Polyisoprenylated Benzophenones in Cuban Propolis; Biological Activity of Nemorosone[§]

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Propolis, Prenylated Benzophenones, Nemorosone

The Copey tree (*Clusia rosea*) has a large distribution in Cuba and its floral resin is a rich source of polyisoprenylated benzophenones. To determine the presence of these natural products, we carried out a study by HPLC of 21 propolis samples produced by honey bees (*Apis mellifera*) from different provinces of Cuba. Nemorosone resulted to be the most abundant polyisoprenylated benzophenone and the mixture of xanthochymol and guttiferone E was also observed, but in minor proportion. We studied the biological activity of the pure natural product nemorosone and its methyl derivatives. We found that nemorosone has cytotoxic activity against epitheloid carcinoma (HeLa), epidermoid carcinoma (Hep-2), prostate cancer (PC-3) and central nervous system cancer (U251). It also exhibited antioxidant capacity. Methylated nemorosone exhibited less biological activity than the natural product.

Introduction

Flavonoids, organic acids and its derivatives have been isolated as the major components in propolis from temperate regions; the flavonoids pinocembrin, galangin, chrysin and the caffeic, ferulic, and cinnamic acids are the most frequently reported (Marcucci, 1995; González and Orzaez 1997; Marcucci *et al.*, 2000;). The influence of the genus *Populus* in the chemical constitution of propolis, has been reported in the propolis obtained in different regions of the world (Bankova *et al.*, 1994; Kurkin *et al.*, 1992; Greenaway *et al.*, 1988). Polyisoprenylated benzophenones in propolis samples from the tropical regions of Venezuela and Cuba have been isolated (Tomás-Barberán *et al.*, 1993; Cuesta *et al.*, 1999). The isolation of propolona A (**1**) from a Cuban propolis sample, suggested that bees collect floral resins from genus *Clusia* to produce propolis. This possibility has been proposed for the Venezuelan propolis (Tomás-Barberán *et al.*, 1993). Considering the large distribution of the Copey tree (*Clusia rosea*) in Cuba and that its floral resin is a rich source of polyisoprenylated benzophenones (Bisse, 1988; De Oliveira *et al.*, 1996; De Oliveira *et al.*, 1999), we carried out an HPLC study in order to determine the presence of these kind of compounds in 21 samples of propolis produced by *Apis mellifera* from different provinces of Cuba. Nemorosone (**2**)[‡] was found to be the most abundant polyisoprenylated benzophenone and the mixture of xanthochymol (**4a**) and guttiferone E (**4b**) was also observed but in minor amounts. Recently, it has been reported the bactericide activity of the methylated isomer of nemorosone **3b** (known as nemorosone II, Lokvam *et al.*, 2000), showing that this product could be responsible of the bactericide activity of propolis. With this idea in mind, we studied the cytotoxic and antioxidant activity of the pure natural product nemorosone (**2**) and the mixture of methyl derivatives **3**.

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[‡] The natural product nemorosone is a mixture of the tautomers **2a** and **2b** which can not be separated, see Cuesta-Rubio *et al.*, 2001.

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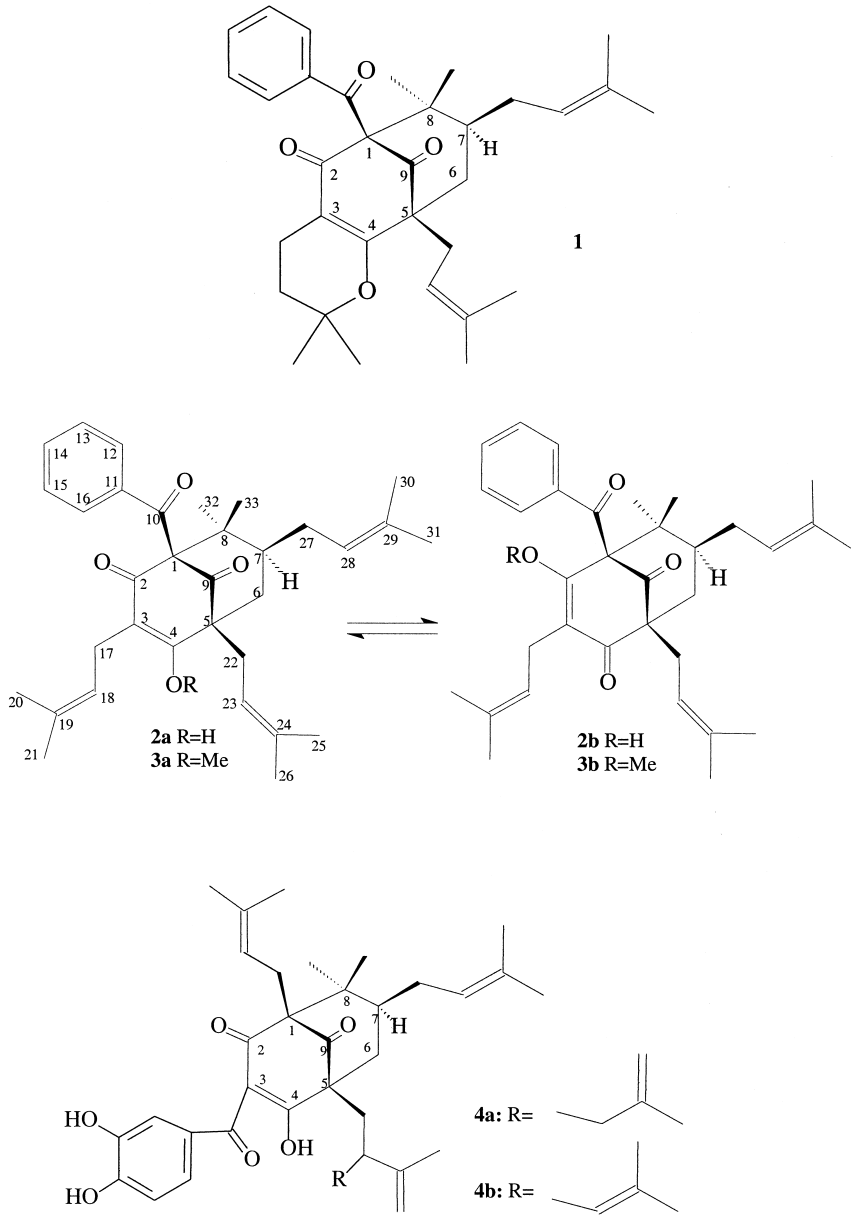

Scheme 1

Experimental

Propolis samples were provided by La Estación Apícola Experimental "La Habana" or collected by one of us (O. C.) in the studied provinces. The HPLC analyses were performed by a Waters Delta prep 4000 equipped with a 486 UV-Vis detector. The chromatographic column was a μBondapak C-18 (300 × 3.9 mm). Organic solvents were HPLC grade from J. T. Baker (55320, Xalostoc, Edo. de Méx., México) and water was purified with the Milli-Q Water System (Millipore Corp., Bedford, MA, USA).

Standard isolation

Propolis samples 1 and 5 (*ca.* 50 g) were extracted three times individually with MeOH (100 ml) for 1 hr. The extracts (*ca.* 7 g) were filtered and subjected to vacuum liquid chromatography on silica gel with mixtures of hexane:EtOAc (100:0 to 0:100 v/v) to give 61 fractions (50 ml): 60–61 (315 mg; mixture of xanthochymol **4a** and guttiferone E, **4b**). Fractions 10–15 were subjected to exhaustive column chromatography with mixtures of hexane:EtOAc. From the fractions 47–51 (520 mg) nemorosone (**2**) was isolated. The mixture of methyl-nemorosone derivatives (**3**) was obtained by methylation with diazomethane (Cuesta-Rubio *et al.*, 2001). The compounds found were as expected according to the chemical and physical characteristics reported in literature (Cuesta-Rubio *et al.*, 2001; Gustafson *et al.*, 1992; Blount and Williams, 1976).

HPLC analyses

All propolis samples for HPLC analyses (*ca.* 5 g) were extracted three times with EtOAc: hexane (1:1 v/v) for 1 hr. The residues were treated with boiling MeOH (3 × 100 ml) for 10 min and filtered. The extracts obtained were concentrated and analyzed by HPLC. The HPLC analyses were achieved using a gradient from MeOH:AcOH 2% (50:50) to MeOH 100% in 15 min with a solvent flow rate of 1 ml min^{-1} at 20 °C. The UV detector was set at 280 nm. Nemorosone (**2**) showed a retention time of 16.4 min under these experimental conditions. Quantification of nemorosone was carried out by interpolation with a standard curve generated by injection of nemorosone samples of known concentration. Detector response was linear over the range of concentration used. In Table I mean and the standard error (SE) of three independent experiments are listed.

Cell culture and assay for cytotoxic activity

The human cervix carcinoma (HeLa), the human larynx carcinoma (HEp-2), prostate carcinoma (PC-3) and central nervous system carcinoma (U251) cell lines were supplied by Facultad de Medicina, UNAM, México and National Cancer Institute (NCI), USA. The human tumor cytotoxicities were determined following protocols established by the NCI (Monks *et al.,* 1991). The cell lines HeLa and Hep2 were cultured in a minimal essential medium with Earle's salts (MEN) and PC-3 and U251 cell lines in RPMI 1640 medium, all supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acids. They were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity. For the assay, cells were detached with 0.1% trypsin-EDTA to make single-cell suspensions, and viable cells were counted using a hematocytometer and diluted with medium to give 7.5×10^4 cells/ml. 100 μl/well of these cells suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 hr the cells were treated with logarithmic concentrations of the test products and the positive controls, doxorubicin (2.5×10^{-5} to 2.5×10^{-9} M) and actinomycin D (2.5×10^{-5} to 2.5×10^{-9} M). They were initially dissolved in DMSO (20 mM) and further diluted in medium to produce six concentration test solutions (100, 31, 10, 3.1 1.0 and 0.31 μM). 100 μl of each test solution with the compound to evaluate were added to each well. The final volume in each well was 200 μl and the plates were incubated for 48 hr.

Sulforhodamine B assay: After the 48 hr, adherent cell cultures were fixed *in situ* by adding 50 μl of cold 50% (wt/vol) trichloroacetic acid (TCA) and incubated for 60 min at 4 °C. The supernatant was discarded and the plates were washed three times with water and air-dried. 100 μl of SRB solution (0.4% wt/vol in 1% acetic acid) was added to each microtiter well and the culture was incubated for 30 min at room temperature. Unbound SRB was removed by washing three times with 1% acetic acid and the plates were air-dried. The bound and stained SRB was solubilized with Tris (tris[hydroxymethyl]aminomethane) buffer, and the optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 515 nm. The IC_{50} (concentrations required to inhibit cell growth by 50%) was calculated according to the protocol previously established (Monks *et al.,* 1991). Mean and the standard error (SE) of three independent experiments for each selected concentration of the studied compound are listed in Table II.

Assay for antioxidant activity

The potential antioxidant activity was assessed on the basis of the scavenging effect of the tested compound on the stable free radical of 1,1-diphenyl-picrylhydrazyl (DPPH) (Cottele *et al.,* 1996). In 96-well microtiter plates, the reaction mixtures containing different concentrations of the test samples in EtOH and 100 mM DPPH in ethanolic solution, were incubated at 37 °C for 30 min. The absorbances were subsequently measured at 515 nm. Measurements were performed in triplicate in at least three independent experiments. The percent of inhibition of each compound was determinated by comparison with a DPPH ethanolic blank solution. IC_{50} values are the concentration of sample required to scavenge 50% of DPPH free radicals. The data shown in Table III represents the mean plus and standard error (SE) of three independent experiments for each selected concentration of the studied compound.

Results and Discussion

Chemical composition of Cuban propolis

The two standards, nemorosone (**2**) and the mixture of xanthochymol (**4a**) and guttiferone E (**4b**) were isolated by conventional procedures from the MeOH extracts of two propolis samples (Sample 1 and Sample 5, see Table I). The structural identification was carried out by comparison with the NMR data reported previously for these compounds (Cuesta-Rubio *et al.*, 2001; Gustafson *et al.*, 1992; Blount and Williams, 1976). Propolis samples from different provinces of Cuba were investigated by HPLC, in order to evaluate the presence of **2**. The HPLC study of nemorosone (**2**) showed a retention time of 16.4 minutes for this product, with the experimental conditions described in the experimental section. The chromatographic signal of nemorosone was an asymmetric peak, with a little shoulder on the tail of the peak. We attributed this shoulder to the presence of the two nemorosone keto-enol isomers (**2a** and **2b**) from the enolizable 1,3-diketone system present in the compound (Cuesta-Rubio *et al.*, 2001).

For the HPLC analyses, the propolis samples were extracted with hexane: EtOAc (1:1 v/v) to facilitate the selective extraction of the benzophenones and the solvent was evaporated *in vacuo*. The residues obtained were dissolved in MeOH to eliminate the beeswax and the methanolic extract was injected to the HPLC equipment. Samples 1 and 5 were included as positive controls of the presence of nemorosone. The HPLC analyses of the extracts obtained in this way, showed a very simple chromatogram with only few signals. The biggest signal corresponds in shape and retention time with the observed for the nemorosone standard. This result confirms the selectivity for the extraction of the prenylated benzophenones by the method previously described and the large quantity of nemorosone present in the sample. The same chromatographic characteristics of nemorosone were observed with 17 propolis samples.

Some crystals of nemorosone were added in all the samples that showed the presence of a chromatographic signal with the characteristic of nemorosone. We observed an increment of absorbance value without lose of the observed shape of the peak. In all the cases where nemorosone was detected, both facts were observed confirming the presence of nemorosone in the analyzed samples.

The nemorosone standard used previously, was employed to construct a standard curve to interpolate the observed absorbance values, which led us to perform the quantitative study of nemorosone in Cuban propolis. The same experimental conditions used for the qualitative analysis were employed in the quantitative study. The results are summarized in Table I.

Interestingly, different samples from the same province (1, 3 and 6) either showed or did not show the presence of nemorosone **2** in propolis. Apparently this is due to different geographic regions with different vegetation. Four samples of propolis (3, 4, 11 and 18) did not contain nemorosone. We observed that nemorosone was contained in 17 propolis samples of the 21 evaluated. The quantities of nemorosone were in a range between 3.7 and 46% in relation to the weight of the propolis sample. The differences can be attributed to factors such as: number of Copey trees near to the beehive, their flowering, the resin recollection season, other available plant exudates. These results suggest that the nemorosone is one of the major constituents of the Cuban propolis and that it can be produced initially from floral resins of *C. rosea*, from where it has been isolated (Cuesta-Rubio *et al.*, 2001).

Table I. Nemorosone quantification in Cuban propolis.

Sample	Province of Cuba	Mass of crude propolis [g]	Mass of the methanolic residue [g]	Quantity extracted (%)	Quantity injected [mg/ml]	(%) Nemorosone* mean ± SE
1	Holguín	5.001	2.470	49.5	0.97	+
2	Ciudad Habana	5.010	2.086	41.6	1.66	6.7 ± 0.22
3	Holguín	5.070	2.150	42.4	2.15	–
4	Ciudad Habana	5.100	1.006	19.7	40.24	–
5	Guantánamo	5.060	2.560	50.5	1.02	+
6	Holguín	5.040	1.620	32.1	1.94	5.2 ± 0.01
7	Santi Spíritus	5.100	2.373	46.5	0.94	13.1 ± 0.02
8	Santiago de Cuba	5.000	1.316	26.3	0.52	9.0 ± 0.10
9	Las Villas	5.070	2.634	51.9	0.84	12.2 ± 0.39
10	Cienfuegos	5.007	3.130	62.5	0.62	28.5 ± 0.53
11	Habana	5.009	1.179	23.5	47.16	–
12	Guantánamo	5.001	3.218	64.3	0.64	22.3 ± 1.9
13	Habana	5.011	1.383	27.5	2.76	3.7 ± 0.23
14	Pinar del Río	5.040	3.830	75.9	0.76	35.2 ± 0.01
15	Santiago de Cuba	5.006	3.910	78.1	0.78	18.8 ± 0.18
16	Pinar del Río	5.001	1.900	37.9	3.80	+
17	Santiago de Cuba	5.018	2.912	58.0	0.58	18.4 ± 0.07
18	Santiago de Cuba	5.029	0.581	11.5	23.24	–
19	Pinar del Río	5.009	3.822	76.3	0.76	36.8 ± 0.11
20	Pinar del Río	5.014	4.660	92.9	0.46	46.4 ± 0.85
21	Pinar del Río	5.003	2.049	40.9	0.81	11.3 ± 0.15

(+) non quantified. (–) non detected. * Each dilution in standard curve and each propolis sample determination was injected in triplicate and the result corresponds to the mean ± SE.

Nemorosone was identified in propolis samples obtained from the occidental, central and oriental regions of Cuba, suggesting that this polyisoprenylated benzophenone is a common compound in Cuban propolis. In these cases, the Copey trees (*C. rosea*) are the principal sources of resins to produce propolis as confirmed by the recollection of this floral resin by the bees.[§] The polyprenylated benzophenones xanthochymol (**4a**) and guttiferone E (**4b**) have not been isolated from the floral resins of *C. rosea*, but they have been found in the yellow resins that secrete the young fruits of *C. rosea* (Gustafson *et al.*, 1992). Our observations and those of the beekeepers confirm, that bees also recollect this resin.

These facts establish the relationship between the Copey tree (*Clusea rosea*) and the presence of polyisoprenylated benzophenones in Cuban propolis. It seems that the floral and fruit resins of *C. rosea* are the source of choice for propolis production when these are available. We have quantified between 0.3 and 0.5 g of resin in each flower, suggesting that only an adult Copey tree near to the bee-hive can be a very important source of starting material for the propolis production.

The role of *C. rosea* in the chemical composition of Cuban propolis should not be considered as essential, because it is known that bees can recollect plant exudates from other plant species. This fact can explain the absence of nemorosone in four of the propolis samples (samples 3, 4, 11, and 18).

The quantities of polyisoprenylated benzophenones in the floral resins of *Clusia* species have been determined using methyl derivatives (De Oliveira *et al.*, 1996; De Oliveira *et al.*, 1999; Lokvam *et al.*, 2000). Our study propose an alternative to quantify this kind of benzophenones without derivatization. The reported data and the results obtained in our investigation suggested, that nemorosone (**2**) is a major component in the majority of the Cuban propolis.

*Biological activity of nemorosone (**2**) and methyl derivatives (**3**)*

We found that the natural product **2** was active against the four cell lines (Table II). When the

[§] Our observation and confirmed by the beekeepers.

Table II. Cytotoxicity activity of nemorosone and methyl derivative (IC_{50} mean ± SE n = 3).

Cellular line:	HELA		HeP-2		PC-3		U25l	
	μM	μg/ml	μM	μg/ml	μM	μg/ml	μM	μg/ml
Nemorosone (**2**)	3.3 ± 0.17	1.6 ± 0.08	3.1 ± 0.17	1.5 ± 0.08	7.2 ± 1.3	3.6 ± 0.65	3.9 ± 1.40	1.9 ± 0.70
Methyl-nemorosone (**3**)	57.1 ± 0.10	29.4 ± 0.05	94.5 ± 0.15	48.7 ± 0.07	85.1 ± 1.5	43.9 ± 0.77	32.9 ± 4.10	17.0 ± 2.11

Reference: Doxorubicin (HELA: $1.9 \times 10^{-3} \pm 4.3 \times 10^{-4}$ mM; HeP-2: $2.06 \times 10^{-3} \pm 3.4 \times 10^{-4}$ mM; U251: $9.28 \times 10^{-4} \pm 2.2 \times 10^{-4}$ mM; PC-3: $3.2 \times 10^{-3} \pm 1.7 \times 10^{-3}$ mM).
Reference: Actinomycin D (HELA: $1.9 \times 10^{-6} \pm 2 \times 10^{-7}$ mM = $2.38 \times 10^{-3} \pm 2.5 \times 10^{-4}$ μg/ml ; HeP-2: $1.3 \times 10^{-6} \pm 2 \times 10^{-8}$ mM = $1.6 \times 10^{-3} \pm 2.5 \times 10^{-4}$ μg/ml).

mixture of methyl derivatives **3** was used, the required concentration to reach the IC_{50}, in the cellular lines, was between ten times and thirty times more concentrated, showing a very important decrease for the cytotoxic activity. Following the recommendation of the Natl. Cancer Institute-USA, we consider that a product is active when the IC_{50} is reached with a concentration lower than 4 μg/ml for pure compounds (Cordell *et. al.*, 1993).

The antioxidant properties of nemorosone (**2**) and it's methyl derivative (**3**) were also studied. We observed that **2** is active to scavenge the DPPH free radical in the same order of magnitude than the reference α-tocopherol (Table III). As observed in the cytotoxic assay, the methylation of nemorosone abolishes the antioxidant properties of this natural compound.

These studies showed that the 1,3-diketone system is a very important molecular region for the activity of **2**. Probably lack of the tautomeric equilibrium or an hysterical hindrance in this region affect severely the biological behavior of nemorosone. This fact is very important, because the previously reported biological tests for nemorosone have been performed only with the methyl derivatives of this compound (Lokvam *et al.*, 2000), because the separation of the compounds is facilitated by forming these methyl derivatives during the phytochemical study.

Table III. Antioxidant activity of nemorosone and methyl derivative (IC_{50} mean ± SE n = 3).

	Scavenging of the free radical of DPPH	
	μM	μg/ml
Nemorosone (**2**)	44.1 ± 0.08	22.2 ± 0.04
Methyl-nemorosone (**3**)	>200	>103.2
Reference: α-tocopherol	24.4 ± 0.33	10.5 ± 0.14

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