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Chemical Characterization of Cuban Propolis by HPLC–PDA, HPLC–MS, and NMR: the *Brown*, *Red*, and *Yellow* Cuban Varieties of Propolis

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Sixty-five samples of propolis were collected from eleven regions of Cuba; methanolic extracts of propolis were prepared from all samples, and a classification method was developed using a combination of NMR, HPLC−PDA, and HPLC−ESI/MS techniques. The analysis of ^{1}H and ^{13}C NMR spectra and chromatographic profiles of all propolis extracts allowed the definition of three main types of Cuban propolis directly related to their secondary metabolite classes: *brown* Cuban propolis (BCP), rich in polyisoprenylated benzophenones, *red* Cuban propolis (RCP), containing isoflavonoids as the main constituents, and *yellow* Cuban propolis (YCP), probably with aliphatic compounds. Subsequently, the principal compounds of the *brown* and *red* types were characterized by HPLC−ESI/MS analysis. Instrumental techniques used are complementary; NMR was shown to be a quick and informative tool for the rapid analysis of crude propolis polar extracts and allowed the identification of the main class of secondary metabolites, while LC−PDA and LC−MS techniques were useful tools for qualitative and quantitative analysis of marker compounds of Cuban propolis.

KEYWORDS: Cuban propolis; *brown*, *red*, and *yellow* type; isoflavonoids; pterocarpans; polyisoprenylated benzophenones; 1D NMR spectroscopy; HPLC−PDA; HPLC−ESI/MS.

INTRODUCTION

Propolis is a natural resinous substance produced by honeybees from parts of the plants, buds, and exudates and possesses various biological activities (*1*,*2*). In recent years, propolis has gained popularity as a health drink, has also been used extensively in food and beverages, and is thought to improve human health and to prevent diseases such as inflammation, heart disease, diabetes, and even cancer (*3*). Because of its broad spectrum of biological activities and uses in health food as well as in folk medicine, there is a renewed interest in the composition of propolis and its biological activities.

The chemical composition of propolis is very complex, and flavonoids, terpenes, aromatic acids, and their esters have been considered the primary biologically active compounds (*2*). However, its composition is highly variable and depends on the local flora at the site of collection (*4*,*5*). Remarkable is the difference of chemical composition observed between propolis of tropical and temperate regions. Propolis originating from temperate zones (West Asia, Europe, and North America) possesses a similar chemical composition, the main constituents being phenolic compounds (flavonoids, cinnamic acids, and derivatives). In these regions, exudates of different poplar buds (*Populus* spp.) are the main sources of propolis, together with other trees, birch (*Betula alba*), beech (*Fagus sylvatica*), horse chestnut (*Aesculus hippocastanum*), alder (*Alnus glutinosa*), and various conifers. (*1*, *5*). In tropical regions, there are no *Populus* spp., and bees use other plant sources for the production of propolis (*5*,*6*). Thus, the chemical composition of propolis from tropical zones is very different from those of temperate zones because of the difference in vegetation. Different compounds have been reported in tropical propolis, terpenoids and prenylated derivatives of *p*-coumaric acids in Brazilian propolis (*4*, *7*), lignans in Chilean propolis (*8*), and polyisoprenylated benzophenones in Venezuelan, Brazilian, and Cuban propolis (*9*−*14*). The resins of *Clusia* spp., *Araucaria heterophylla*, and different *Baccharis* spp. were reported to be the dominant sources of tropical propolis (*5*, *9*, *15*). Interestingly, despite the

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Table 1. Cuban Propolis Samples, Their Origin, and Classification

samples	province	type	samples	province	type
1	Ciudad Habana, Jardín Botánico	*brown*	**34**	Habana, San Antonio	*yellow*
2	Las Tunas, Puerto Padre	*yellow*	**35**	Pinar del Río, La Coloma	*red*
3	Granma, Buey arriba	*brown*	**36**	Pinar del Río, Güanes	*brown + red + yellow*
4	Granma, Buey arriba	*brown*	**37**	Pinar del Río, Güanes	*brown + red*
5	Guantánamo, Imías	*brown*	**38**	Pinar del Río, Guanacabibes	*red + yellow*
6	Guantánamo, Salvador	*brown*	**39**	Pinar del Río, Candelaria	n. c.
7	Granma, Guisa	*brown*	**40**	Pinar del Rio, Consolación del Sur	*red + yellow*
8	Santiago de Cuba, Guamá	*red + yellow*	**41**	Pinar del Río, Bahía Honda	*yellow*
9	Pinar del Río, Cabo de San Antonio	n. c.[a]	**42**	Pinar del Río, Consolación del Sur	*red + yellow*
10	Granma, Bayamo	n. c.	**43**	Pinar del Río, Bahía Honda	*brown + red*
11	Las Tunas, Jobabo	*yellow*	**44**	Matanzas, Jagüey Grande	*yellow*
12	Holguín, Báguanos	*brown*	**45**	Matanzas, Jagüey Grande	*red*
13	Ciudad Habana, Jardín Botánico	n. c.	**46**	Matanzas, Jagüey Grande	n. c.
14	Guantánamo, Salvador	*brown*	**47**	Matanzas, Limonar	*red + yellow*
15	Ciudad Habana, Jardín Botánico	*brown*	**48**	Matanzas, Unión de Reyes	*yellow*
16	Las Tunas, Puerto Padre	*brown*	**49**	Matanzas, Limonar	*yellow*
17	Guantánamo, Salvador	*brown*	**50**	Matanzas, Unión de Reyes	n. c.
18	Ciudad Habana, Jardín Botánico	*red + yellow*	**51**	Matanzas, Limonar	*yellow*
19	Granma, Manzanillo	*brown*	**52**	Holguín, Banes	*brown*
20	Granma, Bayamo	*yellow*	**53**	Holguín, Banes	*brown*
21	Habana, Madruga	*yellow*	**54**	Holguín, Cuesto	*brown*
22	Granma, Manzanillo	*brown*	**55**	Holguín, Banes	*brown*
23	Granma, Manzanillo	*brown*	**56**	Holguín, Cueto	*brown*
24	Habana, Madruga	*yellow*	**57**	Holguín, Cueto	*brown*
25	Villa Clara, Manicaragua	*brown + red + yellow*	**58**	Holguín, Cueto	*brown*
26	Villa Clara, Manicaragua	*brown + red + yellow*	**59**	Holguín, Báguano	*brown*
27	Ciudad Habana, El Cano	*red + yellow*	**60**	Holguín, Báguano	n. c.
28	Villa Clara, Remedios	*brown + red + yellow*	**61**	Pinar del Río, Guanacabibes	*brown*
29	Villa Clara, Manicaragua	*red*	**62**	Ciudad Habana, Campo Florido	n. c.
30	Villa Clara, Manicaragua	n. c.	**63**	Ciudad Habana, Campo Florido	*yellow*
31	Guantánamo, Baracoa	*brown*	**64**	Ciudad Habana, Campo Florido	*brown*
32	Santis Spiritus, Zaza	n. c.	**65**	Ciudad Habana, Campo Florido	*brown*
33	Ciego de Ávila, Calvet Baez	*brown*			

[a] Abbreviation: n. c., not classified.

difference of their constituents, propolis from all regions, including the temperate and tropical zones, exhibits similar biological properties (*3*).

In Cuba, propolis alcohol extract is popular as a homemade remedy. Propolis from Cuba has only recently begun to be studied; therefore, relatively little is known about its chemical composition, phytochemical origins, and phytotherapic properties. Our previous study indicated that the chemical composition of Cuban propolis is both unique and variable, qualitatively and quantitatively (*12*−*14*, *16*). The presence of polyprenylated benzophenones in Cuban propolis has been well established; a HPLC study indicated the nemorosone as the major constituent of 18 analyzed samples (*14*). Other prenylated benzophenones identified as minor components of Cuban propolis are propolones A-D, hyperibone B, garcinielliptone I, xanthochymol, and guttiferone E (*12*−*14*). However, we reported also the occurrence of isoflavonoids as constituents of a sample of Cuban propolis, wherein polyprenylated benzophenones were not detected (*16*).

This evidence suggests possible differences in the chemical composition of Cuban propolis. If this hypothesis is true, it is logical to consider that different components should also define different biological activities or, at least, potency levels. Thus, considering the great diversity and rich floristics of Cuba, we decided to continue our research on the chemical composition of Cuban propolis in order to suggest similarities and differences between samples collected in different regions of the country. For this purpose, 65 samples of propolis were collected in 11 provinces of the country.

The aim of this study was to develop a classification system capable of providing chemical characterization of crude alcoholic extracts and to identify both classes and secondary metabolites of Cuban propolis. The pattern of natural products in propolis is extremely complex; it consists of a wide range of compounds of varying polarity. Therefore, Cuban propolis samples were analyzed by 1D NMR (monodimensional nuclear magnetic resonance), HPLC−PDA (high performance liquid chromatography with photodiode array detector), and HPLC−ESI/MS (high performance liquid chromatography with electrospray ionization mass spectrometer) techniques.

MATERIALS AND METHODS

Propolis Samples. Samples of Cuban propolis (65) were provided by La Estación Experimental Apicola or collected by one of us. Samples were collected between October 2003 and December 2004 in 11 provinces of Cuba. Cuban propolis samples, their origin, and classification are reported in **Table 1**. Both propolis samples and extracts were kept at 0−5 °C and protected from light.

Extraction Procedure. All samples were ground prior to extraction. Propolis samples (about 3 g) were extracted by maceration with methanol (10 mL, 3 times) for 1 h at room temperature (25−30 °C) employing agitation. The combined extracts were filtered on paper filters, and solvent was evaporated at 40 °C under reduced pressure to obtain dry extracts.

NMR Analysis. A portion of each extract (about 100 mg) was dissolved employing $CDCl_3$ or $CDCl_3/CD_3OD$ mixtures (ca. 0.5 mL). A Bruker DRX-600 spectrometer operating at 599.19 MHz for ^1H and 150.858 for ^{13}C, using the UXNMR software package, was used for the NMR experiments.

HPLC−PDA Analysis. HPLC analysis of Cuban propolis extracts (5 mg/mL) was performed on a Agilent 1100 series system consisting of a G-1312 binary pump, a G-1328A Rheodyne injector (20 μL loop), a G-1322A degasser, and a G-1315A photodiode array detector (PDA), equipped with a μ-Bondapack C-18 column (250 × 4.6 mm i.d., particle size 10 μm). The elution solvents used were water (solvent A) and

methanol (solvent B). The flow rate was 1 mL/min, and PDA data were recorded with a 200−600 nm range with three preferential channels as the detection wavelength, 320, 280, and 254 nm. The gradients were as follows: (i) a linear gradient where solvent B increased from 50 to 100% over a 30 min period; and (ii) an isocratic elution for 10 min to 100% of solvent B.

HPLC−ESI/MS Analysis. LC−MS analysis was performed using a Surveyor LC pump and a Surveyor Autosampler coupled with a LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with Xcalibur 3.1 software. HPLC separations were accomplished using a binary gradient composed of (solvent A) water and (solvent B) acetonitrile (10/90% v/v), both containing 0.1% (v/v) trifluoroacetic acid. Elution was performed at a flow rate of 0.2 mL/min, and the volume of the injection was 25 μL for all of the propolis samples (3 mg/mL). All analyses were performed with an ESI interface in the positive ion mode. The data were acquired in the full scan and MS/MS scanning modes, the maximum injection time was 50 ms, the number of microscans was three, and for the MS/MS scanning mode, the percentage of the collision energy was 20−45%. Analysis of the *brown* propolis type was performed using a Luna C-8 column (150 mm × 2.0 mm i.d., particle size 5 μm) protected by its Guard Cartridge (4 × 2.0 mm i.d.) using, as the eluent system, a linear gradient of solvents B from 70 to 95% A in 40 min, followed by washing and re-equilibrating the column. The ionization conditions were optimized, and the parameters were maintained as follows: capillary temperature 200 °C; capillary voltage 29 V; spray voltage 4.50 kV; sheath gas flow rate 30 (arbitrary units); auxiliary gas flow rate 5 (arbitrary units); and scan range of *m*/*z* 200−700 amu. N_2 was used as the sheath and auxiliary gas. LC−MS of the *red* propolis type was carried out on a C-18 Hypurity Aquastar column (150 mm × 2.0 mm i.d., particle size 5 μm) protected by its Guard Cartridge (4 × 2.0 mm i.d.). The following gradient was adopted: a linear gradient of B from 20 to 40% for 5 min, from 40 to 60% B for 30 min, from 60 to 95% B for 10 min, followed by washing and re-equilibrating the column. The optimized instrumental parameters were capillary temperature 220 °C, capillary voltage 30 V, spray voltage 5.10 kV, sheath gas flow rate 30 (nitrogen, arbitrary units), auxiliary gas flow rate 10 (arbitrary units), and scan range of *m*/*z* 100−400 amu.

Isolation of Scrobiculatones A and B. The methanolic extract of Cuban propolis sample n.16 (3 g) was fractionated over a Sephadex LH-20 column (1 m × 3 cm i.d.) using methanol as the solvent to furnish four main fractions (1−4). Fractions 2 and 3 were purified by RP−HPLC (Waters 10 μm μ-Bondapak C18 column, 300 × 7.8 mm; methanol 92% at 2.5 mL/min as eluent) to afford a mixture of scrobiculatones A and B (15 mg). ^{1}H and ^{13}C NMR data of compounds were consistent with those previously reported (*11*).

RESULTS AND DISCUSSION

Because of the large numbers of propolis samples to be analyzed (65 samples), we decided to develop an analytical procedure that supplied a general chemical classification and allowed us to identify both the classes and the main secondary metabolites of Cuban propolis. Considering the extremely complex pattern of natural products in propolis, we chose to characterize the crude extracts of propolis samples by 1D NMR spectroscopy, HPLC−PDA, and HPLC−ESI/MS techniques.

The analyzed crude extracts have been obtained by maceration with methanol since the plant exudates' fraction of propolis, which usually contains the bioactive components, is separated from the wax by extraction with this solvent (*17*). Maceration with ethanol is an efficient procedure for the preparation of propolis extracts; methanol shows the same capacity and results more efficiently in the elimination of inert waxes, and it is thus possible to obtain a more purified set of extracts.

1D NMR Analysis. The only technique that can simultaneously examine complex chemical composition is NMR spectroscopy since HPLC−PDA typically only reveals compounds with UV-absorbing chromophores and ESI/MS can only detect compounds that ionize under the conditions used. Thus, 1D NMR represents a simple method for obtaining global information about complex samples, and it provides a fingerprint of the Cuban propolis analyzed.

^{1}H and ^{13}C NMR spectra of analyzed propolis extracts displayed three principal spectral patterns (**Figure 1**). These results allow grouping of the Cuban propolis in three main groups, *brown*, *red*, and *yellow* Cuban propolis types.

Brown Cuban Propolis Type (BCP Type). Among the Cuban propolis samples evaluated in this study, 27 of them were found to contain polyisoprenylated benzophenone derivatives. This propolis group showed mainly the characteristic chemical shifts of nemorosone. In their ^{1}H NMR spectra (**Figure 1a**), the AA′XX′Y spin system associated with the benzoyl group (δ_H 7.26−7.65), vinyl protons (δ_H 5.0−5.22), methyl groups of isoprenyl chains (δ_H1.56−1.72), and methylene between two double bonds (δ_H 3.10−3.30), a structural characteristic common to the type-A prenylated benzophenones (*18*), was in accordance with the structure of nemorosone (*19*). Also, ^{13}C NMR spectra of BCP samples (**Figure 1b**) displayed the typical signals of nemorosone: benzoyl group (δ_C 127.7 (C-13 and C-15), 128.2 (C-12 and C-16), and 133.2 (C-14)), carbon atoms included in the bicyclo[3.3.1]nonane moiety (δ_C 43.2 (C-7), 48.3 (C-8), 57.4 and 64.9 (C-5 of tautomers), 71.4 and 79.0 (C-1 of tautomers), 167.3 and 170.2 (C-4 of tautomers), and 207.3 (C-9)) (*19*). In addition to nemorosone signals, many ^{1}H NMR spectra of the BCP group (**Figure 1a**) showed two sets of doublets at δ_H 5.24, 5.40, 6.44, and 6.48 (^3J ca. 10 Hz) corresponding to AX spin system signals of the 2,2-dimethylpiran moiety of scrobiculatones A and B, two prenylated benzophenones closely related to nemorosone (*11*). The ^{13}C NMR signals at δ_C 81.8 and 83.2 are clearly associated with the C-19 of scrobiculatones A and B, respectively, and C-2 (δ_C 189.0) of scrobiculatone A was also detected (**Figure 1b**) (*11*).

Red Cuban Propolis Type (RCP Type). The NMR pattern of the RCP type is quite different from the BCP group (**Figure 1**). Three propolis samples (29, 35, and 45) display exclusively characteristic signals of isoflavonoids, while the other 13 samples exhibit, together with these resonances, additional NMR signals, in particular, a strong aliphatic region. The characteristic ^{1}H NMR signals of the RCP group were present between 2.6 and 8.1 ppm, and they were divided in two well-separated zones (δ_H 3.75−3.82 and 6.34−8.1) (**Figure 1c**). The principle differences with respect to the BCP type were the very intense aromatic methoxyl signals (δ_H 3.75−3.82) and a wide and complex aromatic regions (δ_H 6.34−8.1). Our previous report has shown that some isoflavonoids such as pterocarpans, isoflavans, and isoflavones are the characteristic constituents of a *red* Cuban propolis sample (*16*). In accordance with this paper, the NMR signals of the main compounds in the RCP extracts were assigned to isoflavonoids. The protons of the C ring of isoflavans that usually appear in the ranges of δ_H 2.7−2.9 and 4.1−4.3 were clearly visible in all ^{1}H NMR spectra of the RCP samples (**Figure 2a**). Doublets near 5.4 ppm, typical of pterocarpans (H-11a), and signals at ca. 8.0 ppm, corresponding to H-2 of isoflavones, were also found to be characteristic ^{1}H NMR signals of this propolis type (*16*). The presence of two aromatic regions in the ^{13}C NMR spectra of this propolis type (δ_C 96.6−131.7 and 152.5−160.8) were in agreement with the NMR data of isoflavonoids isolated from *red* Cuban propolis, such as the signals in the range of 60−80 ppm with C-ring aliphatic signals of isoflavans, isoflavanones, and pterocarpans (*16*) (**Figure 1d**). In addition, the ^{13}C NMR spectra exhibited a group of signals between 54.9 and 55.9 ppm

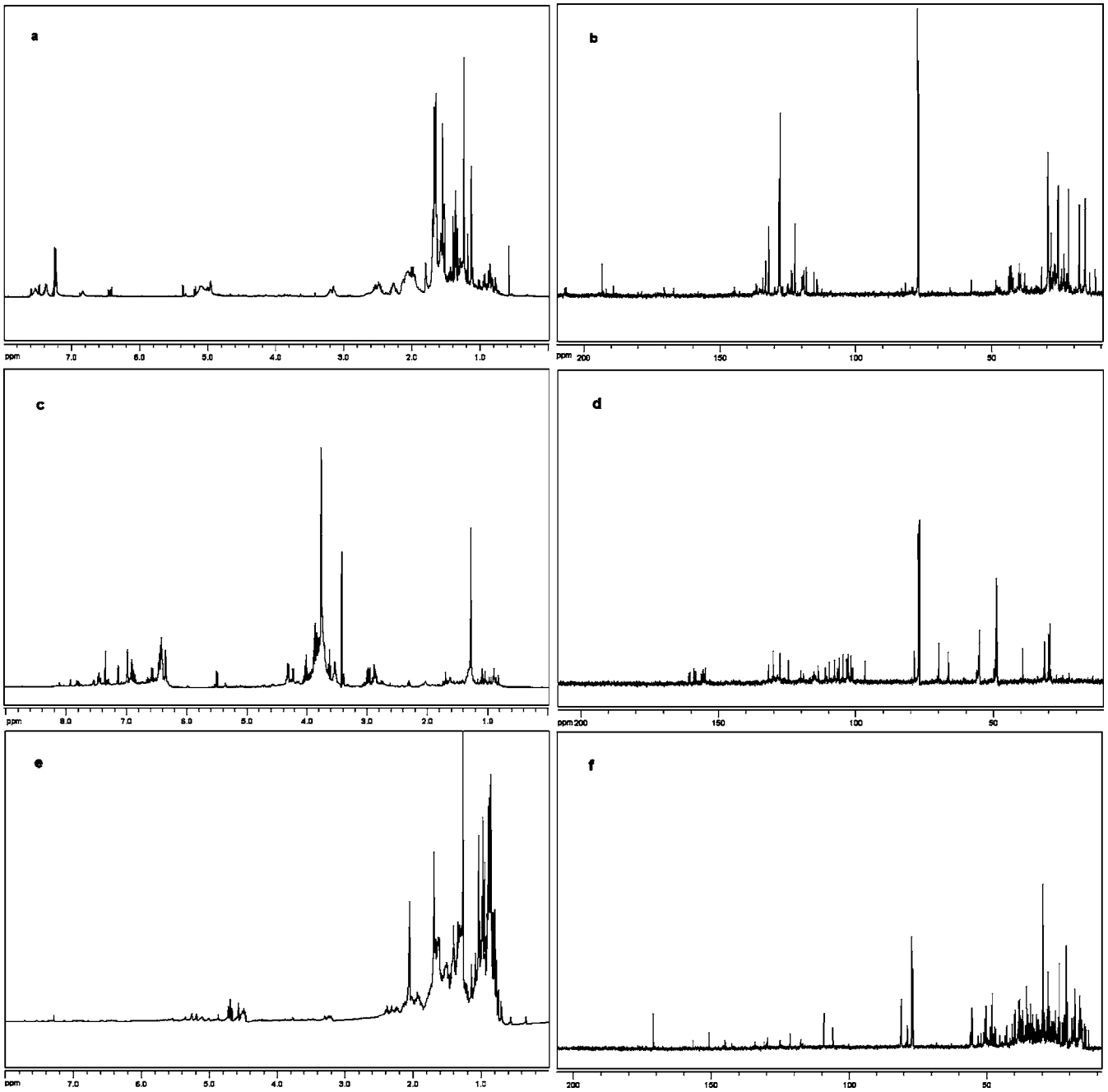

Figure 1. Typical 1D NMR spectra of *brown*, *red*, and *yellow* Cuban propolis; ^{1}H NMR (**a**) and ^{13}C NMR (**b**) spectra of the BCP type, ^{1}H NMR (**c**) and ^{13}C NMR (**d**) spectra of the RCP type, and ^{1}H NMR (**e**) and ^{13}C NMR (**f**) spectra of the YCP type.

corresponding to the methoxyl groups identified in almost all isoflavonoids isolated previously (**Figure 1d**).

Yellow Cuban Propolis Type (YCP Type). ^{1}H NMR spectra of 23 Cuban propolis samples (**Figure 1e**) showed the main signals almost exclusively in the aliphatic region (δ_H 0.75−2.1). Downfield signals of variable intensities between 3.0 and 6.7 ppm were present in all YCP samples, while signals in the aromatic region over 7.0 ppm were absent or very weak. ^{13}C NMR spectra were consistent with the results observed in the ^{1}H NMR spectra. A very complex zone attributed to sp^3 carbon atoms was observed between 17 and 55 ppm, essentially (**Figure 1f**). However, some signals belonging to sp^2 carbon atoms (between 100 and 150 ppm) were observed, and these could be assigned to sp^2 carbon atoms of alkenes substituted mainly by alkyl groups. Moreover, in many YCP samples, two strong signals at ca. δ_C 80 and 172 revealed the presence of carbon atoms bearing oxygen. The simultaneous comparison of the ^{1}H and ^{13}C NMR spectral data suggested the presence of aliphatic compounds (terpenoids and sterols) as the main constituents of this propolis group.

HPLC−PDA Analysis. In this study, a suitable HPLC method has been developed for analysis of a large number of Cuban propolis samples with different chemical compositions and a wide range of compounds of varying polarity. The chromatographic conditions used enable differentiation of the propolis samples according to the polarity of their constituents. Chromatographic profiles of 65 Cuban propolis samples were carried out by HPLC−PDA on a C-18 reversed-phase column. The absorption spectra resulting from diode-array detection were used to identify peaks or to characterize the class of secondary

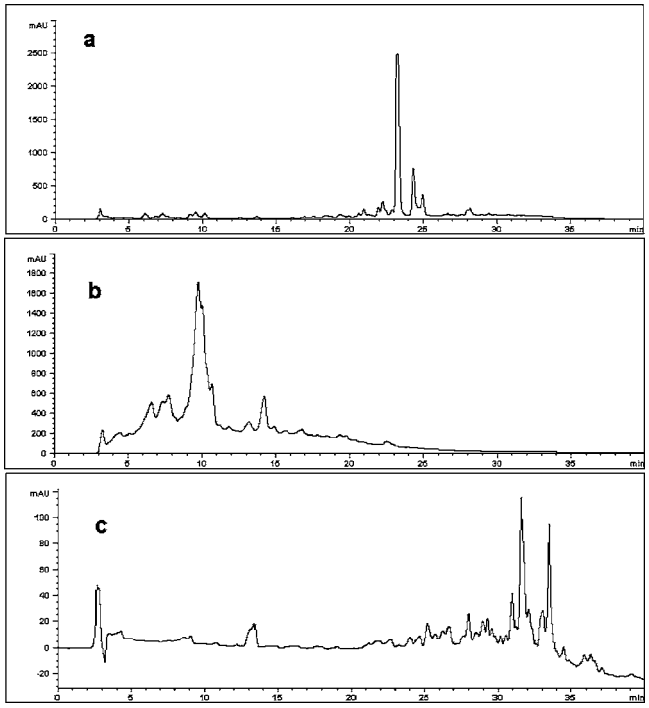

Figure 2. HPLC–PDA chromatograms of methanolic extracts of Cuban propolis, (**a**) BCP type (280 nm), (**b**) RCP type (280 nm), and (**c**) YCP type (250 nm).

metabolites. Identification of compounds, when possible, was carried out by direct HPLC comparison with authentic standards. As is evident from the chromatographic profiles of the BCP, RCP, and YCP types (**Figure 2**), the chromatogram can be easily divided into three areas on the basis of retention times, inversely correlated with the polarity of the constituents' propolis type.

Brown Cuban Propolis Type (BCP Type). The HPLC chromatogram of these samples (**Figure 2a**) showed very similar profiles, and they appeared extremely simple with only a few peaks. The UV spectra (λ_{MAX} 278 nm) and retention time (t_R 23.2 min) of the main peak corresponded to that of nemorosone (**1**). The presence of this compound was confirmed by direct HPLC−PDA comparison with an authentic standard (*19*). Nemorosone was found to be the main compound in many Cuban propolis samples also in our previous study (*14*). In addition, HPLC chromatograms of many BCP samples displayed another two peaks (t_R 24.3 and 24.9 min) with the same UV spectra (λ_{MAX} 278 and 330 nm). The characteristic absorbance bands of these compounds were in accordance with structure and spectroscopic data of scrobiculatones A (**2**) and B (**3**) (*11*). To confirm the presence of these two polyisoprenylated benzophenones in *brown* Cuban propolis, the mixture of scrobiculatones A and B was isolated by preparative chromatographic techniques from the methanolic extract of a *brown* propolis sample (see Materials and Methods). The structural elucidation was carried out by comparison with the NMR data reported in the literature (*11*).

Red Cuban Propolis Type (RCP Type). **Figure 2b** shows a typical HPLC chromatogram of RCP samples that exhibited peaks eluting with retention times less than 20 min, indicating constituents more polar than the BCP type. As can be seen, the most complex region of the chromatogram between 10 and 20 min contains overlapping peaks, making their identification difficult. However, these peaks could be correlated to isoflavonoids from the UV spectra by two absorbance bands around 250 and 280 nm. The principle isoflavonoids isolated from *red* Cuban propolis (*16*) have been used as marker compounds in order to confirm the presence of this class of natural product in the RCP type. These compounds (medicarpin, vestitol, and formononetin) have given, under the experimental conditions used, retention times and UV spectra in agreement with the HPLC−PDA profiles of the RCP type.

Yellow Cuban Propolis Type (YCP Type). As is evident from the HPLC−PDA profiles of YCP extracts (**Figure 2c**), peaks having retention times greater than those of polyisoprenylated benzophenones were detected. The chromatograms of this group appeared qualitatively different between themselves depending on the propolis sample as evaluated by retention times and UV spectra of peaks. Chromatographic homogeneity such as that observed for the first two propolis types was not evident in this type. Due to their HPLC behavior, they are strongly retained by the hydrophobic column, and we assume that these compounds are indeed hydrophobic compounds, in particular, aliphatic compounds, probably mainly triterpenoids and steroids. These last compounds are common products of plants, and most of them exhibit UV spectra with low-intensity bands in the region of 250−330 nm as a result of their having little or no conjugation between the chromophores. This fact supports the variety of chromatograms and low absorbance observed in their HPLC−PDA analysis.

HPLC−ESI/MS Analysis. On the basis of the information supplied by NMR, HPLC−PDA data, and our previous chemical investigation of characteristic samples of Cuban propolis (*12*−*14*, *16*), we have developed two specific HPLC−ESI/MS methods in order to validate the chemical classification obtained by 1D NMR and HPLC−PDA techniques, to characterize the main compounds of *brown* and *red* Cuban propolis types and to supply tools for analysis of the Cuban propolis that complete and support the 1D NMR and HPLC−PDA information.

Brown Cuban Propolis Type (BCP Type). The best separation of the polyisoprenylated benzophenone in a HPLC−ESI/MS chromatogram was obtained by using a C-8 column and acquiring data in the positive mode. The BCP type extracts showed approximately the same HPLC−ESI/MS profile (**Figure 3**) due to the presence of the same molecular species. The (+) ESI−MS spectra of main peaks (**1**−**3**) of the BCP type showed pseudomolecular ions $[M + H]^+$ at *m*/*z* 503 (**1**) and 501 (**2** and **3**) with the respective Na adducts $[M + Na]^+$ at *m*/*z* 525 (**1**) and 523 (**2** and **3**) (**Figure 3**). Comparison of retention times and ESI−MS^n spectra of peaks **1**−**3** with standards confirmed that the main constituent of the BCP type are nemorosone (**1**) and scrobiculatones A (**2**) and B (**3**). The fragment ions present in the MS/MS spectra of these compounds corresponded to the loss of prenylated chains of the bicyclo moiety.

Red Cuban Propolis Type (RCP Type). On the contrary, in the BCP group, the chromatograms of the RCP type were more complex, and the chromatographic resolution required to characterize each constituent of the mixture was impossible to obtain. To improve the separation and identification of the single compounds, simultaneous tandem mass spectrometry experiments (HPLC−ESI−MS/MS) in the selected reaction-monitoring (SRM) mode were performed to evaluate the occurrence of marker compounds, previously isolated from a characteristic *red* Cuban propolis sample (*16*), in the RCP variety.

The SRM experiments were carried out in the positive mode selecting as ion precursors the pseudomolecular ions $[M + H]^+$ of the compounds under investigation and the ion fragments characteristic or more abundant in the corresponding MS/MS spectra were selected (*16*). The following scheme was used in these experiments: isoliquiritigenin (**4**) and liquiritigenin (**5**) 257 ($[M + H]^+$) → 239 (**Figure 4a**); formononetin (**6**) 269 ([M

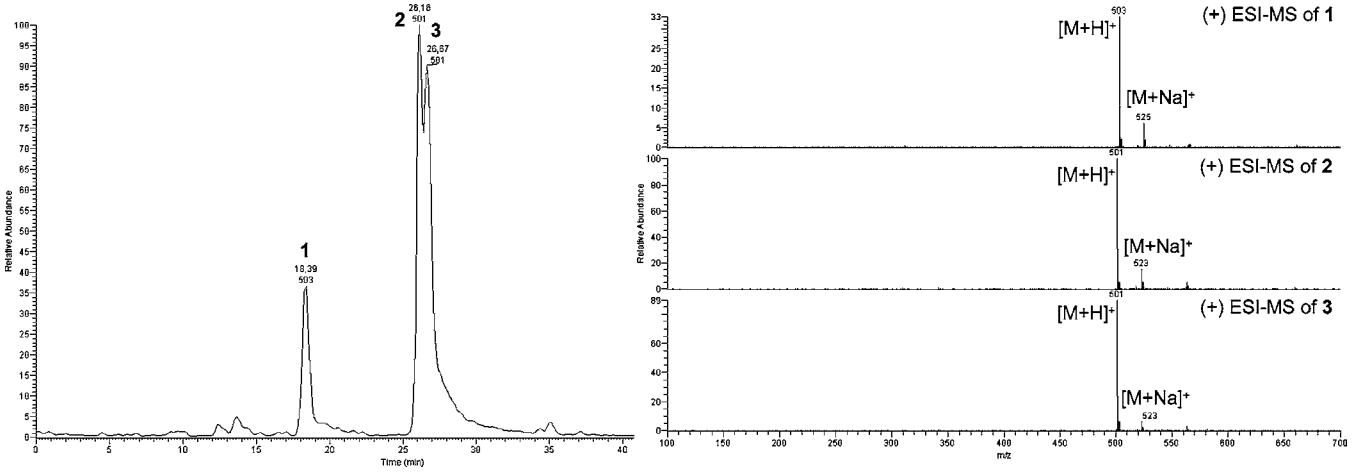

Figure 3. HPLC–ESI/MS chromatogram of a crude extract of the *brown* Cuban propolis type (BCP type) and (+) ESI–MS spectra of nemorosone (**1**) and scrobiculatone A (**2**) and B (**3**).

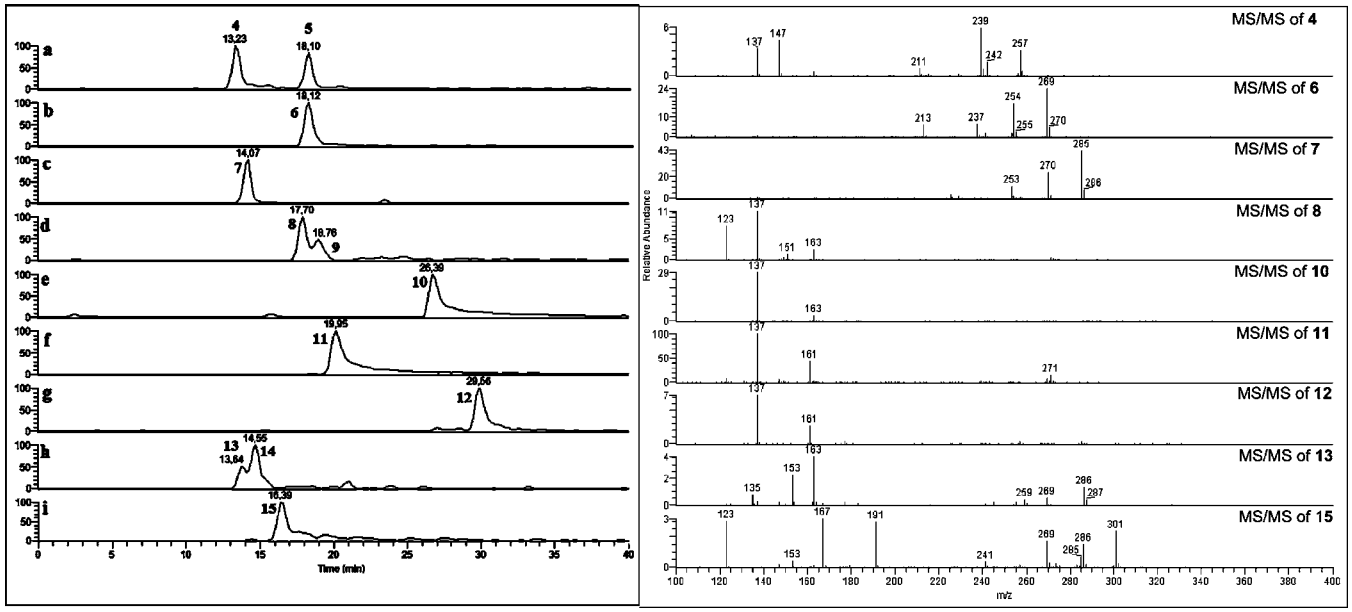

Figure 4. HPLC–ESI-MS/MS chromatogram in the SRM mode of a crude extract of the *red* Cuban propolis type (RCP type) and MS/MS spectra of compounds **4**–**15**. (**a**) **4** and **5**: *m*/*z* 257 → 239; (**b**) **6**: *m*/*z* 269 → 254; (**c**) **7**: *m*/*z* 285 → 270; (**d**) **8** and **9**: *m*/*z* 273 → 123; (**e**) **10**: *m*/*z* 287 → 137; (**f**) **11**: *m*/*z* 271 → 161; (**g**) **12**: *m*/*z* 285 → 161; (**h**) **13** and **14**: *m*/*z* 287 → 153; (**i**) **15**: *m*/*z* 301 → 191.

$+ H]^+$) → 254 (**Figure 4b**); biochanin A (**7**) 285 ($[M + H]^+$) → 270 (**Figure 4c**); vestitol (**8**) and neovestitol (**9**) 273 ($[M + H]^+$) → 123 (**Figure 4d**); 7-*O*-metilvestitol (**10**) 287 ($[M + H]^+$) → 137 (**Figure 4e**); medicarpin (**11**) 271 ($[M + H]^+$) → 161 (**Figure 4f**); homopterocarpin (**12**) 285 ($[M + H]^+$) → 161 (**Figure 4g**); vesticarpan (**13**) and 3,8-dihydroxy-9-methoxypterocarpan (**14**) 287 ($[M + H]^+$) → 153 (**Figure 4h**); 3-hydroxy-8,9-dimethoxypterocarpan (**15**) 301 ($[M + H]^+$) → 191 (**Figure 4i**). Identification of each compounds was carried out by direct HPLC comparison with standards isolated from a RCP sample and identified by spectroscopic techniques (*16*). **Figure 4** shows the chromatographic profile obtained by HPLC−ESI−MS/MS experiments containing all marker compounds (**4**−**15**) of the RCP type and the respective MS/MS spectra.

The developed HPLC−ESI/MS method for analysis of the RCP type allowed detection of the main compounds identified in a *red* propolis sample by extensive preparative analysis (*16*). Compounds **4**−**15** have been used as markers in order to confirm the occurrence of isoflavonoids in the RCP type. The results (**Table 2**) indicated that isoflavonoids are the characteristic class of natural products of this type of Cuban propolis. Particularly, medicarpin (**11**) and formononetin (**6**) are the marker compounds present in all RCP samples analyzed.

The results obtained by analysis of Cuban propolis 1D NMR, HPLC−PDA, and HPLC−ESI//MS data showed satisfactory agreement. In **Table 1**, the chemical classification of propolis samples examined in this study is reported. The methanolic extracts of Cuban propolis, which are distinguished by their brown, red, or yellow colors, display a very characteristic chemical composition and are rather distinct from those of temperate propolis. They also vary significantly among themselves and can be clearly divided into three groups: *brown* Cuban propolis type (BCP type) shown to be rich in polyisoprenylated benzophenones, *red* type (RCP type), containing isoflavonoids as the main constituents, and *yellow* type (YCP type), which seems to be rich in aliphatic compounds.

The greatest number of samples belongs to the BCP group (41.5%). Nemorosone and the mixture of scrobiculatones A and B were found to be the major compounds of this propolis type. The only differences observed within the group are exclusively of quantitative type. The simple and constant chemical composi-

Table 2. Occurrence of Marker Compounds **4**–**15** in *Red* Cuban Propolis Samples

	samples	marker compounds											
	province	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
18	Ciudad Habana, Jardín Botánico			+	+	+	+		+				
27	Ciudad Habana, El Cano		+	+	+	+		+	+	+	+		
45	Matanzas, Jagüey Grande	+	+	+	+	+	+	+	+	+	+	+	+
47	Matanzas, Limonar	+	+	+	+	+	+	+	+				+
40	Pinar del Rio, Consolación del Sur			+	+	+	+	+	+	+			
43	Pinar del Río, Bahía Honda			+	+	+	+		+	+	+		
42	Pinar del Río, Consolación del Sur			+	+	+	+	+	+	+			
38	Pinar del Río, Guanacabibes			+	+	+	+		+				+
36	Pinar del Río, Güanes	+	+	+	+	+	+	+	+	+	+	+	+
37	Pinar del Río, Güanes.	+	+	+	+	+	+	+	+	+	+	+	+
35	Pinar del Río, La Coloma	+	+	+	+	+	+	+	+	+	+	+	+
8	Santiago de Cuba, Guamá	+	+	+	+			+	+				
25	Villa Clara, Manicaragua			+					+				
26	Villa Clara, Manicaragua			+	+	+			+				
29	Villa Clara, Manicaragua	+	+	+	+	+	+	+	+	+	+	+	+
28	Villa Clara, Remedios		+	+	+	+	+	+	+	+			

tion of the BCP type suggests that a single plant species, or a group of related species, were the main source for the major group of Cuban propolis. Nemorosone is the main polyisoprenylated benzophenone of floral resins of *Clusia rosea*, a tree widely diffused in Cuba (*19*). Scrobiculatones A and B have only been found in the floral resin of *Clusia scrobiculata* (*11*) and in Venezuelan propolis samples collected in regions where this plant material was used by honeybees as a propolis source (*10*). Until now, *Clusia rosea* has been considered the main source of Cuban propolis (*12*, *14*, *19*). This suggests the contribution of some other species producing resins that bees combine in order to produce propolis. On the other hand, the scrobiculatones probably also originate from nemorosone either during processing of the resin by the bees, storage of the propolis in the beehive, or via extraction procedures.

Unlike the *brown* type, the RCP type presented a more complex composition, and most of the these samples were shown to be mixtures with BCP and YCP types (**Table 1**). The *red* Cuban propolis type was collected in occidental and central regions of Cuba only. These samples seem to have a relationship with plants that grow near the coast, but the vegetable source of the RCP type has not been identified as of yet. However, the characteristic compounds of this propolis type have a very restricted distribution in the plant kingdom and occur almost exclusively in the *Leguminosae* family. Thus, the botanical sources of the *red* type probably belong to this family.

Also, the YCP type showed an extremely complex and variable composition. Like *brown* propolis, the YCP type has been collected in occidental, central, and oriental regions of Cuba, suggesting a large distribution of their botanical sources. It has not been possible to identify the main compounds of the *yellow* Cuban propolis type because of the lack of standards. Moreover, these compounds have not been characterize by HPLC−ESI/MS because of their insufficient polarity. For this type of propolis, further chemical studies should be undertaken in order to identify its chemical composition.

Some propolis samples showed a chemical profile of two or/and three types of Cuban propolis (**Table 1**). Therefore, these samples are a mixture of different propolis types, suggesting the contribution of different vegetal sources present at the site of collection.

A small number of samples have not been classified since they did not show the typical chemical profiles of *brown*, *red*, and *yellow* Cuban propolis types and not even other common characteristics for being able to identify further types of Cuban propolis. However, our classification system has allowed us to characterize the 85% of samples examined, demonstrating its validity and applicability.

In this study, the NMR spectroscopy has shown to be a powerful method to differentiate among Cuban propolis samples. In fact, in the Cuban propolis 1D NMR spectra, diverse groups of secondary metabolites have been detected, and the presence of one main compound (BCP type) or a group of compounds with a structural relationship (RCP and YCP types) allowed three very different NMR spectra groups to be obtained. This result proves that NMR techniques are attractive tools for the chemical classification of tropical propolis and may provide alternatives to classical analytical phytochemistry to screen commercial preparations of propolis and to evaluate specific nutraceutical benefits. However, the identification and quantification of individual compounds is difficult due to overlapping signals from all of the constituents present. Thus, for identification of the main constituents of propolis and for qualitative and quantitative analysis of marker compounds of each Cuban propolis types, HPLC−PDA and HPLC−ESI/MS methods must be applied. The two specific HPLC−ESI/MS methods described here allow the successful identification of the principal compounds of *brown* and *red* propolis types, confirming the chemical classification obtained by 1D NMR and HPLC−PDA. Moreover, they supply a full tool for the quality control of BCP and RCP types and their commercial products.

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LITERATURE CITED

(1) Ghisalberti, E. L. Propolis: a review. *Bee World* **1979**, *60*, 59−84.

(2) Burdock, G. A. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem. Toxicol.* **1998**, *36*, 347−363.

(3) Banskota, A. H.; Tezuka, Y.; Kadota, S. Recent progress in pharmacological research of propolis. *Phytother. Res.* **2001**, *15*, 561−571.

(4) Marcucci, M. C. Propolis: Chemical composition, biological properties and therapeutic activity. *Apidologie* **1995**, *26*, 83−99.

(5) Bankova, V. S.; De Castro, S. L.; Marcucci, M. C. Propolis: recent advances in chemistry and plant origin. *Apidologie* **2000**, *31*, 3−15.

(6) Park, Y. K.; Alencar, S. M.; Aguiar, C. L. Botanical origin and chemical composition of Brazilian propolis. *J. Agric. Food Chem.* **2002**, *50*, 2502−2506.

(7) Bankova, V.; Dyulgerov, A.; Popov, S.; Marekov, N. A GC/MS study of the propolis phenolic constituents. *Z. Naturforsch*. **1987**, *42*, 147−151.
(8) Valcic, S.; Montenegro, G.; Timmermann, B. N. Lignans from Chilean Propolis. *J. Nat. Prod.* **1998**, *61*, 771−775.
(9) Tomas-Barberan, F. A.; Garcia-Viguera, C.; Vit-Olivier, P.; Ferreres, F.; Tomas-Lorente, F. Phytochemical evidence for the origin of tropical propolis from Venezuela. *Phytochemistry* **1993**, *34*, 191−196.
(10) Trusheva, B.; Popova, M.; Naydenski, H.; Tsvetkova, I.; Rodriguez, J. G.; Bankova, V. New polyisoprenylated benzophenones from Venezuelan propolis. *Fitoterapia* **2004**, *75*, 683−689.
(11) Porto, A. L. M.; Machado, S. M. F.; De Oliveira, C. M. A.; Bittrich, V.; Amaral, M. C. E.; Marsaioli, A. J. Polyisoprenylated benzophenones from Clusia floral resins. *Phytochemistry* **2000**, *55*, 755−768.
(12) Marquez Hernandez, I.; Campo Fernandez, M.; Cuesta-Rubio, O.; Piccinelli, A. L.; Rastrelli, L. Polyprenylated benzophenone derivatives from cuban propolis. *J. Nat. Prod.* **2005**, *68*, 931−934.
(13) Cuesta-Rubio, O.; Cuellar Cuellar, A.; Rojas, N.; Velez Castro, H.; Rastrelli, L.; Aquino, R. A polyisoprenylated benzophenone from Cuban propolis. *J. Nat. Prod.* **1999**, *62*, 1013−1015.
(14) Cuesta-Rubio, O.; Frontana-Uribe, B. A.; Ramirez-Apan, T.; Cárdenas, J. Polyisoprenylated benzophenones in Cuban propolis; biological activity of nemorosone. *Z. Naturforsch*. **2002**, *57*, 372−378.
(15) Banskota, A. H.; Tezuka, Y.; Prasain, J. K.; Matsushige, K.; Saiki, I.; Kadota, S. Chemical constituents of Brazilian propolis and their cytotoxic activities. *J. Nat. Prod.* **1998**, *61*, 896−900.
(16) Piccinelli, A. L.; Campo Fernandez, M.; Cuesta-Rubio, O.; Marquez Hernandez, I.; De Simone, F.; Rastrelli, L. Isoflavonoids isolated from Cuban propolis. *J. Agric. Food Chem*. **2005**, *53*, 9010−9016.
(17) Greenaway, W.; Scaysbrook, T.; Whatley, F. R. The composition and plant origin of propolis: a report of work at Oxford. *Bee World* **1990**, *71*, 107−118.
(18) Cuesta-Rubio, O.; Piccinelli, A. L.; Rastrelli, L. Chemistry and biological activity of polyisoprenylated benzophenone derivatives. *Stud. Nat. Prod. Chem.* **2005**, *32*, 671−720 (Bioactive Natural Products (Part L)).
(19) Cuesta-Rubio, O.; Velez-Castro, H.; Frontana-Uribe, B. A.; Cardenas, J. Nemorosone, the major constituent of floral resins of Clusia rosea. *Phytochemistry* **2001**, *57*, 279−283.

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