Phytochemical Analysis and *in vitro* Free-Radical-Scavenging Activities of the Essential Oils from Leaf and Fruit of *Melaleuca leucadendra* L.

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The phytochemical profile of *Melaleuca leucadendra* L. leaf and fruit oils from Cuba was investigated by GC and GC/MS. Forty-one and sixty-four volatile compounds were identified and quantified, accounting for 99.2 and 99.5% of the leaf-oil and fruit-oil total composition, respectively. The main components were 1,8-cineol (43.0%), viridiflorol (24.2%), α-terpineol (7.0%), α-pinene (5.3%), and limonene (4.8%) in the leaf oil, and viridiflorol (47.6%), globulol (5.8%), guaiol (5.3%), and α-pinene (4.5%) in the fruit oil. The antioxidant capacity of these essential oils was determined by three different *in vitro* assays (2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, thiobarbituric acid reactive species (TBARS), and 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation), and significant activities were evidenced for all of them.

1. Introduction. – Since ancient times, herbs and plant species have been added to different types of food to improve their flavor and organoleptic properties. Currently, there is much research performed on antioxidant compounds from plant extracts and essential oils, the aim being to identify novel lead structures with significant biological activities. Among these various natural compounds, essential oils from aromatic plants are receiving special attention [1].

Many essential oils have been qualified as natural antioxidants and proposed as potential substitutes for synthetic antioxidants in specific sectors of food preservation. Free radicals, *e.g.*, superoxide ions, hydroxyl radicals, and non-free radical compounds, can be responsible for lipid peroxidation (deterioration) in foods, and for various diseases such as malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, cancer, *etc*. Therefore, investigations on natural antioxidants have become a very important field [2].

Melaleuca leucadendra L. (Myrtaceae), commonly named *cajuput*, is a traditional low-growing tree the leaves of which have been used in folk medicine as an inhalant in the treatment of nasal catarrh and purulent skin lesions, as mosquito repellent, and to relieve gout. This plant, which grows naturally in Australia and Southeast Asia, is frequently found in marshy areas of Cuba [3]. For *M. leucadendra*, five leaf volatile-oil chemotypes have been reported: one with 1,8-cineole (>48%) as the major component [4–8], two chemotypes characterized by very high contents of the phenylpropanoids, *viz.* methyl eugenol and (*E*)-methyl isoeugenol (up to 99 and 88%, resp.) [9][10], one containing significant quantities of both viridiflorol (28.2%) and 1,8-cineol (21.3%)

[11], and another one containing significant quantities of 1,8-cineole (19.9%), β-eudesmol (15.8%), α-eudesmol (11.3%), viridiflorol (8.9%), and guaiol (9.0%) [12]. The bioactivity of the leaf oil as antimicrobial, antiviral, and antioxidant agents in order to evaluate its medicinal potential had also been studied [8][13–15]. To the best of our knowledge, there is no report in the literature regarding the chemical analyses and antioxidant activity of the fruit volatile oil from this species.

In many species of aromatic plants, variations in the chemical composition of the volatile oils are used for the identification of different chemotypes. Modern theories have established that secondary metabolites are expressed as a result of external stimuli. According to this theory, an organism can produce completely different groups of metabolites depending on the environmental conditions, duration and intensity of stress, composition, and genetic plasticity of plants [16]. *Melaleuca* species grown in Cuba may, therefore, express chemotypes different from those found in other environments such as in Australia and Brazil.

In this context, the present work describes the chemical composition and antioxidant activities of the volatile-oil constituents isolated from the leaves and fruits of cajuput (*Melaleuca leucadendra* L.).

2. Results and Discussion. – 2.1. *Chemical Composition*. The hydrodistillation of leaves and fruits of *M. leucadendra* gave volatile oils with 0.7±0.2% (*v*/*m*) and 0.4± 0.1% (*v*/*m*) yields, respectively. To identify the chemical constituents, the volatile oils were submitted to GC-FID and GC/MS analyses.

Forty-one and sixty-four volatile compounds, representing more than 99% of the total composition, were identified in the leaf oil and fruit oil, respectively (*Table 1*). All of them are reported for the first time in the fruit oil of *M. leucadendra*, while many compounds were found previously in the leaf oil [4–8][12]. Among all compounds identified and quantified, the major components included 1,8-cineol (**10**; 43.0%), viridiflorol (**48**; 24.2%), terpineol (**23**; 7.0%), α-pinene (**2**; 5.3%), and limonene (**9**; 4.8%) in the leaf oil, and viridiflorol (**48**; 47.6%), globulol (**50**; 5.8%), guaiol (**49**; 5.3%), and α-pinene (**2**; 4.5%) in the fruit oil. Also some other important components such as viridiflorene (**37**; 2.9%, fruit oil), β-eudesmol (**57**; 2.7%, fruit oil), α-eudesmol (**58**; 2.5%, fruit oil), muurolol (**56**; 1.8%, fruit oil), β-caryophyllene (**30**; 1.8%, fruit oil), and α-terpinyl acetate (**27**; 1.6%, leaf oil) were identified (*Fig.*).

The chemical components of these oils exhibited marked differences in quantity as well as quality. The major families in the leaf and fruit oil were: monoterpene hydrocarbons, 13.9 and 9.7%; oxygenated monoterpenes, 54.8 and 5.8%; sesquiterpene hydrocarbons, 0.4 and 9.3%; and oxygenated sesquiterpenes, 30.5 and 75.0%, respectively.

A previous study of the composition of a leaf oil obtained from plants of another Cuban region showed the same two compounds as the most prominent but in an opposite quantity ratio [11]. One economic potential of the fruit oil arises from its woody-floral scent (imparted by viridiflorol), which could be exploited in perfumery.

2.2. *Biological Studies.* So far, it has been well established that some essential oils are rich sources for natural antioxidants [17].

To evaluate the antioxidant activities of these volatile oils, three well-established *in vitro* assays were used. The first is based on the free-radical-scavenging capacity of the

Table 1. *Chemical Composition* [%] *of* M. leucadendra *Volatile Oils from Cuba*[a])

No.	Compound	$KI_{DB\text{-}5}$	Leaf oil	Fruit oil
1	α-Thujene	929	tr[b])	tr
2	α-Pinene[c])	940	5.3	4.5
3	Camphene	952	0.2	0.1
4	Benzaldehyde[c])	961	0.3	0.3
5	β-Pinene[c])	979	2.7	0.9
6	Myrcene[c])	992	0.2	tr
7	α-Terpinene	1017	tr	tr
8	*p*-Cymene[c])	1024	tr	0.1
9	Limonene[c])	1029	4.8	3.9
10	1,8-Cineole[c])	1032	43.0	3.6
11	(*Z*)-β-Ocimene	1037	nd[d])	tr
12	γ-Terpinene[c])	1062	0.3	0.1
13	Terpinolene[c])	1089	0.2	0.1
14	Linalool[c])	1098	0.4	tr
15	*endo*-Fenchol	1117	0.2	0.1
16	α-Campholenal	1124	nd	tr
17	*cis*-*p*-Mentha-2,8-dien-1-ol	1136	tr	nd
18	*trans*-Pinocarveol	1139	0.2	0.1
19	neo-Isopulegol	1147	0.2	tr
20	Camphene hydrate	1150	0.1	tr
21	Borneol[c])	1169	0.4	0.1
22	Terpinen-4-ol[c])	1177	1.1	0.2
23	α-Terpineol[c])	1189	7.0	0.9
24	1-Phenylethyl acetate[c])	1195	tr	tr
25	*trans*-Carveol	1217	0.1	nd
26	Bornyl acetate[c])	1289	tr	tr
27	α-Terpinyl acetate[c])	1349	1.6	0.3
28	α-Copaene	1377	nd	0.1
29	Longifolene	1407	nd	0.1
30	β-Caryophyllene[c])	1419	0.1	1.8
31	Aromadendrene	1441	nd	0.1
32	α-Humulene[c])	1455	tr	0.3
33	*allo*-Aromadendrene	1460	tr	0.3
34	γ-Muurolene	1478	nd	0.2
35	β-Selinene	1490	nd	1.3
36	*cis*-β-Guaiene	1493	nd	0.3
37	Viridiflorene	1497	0.3	2.9
38	α-Muurolene	1502	nd	0.2
39	γ-Cadinene[c])	1514	nd	0.7
40	δ-Cadinene[c])	1523	nd	1.0
41	*trans*-Calamenene	1529	nd	tr
42	*trans*-Cadina-1(2),4-diene	1534	nd	tr
43	α-Cadinene	1539	nd	0.1
44	α-Calacorene	1546	nd	tr
45	Ledol[c])	1569	0.2	0.7
46	Caryophyllenyl alcohol[c])	1572	nd	tr
47	Caryophyllene oxide[c])	1582	1.8	2.8
48	Viridiflorol[c])	1593	24.2	47.6
49	Guaiol	1600	0.2	5.3
50	Globulol[c])	1605	2.4	5.8

Table 1 (cont.)

No.	Compound	$KI_{DB\text{-}5}$	Leaf oil	Fruit oil
51	Humulene epoxide II	1608	0.3	0.5
52	1-*epi*-Cubenol	1628	nd	tr
53	Eremoligenol	1630	nd	1.1
54	γ-Eudesmol	1632	tr	1.2
55	Caryophylla-4(14),8(15)-dien-5α-ol	1641	0.2	0.7
56	α-Muurolol	1646	0.4	1.8
57	β-Eudesmol[c])	1651	0.2	2.7
58	α-Eudesmol[c])	1652	0.2	2.5
59	α-Cadinol	1654	0.2	0.6
60	α-Bisabolol oxide B	1658	nd	0.4
61	Bulnesol	1672	nd	0.6
62	Caryophyllene acetate[c])	1701	nd	0.2
63	(*E*)-Nerolidol acetate[c])	1718	nd	tr
64	(2*E*,6*E*)-Farnesol	1725	nd	0.2
65	β-Eudesmol acetate	1794	nd	tr
66	(2*E*,6*Z*)-Farnesyl acetate	1825	nd	0.1
Monoterpene hydrocarbons			13.9	9.7
O-Containing monoterpenes			54.8	5.4
Sesquiterpene hydrocarbons			0.4	9.3
O-Containing sesquiterpenes			30.5	75.0
Total			99.5	99.4

[a]) For details, see *Exper. Part.* [b]) tr = Trace (< 0.1%). [c]) Identification by injection of an authentic sample and mass spectra. [d]) nd = Not detected.

stable DPPH (= 2,2-diphenyl-1-picrylhydrazyl) radical, the second concerns the spectrophotometric detection of thiobutyric acid reactive species (TBARS), malonaldehyde (MDA) being one of the secondary lipid peroxidation products, the quantification of which gives a measure of the extent of lipid degradation. The third radical-scavenging method involves the determination of the antioxidant ability of each volatile oil in scavenging the colored 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) relative to the radical-scavenging ability of *Trolox* (*Table 2*).

For the first assay, solutions with essential-oil concentrations of 0.3–5.0 mg/ml (leaves), 0.2–5.0 mg/ml (fruits), and different doses of ascorbic acid (positive control) were prepared to evaluate the DPPH radical-scavenging capacity. The respective scavenging capacities ranged from 9.9 ± 0.3 to $76.6 \pm 0.4\%$ and 6.1 ± 0.5 to $78.8 \pm 0.2\%$ with EC_{50} values of 2.4 ± 0.4 and 2.3 ± 0.2 mg/ml for the oil from the leaves and fruits, respectively.

On the other hand, in the second test, different concentration of the volatile oils (20–250 μg/ml) and BHT as positive control also showed antioxidant activities in a dose dependent manner and had 7.54 ± 0.04 to 60.66 ± 0.03 and 9.46 ± 0.07 to $61.89 \pm 0.04\%$ inhibition on lipid peroxidation, the IC_{50} values were found to be 0.23 ± 0.05 mg/ml for the leaf oil and 0.19 ± 0.02 mg/ml for the fruit oil.

By the third method, the antioxidant power of each volatile oil was evaluated based on $ABTS^{\bullet+}$ radical scavenging compared to that of with *Trolox* as a reference

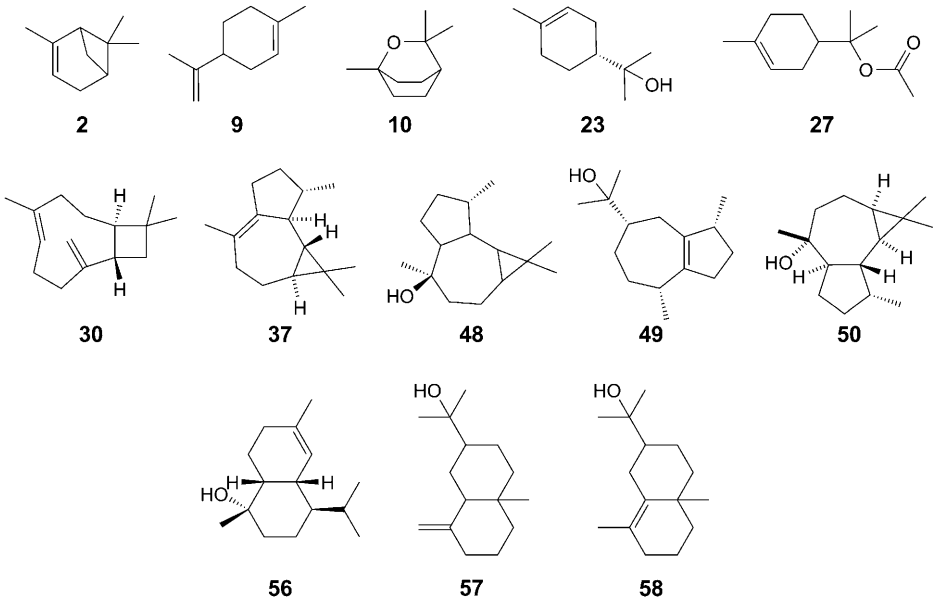

Figure. *Representative volatile compounds of the essential oils from leaf and fruit of* M. leucadendra

Table 2. *Antioxidant Effectiveness of Essential Oils from* Melaleuca leucadendra *Leaf and Fruit*[a])

Sample	EC_{50} [mg/ml][b])	IC_{50} [μg/ml][c])	TEAC [mM][d])
Leaf oil	2.4 (0.4)	230 (15)	448 (9)
Fruit oil	2.3 (0.2)	190 (12)	565 (11)
Ascorbic acid	0.025 (0.004)	nt[e])	nt
BHT	nt	0.15 (0.03)	nt

[a]) Antioxidant effectiveness expressed as EC_{50}, IC_{50}, and *Trolox* equivalent antioxidant capacity (TEAC), and values represent average of three determinations with ± standard deviation (S.D.) given in parentheses. [b]) DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical-scavenging assay. Ascorbic acid was used as positive control. [c]) TBARS (thiobarbituric acid reactive species) assay. Butylated hydroxytoluene (BHT) was used as positive control. [d]) ABTS (2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation-scavenging assay. *Trolox* (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as standard, and the results are expressed in terms of TEAC. [e]) nt: Not tested.

antioxidant. The respective total antioxidant activities were 448 ± 9 and 565 ± 11 mM for the leaf and fruit volatile oils, respectively. A perusal of literature shows that these values were moderate in comparison to those of *Origanum vulgare* (1105 mM) and *Ocimum basilicum* (997 mM) volatile oils [18].

These results demonstrated that the *M. leucadendra* essential oils have significant activities as antioxidants by reacting with the free radicals in the three assays. Nevertheless, the essential oil obtained from the fruits showed a higher antioxidant capacity with respect to the leaf essential oil. It is known that most natural antioxidants often work synergistically to produce a broad spectrum of antioxidative activity that

creates an effective defence system against free-radical attack. These volatile oils could, thus, be used as natural antioxidants in place of synthetic ones.

Terpenes such as 1,8-cineol, α-terpineol, α-pinene, limonene, globulol, and guaiol, which are the representative components in *M. leucadendra* leaf and fruit essential oils have been reported to exhibit significant antioxidant effects by several radical-scavenging assays, including the TBARS method [19][20]. This evidence indicates that these major components contribute to the antioxidant capacities of *M. leucadendra* essential oils. However, it is difficult to assign the activity of a complex mixture to a single or particular constituent. Major or trace compounds might give rise to the biological activities exhibited.

3. Conclusions. – The present results demonstrate that *M. leucadendra* leaf and fruit volatile oils have significant antioxidant properties, and their capacity of scavenging free radicals by three different methods indicates their potential to be used against diseases caused by over-production of these reactive species. Further studies are needed to evaluate the *in vivo* potential of these oils.

Experimental Part

Plant Material. The aerial parts of *M. leucadendra* were collected in Ciénaga de Zapata, Cuba, in February 2009. Taxonomical identification was performed by Dr. *Victor Fuentes* (Instituto de Fruticultura Tropical, Cuba). A voucher specimen was deposited with the Herbarium of Instituto de Ecología y Sistemática, Havana, Cuba.

Isolation of Essential Oil. Leaves and fruits of *M. leucadendra* were harvested, washed with dist. H_2O, and air-dried for *ca.* 3 d. The oils were obtained from 100 g ($3\times$) of each material by hydrodistillation for 3 h in a *Clevenger*-type apparatus. The yields were calculated according to the weights of oils and plant material before distillation.

GC-FID and GC/MS Analyses. GC-FID Analyses on *DB-5* fused silica column (30 m × 0.25 mm, 0.25 μm film thickness) were performed with a *Konik 4000A* GC using the following conditions: injection mode, split ratio 1:20; oven temp., 60–230° at 3°/min and then held isothermal for 30 min; carrier gas, H_2 (1 ml/min); injector and detector temp., 230°.

GC/MS Analyses on *DB-5* fused silica column (30 m × 0.25 mm, 0.25 μm film thickness) were performed with a *Shimadzu QP 500* GC/MS (EI mode at 70 eV, mass range of 35–400 amu), using the following conditions: injection mode, split ratio 1:50; oven temp., 60–230° at 3°/min and then held isothermal for 30 min; carrier gas, He (1 ml/min); injector and transfer line temp., 230°.

Compound Identification. The linear retention indices of the compounds were determined relative to the retention times of a series of *n*-alkanes (C_7–C_{28}) on the three columns, and the percentage compositions were obtained from electronic integration measurements without taking into account relative response factors. Peak identification was carried out by comparison of the mass spectra obtained with those available on database of NIST, NBS, Adams 2001, *Wiley* libraries, and in-house Flavorlib library. The compound identification was finally confirmed by comparison of the relative retention indices in the three columns with those of relative standards or with published data [21–25].

2,2-Diphenyl-1-picrylhydrazyl (DPPH) *Radical-Scavenging Assay.* The antioxidant activity of the essential oils was measured in terms of free-radical-scavenging ability according to DPPH method described in [26] with minor modifications. Basically, a 60 μM MeOH soln. of DPPH (980 μl; *Sigma–Aldrich Co.*, St. Louis, MO, USA), prepared daily, was placed in a spectrophotometer cuvette, and cajeput essential oils of concentrations of 0.3, 0.6, 0.9, 1.35, 1.8, 2.4, 3.0, 4.0, and 5.0 mg/ml (leaves), and 0.2, 0.6, 0.8, 1.2, 2.4, 3.0, 4.0, and 5.0 mg/ml (fruits), or ascorbic acid (standard) (0.16, 0.26, 0.6, 1.0 and 1.30 mg/ml) in MeOH (*v*/*v*) soln. (20 μl) were added. The decrease in absorbance at 515 nm was determined until the reaction plateau step was reached. Methanol was used to zero the spectropho-

tometer. EC_{50} Values were determined from the plotted graph of scavenging activity against the concentration of samples, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. Triplicate measurements were carried out, and their scavenging effect was calculated based on the percentage of DPPH scavenged.

TBARS (Thiobarbituric Acid Reactive Species) *Assay.* The lipid peroxidation assay as TBARS was carried out by a modified method [27]. The reaction mixture containing, in a final volume of 1.1 ml, 100 µl cerebral tissue (whole brain), and 1 ml (0.05M) of KH_2PO_4/K_2HPO_4 buffer, pH 7.4, in NaCl (0.9%), and six concentrations of the essential oils (20, 50, 100, 150, 200, and 250 µg/ml) was incubated at 37° for 1 h. Then, 1 ml of thiobarbituric acid (0.5%) and 1 ml of Cl_3CCOOH (20%) were added to the test tubes and were incubated at 100° for 60 min. After cooling, absorbance was measured at 532 nm against control and buffer, BHT being used as reference compound. All the experiments were performed in triplicate and the results were averaged. The inhibition percentage was determined by comparison of the results between the samples and control.

2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic Acid) (ABTS) *Radical Cation-Scavenging Assay.* The antioxidant activity of *M. leucadendra* essential oils was measured in terms of free-radical-scavenging ability according to ABTS method reported in [28] with minor modifications. A 7.4 mM soln. of ABTS (*BDH Chemicals Ltd.*, England) was prepared in phosphate buffer saline, pH 7.4, and oxidized using a 2.45 mM soln. of potassium persulfate for at least 12–16 h in dark. The $ABTS^{\bullet+}$ soln. was diluted with MeOH to an absorbance of 0.70 ± 0.02 at 734 nm. For measuring antioxidant capacity, 100 µl of the sample (essential oil diluted in MeOH) was mixed with 1.0 ml of $ABTS^{\bullet+}$ soln. The absorbance of the above mixture was measured at 734 nm after 10 min. Appropriate blank measurements were carried out, and the values were recorded. MeOH solns. of known 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (*Trolox*; *BDH Chemicals Ltd.*, England) concentrations were used for calibration. The absorbance of the reaction mixture of ABTS and an antioxidant is compared to that of the *Trolox* standard, and the results are expressed in terms of *Trolox* equivalent antioxidant capacity (TEAC).

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