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Integrated Analysis by GC(RI), GC-MS and ^{13}C NMR of *Fortunella japonica* Leaf Volatiles Obtained by Hydrodistillation, Microwave- assisted Hydrodistillation and Hydrolate Extraction

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The chemical composition of the essential oil (EO), microwave extract (ME) and hydrolate extract (HE) from the same batch of leaves of *Fortunella japonica*, was investigated by combination of chromatographic (GC, CC) and spectroscopic techniques (GC-MS, ^{13}C NMR). *F. japonica* essential oil and extracts are complex mixtures of 28-60 compounds being mainly oxygenated sesquiterpenes. The EO composition was dominated by germacrene D (14.9%), β -elemol (9.1%), *cis*-guai-6-en-10 β -ol (6.3%), β -eudesmol (5.5%), and δ -elemene (5.2%). Limonene was the unique monoterpene identified at appreciable amount (7.1%). The extract obtained by microwave assisted hydrodistillation contained as main components: β -elemol (12.4%), germacrene D (9.9%), *cis*-guai-6-en-10 β -ol (9.0%), β -eudesmol (8.2%), germacra-1(10),5-dien-4 α -ol (7.1%) and α -eudesmol (6.4%). Finally, the highest content of oxygenated sesquiterpenes (near 92%) was found in the hydrolate extract displaying cryptomeridiol (23.3%, but totally absent in the EO and ME), β -eudesmol (20.6%) and α -eudesmol (10.7%). Combined analysis by chromatographic and spectroscopic techniques appeared useful for identification of various sesquiterpenols bearing a tertiary alcohol function.

Keywords: *Fortunella japonica*, Essential oil, Microwave extract, Hydrolate extract.

The *Citrus* genus belongs to the plant family Rutaceae, which also includes *Poncirus* and *Fortunella*. Fruits from *Fortunella* species are called "kumquat". Although when split they present the typical segmented appearance of a citrus fruit, kumquats are ovoid or oblong and smaller than fruits of *Citrus* species (for example, mandarins, oranges, lemons) [1]. The chemical composition of essential oils (EO) isolated from peel of *Fortunella* species has been substantially investigated and a summary of the results was reported in a previous paper [2]. In short, *Fortunella* peel oils contain mainly limonene (76.7%-87.1-96.9%), as do peel oils of *Citrus* species. In contrast, only a few papers have reported on the chemical composition of kumquat leaf oil. As soon as 1969, Scora *et al.* mentioned a leaf oil sample from *F. crassifolia* containing α -terpineol, beside other oxygenated monoterpenes [3]. Leaf oil of *F. margarita* of Chinese origin was dominated by linalool and terpenol beside methyl *N*-methylantranilate [4]. In contrast, γ -eudesmol, β -elemol, and β -eudesmol were the most abundant compounds of Colombian *F. margarita* leaf oil [5]. Similarly, leaves of *F. margarita* grown in Egypt produced an essential oil that contained mainly β -eudesmol, α -muurolene and β -gurjunene [6]. Major compounds of leaf oils from three *Fortunella* species of Greek origin were germacrene D and elemol [7].

Concerning *F. japonica* (Thunb.) Swing., the main components of the leaf oil from Nepal were the monoterpenes linalool (35.1%) and geraniol (12.7%), beside eugenol (14.8%) [8], while leaf oil from Thailand contained mainly β -pinene (47.4%), limonene (10.2%) and linalool (9.8%) [9]. In contrast, the main components of EO isolated from leaves of *F. japonica* grown in Vietnam were elemol (17.7%), β -eudesmol (16.6%) and epi-bicyclosesquiphellandrene (16.6%) [10]. EOs isolated from leaves of *F. japonica* cultivated in Egypt, harvested at different periods of the year, contained elemol (up to

19.4%), δ -cadinene (up to 16.0%), γ -eudesmol (up to 11.9%), guaiol (up to 11.7%), and τ -cadinol (up to 11.1%) [11]. Recently, our attention was focused on the identification, in the EOs of *Fortunella* species, of germacra-1(10),5-dien-4 α -ol whose confusing spectral data and retention index values were reported in the literature [12]. In parallel, we reported the main components contained in the EOs as β -elemol (8.7-30.9%), germacrene D (2.1-16.3%), β -eudesmol (4.6-13.1%), eremophil-9-en-11-ol (1.4-11.2%), valerianol (1.4-8.9%) and α -eudesmol (3.1-8.8%).

Hydrodistillation (HD) is the most common approach to extract volatile compounds from medicinal herbs and plants. However, other extraction techniques have been applied to improve mainly extraction time, yield and sample preparation costs. They are mainly used to enrich extracts with valuable compounds. Among these techniques, microwave assisted hydrodistillation (MAHD), a green chemistry technique, was developed during the past decade and used for the extraction of volatiles from various aromatic plants [13]. In parallel, fragrance and cosmetic industries are more and more interested by water soluble compounds contained in the hydrolate. Indeed, these compounds can be easily incorporated into a cosmetic formulation and hydrolate is a large resource available simultaneously with the EO.

Therefore, the aim of the present work was to investigate the chemical compositions of the microwave extract (ME) and the hydrolate extract (HE) and to compare them with that of the EO obtained from the same batch of leaves of *Fortunella japonica*. Advantages and disadvantages of each mode of extraction will be discussed. Special attention will be brought to the identification of components (particularly sesquiterpenols) by using a combination of chromatographic and spectroscopic techniques.

Table 1: Chemical composition of essential oil (EO), microwave extract (ME) and hydrolate extract (HE) from leaves of *Fortunella japonica*.

N°	Components ^a	RI ^b	RI ^c	HE	ME	HE	Identification
1	α -Pinene	931	1022	tr	tr	0.1	GC(RI), MS
2	Sabinene	965	1124	tr	tr	-	GC(RI), MS
3	β -Pinene	971	1114	tr	tr	-	GC(RI), MS
4	Myrcene	980	1159	0.4	tr	-	GC(RI), MS, ¹³ C NMR
5	α -Phellandrene	994	1164	tr	-	-	GC(RI), MS,
6	3-Carene	1003	1148	0.1	-	0.4	GC(RI), MS, ¹³ C NMR
7	β -Phellandrene*	1021	1210	tr	-	-	GC(RI), MS
8	Limonene*	1021	1217	7.1	0.1	-	GC(RI), MS, ¹³ C NMR
9	(Z)- β -Ocimene	1023	1232	tr	-	-	GC(RI), MS
10	(E)- β -Ocimene	1036	1249	0.6	-	-	GC(RI), MS, ¹³ C NMR
11	Terpinolene	1076	1280	tr	-	-	GC(RI), MS
12	Linalool	1080	1551	tr	-	0.4	GC(RI), MS, ¹³ C NMR
13	δ -Elemene	1339	1467	5.2	2.1	-	GC(RI), MS, ¹³ C NMR
14	α -Cubebene	1345	1454	0.1	tr	-	GC(RI), ¹³ C NMR
15	α -Ylangene	1368	1480	0.1	tr	-	GC(RI), MS, ¹³ C NMR
16	α -Copaene	1373	1488	0.1	tr	-	GC(RI), MS, ¹³ C NMR
17	β -Bourbonene	1381	1515	0.2	0.3	-	GC(RI), ¹³ C NMR
18	β -Elemene	1384	1588	3.3	3.3	-	GC(RI), MS, ¹³ C NMR
19	β -Ylangene	1413	1568	0.3	tr	-	GC(RI), MS, ¹³ C NMR
20	(E)- β -Caryophyllene	1423	1593	1.6	tr	-	GC(RI), MS, ¹³ C NMR
21	β -Copaene	1425	1586	0.3	tr	-	GC(RI), MS, ¹³ C NMR
22	Thujopsene	1435	1618	0.4	tr	-	GC(RI), ¹³ C NMR
23	β -Gurjunene	1441	1593	0.4	0.4	-	GC(RI), ¹³ C NMR
24	(E)- β -Farnesene	1448	1635	0.7	tr	-	GC(RI), MS, ¹³ C NMR
25	α -Humulene	1454	1666	tr	tr	0.2	GC(RI), MS, ¹³ C NMR
26	α -Amorphene*	1475	1686	0.5	1.0	-	GC(RI), ¹³ C NMR
27	β -Chamigrene*	1475	1703	0.1	0.2	-	GC(RI), ¹³ C NMR
28	Germacrene D*	1475	1712	14.9	9.9	0.1	GC(RI), MS, ¹³ C NMR
29	β -Selinene*	1475	1714	0.8	0.3	-	GC(RI), ¹³ C NMR
30	<i>trans</i> - β -Bergamotene*	1475	1718	0.2	0.4	-	GC(RI), ¹³ C NMR
31	γ -Humulene*	1475	1721	0.5	0.3	-	GC(RI), ¹³ C NMR
32	γ -Amorphene	1493	1671	tr	-	-	GC(RI), MS, ¹³ C NMR
33	Bicyclogermacrene	1496	1728	0.8	tr	0.2	GC(RI), ¹³ C NMR
34	α -Selinene	1496	1720	0.2	tr	-	GC(RI), MS, ¹³ C NMR
35	δ -Amorphene	1500	1635	1.6	0.2	-	GC(RI), MS, ¹³ C NMR
36	α -Bulnesene	1502	1711	0.2	0.3	-	GC(RI), ¹³ C NMR
37	δ -Cadinene	1510	1751	1.0	0.5	-	GC(RI), ¹³ C NMR
38	β -Elemol	1532	2094	9.1	12.4	11.1	GC(RI), MS, ¹³ C NMR
39	(E)-Nerolidol	1551	2049	1.1	2.1	tr	GC(RI), MS, ¹³ C NMR
40	Germacrene B	1552	1833	2.4	4.7	-	GC(RI), MS, ¹³ C NMR
41	<i>cis</i> -Guai-6-en-10 β -ol	1575	1993	6.3	9.0	2.4	GC(RI), ¹³ C NMR
42	Amorph-4-en-10 β -ol	1577	2083	tr	1.6	0.1	GC(RI), ¹³ C NMR
43	Guaiol	1580	2083	1.2	1.5	1.3	GC(RI), MS, ¹³ C NMR
44	Eudesm-6-en-4 α -ol	1604	2092	2.2	0.8	2.3	GC(RI), ¹³ C NMR
45	7- <i>epi</i> - γ -Eudesmol	1609	2036	0.4	0.2	1.0	GC(RI), ¹³ C NMR
46	Germacrene-1(10),5-dien-4 α -ol	1611	2188	2.4	7.1	0.2	GC(RI), MS, ¹³ C NMR
47	<i>trans</i> -Guai-6-en-10 β -ol	1612	2158	1.4	1.8	0.6	GC(RI), ¹³ C NMR
48	γ -Eudesmol*	1614	2178	1.4	0.8	4.4	GC(RI), MS, ¹³ C NMR
49	Alismol*	1617	2270	1.2	3.5	1.8	GC(RI), MS, ¹³ C NMR
50	Eremophil-9-11-ol	1623	2198	4.2	4.4	1.0	GC(RI), ¹³ C NMR
51	τ -Muurolol	1630	2200	0.1	1.5	6.7	GC(RI), ¹³ C NMR
52	τ -Cadinol	1630	2173	0.1	0.8	0.2	GC(RI), MS, ¹³ C NMR
53	Amorph-4-en-10 α -ol*	1641	2210	2.2	0.1	0.2	GC(RI), ¹³ C NMR
54	β -Eudesmol [†]	1641	2244	5.5	8.2	20.6	GC(RI), MS, ¹³ C NMR
55	α -Cadinol [†]	1641	2244	0.7	1.1	0.4	GC(RI), ¹³ C NMR
56	Valerianol	1643	2214	2.1	1.8	3.3	GC(RI), MS, ¹³ C NMR
57	α -Eudesmol	1646	2238	4.4	6.4	10.7	GC(RI), MS, ¹³ C NMR
58	7- <i>epi</i> - α -Eudesmol	1649	2228	0.5	0.2	-	GC(RI), MS, ¹³ C NMR
59	Eudesm-7(11)-en-4 α -ol	1682	2310	0.4	0.5	0.2	GC(RI), MS, ¹³ C NMR
60	Cryptomeridiol	1791	2598	-	-	23.3	GC(RI), MS, ¹³ C NMR
61	(Z)-Phytol	2074	2545	-	0.2	-	GC(RI), ¹³ C NMR
62	(E)-Phytol	2104	2620	0.3	3.8	0.1	GC(RI), MS, ¹³ C NMR
Total				91.6	93.8	93.3	

^aOrder of elution and percentages of individual components are given on an apolar column (BP-1), except those with an asterisk (*), percentages on BP-20. ^bRetention indices measured on BP-1. ^cRetention indices measured on BP20 capillary column. tr = trace level (<0.05%). MS: components identified by MS on apolar and/or polar column. NMR (plain) = identification in EO and/or ME and/or HE. NMR (italic) = identification in fractions of CC. [†]Percentages calculated by combination of GC and NMR.

Strategy for analysis of *F. japonica* essential oil (EO), microwave extract (ME) and hydrolate extract (HE): The EO and both extracts were analyzed by GC(RI), GC-MS and ¹³C NMR following a computerized method developed in our laboratory [14]. Major components were identified by comparison of their spectral data (MS and/or NMR) with those of authentic compounds

compiled in our laboratory-made MS and ¹³C NMR spectral data libraries. A few compounds, mainly monoterpenes present in low content, were identified by GC(RI) and GC-MS. In order to identify minor components, the EO and ME were submitted to column chromatography (CC) and all the fractions of CC were analyzed by GC(RI) and ¹³C NMR. ¹³C NMR confirmed the identification of

various sesquiterpenes previously identified by MS and overall permitted the identification, in total, of two dozen components, mainly sesquiterpenols not previously suggested by MS.

Chemical composition of the EO: Leaves of *F. japonica* produced, by HD, a yellow EO (yield: 0.39% v/w, on a dry weight basis). The EO was analysed by GC(RI), GC-MS and ^{13}C NMR, then subjected to CC and the fractions were analyzed by GC(RI) and ^{13}C NMR. In total, 60 components (91.6% of the EO) were identified (Table 1). The EO exhibited a high level of sesquiterpenes bearing various skeletons. The major component was germacrene D (14.9%) accompanied by various oxygenated sesquiterpenes bearing a tertiary alcohol function that accounted for almost half (47%) of the EO composition, for example, β -elemol (9.1%), *cis*-guaia-6-en-10 β -ol (6.3%), β -eudesmol (5.5%), α -eudesmol (4.4%), and eremophil-9-en-11-ol (4.2%). Sesquiterpene hydrocarbons were also represented by δ -elemene (5.2%), β -elemene (3.3%) and (*E*)- β -caryophyllene (1.6%). Limonene is the unique monoterpene identified in appreciable amount (7.1%). Some sesquiterpenols are uncommon components of *Fortunella* EO and more generally of Citrus and they contribute to the originality of *F. japonica* leaf oil: germacra-1(10),5-dien-4 α -ol, amorph-4-en-10 α -ol, amorph-4-en-10 β -ol, and *trans*-guaia-6-en-10 β -ol. The composition of the investigated *F. japonica* leaf oil differed substantially from those of other origins: Vietnam (elemol, 17.7%; β -eudesmol, 16.6%; epibicyclosquiphellandrene, 16.6%) [10]; Egypt [δ -cadinene (up to 16.0%), guaiaol (up to 11.7%), elemol (up to 19.4%), γ -eudesmol (up to 11.9%), and τ -cadinol (up to 11.1%)] [11]. Obviously it differed drastically from the Nepalese leaf oil containing mostly oxygenated monoterpenes, linalool and geraniol [8] and the leaf oil from Thailand whose composition is dominated by β -pinene [9].

Chemical composition of the ME: Leaves of *F. japonica* from the same batch were submitted to MAHD and yielded a dark yellow extract (yield: 0.09%, v/w, on dry weight basis). The ME was treated like the EO. In total 52 components (93.8% of the ME) were identified (Table 1). The content of oxygenated sesquiterpenes, already important in the EO, appears to be higher in the ME (65.8% vs. 46.9%). Indeed, oxygenated compounds possess a high electric dipolar moment. They are also more easily extracted than hydrocarbons possessing a low dipolar moment. However, each sesquiterpene bearing a tertiary alcohol responded in a different manner to MAHD extraction. For instance, the content of some sesquiterpenols increased substantially in the ME compared with the EO: germacra-1(10),5-dien-4 α -ol (7.1% vs. 2.4%), β -elemol 12.4% vs. 9.1%), *cis*-guaia-6-en-10 β -ol (9.0% vs. 6.3%), β -eudesmol (8.2% vs. 5.5%), and (*E*)-phytol (3.8% vs. 0.3%).

Chemical composition of the HE: During the hydrodistillation process, EO is constantly crossed by a water flux. This aqueous phase called hydrolate is permanently enriched with water soluble compounds. During HD, the hydrolate was collected and then extracted with Et₂O. The HE was analysed by GC(RI), GC-MS and ^{13}C NMR. In total 28 components, accounting for 93.3% of the HE, were identified (Table 1). The chemical composition of this extract differed drastically from those of EO and ME. Indeed, cryptomeridiol (23.3%), absent from the EO and ME, became the main component of the HE. The content of other sesquiterpenols was strongly increased: β -eudesmol (20.6%) and α -eudesmol (10.7%). The content of sesquiterpenes bearing a tertiary alcohol function was again increased and reached 92.3%. In parallel, germacrene D, the major compound of the EO (14.9%) accounted for 0.1% of the HE. Solubility is the major phenomenon responsive for qualitative and quantitative variations between EO and HE

compositions. Cryptomeridiol could result from the hydration of eudesmol isomers in the slightly acidic conditions (pH = 5.5) of HD.

In conclusion, EO from *F. japonica* appears to be rich in oxygenated sesquiterpenes that bear a tremendous variety of skeletons. The use of a green extraction technique (MAHD) produced quickly an extract with higher content of oxygenated sesquiterpenes. The high level of oxygenated compounds is often associated with biological activities, which is an interesting key point of this extraction technique. However the low yield observed with MAHD in this study is a disadvantage compared with traditional HD. Hydrolate, a by-product of HD, displayed the highest content of oxygenated compounds and as the major component a sesquiterpene diol totally absent from the EO and ME. From an analytical point of view, combined analysis by chromatographic and spectroscopic techniques (including ^{13}C NMR) appeared useful for identification of sesquiterpenes bearing a tertiary alcohol function and diverse skeletons.

Experimental

Plant material: *F. japonica* trees were grown in the INRA-CIRAD citrus collection (certified as CRB citrus NF96-600, localized in San Ghjulianu, Corsica, France). Details previously reported in reference [2].

Hydrodistillation (HD): About 300 g of mature leaves from spring flush of *F. japonica* were dried one day at room temperature, away from direct sunlight and then they were subjected to water-distillation (3 h), using a Clevenger-type apparatus.

Microwave assisted hydrodistillation (MAHD): MADH was performed using the 'DryDist' microwave oven (multimode microwave reactor, 2.45 GHz, maximum delivered power of 1000 W, variable in 10 W increments). Temperature was monitored using an external infrared (IR) sensor. In a typical MADH procedure performed at atmospheric pressure, 300 g of mature leaves from spring flush of *F. japonica* were heated using a fixed power density of 1 W/g for 45 min with water as extraction solvent. A cooling system outside the microwave cavity condensed the distillate continuously. Condensed water was refluxed to the extraction vessel in order to provide uniform conditions of temperature and humidity for extraction. The following extraction program was used: 20 s at 400 W/200°C - 2 min at 600W/120 °C and 30 mn at 550 W/120°C.

Hydrolate extraction: Fractions of 20 mL of hydrolate were collected every 15 min during the HD process. The total amount of hydrolate (320 mL) was extracted using Et₂O (3 x 30 mL). The organic layer was dried over MgSO₄ and the solvent removed under reduced pressure to obtain the hydrolate extract (HE).

Fractionation of EO and ME: An aliquot (1.845 g) of EO was fractionated by CC (silica gel, 63-200 μm , 80 g) and 11 fractions (F1-F11) were eluted with a gradient of solvents of increasing polarity, *n*-pentane/Et₂O (P/Et₂O): F1-F6: P; 0.142, 0.135, 0.125, 0.126, 0.113, 0.055 g; F7-F11: P:Et₂O, 95:5 to 0:100; 0.280, 0.305, 0.250, 0.122, 0.117 g. An aliquot (2.055 g) of ME leaf extract was fractionated by CC (silica gel, 63-200 μm , 50 g) and 8 fractions (F'1-F'8) were eluted with a gradient of solvents of increasing polarity, *n*-pentane/diethyl ether (P/Et₂O): F'1:P, 0.683g; F'2-F'8 (P/Et₂O from 85/15 to 0/100): F'2-F'8: 0.137; 0.133; 0.265; 0.162; 0.170; 0.458; 0.048 g, respectively.

GC (FID) and ^{13}C NMR analysis: Analyses were carried out as previously reported [15].

GC-MS analysis: The EO, ME and HE were analysed with a Perkin-Elmer TurboMass detector (quadrupole), directly coupled to a Perkin-Elmer Autosystem, equipped with two fused silica capillary columns (50 m x 0.22 mm x 0.25 µm film thickness), BP-1 (polydimethyl siloxane) and BP-20 (polyethylene glycol). Carrier gas, helium at 0.8 mL/min; split, 1/75; injection volume, 0.5 µL; injector temperature, 250°C; oven temperature programmed from 60°C to 220°C at 2°C/min and then held isothermal (20 min); Ion source temperature, 250°C; energy ionization, 70 eV; EI mass spectra were acquired over the mass range 40-400 Da.

Identification of components: Identification of the individual components was based: i) on comparison of their GC retention indices (RI) on apolar and polar columns with those of authentic compounds or with literature data ii) on computer matching with a laboratory-made and commercial mass spectral libraries and comparison of spectra with literature data [16], iii) on comparison of the signals in the ¹³C NMR spectra of the mixtures with those of reference spectra compiled in the laboratory spectral library, with the help of a laboratory-made software [14].

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