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TERPENOID COMPOSITION OF *LIPPIA DULCIS**

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Key Word Index—*Lippia dulcis*; Verbenaceae; yerba (hierba) dulce; tzonpelic xihuitl; chemotaxonomy; chemotype; terpenoids; camphor; hernandulcin; epihernandulcin.

Abstract—The composition of *Lippia dulcis*, an intensely sweet herb endemic to tropical America, was investigated using plants collected in Puerto Rico. Contrary to previous reports, it is characterized by the presence of a high percentage of sesquiterpenoids (79%). Isolation experiments and gas chromatographic characterization using direct injection, show that the intensely sweet sesquiterpenoid (+)-hernandulcin (36%) and its epimer (−)-*epi*-hernandulcin (22%) are the main constituents of these herbs. The oil contains, if any, undetectable amounts of camphor ($\ll$0.01%). These findings are in sharp contrast with the composition reported earlier for 'Hierba dulce', a pungent Mexican plant identified as *L. dulcis*. Only trace amounts of hernandulcin (40 ppm wt/wt, dry weight) were reported in the plants from Mexico. The volatile oil isolated from these plants by steam distillation consisted mainly of monoterpenoids (86%). A bitter monoterpenoid, camphor, made up as much as 53% of the essential oil of the Mexican plants. If the identity of the Mexican plants is confirmed, these results may reveal the existence of two chemotypes of this herb, i.e. an hernandulcin type and a camphor type. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Lippia dulcis Trev. (the name of this taxon is synonymous with *Phyla scaberrima* Mold., *Lippia scaberrima* and *Zapania scaberrima*) is a traditional medicinal plant with a long history [1] of use in tropical America for the treatment of coughs, bronchitis and urinary retention [2, 3]. It is a strongly aromatic, perennial herb with intensely sweet leaves and flowers. Native to tropical America, its habitat extends from southern Mexico to Panama and Colombia, at elevations of up to 1800 m, growing in moist thickets, wooded edges of rivers and ponds, and invading clearings and fields [4]. This species is also found in Puerto Rico, Cuba, Hispaniola and other Caribbean islands [5]. It is cultivated in Puerto Rico as a medicinal plant [6].

Numerous vernacular names are used to denote *L. dulcis* in folkloric medicine [7, 8]: 'orozuz de la tierra' (Cuba); 'tzonpelic xihuitl' and 'hierba buena' (Mexico); 'hierba dulce' and 'yerba dulce' (Mexico, Puerto Rico); 'orozus' and 'oro azul' (Cuba, El Salvador, Costa Rica, Nicaragua); 'orosul' (Nicaragua); 'corronchocho' (El Salvador); 'salvia santa' (El Salvador, Mexico); and 'honey herb' (U.S.A.). In some parts of Mexico (Toluca, Mexico; Tlayacapan, Morelos) the plant decoction is taken as a remedy for coughs, catarrah, bronchitis, asthma and colic [4]. Likewise, it is a common treatment for coughs and colds throughout Central America [9] and has been exported to Europe for similar medicinal uses [3]. In other parts of Mexico (Zitacuaro, Mexico; Patzcuaro, Michoacan; Orizaba, Veracruz; Mexico City), the plant is used as emmenagogue and abortifacient [10]. Puerto Ricans drink the plant infusion for the treatment of bronchitis and as sedative to overcome coughing and gastrointestinal colic [4, 6].

In spite of numerous investigations [11–17], the chemical composition of the whole plant of *L. dulcis* remains uncertain, particularly concerning its camphoraceous constitution. Recent phytochemical analysis of plant samples collected in Mexico (Tlayacapan, Morelos [18]), where the herb is known by the vernacular name 'hierba dulce' and is used against dysentery and abdominal inflamation, led to the identification of the intensely sweet, bisabolane sesquiterpenoid hernandulcin (**1**) as the sweet principle of *L. dulcis* [19]. However, when additional plant samples [20] were studied, they showed only trace amounts of

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*Dedicated to José Luís Martinez Picó, Professor Emeritus and Dean Emeritus of the University of Puerto Rico on the occasion of his 78th birthday.

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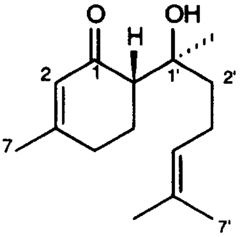

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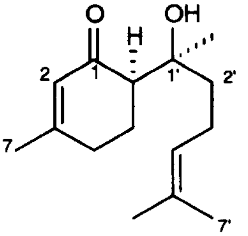

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sweet principle (40 ppm wt/wt dry weight, *ca* 4 ppm fresh weight) [21]. Steam distillation of similarly constituted plant material afforded a volatile oil with a slightly sweet taste [22]. Plant material collected in Tlayacapan (Morelos) was mixed with a large fraction of 'hierba dulce' purchased in Mexico City, where it is mainly sold as an emmenagogue and abortifacient [21].) GC-mass spectrometric analysis showed that this oil was composed mainly of monoterpenoid (86%) and sesquiterpenoid (14%) constituents, with camphor making up as much as 53%. Perplexedly, a bitter monoterpenoid, camphor, was the main constituent of the essential oil from an aromatic herb described in the ancient literature as 'sweeter than honey'. Hernandulcin, though present in the oil, could not be detected.

During the course of a screening programme to identify a competent model system for the *in vitro* production of essential oils [23], we analysed the aerial parts of wild *L. dulcis* collected in Puerto Rico and surprisingly found that its two main constituents were (+)-hernandulcin and its epimer (−)-*epi*-hernandulcin [24]. Camphor was not found in any of the oils extracted, even though we specifically looked for this constituent. Intrigued by these findings, we decided to reinvestigate this species. Here, we provide a detailed report of our initial observations and report for the first time the occurrence in this species of 14 known sesquiterpenes and four known oxygenated sesquiterpenoids.

RESULTS

The search for 'tzonpelic xihuitl' began in 1988 with professional enquiries concerning the botany of this species. In the summer of 1989, an abundant population of a plant, known by the vernacular name 'yerba dulce,' was found in Orocovis (central Puerto Rico). This plant matched the 'tzonpelic xihuitl' of Francisco Hernández in size, number and structure of leaves, inflorescence, organoleptic characteristics and folkloric medicinal uses. (Hernández does not mention any properties as emmenagogue in his accurate description of 'tzonpelic xihuitl.') The plant was indeed identified as *L. dulcis* by a professional taxonomist. Representative plant samples were collected from this population. To preserve the germplasm, seeds from this material were germinated *in vitro* in 1990 [25] to initiate a stock of axenic plantlets. The stock has been maintained since by vegetative propagation using single-node culture techniques [26].

Isolation of hernandulcin and epihernandulcin

Plants collected in Orocovis were air dried and their leaves and flowers chopped and milled, to provide material for isolation of the volatile constituents. The dry powder was extracted with a succession of solvents in a Soxhlet apparatus. Pentane was used for the removal of the least polar components, and more polar solvents (CH_2Cl_2, Et_2O, Me_2CO, CH_3OH and H_2O) were employed for the sequential removal of polar compounds. Each crude extract was analysed by TLC and screened for volatile constituents by capillary GC with flame ionisation detector (FID) and mass selective detector (MSD). Two main components were found in the pentane and CH_2Cl_2 extracts. (Detection of hernandulcin or *epi*-hernandulcin was not possible using the GC conditions of Kinghorn and co-workers [21, 22].) Samples were spotted on normal phase silica plates and developed with hexane–Me_2CO (17:3). A prominent spot with R_f 0.50 was observed and when tested organoleptically was found to have an intense sweet taste. This spot was undoubtedly hernandulcin. Below the spot of hernandulcin, there was a second prominent spot with R_f 0.35 which was subsequently identified as *epi*-hernandulcin.

The pentane and CH_2Cl_2 extracts were concentrated under vacuum yielding 14–20 and 35–50 mg g^{-1} dry weight, respectively, of a yellow–green oil with a refreshing scent. The extracts were separated by prep TLC with hexane–Me_2CO (17:3). Individual fractions were subjected to HPLC-UV analysis and rechromatographed to afford pure (+)-hernandulcin (**1**, 12–18 mg g^{-1}) and (−)-*epi*-hernandulcin (**2**, 7–11 mg g^{-1}). It should be noted that, although (±)-*epi*-hernandulcin is well known as a synthetic analogue, it was unknown as a major constituent of *L. dulcis* when we performed these studies in 1991 [27]. The structure of **2** was established from its chromatographic behaviour and spectral characteristics. The mass spectrum of **2** (GC-mass spectrometry, EI 70 eV) did not show a $[M]^+$ at *m/z* 236, but its fragmentation pattern was identical to that of **1**, suggesting a diastereoisomeric structure. The compound was identified as (−)-*epi*-hernandulcin by comparison of spectral data ($[\alpha]_D$, IR, UV, ^1H and ^{13}C NMR) with synthetic (+) and (−) and (±) forms of this compound. The spectra exhibited by **2** isolated from *L. dulcis* were identical to those previously reported for the synthetic analogues [28]. These findings established for the first time the identities of **1** and **2** as the main constituents of *L. dulcis*.

Extraction of essential oil

Microscale solid–liquid extraction with different solvents was used effectively to optimize the conditions for isolation of concretes rich in volatile constituents. A new extraction procedure was developed by adapting the microfiltration Craig-tube technique [29]. This permitted very clean and rapid screening of major volatile constituents using small quantities of dry plant material. Considering the pharmacological value of essential oils and their economic importance, the Craig solvent microscreening procedure is detailed in the experimental part.

Concretes obtained with different solvents (pentane, hexane, CH_2Cl_2, Et_2O, Me_2CO, MeOH or H_2O) using the microextraction Craig-tube technique were concentrated under vacuum and analysed using TLC and capillary GC. Significant oil yields with a minimum of high M_r substances were obtained with CH_2Cl_2, and this was thus the solvent of choice for macroextraction experiments. The oil was isolated subsequently in larger scale experiments by continuous extraction with CH_2Cl_2 in a Soxhlet apparatus. Concentration of the CH_2Cl_2 extracts yielded 40–60 mg g^{-1} of a yellow–green oil with a refreshing scent (4–6% based on dry weight). Sample sizes varied between 1, 10 and 100 g dry weight. The variation observed in oil yield is probably due to unavoidable experimental errors and to the heterogeneity of the plant population regarding maturity and size.

Essential oil composition

This was determined by a thorough analytical study using capillary GC. The conditions used in the published GC study [21, 22] of plants from Mexico were not adequate for analysis of the isomeric hernandulcins, which are relatively non-volatile, thermally sensitive compounds. Substantial modification of these conditions during the present study have allowed their GC-mass spectrometric quantitation with minimal peak-shape deformation and mass-spectrum distorsion.

Optimum GC conditions were found in the following way. The starting parameters used were those published by Kinghorn and co-workers [21, 22], which consist of the application of low injector temperatures (70–80°), to preserve thermally sensitive hernandulcin, and the use of an open split outlet (split ratio 17:1) to concentrate the sample. We inserted a typical split liner in the injection inlet, although no details were available concerning this crucial parameter (see Experimental, GC section). Oil samples were tested using both a 25-m HP Ultra-2 column and a 60-m Supelco SPB-5 column, with split ratios of 40:1 and 15:1, respectively. The oven temperature was set at 35° for 4 min and programmed to increase to 300° at 4° min^{-1}, as published. After several unsuccessful trials we concluded that with these conditions it was impossible to quantitate hernandulcin due to incomplete vapourization in the cold inlet. Without complete sample vaporization, the less volatile constituents failed the column transfer step during the hold time and were slowly vented during the analysis time.

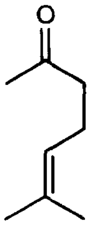
Flash vapourization in a hot inlet, a useful method for samples with thermally labile components, provided better results. To implement this technique, the sample injector was set to much higher temperatures (t_{inj} = 180–250°) than the initial column temperatures (t_{cl} = 35–70°) [30]. The following injection modes were tried.

Split injection

We found that the isomeric hernandulcins could not be quantitated using split injection due to extensive pyrolysis in the hot inlet. Higher injector temperatures facilitated sample vapourization but the column packing in the split liner did not allow short residence times, causing extensive pyrolysis. In fact, the most prominent peaks in the chromatograms were the thermal fragments of hernandulcin, 6-methyl-5-hepten-2-one (**3**) and 3-methyl-2-cyclohexen-1-one (**4**).

Splitless direct injection

To implement this injection mode, we inserted in the capillary inlet different open glass liners, without any packing material, and tried them in turn. The object was to minimize sample and carrier gas mixing while allowing short residence times. Excellent results were obtained with a special small-bore liner (~1.3 mm internal diameter, 140 μl) capable of very rapid on-column sample transfers using small amounts (0.1–0.2 μl) of concentrated solutions. The sample was vapourized in the hot (~220°) injector and carried directly to the column during the hold-time (30–60 s). To use the full efficiency of the column, the sample was reconcentrated on the column head prior to separation. The initial column temperature was set near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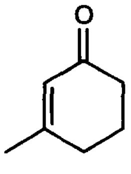
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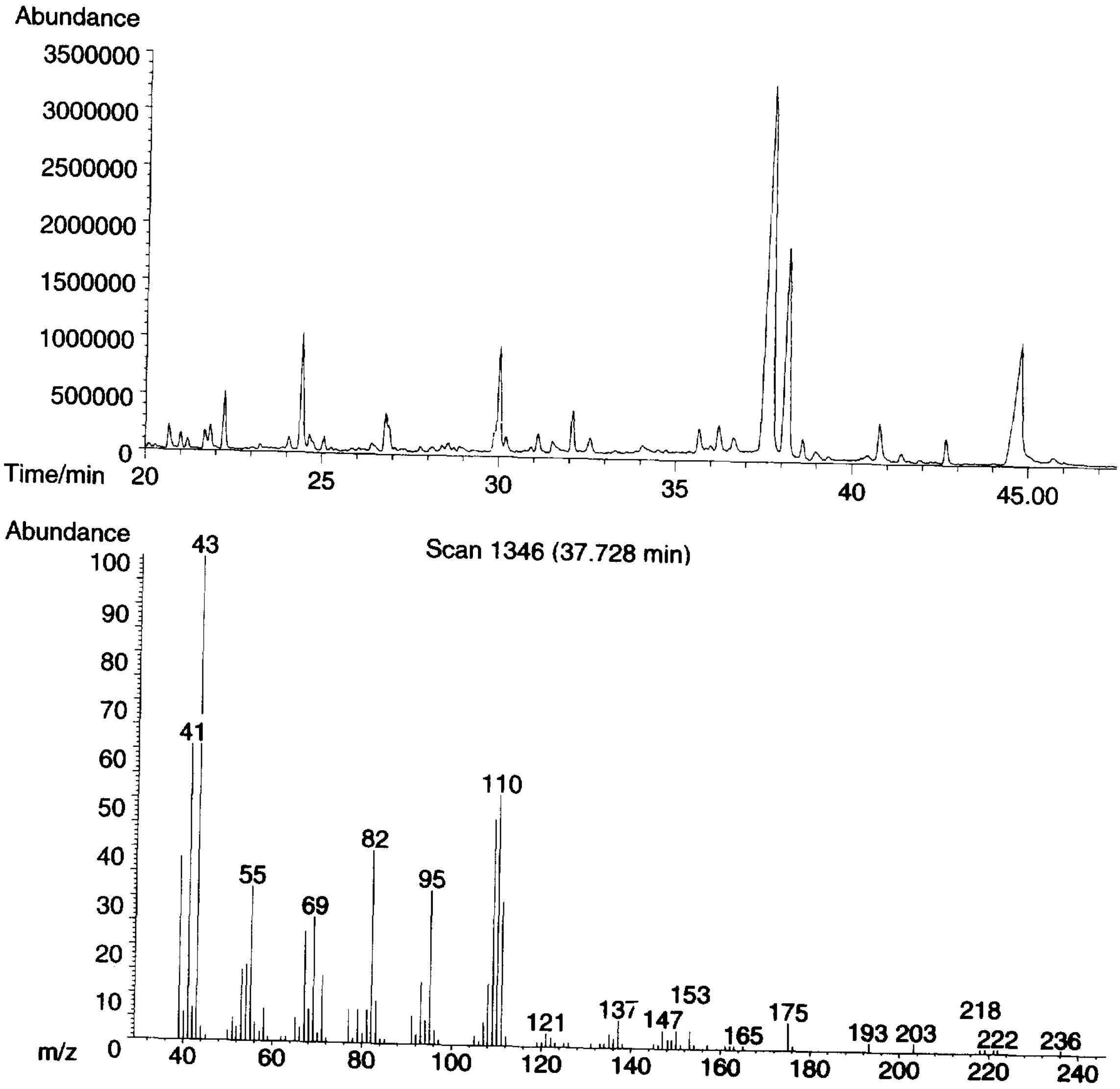

Fig. 1. Section of total ion reconstructed chromatogram of essential oil from wild *Lippia dulcis* extracted with CH_2Cl_2 showing the hernandulcin region. The full mass spectrum of hernandulcin at the maximum peak intensity is displayed. For analytical conditions see Experimental.

the boiling point of the solvent (60–70°, 2–4 min) to achieve a good solvent effect. In this way, it was possible to make a quantitative transfer of the sample in about 30 s. Longer hold times did not lead to an increase in peak sizes but they allowed more solvent to enter the column, obscuring early peaks of interest. Following sample reconcentration on the column head, we applied a two-level temperature programme. The first ramp was steep (10° min^{-1}), to bring up the oven to analysis temperature (125°, 5 min); the second ramp was shallower (2° min^{-1} to 250°) to permit analysis (60 min). This combination provided excellent chromatographic results with minimum thermolysis of the isomeric hernandulcins, very little band shape deformation and minimum mass-spectrum distortion. Figure 1 shows the hernandulcin region of a typical total ion reconstructed chromatogram (TIC). The full mass spectrum of hernandulcin at the maximum peak intensity is displayed.

Essential oil constituents

The formula and retention indices of the constituents identified, together with the *CAS* registry numbers associated with these compounds in the *Dictionary of Natural Products on CD-ROM* [31] are listed in Table 1. Each oil component was tentatively identified by comparison with the relative retention times of authentic samples and by computer library search programs that include mass spectral data and GC retention times. Confirmation of the identifications, excluding the noted exceptions, was accomplished by visual comparison of the full mass spectrum with standards from *The Wiley/NBS Registry of Mass Spectral*

Table 1. Composition of *Lippia dulcis* collected in Orocovis (Puerto Rico). The composition of the oil from the mixture of plants collected in Tlayacapan (Morelos) and purchased in Mexico City is shown for comparison [22]

				Total ion chromatogram		
					Area (%)	
Compound no.	Formula	Name	CAS*	RR_t†	Orocovis	Mexico
1	$C_6H_{10}O$	(*E*)-2-Hexenal	6728-26-3	0.517	0.14	0.00
2	$C_6H_{12}O$	(*E*)-2-Hexen-1-ol	928-95-0	0.727	0.11	0.00
3	$C_{10}H_{16}$	α-Thujene	2867-05-2	0.819	0.05	0.00
4	$C_{10}H_{16}$	α-Pinene‡	80-56-8	0.853	0.00	2.49
5	$C_{10}H_{16}$	Camphene‡	79-92-5	0.904	0.12	15.99
6	$C_8H_{14}O$	6-Methyl-5-hepten-2-one‡	110-93-0	1.000	2.77	0.51
7	$C_{10}H_{16}$	*β*-Pinene‡	127-91-3	1.011	0.00	0.59
8	$C_{10}H_{16}$	Myrcene‡	123-35-3	1.061	0.00	1.45
9	$C_{10}H_{16}$	Limonene‡	138-86-3	1.197	0.00	7.47
10	$C_7H_{10}O$	3-Methyl-2-cyclohexen-1-one	1193-18-6	1.207	2.81	0.00
11	$C_{10}H_{16}$	Terpinolene	586-62-9	1.351	0.45	2.79
12	$C_8H_{14}O_2$	(*E*)-2-Hexenyl acetate	2497-18-9	1.399	0.58	0.00
13	$C_8H_{14}O_2$	(*Z*)-3-Hexenyl acetate	3681-71-8	1.408	0.78	0.00
14	$C_{10}H_{18}O$	Linalool‡	78-70-6	1.427	0.06	0.43
15	$C_{10}H_{16}O$	**Camphor‡**	76-22-2	**1.553**	**0.00**	**53.24**
16	$C_{10}H_{18}O$	Borneol‡	10385-78-1	1.622	0.12	1.12
17	$C_{10}H_{18}O$	α-Terpineol‡	98-55-5	1.727	0.00	0.21
18	$C_7H_9NO_2$	3-Ethyl-4-methyl-1*H*-pyrrole-2,5-dione	20189-42-8	1.802	0.15	0.00
		Total monoterpenoids (%)			**8.14**	**86.29**
19	$C_{15}H_{24}$	α-Copaene	3856-25-5	2.735	0.73	1.60
20	$C_{15}H_{24}$	*β*-Bourbonene	5208-59-3	2.808	0.12	0.00
21	$C_{15}H_{24}$	*β*-Cubebene	13744-15-5	2.832	0.14	0.00
22	$C_{15}H_{24}$	Eremophilene	10219-75-7	2.933	0.05	0.00
23	$C_{15}H_{24}$	*trans*-Caryophyllene	87-44-5	3.023	0.75	3.00
24	$C_{13}H_{22}O$	(*E*)-Geranylacetone	3796-70-1	3.147	0.05	0.00
25	$C_{15}H_{24}$	*trans*-α-Bergamotene	13474-59-4	3.158	0.10	0.00
26	$C_{15}H_{24}$	Isocaryophyllene	118-65-0	3.179	0.15	0.00
27	$C_{15}H_{24}$	(*E*)-*β*-Farnesene	18794-84-8	3.288	0.40	0.00
28	$C_{15}H_{24}$	(*Z*)-*β*-Farnesene	28973-97-9	3.319	0.35	0.00
29	$C_{15}H_{24}$	Aromadendrene	489-39-4	3.364	0.05	0.00
30	$C_{15}H_{22}$	*ar*-Curcumene	644-30-4	3.394	0.11	0.00
31	$C_{15}H_{24}$	*cis*-*β*-Guaiene	88-84-6	3.432	0.27	0.00
32	$C_{11}H_{16}O_2$	Dihydroactinidiolide	15356-74-8	3.529	0.17	0.00
33	$C_{15}H_{24}$	*γ*-Cadinene	1460-97-5	3.547	0.13	1.40
34	$C_{15}H_{24}$	Germacrene B	15423-57-1	3.586	0.43	0.00
35	$C_{15}H_{24}$	α-Muurolene	31983-22-9	3.618	0.10	0.00
36	$C_{15}H_{24}$	*β*-Bisabolene	495-61-4	3.706	0.19	0.00
37	$C_{15}H_{24}$	Unknown	–	3.755	0.11	0.00
38	$C_{15}H_{24}$	*δ*-Cadinene	483-76-1	3.798	0.65	0.00
39	$C_{15}H_{24}$	Unknown		3.971	0.10	0.00
40	$C_{15}H_{26}O$	Unknown	–	4.085	0.12	0.00
41	$C_{15}H_{26}O$	(*Z*)-Nerolidol	142-50-7	4.110	0.21	0.00
42	$C_{15}H_{24}O$	Spathulenol	6750-60-3	4.171	1.24	0.00
43	$C_{15}H_{26}O$	Globulol	489-41-8	4.848	0.49	0.00
44	$C_{15}H_{26}O$	α-Bisabolol	515-69-5	5.127	2.91	0.00
45	$C_{15}H_{24}O_2$	Isomers related to hernandulcin	–	5.957	11.40	0.00
46	$C_{15}H_{24}O_2$	**(+)-Hernandulcin**	95602-94-1	6.431	36.00	0.00
47	$C_{15}H_{24}O_2$	**(−)-*epi*-Hernandulcin**	110715-85-0	6.518	22.00	0.00
		Total sesquiterpenoids (%)			**79.52**	**6.00**
		Total volatile fraction (%)			**87.66**	**92.29**

* CAS registry numbers are listed for structure identity and to avoid synonym confusion.

† Relative retention time with respect to 6-methyl-5-hepten-2-one $RR_t = 1.000$.

‡ Standard.

Data, in hard-copy [32] and electronic versions [33], and by comparison with authentic samples, when available.

DISCUSSION

The gas chromatogram of the essential oil from *L. dulcis* collected in Puerto Rico consists of two main sections. A light oil fraction, composed mainly of monoterpenoid (C_{10}) and sesquiterpenoid (C_{15}) substances (Table 1), and a heavy oil fraction, made up of higher M_r materials (C_{16} to C_{30}). The more volatile fraction is similar to essential oils from other *Lippia* spp. that have a primarily sesquiterpenoid composition [34].

The isolation experiments and the GC data confirm that the main constituents of this volatile oil are hernandulcin (**1**, 36%) and its stereoisomer *epi*-hernandulcin (**2**, 22%). The thermal fragments **3** and **4** seem to be true extract constituents, since they are found in noticeable yields even under minimum thermolysis conditions. Additional oxygenated constituents are reported here for the first time, namely the known sesquiterpenoids α-bisabolol, globulol, spathulenol and nerolidol. We report, also for the first time, the following known sesquiterpenes as constituents of *L. dulcis*: β-bourbonene, β-cubebene, eremophilene, α-bergamotene, isocaryophyllene, (*E*)- and (*Z*)-β-farnesene, aromadendrene, α- or *ar*-curcumene, *cis*-β-guaiene, γ-cadinene, germacrene B, α-muurolene, β-bisabolene and δ-cadinene. We must stress that the toxic and bitter monoterpenoid camphor was not detected in any of the oils extracted, even though we specifically looked for this constituent.

Lippia dulcis plants collected in Mexico afforded very low yields (1.0–7.4 mg g^{-1}) of essential oils that exhibited a predominant monoterpenoid composition, with camphor alone representing as much as 53%. Some differences in composition might be due to the use of different methodologies for phytochemical analysis. Firstly, differences in the extraction technique. Essential oils are extracted from plant materials by two main methods: simple expression or extrusion with pressure; or steam, water or dry distillation [35]. Presumably, because the official method of essential oil extraction is based on steam distillation [36], Kinghorn and co-workers extracted the Mexican plants using this method [22]. We opted to obtain concretes by low-temperature solvent extraction rather than distillation or expression. This was necessary because the essential oil was affected adversely by hot water and steam, and because a more complete extraction of constituents was highly desirable.

Secondly, differences in analytical methodology, particularly in handling the GC capillary inlet, may also be responsible. The GC conditions reported by Kinghorn (a low injector temperature with an open split outlet) suggest a *solvent split injection*, in which solvent evaporation takes place at low injector temperatures with an open split outlet. However, since the injector temperature is not raised, the sample, deposited as a liquid plug onto the liner packing connecting the column and injector, is bound to fail the column transfer step in the splitless period. This could be described as an *on-column split* injection, which is an uncommonly used injection method [30]. The end result under these conditions is that most of the solute is released belatedly and lost through the split outlet. This made the detection of hernandulcin impossible.

Splitless direct injection, the technique we have optimized for hernandulcin, has two advantages over the other injection modes. Firstly, this is a flash vaporization technique and, with the use of a small-bore insert, the higher injector temperatures required for complete sample volatilization can be used effectively, even with thermally sensitive components. Secondly, the much smaller volume (~ 140 μl) of the direct injection liner allows, with a standard flow rate of 1 ml min^{-1} and a hold time of 30 s, to sweep the insert about three times before the solvent is purged. Thus, a very rapid on-column sample transfer can be achieved avoiding thermal degradations. In fact, we calculate that with an injection volume of 0.1 μl, at 225° and 15–25 psi, a 25-μl vapourization volume is generated; this can be swept into the column in <2 s at a flow rate of 1 ml min^{-1} (16.6 μl s^{-1}).

This GC methodology, when combined with the Craig-tube microextraction technique, provides an almost ideal method for rapid phytochemical screening of essential oils extracted with a succession of solvents. Numerous screening and isolation methods are available for the detection and extraction of phytochemical constituents from a given homogeneous and representative plant sample [37]. Most of these are based on tedious procedures and require large amounts of plant material for chemical analysis. For instance, the official method of essential oil extraction is based on steam distillation for 4 hr and requires 10–50 g of dry plant material. In the abridged official method, the distillation period is reduced to 1 hr, but it still requires 1 g of powdered plant material. With the Craig-tube technique, just 50–100 mg of dry plant material is sufficient for rapid phytochemical screening.

Nevertheless, in spite of all these differences in analysis and extraction techniques, it still remains to be clarified why (i) the amounts of hernandulcin were so low in the plants collected in Mexico as *L. dulcis*, and (ii) the concentrations of the bitter monoterpenoid camphor were so high in an intensely sweet herb. The large amounts of hernandulcin isolated from the extracts of *L. dulcis* collected in Puerto Rico, together with the complete lack of camphor in any of the extracts analysed, proves beyond a reasonable doubt that at last the 'tzonpelic xihuitl' of Francisco Hernández has been found. The plant known in Puerto Rico by the vernacular name 'yerba dulce' [6] has all the characteristics which the Aztec physicians described to him more than 400 years ago [38]. As

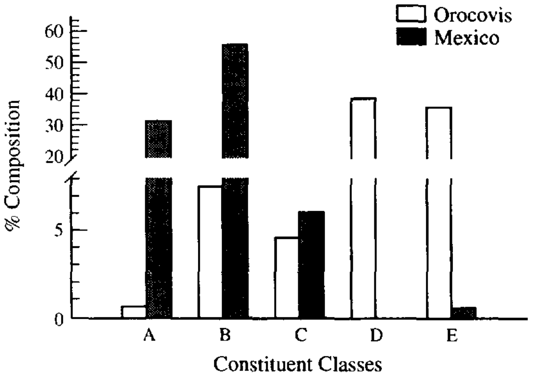

Fig. 2. Comparison of the distribution of constituents found in *Lippia dulcis* plants from Orocovis (Puerto Rico) and the mixture of plants from Tlayacapan and Mexico City (Mexico): (A) monoterpenes, (B) monoterpenoids, (C) sesquiterpenes, (D) sesquiterpenoids excluding hernandulcin, (E) hernandulcin.

such, it is cultivated as a medicinal plant for the treatment of bronchitis or as sedative to overcome coughing and gastrointestinal colic. No records are available here supporting any emmenagogue properties or the alleged abortifacient activity attributed in Mexico to 'hierba dulce'.

The contrast between *L. dulcis* from Puerto Rico and Mexico is striking (Fig. 2). These plants differ sufficiently in medicinal uses and in essential oil yield and composition to raise serious questions about the real identity of the Mexican plants (Table 2). Initially, we tried to explain the differences by assuming that the samples contained different populations of the same plant species. It is well known that oils obtained from different varieties of the same species may differ widely in composition. This can be due to environmental pressures that lead to adaptation to new conditions by spontaneous mutation [39]. In this sense, the marked differences in the accumulation of camphor and hernandulcin between two populations of *L. dulcis* would suggest the existence of two chemotypes, an hernandulcin type and a camphor type. However, this implies a high plasticity for this plant in response to varying environmental conditions, for which we can find no published evidence. Alternatively, the collection of Mexican plants is suspect since material collected in Tlayacapan (Morelos) was mixed with a large fraction of 'hierba dulce' purchased in Mexico City [21]. The differences in composition can have a simple explanation: the Mexican plants purchased in Mexico City at the stands of medicinal plant vendors may not be authentic *L. dulcis*.

The information available is not sufficient to identify the relationship between 'tzonpellic xihuitl,' the hernandulcin-producing chemotype of *L. dulcis*, and the plants purchased in Mexico City. However, the evidence presented here is conclusive in that 'tzonpellic xihuitl', *L. dulcis* from Puerto Rico, is not a camphoraceous Verbenaceae. The apparent paradox concerning the large quantities of camphor in an intensely sweet herb is thus solved. The common practice of loosely using the name 'lippia mexicana,' a camphoraceous herb, to denote *L. dulcis* and its association with 'tzonpellic xihuitl', may have caused the present confusion, leading some to believe that *L. dulcis* is also a camphoraceous herb with pungent leaves.

EXPERIMENTAL

General. Optical rotations were recorded in CHCl$_3$ (*ca* g/100 ml) using a 10-cm quartz cell thermostatted at 25°. ^{1}H and ^{13}C NMR: measured in CDCl$_3$ at 300 MHz (^{1}H) and 75 MHz (^{13}C), with TMS as int. standard. UV spectra: recorded in EtOH sol. IR spectra: obtained using thin films on AgCl plates. HPLC, analytical and prep.: done using an instrument equipped with automated gradient, column temp. control, digital data module and fixed wavelength UV absorbance detector (λ = 254 and 313 nm). Capillary GC was carried out using FID detector set at 250°. Mass spectra were recorded by GC-MS with a quadrupole mass-selective detector (EI, 70 eV, electron multiplier 1600–1800 V, scan rate 1.21 scan s^{-1}, scanned mass range 45–400 mu, ion source 200°, transfer line 180°). The

Table 2. Distribution of constituents and ethnopharmacology of *Lippia dulcis* plants from Orocovis (Puerto Rico) and Mexico City (Mexico)

Habitat	Vernacular names, medicinal uses	Number of constituents (% composition)				
		Monoterpenes	Monoterpenoids	Sesquiterpenes	Sesquiterpenoids	Hernandulcins
Mexico	Hierba dulce, salvia santa. Emmenagogue, abortifacient	6 (30.78)	5 (55.51)	3 (6.00)	0 (0)	1 (0.5)
Puerto Rico	Yerba dulce. Coughs, urinary retention, bronchitis	3 (0.62)	9 (7.52)	19 (4.93)	8 (16.59)	2 (58.0)

MS of the main constituents were interpreted using *MassSpec* 2.01, a graphics-based mass-spectrum analyser from Trinity Software. Peaks arising from the gain or loss of one hydrogen atom and their corresponding abundances are reported here in italic Arabic numerals: m/z (rel. int.) *151*, 150, *149*, [219-C_5H_9, $C_{10}H_{14}O$] (*2*, 8, *3*). All solvents used were of the best analytical grade available.

Plant material. Field work was carried out in Puerto Rico for the first time during the summer of 1988 with the purpose of collecting authentic samples of the plant described by Francisco Hernández as 'tzonpelic xihuitl' [1]. Prof. Miguel Vives, a plant taxonomist at Quebradillas, immediately brought to our attention a plant known by the vernacular name 'yerba dulce', which he identified as *L. dulcis* [4, 7]. Mr Benigno Rodríguez, a naturalist and botanical chemist at Quebradillas, provided specimens of different *Lippia* spp. growing in this region, namely, *L. alba*, *L. helleri*, *L. micromera*, *L. grandiflora* and *L. dulcis*. Additional specimens of *L. dulcis* were obtained from the collection of medicinal plants of Mr Francisco López, a herbalist at Urbanización El Comandante, Rio Piedras. Collections of *Lippia* spp. deposited at the Herbarium of the Botanical Garden of Puerto Rico were surveyed to find natural populations of *L. dulcis*. Guided by these enquiries, in the summer of 1989 we conducted field studies in Central Puerto Rico. In Sector Toro Negro, Orocovis, we found abundant samples of this species at the country estate of Mrs María Ortolaza, Road No. 143, km. 30.7. A specimen of this population is deposited at the Herbarium of the Biology Department, UPRM. Prof. Duane A. Kolterman, the plant taxonomist in charge, identified the plants as *L. dulcis*. The size, number and structure of leaves, the inflorescence, and the organoleptic characteristics of *individual plants* matched the 'tzonpelic xihuitl' of Francisco Hernández. Additional plant samples were collected during the period 1989–95. Immediately after collection, the plants were surface-sterilized in a soln containing 20% household bleach (1% NaOCl) and 0.1% (v/v) Tween 20 for 10 min, air-dried for 3–4 weeks (25°, 70% RH), and their leaves and flowers chopped and milled. The dry powder was preserved deep-frozen and, prior to use, was defrosted in a desiccator.

TLC. Plant extracts were analysed with standard Analtech GHLF (normal phase) and RPSF (C_{18} reverse phase) silica gel plates with fluorescent indicator. Some separations were improved using Analtech HLF silica gel channelled plates (250 μm, preadsorbent, 10 × 20 cm). The plates were developed using the following solvent systems. Normal phase silica: hexane–Me_2CO (17:3 and 7:3), $CHCl_3$ and $CHCl_3$–Me_2CO (1:1 and 1:4). Reverse phase: Me_2CO–H_2O (49:1 and 4:1), *iso*-PrOH–H_2O (49:1 and 1:1), CH_3CN and MeCN–H_2O (4:1, 3:2 and 1:1). Prep. TLC to isolate **1** and **2** was with Analtech GHLF silica gel plates (1–2 mm, 20 × 20 cm) with fluorescent indicator, 20–40 mg each plate, eluted with hexane–Me_2CO (17:3). Visualization of the separations was by UV-activated fluorescence quenching. Compounds were recovered with hexane–Me_2CO (7:3).

HPLC. Analysis of selected plant extracts and the purity check of the prep. TLC bands were done using a Waters Nova-Pack C_{18} column (3.9 mm × 150 mm), eluting with MeCN–H_2O (1:1) and with a Resolve Silica column (3.9 mm × 150 mm), eluting with hexane–Me_2CO (17:3). The flow rate was set at 1 ml min^{-1} with a back-pressure of 2000 psi at 30°. UV-active frs were detected at 254 nm.

GC. Plant extracts and TLC frs were analysed by GC with the following fused silica capillary columns: HP Ultra-2 with 5% diphenyldimethylsilicone cross-linked film (25 m long × 0.2 mm ID × 0.33 μm film thickness), Supelco SPB-5 with 5% diphenyl 94% dimethyl 1% vinylsilicone cross-linked film (60 m × 0.25 mm × 1 μm) and SGE BP-1 with dimethylsilicone cross-linked film (25 m × 0.22 mm × 0.25 μm). He was used as carrier gas with a column head pressure of 15–25 psi. The inj. temp. was set at 80° [22] but was later changed to the optimum value of 220–225°. Samples were injected using split (split ratios of 40:1 for the Ultra-2 column and 15:1 for the SPB-5 column), splitless and direct injection modes. The split liner is a borosilicate glass tube (4-mm ID, nominal volume 500 μl) with a nozzle-cup mixing chamber and a small amount of column packing (10% OV-1 on 80/100 Chromosorb-WHP) retained between silanized glass wool plugs. The split/splitless liner (4 mm ID, ~990 μl) was used without any packing material. A plug of silanized glass wool present in the liner was removed for splitless injections (this packing was found to cause adsorption and decomposition of thermally sensitive hernandulcins). The direct injection liner (1.3 mm ID, ~140 μl) is a special, small-bore, hollow tube. A typical analysis was done injecting 0.1–0.2 μl of 10% oil solns in $CHCl_3$ unless otherwise stated. Oil components were tentatively identified by comparison with the RR_ts of known components using GC-FID. Selected samples were analysed by GC-MS. Optimum temp. programmes: 25-m columns, 4 min at 70°, 10° min^{-1} to 125°, 5 min at 125°, 2° min^{-1} to 250°, and 2 min at 250°; 60-m SPB-5 column, 4 min at 25°, 4° min^{-1} to 250°, and 10 min at 250°.

Isolation of hernandulcin and epi-*hernandulcin.* A major portion of the wild plants collected in 1989 provided the material used for the isolation of hernandulcin and *epi*-hernandulcin. Expts were carried out on 10, 100 and 600-g scales. In a typical isolation expt, a 10 g sample of milled plant material was brought to constant wt in a desiccator and extracted in a Soxhlet apparatus with refluxing pentane (100 ml) for 4 hr, followed by a second 4-hr extraction with CH_2Cl_2. Individual extracts were analysed by TLC, HPLC and capillary GC with FID and MSD. The pentane and CH_2Cl_2 extracts were concd in a rotary evaporator at 25° under red. pres., yielding 142 and 350 mg, respectively, of a yellow–green oil with a refreshing scent. The oils dissolved in 1–3 ml $CHCl_3$ were spotted (1–10 μl) on normal phase silica plates and developed with the same mixed solvent system used by Kinghorn and co-workers. A sample of hernandulcin provided by Kinghorn is acknowledged. Hernandulcin has R_f 0.5 in normal phase silica developed with hexane–Me_2CO (17:3). Hernandulcin gave a prominent spot with R_f 0.50. Below the spot of hernandulcin, there was a second prominent spot (R_f 0.35), which was identified subsequently as (−)-*epi*-hernandulcin by comparison with the spectroscopic characteristics of the synthetic (+) and (−) and (±) forms of this compound. Pure (+)-hernandulcin (32 mg) and (−)-*epi*-hernandulcin (20 mg) were isolated from the pentane extract by prep. TLC (normal phase silica) using hexane–Me_2CO (17:3). Additional quantities of both compounds were worked-up by prep. TLC and semiprep. HPLC using normal phase silica.

(+)-*Hernandulcin* (**1**). Oil, bp 125° (0.1 mmHg). $[\alpha]_D^{25}$ $+115° \pm 5°$ ($CHCl_3$; *c* 0.640). UV λ_{max}^{EtOH} nm (log ε): 236 (4.12). IR (film) ν_{max} cm^{-1}: 3470, 3050, 2960, 2870, 1650, 1220, 1120, 1020, 940, 880. ^1H and ^{13}C NMR ($CDCl_3$) spectra were identical within experimental error to spectra published elsewhere [19, 21, 28]. EIMS (GC) 70 eV, *m*/*z* (rel. int.): 236 [M^+, $C_{15}H_{24}O_2$] (2), *222*, 221 [M^+-CH_3, $C_{14}H_{21}O_2$] (*1*, 7), 219, *218* [M^+-OH, $C_{15}H_{23}O$] (1, *11*), 204, *203* [221-OH, $C_{14}H_{20}O$] (1, 4), 193 [221-CO, $C_{13}H_{21}O$] (3), 176, *175* [193-OH, $C_{13}H_{20}$] (2, *11*), 161, *160* [176-CH_3, $C_{12}H_{16}$] (8, *1*), *153*, 152 [221-C_5H_9, $C_9H_{12}O_2$] (*15*, 1), *151*, 150, *149* [219-C_5H_9, $C_{10}H_{14}O$] (*2*, 8, *3*), *139*, 138, *137* [221-C_6H_{11}, $C_8H_{10}O_2$] (*1*, 1, *10*), *136*, 135, *134* [204-C_5H_9, $C_9H_{11}O$] (*2*, 5, *1*), *123*, 122, *121* [150-CO, C_9H_{14}] (*1*, 2, *6*), 112, *111* [221-CH_3-*c*-C_6H_6O, $C_7H_{12}O$] (3, *44*), *110*, 109, *108* [152-$CH_3C{=}O$, C_7H_9O] (*78*, 77, *16*), *95*, 94, *93* [109-CH_3, C_6H_6O] (*40*, 7, *15*), *84*, 83 [112—CHO, C_6H_{11}] (*1*, 10), 82, *81* [C_5H_6O] (33, *11*), *72*, 71 [C_4H_7O] (*1*, 16), 69 [C_5H_9] (31), 68, *67* [C_5H_8] (8, *27*), *59*, 58, *57*. [C_3H_6O] (*2*, 6, *3*), *55*, 54, *53* [C_4H_6] (*35*, 15, *15*), *45*, 44, *43*. [C_2H_4O] (2, 4, *100*), *41*, 40, *39* [C_3H_4] (*60*, 6, *33*).

(−)-epi-*Hernandulcin* (**2**). Oil, bp 130° (0.1 mmHg). $[\alpha]_D^{25}$ $-192° \pm 6°$ ($CHCl_3$, *c* 0.400). UV λ_{max}^{EtOH} (log ε) 236 nm (4.11). IR (film) ν_{max} cm^{-1} 3460, 3050, 2950, 2860, 1650, 1220, 1190, 1085, 1020, 930, 880. ^1H and ^{13}C NMR ($CDCl_3$) were identical within experimental error to spectra published elsewhere [21, 28]. EIMS (GC) 70 eV, *m*/*z* (rel. int.): 219, *218* [M^+—OH, $C_{15}H_{23}O$] (3, *16*), 204, *203* [221—OH, $C_{14}H_{20}O$] (1, *5*), 176, *175* [221—CO,—OH, $C_{13}H_{20}$] (1, *8*), 161, *160* [176—CH_3, $C_{12}H_{16}$] (8, *1*), *153*, 152 [221—C_5H_9, $C_9H_{12}O_2$] (*17*, 1), *151*, 150, *149* [219—C_5H_9, $C_{10}H_{14}O$] (*1*, 7, *3*), *139*, 138, *137* [221—C_6H_{11}, $C_8H_{10}O_2$] (*1*, 1, *11*), *136*, 135, *134* [204—C_5H_9, $C_9H_{11}O$] (*3*, 5, *1*), *123*, 122, *121* [150—CO, C_9H_{14}] (*1*, 2, *5*), 112, *111* [221—CH_3-*c*-C_6H_6O, $C_7H_{12}O$] (3, *52*), *110*, 109, *108* [152—$CH_3C{=}O$, C_7H_9O] (*82*, 79, *13*), *95*, 94, *93* [109—CH_3, C_6H_6O] (*39*, 7, *15*), *84*, 83 [112—CHO, C_6H_{11}] (*2*, 10), 82, *81* [C_5H_6O] (28, *11*), *72*, 71 [C_4H_7O] (*1*, 17), 69 [C_5H_9] (31), 68, *67* [C_5H_8] (7, *24*), *59*, 58, *57* [C_3H_6O] (*1*, 6, *3*), *55*, 54, *53* [C_4H_6] (*30*, 11, *14*), *45*, 44, *43* [C_2H_4O] (*2*, 3, *100*), *41*, 40, *39* [C_3H_4] (*59*, 6, *30*).

Essential oil isolation and analysis. Microscale solid–liquid extraction with different solvents was performed in 3-ml Craig tubes [29]. The sample (50–100 mg dry plant tissue) was placed in a recrystallization tube and 1–2 ml of solvent (pentane, hexane, CH_2Cl_2, Et_2O, Me_2CO, MeOH or H_2O) added. The upper section of the Craig tube was set in place and the assembly shaken in a vortex rotator to form a suspension of plant material. The extraction was completed by placing the system in an ultrasonic bath at 35–50° for 10 min. Solvent was collected by inverting the Craig-tube assembly into a centrifuge tube and spinning the solvent extract away from the remaining solid material. Following removal from the centrifuge, the Craig tube was disassembled and the process repeated a total of 4 times. The combined extracts (4–8 ml) were concd and analysed by TLC and capillary GC with FID and MSD.

The volatile essential oil was isolated by solid–liquid continuous extraction in a Soxhlet apparatus. Experiments were done on 1, 10 and 100-g scales. In a typical procedure, a sample of dry powder from wild plant material (1.0 g) was extracted in refluxing CH_2Cl_2 (25 ml) for 4 hr. The extract was concd in a rotary evaporator (25°, 150 mmHg) yielding 40–50 mg of a yellow–green oil with a refreshing scent. Composition was determined by capillary GC injecting 0.1–0.2 μl of 10% sols of oil in $CHCl_3$.

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