New Bioactive Chalcones in Propolis from El Salvador

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2′,3′-Dihydroxy-4,4′-dimethoxychalcone (**1**) and 2′,3′,4-trihydroxy-4′-methoxy-chalcone, two new chalcones, were isolated from propolis from El Salvador. The compounds showed significant antibacterial ana antifunfag activity and moderate toxicity to *Artemia salina* nauplii.

Introduction

Propolis (bee glue) is a resinous hive product collected by bees from plant exudates. It is widely used in medicine, cosmetics and food industry (Marcucci 1995; Matsuda 1994), due to its versatile biological activities. These include antimicrobial, fungicidal, antiviral, immunostimulating, cytostatic and radical-scavenging activities (Marcucci, 1995; Krol *et al.*, 1996). For a long period, investigations on propolis were focused on samples originating from the temperate zones (Ghisalberti, 1979; Marcucci, 1995). However, the chemical composition of bee glue depends on the flora at the site of collection and varies at different geographic locations (Bankova *et al.*, 2000). For this reason the composition of tropical propolis is quite different form propolis from temperate regions. Recently, tropical propolis has become a subject of increasing interest for chemists and biologists (Banskota *et al.*, 2000a, Marcucci and Bankova, 1999; Valcic *et al.*, 1999, Bankova *et al.*, 1998). It turned out to be a source of new biologically active compounds (Banskota *et al.*, 2000b; Claus *et al.*, 2000, Hirota *et al.*, 2000; Kimoto *et al.*, 1998). In this paper we report the isolation and characterization of two new chalcones from propolis originating from El Salvador, Central America, and their biological activity.

Experimental

Propolis was collected in the eastern region of El Salvador, near Usulutan, in January 2000.

Extraction of propolis

Propolis (50 g) was cut into small pieces and extracted with 70% ethanol (1:10, w:v) at room temperature for 24 h. A small part of this extract (10 ml) was evaporated to dryness and used in biological tests. The ethanol extract was concentrated *in vacuo* and extracted successively with *n*-hexane (3 times) and with ethyl ether (3 times). The hexane extract was evaporated to give 9.1 g dry residue and the ethyl ether extract gave13.0 g dry residue after evaporation.

Isolation of compounds

The ethyl ether extract was subjected to column chromatography on silica gel with an acetone – *n*-hexane gradient to produce several fractions. After repeated column chromatography and preparative TLC on silica gel, *n*-hexane – methyethyl ketone as the mobile phase, compounds **1** and **2** were isolated.

2′,3′-dihydroxy-4,4′-dimethoxychalcone (**1**). 51 mg. M.p. 143–145 °C. UV (MeOH) λ_{max} 294, 308, 366 nm. EIMS (70 eV) *m/z* 300 ($M^{+\cdot}$, 100%), 285 ($[M\text{-}15]^+$, 7%), 166 ($A_1^{+\cdot}$, 57%), 161 (B_5^+,

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8%), 138 ([A_2 – CO]$^+$, 59%) 134 ($B_1^{+\cdot}$, 78%), 121 (B_4^+, 17%). For ^{1}H and ^{13}C NMR – see Table I.

2',3'4-trihydroxy-4'-methoxychalcone (**2**). 153 mg. M.p. 179–184 °C. UV (MeOH) λ_{max} 305, 372 nm. EIMS (70 eV) *m/z* 286 ($M^{+\cdot}$, 100%), 271 ([M-15]$^+$, 9%), 167 (A_2^+, 69%), 138 ([A_2 – CO]$^+$, 63%), 120 (B_1^+, 26%). For ^{1}H and ^{13}C NMR – see Table I.

Cytotoxicity assay

Artemia salina (nauplii) lethality (Soils *et al.*, 1993) was determined using caffeic acid phenethyl ester (CAPE) as active reference compound. Concentrations of 1000, 100, 10 and 1 ppm were used (3.33, 0.33, 0.033 and 0.0033 nM for **1** and 3.5, 0.35, 0.035 and 0.0035 nM for **2**). 10 *A. salina* per concentration plus control. The activity of the total extract and of the individual compounds was measured.

Antibacterial activity

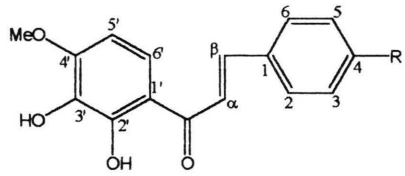
For the investigation of the antibacterial activity, the agar cup method (Spooner and Sykes, 1972) was used with test strains *Staphylococcus aureus* 209 (obtained from the Bulgarian Type Culture Collection, Institute for State Control of Drugs, Sofia) and *Escherichia coli* WF+ (obtained from the Collection of ZIMET, Central Institute of Microbiology and Experimental Therapy, Jena). An inhibitory zone with a diameter less than 10 mm corresponds to lack of activity (10 mm is the diameter of the cup). 0.1 ml of test solution containing 0.4 mg of each substance in ethanol was applied to every cup.

Antifungal activity

For the investigation of the antifungal activity the agar cup method was used (Spooner and Sykes, 1972). As test microorganism, *Candida albicans* 562 (obtained from the Bulgarian Type Culture Collection, Institute for State Control of Drugs, Sofia) was used. The antifungal activity was measured as diameter of the inhibitory zones. An inhibitory zone with a diameter less than 10 mm corresponds to lack of activity (10 mm is the diameter of the agar cup), 0.1 ml of test solution containing 0.5 mg of each substance in ethanol was applied to every cup. Solvents showed no activity.

Results and Discussion

The ether fraction from the ethanolic extract of the investigated propolis sample was subjected to repeated column chromatography and preparative TLC on silica gel and afforded two yellow crystalline compounds (**1** and **2**). Their structures were determined by UV, EIMS, ^{1}H and ^{13}C NMR spectra, DEPT, HSQC, HMQC, HMBC.



1: R = OMe
2: R = OH

The UV spectrum of compound **1** was characteristic for chalcones (Mabry *et al.*, 1970), with a dominant band I absorption at 366 nm, a shoulder at 308 nm and a relatively minor band II (294 nm). This compound afforded a $M^{+\cdot}$ ion peak at *m/z* 300, according to EIMS. Additional peaks at 166 ($A_1^{+\cdot}$), 161 (B_5^+), 138 ([A_2 – CO]$^+$) and 134 ($B_1^{+\cdot}$, 78%) suggested the presence of methoxylated ring B and ring A with two hydroxy and one methoxy group. Analysis of the 1D and 2D NMR spectra with homo- and heteronuclear direct or long-range correlation allowed assignment of ^{1}H and ^{13}C NMR signals, as listed in Table I. The ^{1}H NMR revealed 6 aromatic proton signals, belonging to two aromatic nuclei. The two doublets at δ 7.02 and 7.87 (2H each, J = 8.8 Hz) could be attributed to a 1,4-disubstituted benzene ring, and the doublets at δ 6.48 and 7.96 (1H each, J = 9 Hz) to a 1,2,3,4-tetrasubstituted one. Two more singlet signals at δ 7.80 and 7.81 were obserevd in this spectrum; they were assigned to H-α and H-β by analysis of the HMBC data. Correlations between these protons and C-2, C-6, and the conjugated carbonyl carbon (δ 192.2), showed that they belonged to the E-double bond of the chalcone unit. The coupling constant J_E= 16 Hz, indicative of the E-configuration of the double bond, could only be observed in the HSQC spectrum without decoupling. Cross peak was observed also between H-6′ and the carbonyl carbon. The two methoxy groups were positioned at C-4′ and C-4 because the methyl protons at these groups (δ 3.73 and 3.82)

Table I. ^{1}H and ^{13}C NMR data of chalcones **1** and **2**.[a]

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1		127.3		127.0
2	7,87 d (8.8)	131.2	7.77 d (8.6)	131.4
3	7.02 d (8.8)	114.6	6.84 d (8.6)	115.9
4		161.7		160.4
5	7.02 d (8.8)	114.6	6.84 d (8.6)	115.9
6	7,87 d (8.8)	131.2	7.77 d (8.6)	131.4
C=O		192.2		192.2
α	7.81 s	118.6	7.76 s	117.4
β	7.80 s	144.1	7.76 s	144.6
1′		134.9		134.8
2′		158.7		158.6
3′		113.8		113.8
4′		157.6		157.3
5′	6.48 d (9)	108.2	6.52 d (9)	108.0
6′	7.96 d (9)	127.2	7.96 d (9)	125.8
OMe (C-4)	3.82 s	55.5		
OMe (C-4′)	3.73 s	59.9	3.72	59.9

[a] ^{1}H and ^{13}C NMR were measured at 250 and 62.9 MHz, respectively, in DMSO-d_6 and coupling constants (parentheses) are in Hz.

showed diagnostic HMBC correlations to C-4′ and C-4, respectively. Other significant long-range correlations are shown in Fig. 1. and thus **1** was characterized as 2′,3′-dihydroxy-4,4′-dimethoxychalcone.

The spectral characteristics of **2** were similar to those of **1**. The difference between the two compounds appeared to be the presence of only one methoxy group in the molecule of **2**. This was obvious from the comparison of NMR and MS spectral data. The signals corresponding to the methoxy group at C-4 (at δ 3.82 in the ^{1}H NMR spectrum and at δ 55.5 in the ^{13}C NMR spectrum) were absent in the spectra of **2**. The mass spectrum of **2** indicated the presence of a hydroxy group instead of a methoxy in ring B. The fragments originating from ring A were the same in the mass spectra of **1** and **2**. Based on these data, the structure of **2** was elucidated as 2′,3′,4-trihydroxy-4′-methoxychalcone.

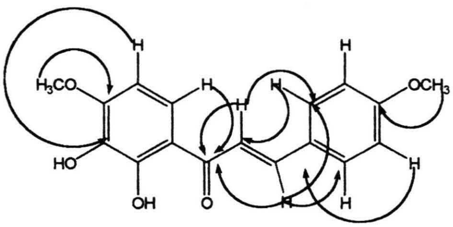


Fig. 1. Important HMBC correlations in **1**.

To the best of our knowledge, **1** and **2** are new natural compounds. Both compounds showed good activity against *Staphylococcus aureus* and *Candida albicans*, which was found significantly higher than that of the total extract (Table II). No activity against *Escherichia coli* was observed. The new chalcones exhibited moderate activity against *Artemia salina* nauplii (Table II). Obviously, **1** is partially responsible for the toxicity of the ethanol extract.

The plant origin of the chalcones **1** and **2** in the investigated bee glue is unclear. The identification of new natural compounds in propolis samples provides suitable markers because the identification of **1** and **2** in some plant exudate will be an indication that this exudate is one of the sources of propolis in El Salvador.

Table II. Biological activity of propolis extract and isolated compounds.

Sample	Cytotoxicity assay	Antibacterial and antifungal activity	
	$LC_{50}\pm SD$ (mg/ml)[a]	Diameter of the inhibitory zone ± SD (mm)[a]	
		S. aureus	*C. albicans*
Alcohol extract	39±9	12±1	11±1
1	38±8	29±3	19.3±0.6
2	327±47	23±1	29±1
CAPE	0.45±0.05	–	–

[a] Mean of three measurements.

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