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Complete ^{1}H and ^{13}C NMR assignments and antifungal activity of two 8-hydroxy flavonoids in mixture

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ABSTRACT

A mixture of the two new flavonols 8-hydroxy-3,4',5,6,7-pentamethoxyflavone (**1**) and 8-hydroxy-3,3',4',5,6,7-hexamethoxyflavone (**2**) was isolated from a commercial sample of *Citrus aurantifolia*. An array of one- (^{1}HNMR, {^{1}H}-^{13}C NMR, and APT-^{13}C NMR) and two-dimensional NMR techniques (COSY, NOESY, HMQC and HMBC) was used to achieve the structural elucidation and the complete ^{1}H and ^{13}C chemical shift assignments of these natural compounds. In addition, the antifungal activity of these compounds against phytopathogenic and human pathogenic fungi was investigated.

Key words: polymethoxyflavones, antifungal activity, *Citrus aurantifolia*.

INTRODUCTION

Flavonoids constitute one of the most important classes of naturally occurring phenols with interesting properties, to the plants as protective agents and human health (Ezeonu et al. 2001, Del Río et al. 1998, Bohm 1998, Chen et al. 1997, Harborne et al. 1975, Mayer 1998). Many analytical procedures have been developed for flavonoids analysis in mixture, although the most successful are based on chromatographic techniques such as high-performance liquid chromatography (HPLC) and Gas Chromatography (GC) (Oliveira et al. 2001, Robards et al. 1997, Robards K. and Antolovich 1997, Branco et al. 1998, 2001, Stremple 1998).

Nuclear magnetic resonance (NMR) spectroscopy is increasingly used as a technique to provide insight into mixture of natural products belonging to the same or different chemical classes without previous separation of the individual components. The sample preparation for NMR is simpler and nondestructive. In this context, NMR methods have been used with success in the structural identification of natural products in mixture such as alkanes (Loaiza et al. 1997), essential oil (Al-Burtamani et al. 2005), furanosesquiterpenes (Gaspar et al. 2005), diterpenoids (Appendino et al. 1992), triterpenoids (Olea and Roque 1990), sterols (Zollo et al. 1986), saponins (Young et al. 1997), anthocyanins (Kosir and Kidric 2002), glycerol esters (Gusntone 1991) and phenolic acids (Gerothanassis et al. 1998).

In this paper, we report the structural elucidation of two new 8-hydroxypolymethoxyflavonols (**1** and **2**) isolated from a commercial sample of *Citrus auranti-*

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folia. The structural determination of these compounds, as components of a mixture, was based on spectral data, including 2D NMR techniques, such as heteronuclear correlation ^{1}H-^{13}C-COSY-$^{n}J_{CH}$ (n = 1, HMQC = ^{1}H-detected Heteronuclear Multiple Quantum Coherence; n = 2 and 3, HMBC = ^{1}H-detected Heteronuclear Multiple Bond Connectivity) and gas chromatography mass spectrometry, together with chemical transformation and comparative analysis of chemical shifts described in the literature. In addition, the antifungal activity of these compounds against phytopathogenic and human pathogenic fungi was investigated.

MATERIALS AND METHODS

General Experimental Procedures

Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a JEOL Eclipse+ 400 spectrometer, using TMS as internal standard or by reference to solvent signals. GC- EIMS spectra were run at 70 eV on a Shimadzu QP-2000 spectrometer.

Plant Material

The fruits of *Citrus aurantifolia* ("lime of Persia") were purchased from local supermarket in Florianópolis, Santa Catarina State, Brazil.

Extraction and Purification

The peels of the fruit were removed manually. The peels of *C. aurantifolia* (600 g) were extracted by maceration with hexane (1 L) at room temperature for 72 h. The peels were later removed by filtration and the hexane extracts were concentrated under reduced pressure. The addition of acetone to the extracts furnished the compounds **1** and **2** as precipitates.

Mixture of 8-hydroxy Flavonols **1** and **2**

IR $_{max}$/cm^{-1}: 3408 (OH), 1651, 1602, 1559 (KBr). GC-EIMS 70 eV m/z (rel. int.): **1** [R_t 17.1 min], 388 ([M]$^+$, 100), 373 ([M-CH_3]$^+$, **1d**, 98), 345 ([M-CH_3-CO]$^+$, **1e**, 12), 327 (39), 135 (**1f**, 58); **2** [R_t 19.8 min], 418 ([M]$^+$, 100), 403 ([M-CH_3]$^+$, **2d**, 89), 375 ([M-CH_3-CO]$^+$, **2e**, 9), 357 (24), 165 (**2f**, 25). ^{1}H NMR (400 MHz, $CDCl_3$): **1**) δ_H 8.24 (*d*, *J* 9.2 Hz, H-2' and 6'), 7.05 (*d*, *J* 9.2, H-3'and 5'), 4.12 (*s*, CH_3O-7), 4.03 (*s*, CH_3O-3), 3.99 (*s*, CH_3O-6), 3.96 (*s*, CH_3O-5), 3.89 (*s*, CH_3O-4'); **2**) 7.90 (*dd*, *J* 1.8 Hz, H-2'), 4.04 (*s*, CH_3O-3), 3.99 (*s*, CH_3O-3'), 3.96 (*s*, CH_3O-4'). ^{13}C NMR (100 MHz, $CDCl_3$): **1**) δ_C 143.21 (C-2), 137.89 (C-3), 171.80 (C-4), 147.51 (C-5), 143.47 (C-6), 151.51 (C-7), 137.23 (C-8), 146.85 (C-9), 111.73 (C-10), 123.61 (C-1'), 160.88 (C-4'), 129.04 (CH-2'), 114.09 (CH-3'), 114.09 (CH-5'), 129.04 (CH-6'), 61.94 (CH_3O-3), 62.25 (CH_3O-5), 61.76 (CH_3O-6), 61.61 (CH_3O-7), 55.36 (CH_3O-4'); **2**) 142.75 (C-2), 137.81 (C-3), 171.80 (C-4), 147.57 (C-5), 143.47 (C-6), 151.60 (C-7), 137.36 (C-8), 146.80 (C-9), 111.67 (C-10), 123.83 (C-1'), 148.88 (C-3'), 150.53 (C-4'), 110.27 (C-2'), 111.07 (C-5'), 120.97 (C-6'), 61.86 (CH_3O-3), 62.25 (CH_3O-5), 61.76 (CH_3O-6), 61.61 (CH_3O-7), 55.90 (CH_3O-3'), 55.81 (CH_3O-4'). ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR in benzene-d_6: Tables I and II.

Mixture of 8-O-methyl Derivatives **1a** and **2a**

A mixture of **1** and **2** (26 mg) was treated with CH_2N_2 as usual to yield **1a + 2a** (26 mg). ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR in benzene-d_6: Tables I and II.

Antifungal Activity

The assays were carried out with three phytopathogenic fungi (*Penicillium digitatum*, *Colletotrichum* sp. and *Curvularia* sp.) and two species of human pathogenic fungi (*Trichophyton mentagrophytes* and *Microsporum canis*). Fungal strains were maintained in potato dextrose agar at 4°C and the inoculum was a suspension of each strain, in nutrient broth, containing approximately 5.10^4 spores/mL.

Bioautography Method

50μL of each solution of extract or substance prepared in hexane-acetone (1:1) (100μg/mL) were applied on TLC plates ($60F_{254}$; Merck), as well as 50μL of amphotericin B (1.60μg/ml) (positive control). The plates were submerged on the fungal inoculum and incubated for 72 h at 30°C in a humid camera. The plates inoculated with dermatophite fungi were then sprayed with p-iodonitrotetrazolium violet (INT) and once more incubated for 4 hour at 30°C. The plates where fungal growth occurred, the INT changed from yellow to purple, while persistence of the yellow color indicated no

TABLE I

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR for flavonols *1* and *2* and their 8-O-methyl ether derivatives (*1a* and *2a*), in benzene-d_6 as solvents. Residual C_6D_6 was used as internal reference (δ_H 7.16 and δ_C 128.00, respectively). Chemical shifts in δ ppm and coupling constants (J, in parenthesis) in Hz.[a]

	1		***2***		***1a***		***2a***	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
				C				
2	143.1	–	143.1	–	152.9	–	152.9	–
3	138.1	–	138.5	–	138.5	–	138.5	–
4	172.0	–	173.0	–	173.3	–	173.3	–
5	148.4	–	148.5	–	149.0	–	149.1	–
6	144.2	–	144.7	–	144.5	–	144.5	–
7	151.9	–	151.9	–	151.5	–	151.6	–
8	137.9	–	138.7	–	141.3	–	141.4	–
9	147.4	–	147.4	–	147.2	–	147.2	–
10	112.7	–	112.6	–	116.4	–	116.4	–
1'	124.5	–	125.0	–	124.2	–	124.2	–
3'	–	–	150.0	–	–	–	149.9	–
4'	161.3	–	151.6	–	161.7	–	151.9	–
				CH				
2'	129.5	8.40 (d, 9.1)	111.2	8.02 (d, 1.8)	130.3	8.22 (d, 8.8)[b]	112.0	7.85 (d, 1.8)
3'	114.5	6.85 (d, 9.1)	–	–	114.3	6.80 (d, 8.8)[b]	–	–
5'	114.5	6.85 (d, 9.1)	111.9	6.65 (d, 8.5)	114.3	6.80 (d, 8.8)[b]	111.7	6.59 (d, 8.4)
6'	129.5	8.40 (d, 9.1)	121.5	8.11 (dd, 8.5, 1.8)	130.3	8.22 (d, 8.8)[b]	122.2	7.95 (dd, 8.4, 1.8)
				CH_3O				
3	61.5	3.69 (s)	61.5	3.71 (s)	61.5	3.68 (s)	61.5	3.69 (s)
5	62.4	3.99 (s)	62.4	4.00 (s)	62.4	4.04 (s)	62.4	4.05 (s)
6	61.6	3.73 (s)	61.6	3.74 (s)	61.6	3.74 (s)	61.6	3.75 (s)
7	61.3	3.77 (s)	61.4	3.79 (s)	61.4	3.77 (s)	61.3	3.78 (s)
8	–	–	–	–	59.6	3.85 (s)	59.6	3.88 (s)
3'	–	–	55.5	3.62 (s)	–	–	55.5	3.55 (s)
4'	54.9	3.28 (s)	55.4	3.39 (s)	54.1	3.27 (s)	55.4	3.37 (s)

[a]Number of hydrogens bound to carbon atoms deduced by comparative analysis of {^{1}H}- and APT-^{13}C NMR spectra. Chemical shifts and coupling constants (J) obtained from 1D ^{1}H NMR spectrum. Heteronuclear ^{1}H-^{13}C-COSY-$^nJ_{CH}$ (n = 2 and 3, HMBC, Table II) and homonuclear ^{1}H-^{1}H-COSY and ^{1}H-^{1}H-NOESY spectra were also used in these assignments. [b]AA'XX' system.

growth. The diameter of growth inhibition was expressed in millimeters.

Minimal Inhibitory Concentration

The minimal inhibitory concentration was determined using the method described in the literature (Smânia et al. 1995, Pizzolatti et al. 2002) and the results are expressed in μL/mL.

RESULTS AND DISCUSSION

^{1}H and ^{13}C NMR spectral data of the polymethoxylated flavonoids **1** and **2**, recorded in $CDCl_3$ (See Experimental), are in good agreement with those described in the literature (Calvert et al. 1979, Chen et al. 1997). The resonances of the aromatic methoxyl groups attached to *ortho*-disubstituted carbons occur considerably downfield (ca δ_C 60 ppm) when compared with aromatic methoxyl groups attached to carbons bearing only

TABLE II

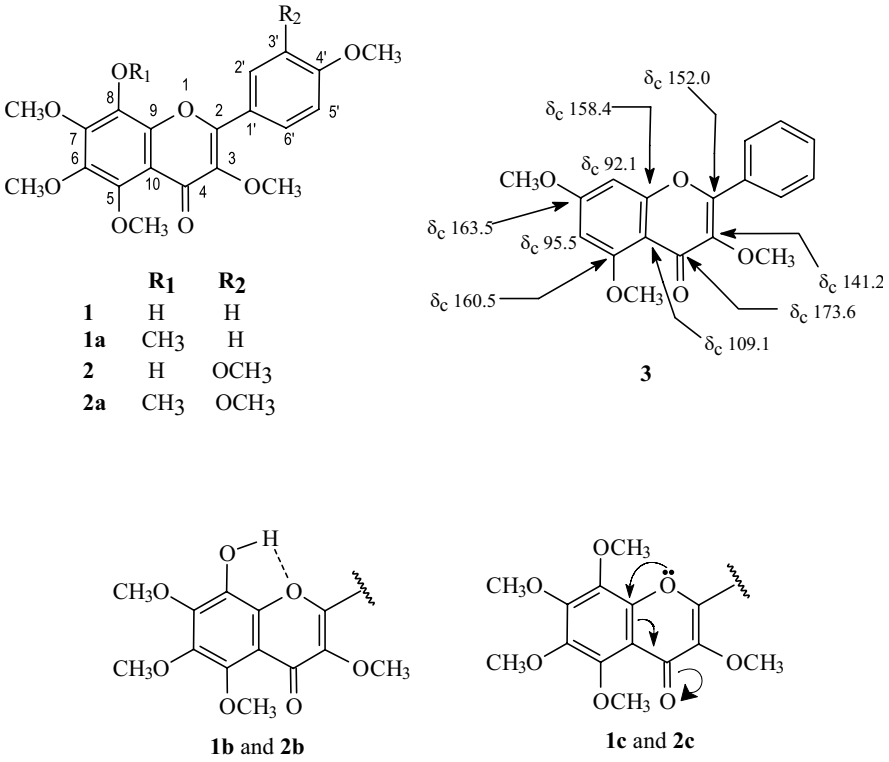
Long-range couplings of hydrogen and carbon atoms observed in the HMBC ($^{n}J_{CH}$, n = 2 and 3) spectra of flavonols *1* and *2* and their 8-O-methyl ether derivatives (*1a* and *2a*), in benzene-d_6 as solvents. Residual C_6D_6 was used as internal reference (δ_H 7.16 and δ_C 128.00, respectively). Chemical shifts (δ, in ppm).*

	1		*2*		*1a*		*2a*	
	δ_C	$^{3}J_{CH}$	δ_C	$^{3}J_{CH}$	δ_C	$^{3}J_{CH}$	δ_C	$^{3}J_{CH}$
C								
2	143.1	2H-2',6'	143.1		152.9	2H-2',6'	152.9	
3	138.6	MeO-3	138.5		138.5	MeO-3	138.5	
4	172.0	–	173.0		173.3	–	173.3	
5	148.4	MeO-5	148.5		149.0	MeO-5	149.1	
6	144.2	MeO-6	144.2		144.5	MeO-6	144.5	
7	151.9	MeO-7	151.9		151.5	MeO-7	151.6	
8	137.9	–	138.1	–	141.3	MeO-8	141.4	
9	147.3	–	147.4	–	147.2		147.2	
10	112.7	–	112.7	–	116.7		116.4	
1'	124.5	2H-3',5'	125.0	H-5'	124.2	2H-3',5'	124.2	H-5'
3'	–	–	150.0	H-5'; MeO-3'	–	–	149.9	H-5'; MeO-3'
4'	161.3	2H-2',6'; MeO-4'	151.6	H-2'; MeO-4'	161.7	2H-2',6'; MeO-4'	151.9	H-2'; H-6'; MeO-4'
CH								
2'	129.5		111.2	H-6'	130.7		112.0	H-6'
3'	114.5		–	–	114.3		–	–
5'	114.5		111.9	–	114.3		111.7	–
6'	129.5		121.7	H-2'	130.3		122.7	H-2'

[a]Number of hydrogens bound to carbon atoms deduced by comparative analysis of HBBD- and APT-^{13}C NMR spectra. Heteronuclear ^{1}H-^{13}C-COSY-$^{1}J_{CH}$ (HMQC, Table I) and homonuclear ^{1}H-^{1}H-COSY and ^{1}H-^{1}H-NOESY spectra were also used in these assignments.

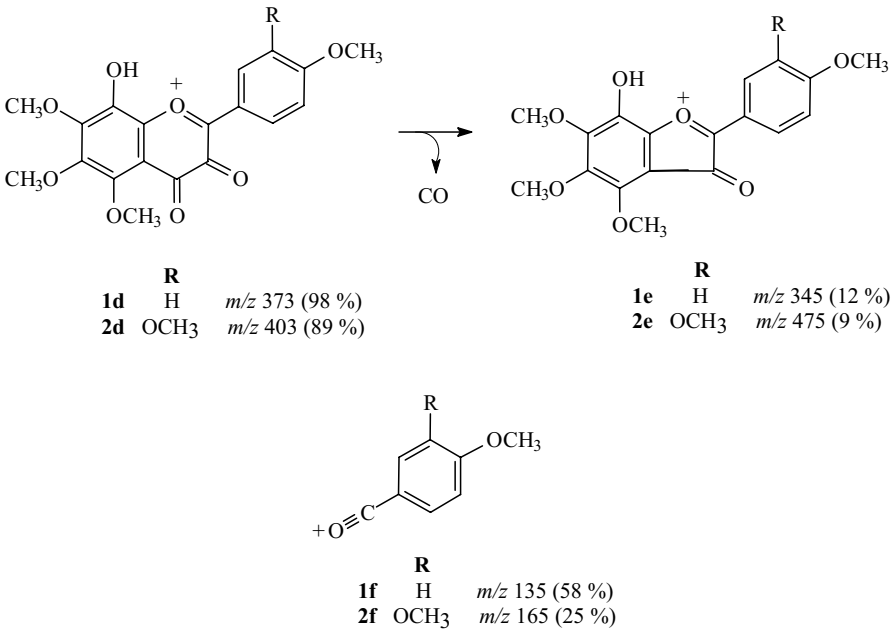
one or no *ortho* substituent (ca δ_C 55 ppm). Unequivocal ^{1}H and ^{13}C chemical shift assignments of these natural compounds were also carried out by 2D ^{1}H-^{13}C correlation techniques (HMQC and HMBC) involving comparison with literature data (Calvert et al. 1979, Chen et al. 1997). The mass spectra of these flavonoids, obtained by high-resolution gas chromatography (HRGC) mass spectral analysis, showed intense molecular ions and $[M-15]^{+}$ fragments characteristic of flavonoids with methoxyl groups at C-6 and/or C-8 (Bohm 1998). The ionic fragments attributed to principal peaks observed in mass spectra of the flavonoids **1** and **2** are showed in Figure 2.

Exhaustive analysis of 1D and 2D NMR spectra ($CDCl_3$, Experimental; benzene-d_6, Tables I and II) of the mixture of **1** and **2** and of their *O*-methyl ether derivatives **1a** and **2a** obtained by methylation with CH_2N_2, recorded in benzene-d_6 (Tables I and II), was used to confirm the presence of a hydroxyl group at carbon C-8. This analysis was facilitated by consid-



	R_1	R_2
1	H	H
1a	CH_3	H
2	H	OCH_3
2a	CH_3	OCH_3

Fig. 1 – Structure of the new 8-hydroxy flavonols **1** and **2**, derivatives **1a** and **2a** and model flavonol **3**.



	R	
1d	H	*m/z* 373 (98 %)
2d	OCH_3	*m/z* 403 (89 %)

	R	
1e	H	*m/z* 345 (12 %)
2e	OCH_3	*m/z* 475 (9 %)

	R	
1f	H	*m/z* 135 (58 %)
2f	OCH_3	*m/z* 165 (25 %)

Fig. 2 – Ionic fragments attributed to principal peaks observed in the mass spectra of the 8-hydroxyflavonols **1** and **2**.

ering electronic effects (inductive and mesomeric) and anisotropic effects as responsible for usual shift-parameters (Günther 1995), solvent effects induced by benzene-d_6 (Horie et al. 1998) and comparison with model compounds, e.g. **3** (Agrawal et al. 1989). In benzene-d_6 as solvent, all the methoxyl signals in the ^{1}H NMR spectra of the mixtures of **1 + 2** and **1a + 2a** appeared clearly separated, which in combination with the relative intensities (**1** in major percentage than **2**) allowed to assign methoxyl groups of each component (Table I). The ^{1}H-^{1}H-NOESY spectra of mixtures of **1 + 2** and **1a + 2a** showed dipolar interactions (NOE effect) between: MeO-3 and H-2'/H-6', and MeO-4' and H-3'/H-5' of **1** and **2a**; MeO-3' and H-2' and MeO-4' and H-5' of **2** and **2a**. Consequently, these results allowed unequivocally to assign the ^{1}H chemical shifts of the signals corresponding to MeO-3 and MeO-4' of **1** and **1a** as well as those of MeO-3' and MeO-4' of **2** and **2a** (Table I). Subsequently, the assignments of the ^{13}C signals attributed to quaternary carbon atoms C-2, C-3 and C-4' of **1** were based on the heteronuclear long-range coupling with hydrogens H-2'/H-6', MeO-3 and MeO-4', respectively, observed in the HMBC spectrum, which also revealed couplings ($^3J_{CH}$) of C-3' with both H-5' and MeO-3' and C-4' with H-2', H-6' and MeO-4' of **2** (Table II). Comparative analysis of the ^{1}H NMR spectra (in C_6D_6) of the mixtures **1 + 2** and **1a + 2a** showed additional singlet signals for MeO-8 at δ_H 3.85 (**1a**) and 3.88 (**2a**), which revealed correlation with the ^{13}C signals corresponding to C-8 at δ_C 141.3 (**1a**) and 141.4 (**2a**) in the HMBC spectrum (Table II). These ^{13}C chemical shifts compared with the remaining signals corresponding to quaternary oxygenated carbon atoms of the A ring of flavonoids may be attributed only to C-8, as anticipated by a mesomeric effect (^{13}C chemical shifts: C-7C-5C-9, conjugated with carbonyl group C-4) and also comparison with model flavonol **3** (e.g.) showing C-8 with a smaller chemical shift than C-6 (Agrawal et al. 1989). Finally, our attention was drawn to the significant ^{13}C chemical shift difference ($\Delta\delta_C$: 9.8 ppm) observed between the signals corresponding to C-2 of 8-hydroxy- (**1** and **2**: δ_C 143.1) and 8-methoxy- (**1a** and **2a**: δ_C 152.9) derivatives. These assignments are summarized in Tables I and II. Most probably, this difference may be attributed to an intramolecular hydrogen bond involving the pyran oxygen (**1b** and **2b**), containing unpaired electrons conjugated with the carbonyl group C-4. A hydrogen bond may be used to justify an attenuation of delocalization (mesomeric effect) of the unpaired electrons of the heterocyclic oxygen atom, which results in a smaller contribution of the canonical structure shown in **1c** and **2c**. Thus, presence of a methoxyl group at C-8 allows major contribution of the corresponding canonical structures (**1c** and **2c**) and, consequently, the partial positive charge at the oxygen atom reduces the electronic density at C-2 by a major inductive electron attracting effect (deshielding).

The fragments **1d-f** and **1e-2f** (Figure 2) attributed to main peaks observed in EIMS are consistent with these structural deductions.

Thus, the new polymethoxylated flavonols isolated as a mixture from commercial *C. aurantifolia* were characterized as 8-hydroxy-3,4',5,6,7-pentamethoxyflavone (**1**) and 8-hydroxy-3,3',4',5,6,7-hexamethoxyflavone (**2**).

TABLE III
Antifungal activity of flavones *1* (8-hydroxy-3,4',5,6,7-pentamethoxyflavone) and *2* (8-hydroxy-3,3',4',5,6,7-hexamethoxyflavone) in mixture by bioautography assay.

Fungi	***1 + 2*** [a]	Anfotericin
P. digitatum	15[b]	15
Curvularia sp.	12	18
Colletotrichum sp.	18	18
T. mentagrophytes	10	30
M. canis	18	24

[a] Compounds tested at 100μL/mL.

[b] Inhibition zone (mm) as an average of two repetitions.

ANTIFUNGAL ACTIVITY OF 8-HYDROXY FLAVONOLS 1 AND 2

The antifungal activity of mixture of the flavonols **1** and **2**, against phytopathogenic and human pathogenic fungi was studied by two different methods. A preliminary analysis was carried out by a bioautography method. The mixture contends **1** and **2** was active against all tested organisms (Table III). However, when these compounds were assayed by a micro dilution method, only a discrete activity was observed. As can be observed in Table IV, the phytopathogenic fungi are more resistant than the

human pathogenic fungi. These results could already be expected because these phytopatogenics were isolated from the same it Citrus species from which the flavonoids were originally obtained.

TABLE IV
Minimal inhibitory concentration (MIC) for *1* (8-hydroxy-3,4',5,6,7-pentamethoxyflavone) + *2* (8-hydroxy-3, 3',4',5,6,7-hexamethoxyflavone, in mixture.

Fungi	***1 + 2*** [a]	Fluconazole
P. digitatum	1000[b]	0.6
Colletotrichum sp.	>1000	0.6
Curvularia sp.	>1000	0.6
T. mentagrophytes	500	1.25
M. canis	>1000	0.6

[a]Compounds tested at 100μL/mL.

[b]Minimal inhibitory concentration expressed in μg/ml.

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RESUMO

Os flavonóis 8-hidroxi-3,4´,5,6,7-pentametoxiflavona (**1**) e 8-hidroxi-3,3´,4´,5,6,7-hexametoxiflavona (**2**) foram isolados em mistura a partir de uma amostra comercial de *Citrus aurantifolia*. A determinação estrutural e a inequívoca atribuição dos sinais de deslocamento químico dos átomos de hidrogênio e carbono destes compostos naturais foram realizadas através da análise dos espectros de RMN 1D e 2D, incluindo COSY, NOESY, HMQC e HMBC. Em adição, a atividade antifúngica destes compostos contra fungos patogênicos também foi investigada.

Palavras-chave: polimetoxiflavonas, atividade antifúngica, *Citrus aurantifolia*.

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