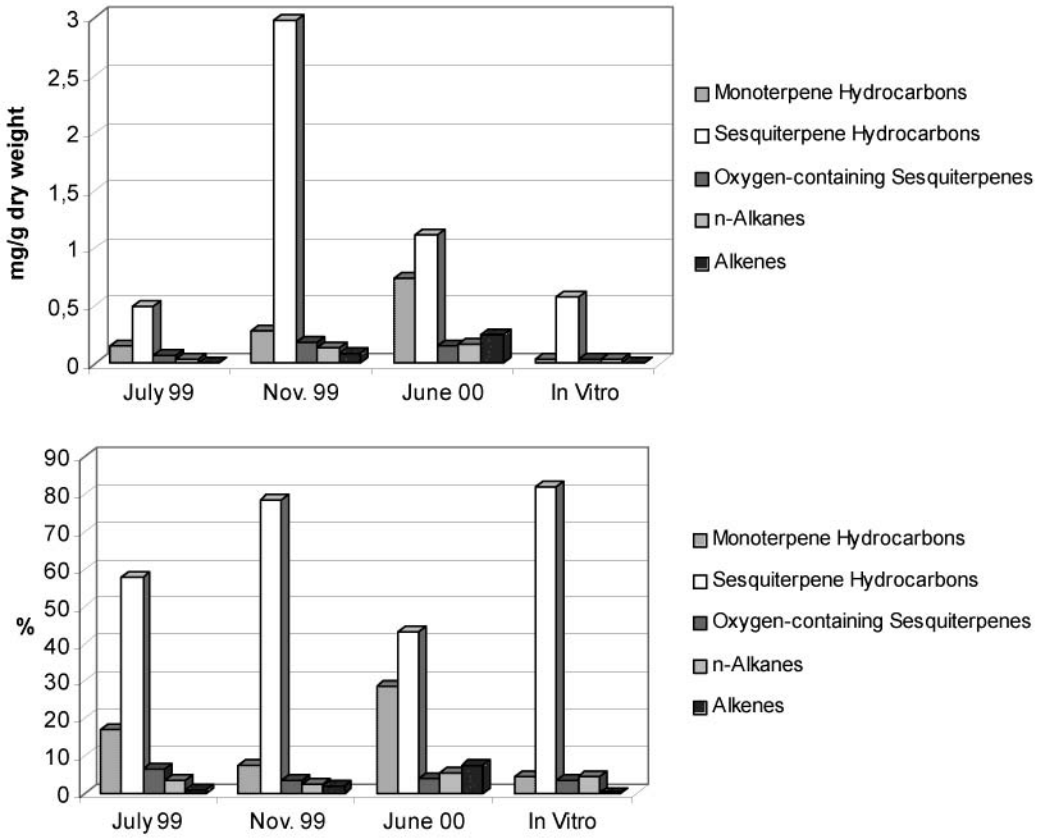

Figure 2. Specific and relative contents of the main compound groups in the essential oils from *H. androsaemum* in vitro shoots and respective in vivo plants cultivated at Arcos de Valdevez and harvested in the months of July and November 1999 and June 2000.

Most of the compounds from the essential oils of *H. androsaemum* can be distributed in five groups: monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxygen-containing sesquiterpenes, *n*-alkanes, and 1-alkenes. **Figure 2** shows the specific and relative amounts of each of these compound groups in the studied *H. androsaemum* essential oils. Independently of the harvest time, the sesquiterpene hydrocarbons constituted the major compound group, accounting for >40−78% of the total essential oil (**Figure 2**). Sesquiterpenes had already been considered to be the main compounds from the essential oils of this species (*11*), and despite the fact that the identity of the most of them was unknown, they were considered to be responsible for the specific essential oil olfactroscopic pattern of *H. androsaemum* L. (*22*). Oxygen-containing monoterpenes are often the main compound group in the essential oils from other species. In the essential oils here studied, this group represented no more than 0.2−1.0%.

The main compounds that accounted for most of the percentage variations in each group were β-pinene, limonene, α-pinene, and terpinolene, in monoterpene hydrocarbons; (E)-caryophyllene, β-gurjunene, γ-muurolene, (*E*)-γ-bisabolene, and the putative γ-elemene, in sesquiterpene hydrocarbons; *epi*-α-muurolol, in the oxygen-containing sesquiterpenes; *n*-nonane and *n*-undecane, in *n*-alkanes; and 1-octene, in 1-alkenes (**Table 1**).

Yield and Composition of the Essential Oil Produced by in Vitro Shoots. The essential oil yield obtained by hydrodistillation of in vitro *H. androsaemum* shoots (0.74 mg/g of biomass dry weight) was lower than the minimum value obtained from the in vivo cultivated plants (**Figure 1**). Either the different conditions of growth or the immaturity of the in vitro shoots compared to those of in vivo plants may be responsible for the respective low content of essential oil. Analyses by GC and GC-MS revealed the presence of 52 constituents, all of them common to the essential oils of in vivo plants (**Table 1**). Sesquiterpene hydrocarbons were the major compound group, representing >80% of the total essential oil, a value higher than that of the same group from the essential oil of in vivo plants harvested in November 1999 (**Figure 2**). The major compound of the in vitro shoots essential oil (γ-muurolene, 15.3%) was not among the five most represented constituents of the essential oils from in vivo plants. The same was true for the second and third most represented compounds (**Table 1**). On the other hand, from the series of *n*-alkanes and 1-alkenes identified in in vivo plants, only *n*-hexacosane, at 0.2%, was found. Differences in the turnover of the compounds due to the immaturity of the shoots and/or the absence of elicitor factors on shoots, namely, contaminant organisms such as the rust fungus *Uromyces* sp. and/or the aphis *A. gossypii* referred to above, would eventually explain the differences in the essential oil composition between the in vitro shoots and the in vivo plants of *H. androsaemum*. Detailed studies are needed, however, to confirm these hypotheses.

The utility of the in vitro shoot cultures in studies on the production of essential oils has not been sufficiently explored. In the case under study, the shoot cultures constitute a stage of the micropropagation process of this species, a specific goal from the practical point of view, and the search on the respective essential oils was performed with the aim to compare its composition with that of in vivo plants. However, as the development and the environment of this type of culture can be maintained under strict control, lowering the sources of variability affecting the composition of the essential oils, we consider that in vitro shoots could be advantageously used as a

tool, for example, in the determination of chemotypes based on the respective essential oil composition. On the other hand, in our view, in vitro shoots or plantlets are the most suitable in vitro system models for studies on the metabolism of terpene compounds because they resemble more closely the in vivo plants.

In conclusion, the work here reported showed that the composition of the essential oils from *H. androsaemum* is complex, having a variable number of compounds which depends on the time of harvest and the origin (in vivo versus in vitro) of the biomass, both cases being dominated by sesquiterpene hydrocarbons.

LITERATURE CITED

(1) Costa, A. F., Ed. *Farmacognosia*, 3rd ed.; Fundação Calouste Gulbenkian: Lisboa, Portugal, 1987; Vol. II, pp 1021−1022.

(2) Seabra, R. M.; Vasconcelos, M. H. Análise de amostras de hipericão existentes no mercado. *Rev. Portuguesa Farmacognosia* **1992**, *17* (3).

(3) Seabra, R. M.; Alves, A. C. Mangiferin and chlorogenic acid from *Hypericum* species. *Planta Med.* **1989**, *55*, 404−405.

(4) Seabra, R. M.; Alves, A. C. Flavonoids from *Hypericum* species. *Fitoterapia* **1990**, *61* (2), 146−147.

(5) Dias, A. C. P.; Seabra, R. M.; Andrade, P. B.; Fernandes-Ferreira, M. The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from *in vivo* and *in vitro* biomass of *Hypericum* species. *J. Liq. Chromatogr. Relat. Technol*. **1999**, *22* (2), 215−227.

(6) Nielsen, H.; Arends, P. Xanthone constituents of *Hypericum androsaemum*. *J. Nat. Prod*. **1979**, *42*, 301−304.

(7) Kitanov, G. M.; Nedialkov, P. T. Mangiferin and isomangiferin in some *Hypericum* species. *Biochem. Syst. Ecol.* **1998**, *26*, 647−653.

(8) Mathis, C.; Ourisson, G. Étude chimio-taxonomique du genre *Hypericum* III. Répartition des carbures saturés et des monoterpènes dans les huiles essentielles d'*Hypericum*. *Phytochemistry* **1964**, *3*, 133−141.

(9) Mathis C.; Ourisson, G. Etude chimio-taxonomique du genre *Hypericum*—IV. Repartition des sesquiterpenes, des alcools monoterpeniques et des aldehydes satures dans les huilles essentielles d'*Hypericum*. *Phytochemistry* **1964**, *3*, 377−378.

(10) Carnduff, J.; Hargreaves, K. R.; Nechvatal, A. Constituents of the *Hypericum androsaemum*. *Phytochemistry* **1966**, *5*, 1029.

(11) Nogueira, T.; Duarte, F.; Venâncio, F.; Tavares, R. Lousã, M.; Bicchi, C.; Rubiolo, P. Aspectos quimiotaxonomicos do género *Hypericum* L. em Portugal. *Silva Lusitanica* **1998**, *6* (1), 55−61.

(12) Dias, A. C. P.; Seabra, R. M.; Andrade, P. B.; Fernandes-Ferreira, M. Xanthone biosynthesis and accumulation in *calli* and suspended cells of *Hypericum androsaemum*. *Plant Sci.* **2000**, *150*, 93−101.

(13) Margara, J. *Bases de la Multiplication Vegetative. Les Méristèmes et l'Organogènese*; INRA, Institut National de la Recherche Agronomique: Paris, France, 1989.

(14) Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473−497.

(15) Santos-Gomes, P. C.; Fernandes-Ferreira, M. Organ- and season-dependent variation in the essential oil composition of *Salvia officinalis* L. cultivated at two different sites. *J. Agric. Food Chem*. **2001**, *49*, 2908−2916.

(16) International Organization of the Flavor Industry. The identification of individual components in flavouring and flavoured foods. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 530−534.

(17) Adams, R. P. *Identification of Essential Oils by Ion Trap Mass Spectroscopy*; Academic Press: San Diego, CA, 1989.

(18) Adams, R. P. *Identification of Essential Oil Components by Gas Chromatography/Quadropole Mass Spectroscopy*; Allured Publishing: Carol Stream, IL, 2001.

(19) Mathis C.; Ourisson, G. Étude chimio-taxonomique du genre *Hypericum* II. Identification de constituants de diverses huiles essentielles d'*Hypericum*. *Phytochemistry* **1964**, *3*, 115−131.

(20) Brondz, I.; Greibrokk, T.; Aasen, A. J. *n*-Alkanes of *Hypericum perforatum*: a revision. *Phytochemistry* **1983**, *22*, 295−296.

(21) Alves-Pereira, I. M. S.; Fernandes-Ferreira, M. Essential oils and hydrocarbons from leaves aand calli of *Origanum vulgare* ssp. *virens*. *Phytochemistry* **1998**, *48*, 795−799.

(22) Nogueira, T.; Duarte, F.; Tavares, R.; Curto, M. J. M.; Capelo, J.; Freitas, A. C. Comparative study of the aromas of *Hypericum* L. species from Portugal using olfactroscopy. *Flavour Fragrance J.* **1999**, *14*, 195−199.

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