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Biological and chemo-diverse characterization of Amazonian (Ecuador) *Citrus* petitgrains

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Summary

Six Amazonian petitgrain samples from *C. nobilis* Lour., *C. aurantium* L., *C. limon* L. and mixture of *Citrus* spp. (Rutaceae), named CN, CA, CL1, CL2, C1 and C2, were chemically characterized by GC-MS and ^{13}C NMR and evaluated for antioxidant acitivity (DPPH and β-carotene bleaching tests), for antimicrobial properties (disk diffusion method) and for antifungal capacity (agar vapour assay). CN, C1, C2 samples evidenced the most interesting results: CN (γ-terpinene/linalool chemotype: 14.3 %/41.6 %, with a considerable amount of thymol: 9.0 %), and C1 (linalool, 18.3 %; sabinene, 11.6 %; thymol, 5.5 %), showed relevant antioxidant activity with both DPPH (IC_{50} = 3.52 and 5.48 mg/ml, respectively) and β-carotene (IC_{50} = 0.387 and 0.491 mg/ml, respectively). Antibacterial properties of CN and C1 against *P. mirabilis* (MIC = 0.61 mg/ml for both) and *B. subtilis* (MIC = 0.61 and 0.44 mg/ml, respectively) were most probably due to thymol. C2 (geranial: 34.7 %, neral: 33.1 %) evidenced a valuable bioactivity against *C. albicans* (MIC = 0.44 mg/ml). The 50 % growth inhibition (IC_{50}) of the dermatophytes *T. mentagrophytes* and *N. cajetani* was reached with amounts of C1, C2 and CN less than 4 µl/plate. Bioactivity of Amazonian *Citrus* spp. CN, C1 and C2 essential oils suggests their potential use as food preservatives or additives in cosmeceuticals as preventive against dermatophytic fungal infections.

Introduction

Herbs and spices have been employed since ancient times as flavouring and preservatives agents for food, but only in the last decade scientific research has focused its interest on essential oils and extracts as natural sources of antimicrobial and antioxidant compounds, as safer alternative additives for food preservations (Burt, 2004; Shaaban et al., 2012).

While *Citrus* essential oils are usually cold-extracted with mechanical systems (as stated in European Pharmacopoeia, VII ed.), the so-called petitgrain oils are obtained by distillation of *Citrus* leaves, buds and small branches from *Citrus* spp. adult plants (Dugo et al., 2010).

The composition of C*itrus* leaves essential oils are not as well defined as the correspondent peel oils (Dugo et al., 2010) and studies concerning these aspects have been often reviewed (Lota et al., 2001a; Lota et al., 2001b; Lota et al., 2002; Dugo et al., 2010). Due to its pleasant and characteristic fragrance, bitter orange (sour orange, bigarade) petitgrain is the most important and appreciated among leaf essential oils: it is widely used in perfumery for preparation of eau de Colognes, lotions and soaps because of its good resistance to alkaline medium. Sour orange plants are cultivated mainly in the Mediterranean countries (France, Italy, Spain) and in Paraguay (Lota et al., 2001a; Dugo et al., 2010). The best known and most employed species is *Citrus aurantium* L. Other *Citrus* spp. petitgrains (lemon, mandarin, etc.) are afforded at small quantities. Lemons (*Citrus limon* in particular) are cultivated in Mediterranean countries, southern California and Argentina while mandarins (other several *Citrus* spp. and hybrids) are cultivated in Mediterranean countries, Japan, Brazil, Argentina, United States (known as tangerines) and Australia (Lota et al., 2000; Dugo et al., 2010).

Although there is extensive literature on *Citrus* spp. petitgrain composition, very few papers concern biological properties or report correlations among phytochemical data, chemodiversity and biological activities (Dugo et al., 2010; Shaaban et al., 2012). In fact it is known that the wide chemodiversity which characterizes this kind of phytocomplexes could be due to several variables, affecting the chemical profile, as geographical origin, time of collection and cultivars (Guerrini et al., 2011). These variables can determine chemical diversities in essential oils obtained from the same species which necessarily could reflect different bioactivities and possible functional uses.

In light of these premises, in Ecuadorian traditional ethnomedicine *Citrus* spp. leaves are used to treat stomachaches (Rios et al., 2007).

The present paper represents the first report about *Citrus* spp. petitgrain essential oils from plants grown at the margin of the Amazonian forest (Ecuador). The aim is to evaluate possible, distinctive bioactivity properties of chemotypes driven due to this peculiar geographical origin. In fact, it is known that the Amazonian biodiverse hot-spot is characterized by an biotic and abiotic set of conditions which can force the secondary plant metabolism to peculiar and unique profiles with corresponding interesting bioactivities (Ryder Wilke et al., 2010; Rossi et al., 2013). Moreover, the ethnobotanical use of *Citrus* spp. leaves is sometimes performed without particular attention to the single species since the shape is similar and they are collected without distinction. This fact contributes to have preparations with mixed-species leaves (mainly as flavouring and anxiolytic agents) (Hanazaki et al., 2000). In the present work, the study of essential oils obtained from mixture of *Citrus* leaves would mime this ethnobotanical evidence. Therefore, different Amazonian *Citrus* spp. petitgrains from single and mixed species leaves were evaluated for their chemical composition through GC, GC-MS and ^{13}C NMR in order to point out the possible chemodiversity aspects related to their geographical origin and to check for functional properties (antimicrobial, antifungal, antioxidant) to valorize their possible applicative uses in food and/or health fields.

Material and methods

Chemicals

All the solvents employed for chemical analyses and bioassays were chromatographic grade. Solvents and pure compounds were all purchased from Sigma-Aldrich Italy (Milano, Italy). *Thymus vulgaris* essential oil, thymol-chemotype employed as reference phytocomplex (Sacchetti et al., 2005), was purchased from Extrasynthese (Genay, France). Microbial culture media were obtained from Oxoid Italia (Garbagnate, Italy).

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Plant material

C. nobilis (named CN), *C. aurantium* (named CA), *C. limon* (named CL1 and CL2) fresh leaves and fresh leaves mixture of genus *Citrus* spp. (named C1 and C2) were purchased by Fundacion Chankuap (Quito, Ecuador), non-governmentive organization which has as main target the valorization of Amazonian sources recovering plant material to directly obtain commercial products from natives, with the cooperation of our research about Ecuadorian Amazonian biodiversity. For what concerns the essential oil mixtures, no information have been given by Fundacion Chankuap regarding the different *Citrus* species employed and their quantitative ratio. Leaves were collected in September 2010 from wild adult plants growing in three different locations on the outskirts of the Wasak'entsa reserve in eastern Ecuador (77°.15" W/2°.35" S) and positively identified by Fundacion Chankuap (Quito, Ecuador). Dried specimens were deposited at the Department of Biology and Evolution, University of Ferrara, Code C1, C2, CA1, CN1, CL1, CL2.

Essential oils isolation

Essential oils were in situ extracted for 8 hours through steam distillation of *C. limon, C. aurantium, C. nobilis,* mixture of *Citrus* spp. fresh leaves (approximately 10 kg) using a mobile essential oil distiller (Essential Oil Company, Portland, OR, USA) set up following the parameters reported in literature (Horwitz, 2003). Essential oil yields have been achieved through three different distillations of fresh plant material belonging to *Citrus* spp. The petitgrains were dried over anhydrous sodium sulfate and stored in airtight glass vials with Teflon-sealed caps at -18.0±0.5 °C in the absence of light until analysis.

Gas Chromatography

Essential oil samples were analyzed and the relative peak areas for individual constituents averaged. The relative percentages were determined using a ThermoQuest GC-Trace gas-chromatograph equipped with a FID detector and a Varian FactorFour VF-5ms poly-5 % phenyl-95 %-dimethyl-siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.15 μm). Operating conditions were as follows: injector temperature 300 °C; FID temperature 300 °C, carrier (Helium) flow rate 1 ml/min and split ratio 1:50. Oven temperature was initially 55 °C and then raised to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min and finally held at that temperature for 15 min. One μl of each sample dissolved in CH_2Cl_2 was injected. The percentage composition of the oils was computed by the normalization method from the GC peak areas, without using correction factors.

Gas Chromatography-Mass Spectrometry

Essential oil constituents were then analyzed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer using electron impact and hooked to NIST library.

The conditions were the same reported for GC analysis and the same column was used. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 10 μAmp; scan rate, 1 scan/s; mass range, 29-400 Da; trap temperature, 150 °C, transfer line temperature, 300 °C. The constituents of the volatile oils were identified by comparing their relative retention time, KI and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns and retention indices with the above mentioned mass spectra libraries and with those in the literature (Adams, 2007). In order to determine the Kovats index of the components, a C_8-C_{22} *n*-alkanes (Sigma-Aldrich) was added to the essential oil before injecting in the GC–MS equipment and analyzed under the same conditions as above.

NMR spectroscopy

^{13}C NMR spectra were recorded at 100.58 MHz and at temperature of 303 K with a Varian Gemini-400 spectrometer. The essential oils were dissolved in $CDCl_3$ (70 mg/0.8 mL) into a 5 mm NMR and solvent signal was used for spectral calibration (central line of triplet at 77.0 ppm). Chemical shifts (ppm) and peak attribution were based on comparisons of the resonances in ^{13}C NMR spectrum of the essential oil with those of pure standards and mixture of these (α-pinene, sabinene, β-pinene, D-limonene, γ-terpinene, linalool, citronellal, 4-terpinenol, citral, thymol) present in our spectral library (Guerrini et al., 2006) or according with those of literature (Kubeczka, 2002), SDBS (Saito et al., 2009).

Biological activities

Antioxidant, antifungal and antimicrobial activities were performed comparing all the data with those obtained with appropriate pure synthetic compounds and/or commercial *Thymus vulgaris* essential oil, in order to have positive control references with single compounds or comparable phytocomplexes reputed for their functional bioactivities. The use of a phytocomplex known for its chemical and biological properties (e.g. thyme essential oil) as a positive reference results particularly indicative of the real functional efficacy of a tested extract (Maietti et al., 2013). Data reported for each assay are the average of three determinations of three independent experiments.

Antifungal and antimicrobial strains

According to previously described methodology (Guerrini et al., 2006; Maietti et al., 2013), *Citrus* petitgrain antifungal and antimicrobial activities were performed with agar vapor method and standard disk diffusion technique respectively.

For antibacterial assays, *Gram*-positive (*Enterococcus foecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* ATCC 7003) and *Gram*-negative (*Escherichia coli* ATCC 4350, *Proteus mirabilis* ATCC 29852 and *Klebsiella oxytoca* ATCC 29516) bacterial strains were employed. Antifungal activity was assessed on yeast *Candida albicans* ATCC 48274, on phytopathogen strains (*Botrytis cinerea* Micheli ATCC 48339, *Pythium ultimum* Trow, kindly supplied by Prof. G. D'Ercole (Institute of Vegetal Pathology, University of Bologna, Italy), *Magnaporthe grisea* ATCC 64413) and dermatophyte strains (*Trichophyton mentagrophytes var. mentagrophytes* (Robin) Blanchard CBS (Centraal Bureau Voor Schimmelcultures, Baarn, the Netherlands) 160.66, *Nannizzia cajetani* Ajello IHME (Institute of Hygiene and Epidemiology-Mycology (IHME) Brussels, Belgium) 3441 and *Trichophyton rubrum* (Castellani) Sabouraud IHME 4321).

Antimicrobial activity: disks diffusion method

Mother cultures of each bacteria were set up 24 h before the assays in order to reach the stationary phase of growth. The tests were assessed by inoculating from the mother cultures Petri dishes with proper sterile media with the aim of obtaining the microorganisms concentration 10^6 CFU/ml. For bacteria, aliquots of dimethyl sulfoxide (DMSO) were added to the essential oils in order to obtain a 0.01-50.0 mg/ml concentration range and then deposited on sterile paper disk (6 mm diameter, Difco).

Bioactivity against the yeast *Candida albicans* was also processed. Mother cultures were set up inoculating 100 ml YEPD liquid medium (Yeast Extract and Potato Dextrose) in 250 sterile flasks and

incubated in the dark at 30 °C in order to assess growth curves. From each mother cultures at the stationary phase of growth, broth dilutions were made to obtain the strain concentration of 10^5 CFU/ml to inoculate Petri dishes with agarized YEPD for bioassays. Then, 10 µl of DMSO-essential oil sample solutions were prepared in order to have an assay range 0.01-50.0 mg/ml, and then deposited on sterile paper disk (6 mm diameter, Difco). The Petri dishes were successively incubated at 30 °C in the dark and checked for evaluating the growth inhibition after 48 h. both for bacteria and *Candida* streams, the lowest concentration of each essential oil showing a clear zone of inhibition was taken as the MIC (Minimum Inhibitory Concentration). Negative controls were set up with 10 µl of DMSO in the test solution, while positive ones were assessed with *T. vulgaris* essential oil.

Antifungal activity: agar vapour assay

Biological activity of *Citrus* petitgrains against three phytopathogenic and three dermatophytic fungi was performed by using the agar vapour method (MAIETTI et al., 2013). They were grown in Petri plates (90 mm) supplemented with 15 ml/plate of potato dextrose agar, inoculated with 6 mm plugs from stationary-phase cultures. The plates were then incubated for 24 h at 26 ± 1 °C. Successively, sterilized filter paper discs (diameter 9.0 mm) were absorbed with different volumes of *Citrus* petitgrains samples ranging from 0.20 to 25.00 µl, and placed inside the upper lid of each plate. Plates were kept in an inverted position, tightly sealed with parafilm, and incubated for 7 days at 26 ± 1 °C. Blanks served as a negative control. Commercial *T. vulgaris* essential oil was prepared as described above for petigrain samples, with volumes ranging from 0.20 to 25.00 µl, and considered as a positive control reference phytocomplex. Three replicates were made for each treatment. After 7 days the results were determined as the inhibition of radial growth and expressed as the amount of essential oil that led to 50 % inhibition of growth in each fungal strain (IC_{50}).

Antioxidant activities

Radical scavenging and antioxidant properties of essential oils were performed through different assays, namely the DPPH assay and the β-carotene bleaching test according to previously described methods. This approach permits the antioxidant effectiveness of an essential oil to be more carefully defined, as it is almost impossible to express the antioxidant activity as an absolute value that is universally recognizable, besides being expressed by only one type of assay (MAIETTI et al., 2013). *T. vulgaris* essential oil was used as positive controls. Essential oils antioxidant activity was considered as the IC_{50}, calculated from inhibition curves obtained by plotting the % inhibition against oil concentration. All the data collected for each assay are the average of three determinations for three independent experiments.

Statistical analysis

Relative standard deviations and statistical significance (Student's t test; $P \leq 0.05$), one-way ANOVA and LSD post hoc Fisher's honest significant difference test, were given, where appropriate, for all data collected. All computations were made using the statistical software STATISTICA 6.0 (StatSoft Italia srl).

Results

Chemical fingerprinting

Steam distillation of *Citrus* spp. leaves provided petitgrains with yield from 0.20 ± 0.02 g/100g for CN to 0.29 ± 0.03 g/100g for CL1 and density that covered a range of 0.82-0.92 g/ml (Tab. 1).

The Ecuadorian CA petitgrain exhibited as major components sabinene (38.3 %), *trans*-E-ocimene (6.7 %), linalool (8.8 %): other minor components were 3-carene (8.9 %), D-limonene (7.9 %), β-myrcene (3.4 %), 4-terpinenol (2.5 %), α-pinene (1.9 %), geranial (1.9 %), β-pinene (1.8 %). CL1 essential oil evidenced an high abundance of limonene (52.7 %) and linalool (15.1 %) and as minor compounds citronellal (3.1 %), sabinene (2.7 %) and carvone (2.6 %), instead in CL2 petitgrain predominated sabinene (36.1 %) followed by limonene (24.1 %), linalool (4.7 %), 4-terpinenol (3.9 %), γ-terpinene (3.9 %), citronellal (3.6 %), *trans*-β-ocimene (3.2 %), α-terpinene (2.8 %), β-myrcene (2.6 %). *Citrus nobilis* petitgrain evidenced high abundance of linalool (41.6 %) and appreciable contents of γ-terpinene (14.3 %) and thymol (9.0 %), followed by *trans*-E-ocimene (10.9 %), p-cymene (4.1 %), α-pinene (3.6 %), β-pinene (3.1 %) limonene (2.8 %) as minor compounds. Finally, linalool (18.3 %), sabinene (11.6 %), limonene (11.1 %), γ-terpinene (10.6 %), thymol (5.5 %), β-pinene (4.9 %), *trans*-E-ocimene (4.8 %) and p-cymene (3.4 %) were the most characteristic compounds for C1 petitgrain samples; C2 petitgrain, instead, evidenced geranial (34.7 %) and neral (33.1 %) followed by β-myrcene (5.4 %), linalool (4.7 %), and geraniol (3.1 %) as the most abudant chemicals (Tab. 1).

To contribute to define a metabolomic fingerprinting of *Citrus* spp. essential oils, ^{13}C-Nuclear Magnetic Resonance (NMR) of the most abundant chemical standard compared with whole essential oil spectrum was performed confirming the evidences emerged by GC-MS (supplementary materials, Tab. 5 and Fig. 1). Mono-dimensional ^{13}C spectrum revealed typical and numerous diagnostic signals for characterizing the chemical makeup of carbons and, therefore, of the functional groups typical of the examined molecules.

Antioxidant activities

The essential oils examined evidenced interesting antioxidant properties with sligthly different among the samples (Tab. 2). In particular, CN petitgrain exhibited a good radical antioxidant activity both with DPPH test (IC_{50}=3.52±0.25 mg/ml) and β-carotene bleaching assay (IC_{50}=0.387±0.021 mg/ml). These results are particularly relevant if compared to that obtained with commercial *Thymus vulgaris* essential oil (IC_{50}=1.24±0.10 mg/ml), taken as reference phytocomplex (SACCHETTI et al., 2005). C1 petitgrain, that showed a relative abundance of γ-terpinene (10.6 %) and thymol (5.5 %), probably to relate to the interesting DPPH activity (IC_{50}=5.48±0.45 mg/ml), displayed instead a lower activity in β-carotene bleaching test (IC_{50}=0.491±0.041 mg/ml). Thymol has been tested as pure compound in DPPH and β-carotene bleaching tests suggesting that the antioxidant capacity displayed by essential oils could be mainly due to the presence and the abundance of this substance.

Antimicrobial activity

Evaluation of antibacterial activity (Tab. 3), expressed as MIC (Minimun Inhibitory Concentration), revealed that CN petitgrain was the most active among the C*itrus* spp. phytocomplexes against *Gram*-negative as well as *Gram*-positive bacteria. The most interesting results were against *P. mirabilis* for CN, C1 and CA, against *B. subtilis* for CN and C1 and finally against *E. coli* for CN and CA since MICs were comparable. The antibacterial properties of CN petitgrain were relevant also against *S. aureus* and *E. foecalis* (0.78±0.08 and 0.95±0.09 mg/ml) if compared to the positive control *T. vulgaris*. No remarkable inhibition activity was observed against *K. oxytoca*. C2 petitgrain was instead particularly active against the yeast *C. albicans*, with a MIC of 0.44±0.05 mg/ml.

Tab. 1: Chemical composition of *Citrus* petitgrains

Compound	**KI[a]**	***RA[b]***					
		CN	CA	CL1	CL2	C1	C2
α-Thujene	930	1.8	0.5	0.2	1.0	1.1	0.1
α-Pinene	939	**3.6**	1.9	0.7	2.7	2.7	0.2
Sabinene	977	0.4	**38.3**	2.7	**36.1**	**11.6**	0.6
β-Pinene	979	**3.1**	1.8	0.9	**3.4**	**4.9**	0.2
6-Methyl-5-hepten-2-one	986	-	-	0.3	0.1	-	1.9
β-Myrcene	991	0.7	**3.4**	0.3	2.6	1.1	**5.4**
α-Phellandrene	1003	0.1	0.6	-	0.2	-	-
p-Mentha-1(7),8-diene	1004	-	-	-	-	0.6	-
3-Carene	1009	-	**8.9**	-	-	-	-
α -Terpinene	1017	0.4	1.0	0.3	2.8	0.4	-
p-Cymene	1025	**4.1**	0.5	1.9	0.3	**3.4**	0.5
D-Limonene	1029	2.8	**7.9**	**52.7**	**24.1**	**11.1**	0.7
1,8-Cineole	1031	-	-	-	0.2	0.6	-
cis-Z-Ocimene	1031	0.8	0.2	-	0.8	0.4	0.4
trans-E-Ocimene	1037	**10.9**	**6.7**	-	**3.2**	**4.8**	0.6
γ-Terpinene	1051	**14.3**	1.6	0.3	**3.9**	**10.6**	0.6
cis-Sabinene hydrate	1060	0.1	0.3	0.4	0.8	0.1	-
trans-Linalool oxide	1073	0.1	-	1.5	-	-	-
cis-Linalool oxide	1087	-	-	1.6	-	-	-
Isoterpinolene	1088	-	0.3	-	-	-	-
Terpinolene	1089	1.6	1.7	0.5	0.8	0.9	0.2
p-Cymenene	1091	0.9	-	-	-	0.4	0.2
Linalool	1097	**41.6**	**8.8**	**15.1**	**4.7**	**18.3**	**4.7**
1,3,8-p-Menthatriene	1110	0.3	-	-	-	-	0.3
cis-p-Ment-2-en-1-ol	1122	-	0.1	0.7	-	-	-
cis-Limonene oxide	1138	-	-	0.5	-	-	-
trans-p-Ment-2-en-1-ol	1141	-	-	0.8	-	-	0.3
trans-Limonene oxide	1142	-	-	-	-	0.1	0.5
Isopulegol	1150	-	-	0.2	0.1	0.8	-
Citronellal	1153	-	1.4	**3.1**	**3.6**	0.8	0.3
cis-Linalyl oxide	1174	-	-	0.2	-	-	-
trans-Linalyl oxide	1176	-	-	0.1	-	-	-
4-Terpinenol	1177	0.2	2.5	0.7	**3.9**	0.6	0.2
α-Terpineol	1189	0.2	0.6	0.5	0.3	0.4	0.7
cis-Dihydrocarvone	1193	-	-	0.6	-	-	-
trans- Dihydrocarvone	1201	-	-	0.3	-	-	-
trans-Carveol	1217	-	-	1.4	-	-	-
Citronellol	1226	-	0.3	0.5	0.7	-	0.3
cis-Carveol	1229	-	-	0.6	-	-	-
Nerol	1230	-	0.3	-	0.7	-	-
Neral	1238	-	1.6		0.1	0.2	**33.1**
Carvone	1243	-	-	2.6	-	-	-
Geraniol	1253	-	0.1	-	-	-	**3.1**
Geranial	1267	-	1.9	-	0.1	0.3	**34.7**
Perillaldehyde	1272	-	-	0.4	-	-	1.0

Compound	KI[a]	*RA*[b]					
		CN	CA	CL1	CL2	C1	C2
Citronellyl formate	1274	-	-	0.9	-	-	-
2-Undecanone	1294	-	-	-	-	-	1.3
Geranyl formate	1298	-	-	0.5	-	-	0.4
Carvacrol	1299	-	-	-	-	-	-
δ-Elemene	1338	-	-	-	0.1	-	-
Citronellyl acetate	1353	-	0.3	0.9	0.2	0.1	-
Neryl acetate	1362	-	0.5	0.8	0.2	0.1	-
Geranyl acetate	1381	-	0.4	-	-	-	0.4
β-Elemene	1391	-	1.4	-	-	0.1	-
Methyl methylantranilate	1406	0.3	0.2	-	-	**13.1**	**3.1**
trans-β-Caryophyllene	1419	0.9	0.8	-	0.6	1.3	0.2
cis-Carvyl propanoate	1422	-	-	0.6	-	-	-
γ-Elemene	1433	-	-	-	-	-	-
trans-α-Bergamotene	1435	-	-	-	-	-	0.2
α-Humulene	1455	0.1	0.4	-	-	0.2	-
β-(E)-Farnesene	1457	-	0.2	-	-	-	-
2-Tridecanone	1470	-	-	-	-	-	0.5
Bicyclogermacrene	1500	0.2	0.2	-	0.3	0.4	-
Germacrene A	1509	-	0.7	-	-	0.2	-
Germacrene B	1561	-	-	-	0.1	-	-
Spathulenol	1578	-	-	-	-	0.1	-
Caryophyllene oxide	1583	-	-	0.5	-	-	-
1-Methoxy-9(E)-octadecen	1651	-	-	-	-	0.7	-
β-Sinensal	1700	-	0.6	-	-	-	-
α-Sinensal	1757	0.3	0.1	-	-	0.2	-
TOTAL IDENTIFIED		99.8	99.0	96.7	98.7	98.8	98.9
Extraction yield (g/100g)		0.20±0.02	0.23±0.01	0.29±0.03	0.29±0.01	0.23±0.01	0.24±0.03

[a] Arithmetic indices calculated on a Varian VF-5ms column
[b] Relative peak area calculated by GC-FID.
The major components (bold letters) of samples were identified by ^{13}C NMR

Tab. 2: Antioxidant activity of *Citrus* petitgrains performed by DPPH, *β*-carotene bleaching assays and compared to commercial *Thymus vulgaris* essential oil and pure compound thymol.

Essential oils	IC_{50}± SD (mg/ml)	
	DPPH	β-carotene bleaching
CN	3.52 ± 0.25	0.387 ± 0.021
CA	7.12 ± 0.50	0.432 ± 0.037
CL1	9.90 ± 0.71	0.986 ± 0.088
CL2	7.45 ± 0.61	0.521 ± 0.038
C1	5.48 ± 0.45	0.491 ± 0.041
C2	8.41 ± 0.71	0.788 ± 0.066
Thymol	0.60 ± 0.05	0.09 ± 0.011
Thymus vulgaris	1.24 ± 0.10	0.164 ± 0.013

Antifungal activity

The most interesting results concerning antifungal activities (Tab. 4) were exhibited by C2 petitgrain, particularly against dermatophytes species (*T. mentagrophytes*, *N. cajetani*), that showed IC_{50} less than 0.20 µl/plate, comparable to positive control *T. vulgaris* essential oil: however, the concentrations corresponding to the 100 % growth inhibition was better for C2. This essential oil was the most active also against phytopathogens, but less active than the reference standard *T. vulgaris*.

C1 and CN petitgrains also showed good activity against all tested fungi reaching values of 50 % inhibition at concentration comprised from 2 to 8 µl/plate. The most sensitive fungal strain, however, appears to be *T. rubrum*.

The study of activity of citral (mixture of neral/geranial) standard, the most abundant component in C2, against phytopathogens and dermatophytes, confirmed the best activities against *T. mentagrophytes*, *N. cajetani*, *T. rubrum*: in particular, *T. mentagrophytes* was the most sensitive fungus since it evidenced 50 % inhibition at concentration less than 0.20 µl/plate.

Tab. 3: Antimicrobial activity of *Citrus* petitgrains compared to commercial *Thymus vulgaris* essential oil, thymol and citral.

Essential oils	MIC (mg/mL) ± SD						
	Gram-positive bacteria			*Gram*-negative bacteria			Yeast
	S. aureus ATCC29213	*B. subtilis* ATCC7003	*E. foecalis* ATCC29212	*K. oxytoca* ATCC29516	*E. coli* ATCC4350	*P. mirabilis* ATCC29852	*C. albicans* ATCC48274
CN	0.78 ± 0.08	0.61 ± 0.05	0.95 ± 0.09	2.17 ± 0.22	0.61 ± 0.06	0.61 ± 0.05	1.74 ± 0.19
CA	8.46 ± 0.64	1.52 ± 0.16	1.06 ± 0.11	3.38 ± 0.35	0.68 ± 0.07	0.68 ± 0.07	3.38 ± 0.34
CL1	4.06 ± 0.41	3.79 ± 0.37	1.99 ± 0.19	8.12 ± 0.61	6.32 ± 0.62	4.06 ± 0.41	0.88 ± 0.09
CL2	3.50 ± 0.31	1.73 ± 0.17	3.89 ± 0.38	6.05 ± 0.55	3.89 ± 0.38	3.89 ± 0.37	3.46 ± 0.33
C1	1.10 ± 0.12	0.44 ± 0.04	1.93 ± 0.18	4.38 ± 0.42	2.19 ± 0.22	0.61 ± 0.06	1.75 ± 0.18
C2	7.94 ± 0.59	1.94 ± 0.18	3.97 ± 0.37	6.17 ± 0.41	1.76 ± 0.17	3.97 ± 0.39	0.44 ± 0.08
thymol	0.31 ± 0.06	0.28 ± 0.03	0.52 ± 0.05	1.10 ± 0.21	0.29 ± 0.03	0.31 ± 0.02	0.90 ± 0.07
citral	0.50 ± 0.09	0.30 ± 0.07	0.58 ± 0.06	0.20 ± 0.03	0.25 ± 0.05	0.70 ± 0.07	0.42 ± 0.04
T. vulgaris	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.40 ± 0.04	0.06 ± 0.01	0.12 ± 0.01	0.06 ± 0.01

Tab. 4: Antifungal activity of *Citrus* spp. petitgrains compared to commercial *T. vulgaris* essential oil, citral and thymol.

Samples	Phytopathogens			Dermatophytes		
	P. ultimum	*M. grisea*	*B. cinerea*	*N. cajetani*	*T. mentagrophytes var. mentagrophytes*	*T. rubrum*
CN	4.0 ± 0.2	6.3 ± 0.4	3.4 ± 0.3	3.7 ± 0.2	3.6 ± 0.2	2.7 ± 0.2
CA	12.7 ± 0.9	16.1 ± 1.1	15.3 ± 1.2	9.3 ± 0.9	>25	14.2 ± 1.2
CL1	8.3 ± 0.7	>25	20.7 ± 1.5	>25	19.9 ± 1.8	12.0 ± 1.1
CL2	10.0 ± 1.0	>25	18.6 ± 1.5	17.6 ± 1.9	18.3 ± 1.7	16.7 ± 1.4
C1	3.2 ± 0.4	7.9 ± 0.5	6.2 ± 0.4	6.7 ± 0.7	4.8 ± 0.3	2.0 ± 0.2
C2	2.2 ± 0.2	2.4 ± 0.3	1.9 ± 0.2	<0.20[a]	<0.20[b]	1.9 ± 0.1
T. vulgaris	0.40 ± 0.10	0.38 ± 0.08	0.23 ± 0.04	<0.20[c]	<0.20[a]	<0.20[c]
Citral	1.2 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	0.44 ± 0.02	<0.20[b]	0.88 ± 0.05
Thymol	1.1 ± 0.1	2.2 ± 0.3	1.6 ± 0.2	1.1 ± 0.2	1.2 ± 0.4	0.8 ± 0.2

All the values are expressed as IC_{50} (ml/plate) ± standard deviation
[a]100% growth inhibition at concentration of 2.0 ml/plate
[b]100% growth inhibition at concentration of 1.0 ml/plate
[c]100% growth inhibition at concentration of 5.0 ml/plate

Discussion

The purpose of the current study was to compare the chemical composition of Amazonian *Citrus* spp. leaves essential oils with those reported in literature to determine possible different chemotypes and biological activities with the final aim to valorize their commercial use.

The distillaton yields were average values among those reported for CA (Blanco Tirado et al., 1995; Lota et al., 2001a) and CN (Lota et al., 2001b); instead for CL petitgrains were lower than those reported for other lemon species (Vekiari et al., 2002).

The Ecuadorian CA petitgrain was comparable to an atypical sabinene/*trans*-E-ocimene chemotype, as previously reported (Lota et al., 2001b). CL1 petitgrain exhibited an atypical composition with high abundance of limonene (52.7 %) and linalool (15.1 %), as reported for the Meyer cultivar (*C. meyeri*) (Lota et al., 2002), CL2 petitgrain could be defined as limonene (24.1 %)/sabinene (36.1 %)/linalool (4.7%) chemotype, standing out the most common lemon chemotype characterized by limonene (17.8-33.5 %), α-pinene (10.5-25.1 %), geranial (8.6-22.6 %), neral (5.9-16.1 %) (Lota et al., 2002). *C. nobilis* petitgrains (CN) evidenced a γ-terpinene/linalool chemotype, because of high abundance of linalool (41.6 %) and appreciable contents of γ-terpinene (14.3 %) and thymol (9.0 %). A similar chemotype was pointed out in a systematic research on petitgrains derived from 58 Corsical mandarin cultivars from different species and 41 cultivars belonging to *C. reticulata* Blanco (Lota et al., 2000; Lota et al., 2001b). Mandarin leaves essential oil composition from plants of different geographical origins, Floridian *C. tangerine* Hort. ex Tan and Israelian *C. reticulata,* evidenced high content of linalool, thymol and γ-terpinene (Attaway et al., 1967; Fleisher and Fleisher, 1990; Fleisher and Fleisher, 1991), while Colombian *C. reticulata* petitgrain was characterized by an high abundance of linalool (52.66 %), but less content of γ-terpinene (1.95 %) (Blanco Tirado et al., 1995).

The chemical composition of *Citrus* spp. leaves essential oils (C1 and C2) does not allow to deduce any consideration about the species employed and their abundance, but it is an important starting point in making suggestions about the comparison between the bio-

Tab. 5: Chemical shifts (^{13}C) of compounds in *Citrus* spp. petitgrains

Compound	Chemical shift ^{13}C
α-Pinene	144.5/116.0/47.0/40.7/38.0/31.4/31.2/26.3/23.0/20.9
Sabinene	154.5/101.5/37.6/32.6/30.1/29.0/27.9/19.8/19.7/16.0
β-Pinene	152.2/105.9/51.6/40.7/40.5/27.0/26.1/23.5/21.8
β-Myrcene	146.2/139.0/131.9/124.3/115.7/113.1/31.6/26.7/25.6/17.6
3-Carene	131.4/119.4/28.3/23.7/20.8/18.4/16.8/16.6/13.2
p-Cymene	145.9/135.1/129.0/126.2/33.7/24.1/20.9
D-Limonene	150.3/133.8/120.6/108.3/41.0/30.8/30.6/27.9/235/20.8
trans-E-Ocimene	1415/133.7/122.1/110.6/ 27.3/25.7/17.7/11.6
γ-Terpinene	140.6/131.2/118.9/116.0/34.5/31.6/27.5/23.0/21.3
Linalool	145.0/132.0/124.3/111.7/73.5/42.0/27.9/25.7/22.9/17.7
Citronellal	202.7/131.8/124.0/51.0/36.9/27.8/25.7/25.4/19.9
4-Terpinenol	133.8/118.4/71.7/36.8/34.6/30.8/27.0/23.3/16.8
Neral	190.9/163.9/133.7/128.6/122.2/32.6/27.0/25.6/25.1/17.7
Geraniol	139.5/131.4/124.1/123.6/59.4/39.4/26.3/25.7/17.6/16.2
Geranial	191.4/163.9/132.9/127.4/122.5/40.6/25.7/25.6/17.7/17.6
Thymol	152.5/136.6/131.8/126.2/121.5/116.0/26.7/22.7/20.9
Methyl methylantranilate	169.0/151.2/134.7/131.8/115.4/110.6/51.5/29.1

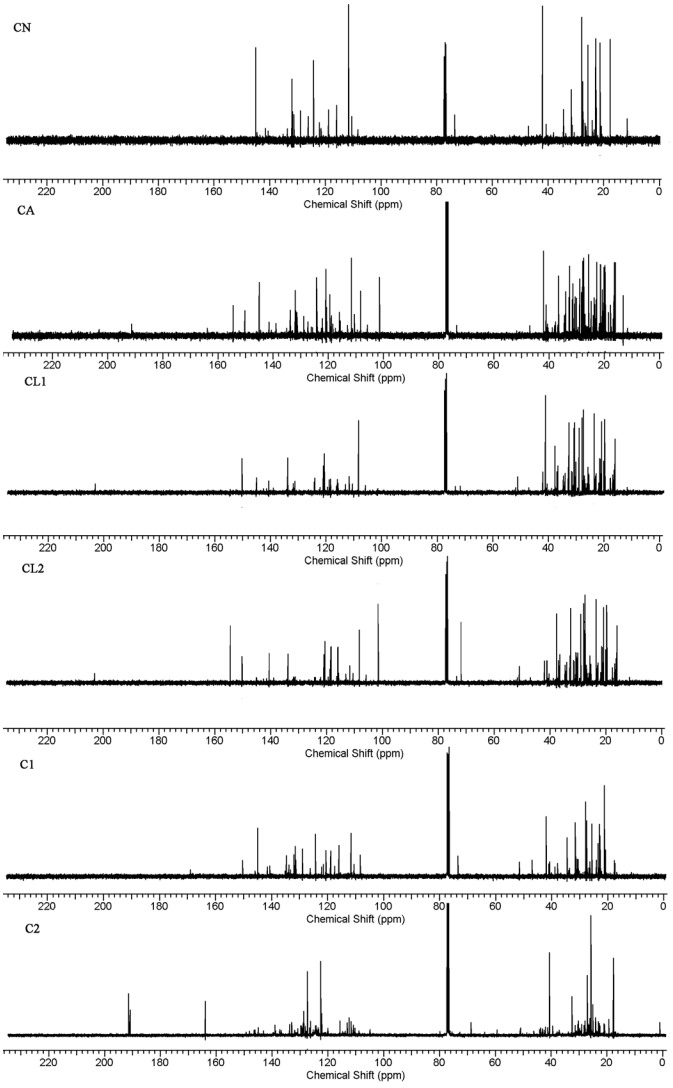

Fig. 1: ^{13}C spectra of *Citrus* spp. petitgrains

activities of the mixtures and the other essential oils.

However, the studies concerning plants growing in Amazonia are particularly interesting since the Amazonian basin is one of the most important biodiversity hotspots where the ecological conditions and high density and diversity of species per unit area drive the plant secondary metabolism to biosynthetic pathways which are particularly rich in different chemical structures (Ryder Wilkie et al., 2010; Rossi et al., 2013). This aspect could explain the slight differences in chemical composition detected for the essential oils, with particular reference to those belonging to *C. limon* (CL) samples.

The confirmation of the gas chromatographic results by NMR experiments suggests this spectroscopic technique as suitable for the identification, quality control, or fraud detection of essential oils providing their good and fast discrimination. Moreover, these kinds of evidences reinforce the role of non-chromatographic approach as potential tool to discriminate chemotypes, cultivar and hybrids as already suggested elsewhere (Lota et al., 2001b; Guerrini et al., 2006; Guerrini et al., 2011). All these chemical profiles obtained through GC-MS and confirmed by NMR spectroscopy, evidence that Amazonian biodiversity does not induce strong chemodiversity among *Citrus* spp. petitgrains examined, if compared to what related literature reports, even if interesting differences regarding minor compounds were found.

The examined essential oils evidenced that CN petigrain revealed the highest antioxidant activity, if compared to results obtained with commercial *T. vulgaris* essential oil, taken as reference phytocomplex (Sacchetti et al., 2005), C1 petitgrain, with relative abundance of γ-terpinene (10.6 %) and thymol (5.5 %), showed also interesting data. The antioxidant capacity displayed by essential oils could be mainly due to the presence and the abundance of thymol, as experimental results evidenced. However, with particular reference to CN sample, the relevant abundance of γ-terpinene (14.3 %) could be also suggested as responsible of this biological property (Choi et al., 2001), together with the presence of thymol (9.0%) (Ruberto and Baratta, 2000), as well as methyl-N-methylanthranilate (13.1 %) (El-Ghorab et al., 2003).

CN petitgrain was the most effective against all the bacteria strains: MIC values of CA and C1 samples were instead lower and comparable. The amounts of thymol in CN (9.0 %) and C1 (5.5 %) petitgrains could be one of the possible reasons for the antibacterial activity (Burt, 2004). C2 petitgrain was instead particularly active against the yeast *C. albicans*, with a MIC of 0.44±0.05 mg/ml probably due to the high abundance of neral (33.1 %) and geranial (34.7 %), previously described as anti-*Candida* spp. agents (Silva et al., 2008) and confirmed by our results. Trying to relate antimicrobial activity with chemical data, thymol has been assayed as pure compound, but no remarkable results were obtained. However, it should be stressed that higher antibacterial capacity of thyme essential oil than that of thymol could be due to a sinergic interaction involving more chemicals, thymol included. This suggestion plays certainly a role in the activities displayed by petitgrains.

The most interesting results concerning antifungal activities (Tab. 4) were exhibited by C2 petitgrain due to the high abundance of citral, as confirmed by experimental data. The good activities of C1 and CN petitgrains could be explained with the relative abundance of thymol, tested by us as pure compound and previously described as antifungal agent *in vitro* and *in vivo* against dermatomycoses (Sokovic et al., 2008). Finally, the particular interesting bioactivity of the essential oil mixtures confirmed the amazonian ethnobotany which

often does not discriminate *Citrus* species in using leaves for traditional preparations, emphasizing synergic expression of different extracts/chemical compounds to have better biological performances.

Conclusions

This first report about Amazonian *Citrus* spp. petitgrains evidenced their chemical characterization by GC and GC-MS and remarked the use of NMR as useful tool to characterize and discriminate chemotype for identification, quality control and fraud detection of essential oils (Lota et al., 2001b; Guerrini et al., 2006; Guerrini et al., 2011). However, no remarkable difference emerged with other C*itrus* spp. petitgrains from other geographical regions, even if interesting differences regarding minor compounds were found. In particular Amazonian CN petitgrain, γ-terpinene/linalool chemotype on the basis of chemical composition defined by GC/MS and NMR, and C1 petitgrain revealed both interesting *in vitro* antibacterial and radical scavenging activities. Result highlights that these two essential oils could be potentially employed as food preservatives or functional constituents in food supplements and/or health herbal products.
Moreover the antidermatophytic properties of C1, CN and the most interesting C2 leaf essential oils suggest their possible application in cosmeceuticals as antidermatophytic additives, not only as single essential oil but also as mixtures.

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