Antimicrobial activity of wax and hexane extracts from *Citrus* spp. peels

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Antibacterial and antifungal properties of wax and hexane extracts of Citrus *spp. peels were tested using bioautographic and microdilution techniques against three plant pathogenic fungi* (Penicillium digitatum, Curvularia *sp.*, *and* Colletotrichum *sp.*), *two human pathogens* (Trichophyton mentagrophytes *and* Microsporum canis), *and two opportunistic bacteria* (Escherichia coli *and* Staphylococcus aureus). *Two polymethoxylated flavonoids and a coumarin derivative, were isolated and identified from peel extracts, which presented antimicrobial activity especially against* M. canis *and* T. mentagrophytes*: 4',5,6,7,8-pentamethoxyflavone (tangeritin) and 3',4',5,6,7,8-hexamethoxyflavone (nobiletin) from* C. reticulata*; and 6,7-dimethoxycoumarin (also known as escoparone, scoparone or scoparin) from* C. limon.

Key words: polymethoxylated flavonoids - antibiosis - pathogens - fungi - bacteria

Citrus spp. are considered an important source of polymethoxylated flavonoids (PMF), a class of secondary plant metabolites (Afek et al. 1986). PMF's are generally found bound to sugar moieties (glycosides) or in exceptional circumstances as free aglycones (Robards et al. 1997). They are often used to establish phylogenetic relationships among plants (Mizuno et al. 2001). However, flavonoids are known to have physiological effects on other organisms. They have been shown to reduce erythrocyte aggregation and sedimentation rates in human blood (Robbins 1976), as well as to exhibit antiviral (Brinkworth et al. 2002), antimutagenic (Iwasse et al. 2001), and antimicrobial properties (Afek et al. 1986, Cushinie & Lamb 2005). Some studies have indicated that foods containing high amounts of flavonoids may reduce the risk of heart diseases, due to their antioxidant properties (Young et al. 1999, Arts et al. 2001, Tripoli et al. 2007).

Studies of the antimicrobial activities of flavonoids have become important because of the increasing occurrence of opportunistic systemic mycosis, as well as the rising prevalence of drug resistance in human pathogenic bacteria (Afek et al. 1986). Drug-resistant bacteria and fungi have complicated the treatment of infectious diseases in immunocompromised AIDS and cancer patients. The evolution of multiple drug resistant human pathogenic microorganisms, has driven the search for new sources of antimicrobial substances, including plant metabolites (Nostro et al. 2002).

The dramatic increase in the resistance of plant pathogens to chemical fungicides has led to the use of repeated applications of chemical fungicides to control plant diseases. As a consequence, public concern has increased the demand for safer and less environmentally harmful agrochemicals (Wedge & Nagle 2000). New antifungal and antibacterial agents are necessary to address this situation.

PMFs with their lipophylic properties, are usually present in vacuoles or in the wax cuticle of plants. Several studies have detected their presence in the wax epicuticular layer on plants where they play a role in protecting these regions against UV radiation and microbial pathogens (Yousef & Tawil 1980, Fang et al. 2001).

In addition, Hamed and Hetta (2005) recorded the ability of *C. reticulata* to reduce the hazardous effect of *Schistosoma mansoni* and reduction of worm burden and ova count. In a previous study we described the isolation and antifungal activity of two new polymethoxylated flavonoids from *C. aurantifolia* (Johann et al. 2007).

The aim of this study was to investigate the effects of wax and hexane extracts of *Citrus* spp. peels on the growth of three common pathogens, *Penicillium digitatum*, *Curvularia* sp., and *Colletotrichum* sp., which are responsible for serious losses in *Citrus* plantations (Rosseti et al. 1993). The extracts and the compounds isolated were also tested against the dermatophyte fungi, *Trichophyton mentagrophytes* and *Microsporum canis*, and the opportunistic bacteria, *Escherichia coli* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Plant material - Fruits from three *Citrus* species: *C. sinensis*, *C. limon*, and *C. reticulata* were obtained from markets in Florianópolis, state of Santa Catarina, Brazil.

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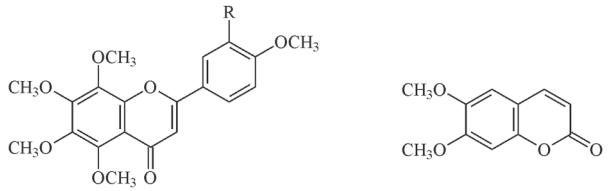
Extraction and isolation - Fruit peels of each species were removed manually and 750 g *C. sinensis*, 520 g *C. limon*, and 495 g *C. reticulata* were separately macerated in hexane (1000 ml) at room temperature for 40 s. The extracts were then filtered and the peels retained. The extracts were concentrated under reduced pressure and the respective wax extracts were collected; further 1000 ml of hexane were added to the peels and they were kept at room temperature for 72 h; once again the hexane extracts were concentrated under reduced pressure.

Purification of the extracts was achieved by recrystalization, providing a mixture of the flavones (210 mg), tangeritin (1) and nobiletin (2), from *C. reticulata*, and a cumarine derivade, escoparone (120 mg, 3) from *C. limon* (Figure).



R
1 H
2 OCH_3

Polymethoxylated 4',5,6,7,8-pentamethoxyflavone (1) and 3',4',5,6,7,8-hexamethoxyflavone (2) from *Citrus reticulata*, and 6,7-dimethoxycoumarin (3) from *C. limon.*

Analyses of the compounds were performed using thin-layer chromatography (TLC), on silica gel SiF_{254} plates (Merck, Darmstadt, Germany). Plates were developed with toluene/acetic acid (4/1) as the eluent and visualized under ultra-violet light (254/366 nm). 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 the internal standard or by reference to solvent signals. GC-EIMS spectra were run at 70 eV on a Shimadzu QP-2000 spectrometer.

*Mixture of the flavones **1** and **2*** - IR n $^{KBr}_{max}$ 2940, 1712, 1646, 1590, 1518, 1410 cm^{-1}. GC-EIMS 70 eV *m/z* (rel. int.): **1** [R_t 16.7 min], 372 ([M]$^{\cdot+}$, 29), 357 ([M-CH_3]$^+$; **2** [R_t 19.2 min], 402 ([M]+, 32), 387 ([M-CH_3]$^+$, **1**) d_H 7.88 (*d*, *J* = 8.8 Hz, H-2' and 6'), 7.02 (*d*, *J* = 8.8, H-3' and 5'), 6.63 (*s*, H-3), 4.11 (*s*, CH_3O-7), 4.02 (*s*, CH_3O-8), 3.94 (*s*, CH_3O-6 and 5), 3.89 (*s*, CH_3O-4'); **2**), 7.57 (*dd*, *J* = 1.8; 8.8 Hz, H-6'), 7.42 (*d*, *J* = 1.8, H-2'), 6.99 (*d*, 8.4, H-5'), 6.66 (*s*, H-3), 4.12 (*s*, CH_3O-7), 4.03 (*s*, CH_3O-8), 3.95 (*s*, CH_3O-6 and 5), 3.98 (*s*, CH_3O-3'), 3.96 (*s*, CH_3O-4').^{13}C NMR (100 MHz, $CDCl_3$): **1**) 161.18 (C-2), 177.39 (C-4), 148.30 (C-5), 144.00 (C-6), 151.33 (C-7), 137.94 (C-8), 147.66 (C-9), 114.72 (C-10), 123.72 (C-1'), 162.23 (C-4'), 106.56 (CH-3), 127.99 (CH-2'), 114.44 (CH-3'), 114.44 (CH-5'), 127.99 (CH-6'), 62.02 (CH_3O-5), 62.21 (CH_3O-6), 61.71 (CH_3O-7), 62.04 (CH_3O-8), 55.43 (CH_3O-4'); **2**) 161.04 (C-2), 177.30 (C-4), 148.34 (C-5), 144.00 (C-6), 151.39 (C-7), 137.94 (C-8), 147.66 (C-9), 114.72 (C-10), 123.91 (C-1'), 149.23 (C-3'), 151.89 (C-4'), 106.76 (CH-3), 108.52 (CH-2'), 111.18 (CH-5'), 119.58 (CH-6'), 62.02 (CH_3O-5), 62.21 (CH_3O-6), 61.71 (CH_3O-7), 62.04 (CH_3O-8), 56.14 (CH_3O-3'), 56.03 (CH_3O-4').

6,7-Dimethoxycoumarin (**3**) - IR n 3402, 1712, 1610, 1496 cm-1. EIMS 70 eV m/z (rel. int.): 206 ([M]+, 100), 178 ([M-CO]+, 94), 163 ([M-CH3-CO]+, 62), 135 (35). 1H NMR (200 MHz, CDCl3): dH 7.96 (d, J = 9.6 Hz, H-4), 6.41 (br s), 6.29 (br s), 6.15 (d, J = 9.6 Hz, H-3), 3.89 (s, CH_3O-6), 3.85 (s, CH_3O-7). 13C NMR (50 MHz, CDCl3): dC 164.4 (C-2), 162.2 (C-7), 157.6 (C-6), 157.5 (C-9), 139.4 (C-4), 111.6 (C-3), 104.7 (C-10), 95.5 (C-5), 93.5 (C-8), 56.6 (CH_3O-7), 56.5 (CH_3O-6).

Microorganisms, growth conditions, and preparation of inoculum - Fungi studied were *Colletotrichum* sp., *P. digitatum*, and *Curvularia* sp., isolated from *Citrus* (Johann 2003), and *T. mentagrophytes* and *M. canis* (clinical isolates). They were maintained on potato dextrose agar (PDA) at 4°C before being tested. Experiments were carried out on PDA and nutrient agar. Inoculum was prepared in order to attain a fungal suspension of 5.10^5 spores/ml, in nutrient broth (Espinel Ingroff et al. 1993).

Bacterial species were *E. coli* ATCC 25922, and *S. aureus* ATCC 2593. The bacteria were maintained in Brain Heart Infusion medium at –20°C and tested in Müeller-Hinton broth. Inoculum was a bacterial suspension adjusted to 10^8 c.f.u./ml (Robbins 1976).

All media were purchased from Difco Laboratories.

Bioautography tests - Extracts and isolated compounds from *Citrus* spp. were dissolved in hexane or dimethylsulfoxide (DMSO) (Merck) depending on the polarity of the sample, to provide concentrations of 100 µg/ml; 50 µl were then applied to silica gel TLC plates of $60F_{254}$ (Merck) with graduated micropipettes (Rahalison et al. 1994). The plates were submerged twice in one of the bacterial or fungal suspensions for 5 min, and then transferred to sterile Petri dishes and incubated for 24 h at 37°C for bacteria, and 72 h at 30-35°C for fungi, in a hermetic bell-jar. Inoculated plates were then sprayed with an aqueous solution of *p*-Iodonitrotetrazolium violet (INT, Sigma®) (1 mg/ml) and incubated for a further 4 h at 36 ± 1°C. Inhibition was observed as clear zones against a rose-red colored background. The diameters of the zones were measured. Solvents (hexane and DMSO) were used as negative controls, and tetracycline (Sigma Chemical Co, US) and fluconazol (Sigma) (1.60 µg/ml) were used as positive antibacterial and antifungal controls, respectively. Aliquots of the fungal and bacterial suspensions were grown on culture media to verify that the microorganisms remained viable. All tests were performed in duplicate.

Minimum inhibitory concentration (MIC) - The MIC was determined through a standard two-fold microdilution technique (Souza et al. 2005). Nutrient broth was used for fungi and Muller Hinton broth for bacteria. Susceptibility was determined by the microbroth dilution

method performed in sterile flat-bottom 96-well microplates (Difco Laboratories, Detroit, MI, US). Extracts and fractions were dissolved in DMSO after the addition of appropriate culture media. Serial dilutions were then performed maintaining a constant volume of 1000 µl per tube. The natural products were tested at eight concentrations from 1000 to 7.8 µg/ml. Tetracycline and fluconazol were also included, at an initial concentration of 0.8 mg/ml.

After inoculation with the microorganisms, plates were incubated at 37°C for 24 h for bacteria, and 35°C for 72 h for fungi. The endpoints were determined visually by comparison with drug-free growth in control wells. The MICs, expressed in µg/ml were defined as the lowest extract concentration for which the well was optically clear. Tests were performed in duplicate.

RESULTS AND DISCUSSION

Compounds isolated in the present study were tested for their antimicrobial activity against human pathogenic fungi and bacteria. Besides microorganisms of medical importance, plant pathogenic microorganisms were also tested. They were *P. digitatum*, *Colletotrichum* sp., and *Curvularia* sp. These fungi were isolated from the same lots of *C. sinensis* fruits used for the extraction of the tested compounds.

Results obtained by bioautography tests against fungi and bacteria are given in Table I. In this assay, bacteria were more sensitive to the citrus extracts than were the fungi. Comparing the bioautographic test results (Table I), for each specific substance, with the MIC test results (Table II), shows that the higher inhibition diameter does not always correspond to the minor value obtained with the MIC test. Similar observations have been reported (Yousef & Tawil 1980). It is possible that the presence of different moieties in the basic structures of the substances were present in the solutions and may have interfered with the diffusion rates of the compounds on the silica gel plates.

Comparing the MICs of the wax extracts with the hexane extracts it shows them to have very similar antimicrobial activities, excepting those of *C. limon* whose hexane extract exhibited a stronger activity against *T. mentagrophytes* and *M. canis* than did the wax extract (Table II). As previously mentioned, plant pathogenic fungi tested in this work were isolated from the same lots of fruits that the extracts were obtained from. This fact partially justifies the low activity of the compounds against the plant pathogenic fungi and the lower activity against the human pathogenic fungi.

Antimicrobial activity of flavonoids obtained from plants extracts other than the *Citrus* fruits has been studied by other authors. Leaf extracts from *Helichrysum italicum*, flower extracts from *Nepeta cataria* (Nos-tro et al. 2000) and epicuticular wax extracts from *Arrabidaea brachypoda* (Alcerito et al. 2002) contained flavonoids that exhibited antimicrobial activity against *S. aureus* when submitted to the bioautographic technique.

The wax and crude hexane extracts from *Citrus* spp. peels were initially analyzed by TLC. By comparing their R_f, the presence of different compounds were observed in the wax and crude hexane extracts, due to the presence of different banding patterns. Three lipophylic substances were obtained (Figure). Peels of *C. reticulata* fruits yielded the already described flavones tangeretin and nobiletin (Tripoli et al. 2007, Wang et al. 2007). The compound escoparone was isolated from peels of *C. limon.* No compound was isolated from peels of *C. sinensis.*

Some studies have related the stage of growth to the production of phenolic compounds by fruits. Ortunõ et al. (1999) studied different species of *Citrus* and reported the presence of nobiletin, sinesetin, and tangeretin at the exponential growth phase and quercetogetin and heptamethoxyflavone during the stationary phase. According to these authors, compounds observed at the exponential phase could be the precursors of those observed during the stationary phase. In the present study, nobiletin and tangeretin were detected in peels of fruits at exponential phase. The presence of 6,7-dimethoxycoumarin in citrus fruits after 6 days of storage was observed by Tatum and Berry (1997). These authors suggested that this compound is produced when fruits are either under stress conditions or at their senescent stage.

TABLE I

Antimicrobial activity of hexane (H), wax extracts (W), flavonoids (1+2), and coumarin (3) from *Citrus* spp. by bioautographic technique [a]

	Inhibition zone (mm)							
	C. reticulata			*C. limon*			*C. sinensis*	
Microorganisms	H	W	1+2	H	W	3	H	W
Staphylococcus aureus	18	15	20	18	12	24	18	12
Escherichia coli	20	24	18	18	18	20	18	20
Penicillium digitatum	12	12	15	6	12	18	12	12
Curvularia sp.	18	18	18	18	18	18	10	12
Colletotrichum sp.	6	12	18	12	6	18	12	10
Trichophyton mentagrophytes	15	15	15	10	10	6	12	12
Microsporum canis	12	12	20	12	12	12	12	6

a: extracts and compounds were tested at 100 mg/ml; inhibition zone (mm) represents average of two replications; 1(tangeritin); 2 (nobiletin); 3 (6,7-dimethoxy-coumarin); 24 h at 37°C for bacteria, and 72 h at 30-35°C.

TABLE II

Minimal inhibitory concentration (μg/ml) of hexane extracts (H), wax extracts (W), flavonoids (1+2), and coumarin (3), isolated from *Citrus* spp. against plant pathogenic fungi and human pathogenic fungi and bacteria

	C. reticulata			*C. limon*			*C. sinensis*			
Microorganisms	H	W	1+2	H	W	3	H	W	TR	FLC
Staphylococcus aureus	1000	1000	500	1000	1000	500	500	< 1000	0.5	NT
Escherichia coli	1000	1000	500	1000	500	1000	1000	< 1000	0.5	NT
Penicillium digitatum	< 1000	< 1000	< 1000	500	< 1000	1000	1000	< 1000	NT	1.0
Curvularia sp.	< 1000	< 1000	< 1000	1000	< 1000	500	< 1000	< 1000	NT	2.0
Colletotrichum sp.	< 1000	< 1000	< 1000	1000	< 1000	< 1000	< 1000	< 1000	NT	1.0
T. mentagrophytes	500	500	500	500	1000	250	500	500	NT	1.0
M. canis	500	500	500	250	1000	250	500	500	NT	1.2

1 (tangeritin); + 2 (nobiletin); 3 (6,7-dimethoxy-coumarin); TR: tetracycline; FLC: fluconazole. NT: not tested; 37°C for 24 h for bacteria, and 35°C for 72 h for fungi; *T*: *Trichophyton*; *M*: *Microsporum*.

A correlation between the production of compounds and stress has been noticed during the course of plant infection by microorganisms. The gradual increase of 6,7-dimethoxy-coumarin content in *C. aurantium, C. limon, C. paradise, C. sinensis, Poncirus trifoliata,* and *Troyer citrange* was observed during the course of infection by *Phytophthora citrophtora*, indicating that this process is part of the plant's responses to invading pathogens (Afek et al. 1986).

Our results indicate that the peels of the *Citrus* species present substantial antimicrobial properties. Antifungal activity of some of the extracts and compounds was of such a level that it would probably be therapeutically useful, and it is possible that some of the extracts may be clinically applicable for the treatment of dermatophyte infections caused by *M. canis*.

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