

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 (Afeq 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 (Afeq et al. 1986, Cushnie & 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 (Afeq 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 lipophilic 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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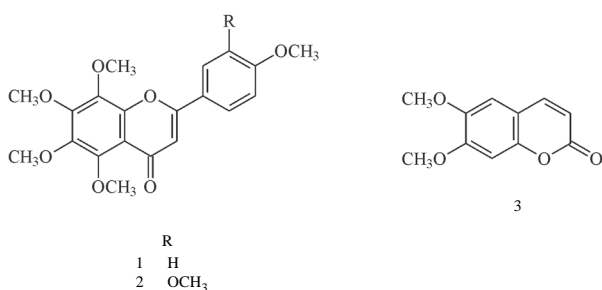
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Extraction and isolation - Fruit peels of each species were removed manually and 750 g *C. sinensis*, 520 g *C. limon*, and 495 g *C. reticulata* were separately macerated in hexane (1000 ml) at room temperature for 40 s. The extracts were then filtered 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 recrystallization, providing a mixture of the flavones (210 mg), tangeritin (1) and nobiletin (2), from *C. reticulata*, and a coumarin derivative, escoparone (120 mg, 3) from *C. limon* (Figure).



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 SiF₂₅₄ 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 ¹H and 100 MHz for ¹³C 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 $\nu_{\text{max}}^{\text{KBr}}$ 2940, 1712, 1646, 1590, 1518, 1410 cm⁻¹. GC-EIMS 70 eV m/z (rel. int.): **1** [R_t 16.7 min], 372 ([M]⁺, 29), 357 ([M-CH₃]⁺); **2** [R_t 19.2 min], 402 ([M]⁺, 32), 387 ([M-CH₃]⁺, **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 , CH₃O-7), 4.02 (s , CH₃O-8), 3.94 (s , CH₃O-6 and 5), 3.89 (s , CH₃O-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 , CH₃O-7), 4.03 (s , CH₃O-8), 3.95 (s , CH₃O-6 and 5), 3.98 (s , CH₃O-3'), 3.96 (s , CH₃O-4'). ¹³C NMR (100 MHz, CDCl₃): **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 (CH₃O-5), 62.21 (CH₃O-6), 61.71 (CH₃O-7), 62.04 (CH₃O-8), 55.43 (CH₃O-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 (CH₃O-5), 62.21 (CH₃O-6), 61.71 (CH₃O-7), 62.04 (CH₃O-8), 56.14 (CH₃O-3'), 56.03 (CH₃O-4').

6,7-Dimethoxycoumarin (3) - IR ν 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): d_{H} 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 , CH₃O-6), 3.85 (s , CH₃O-7). ¹³C NMR (50 MHz, CDCl₃): d_{C} 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 (CH₃O-7), 56.5 (CH₃O-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 5.10⁵ 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 10⁸ 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 60F₂₅₄ (Merck) with graduated micropipettes (Rahaison 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 *Arabidopsis thaliana* (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 lipophilic 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, sinasetin, 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

Microorganisms	Inhibition zone (mm)							
	<i>C. reticulata</i>			<i>C. limon</i>			<i>C. sinensis</i>	
	H	W	1+2	H	W	3	H	W
<i>Staphylococcus aureus</i>	18	15	20	18	12	24	18	12
<i>Escherichia coli</i>	20	24	18	18	18	20	18	20
<i>Penicillium digitatum</i>	12	12	15	6	12	18	12	12
<i>Curvularia</i> sp.	18	18	18	18	18	18	10	12
<i>Colletotrichum</i> sp.	6	12	18	12	6	18	12	10
<i>Trichophyton mentagrophytes</i>	15	15	15	10	10	6	12	12
<i>Microsporium canis</i>	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 (tangeretin); 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 ($\mu\text{g/ml}$) 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

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

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 citrophthora*, indicating that this process is part of the plant's responses to invading pathogens (Afeke 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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REFERENCES

- Afeke U, Szejnberg A, Carmel S 1986. 6,7-dimethoxycoumarin, a *Citrus* phytoalexin conferring resistance against *Phytophthora gummosis*. *Phytochemistry* 25: 1855-1856.
- Alcerito T, Barbo FE, Negri G, Santos DYAC, Meda CL, Young MCM, Chávez D, Blatt CTT 2002. Foliar epicuticular wax of *Arrabidaea brachypoda*: flavonoids and antifungal activity. *Biochem System Ecol* 30: 677-683.
- Arts ICW, Hollman PCH, Feskens EJM, Mesquita HBB, Kromhout D 2001. Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly study. *Am J Clin Nutr* 74: 227-232.
- Brinkworth RI, Stoermer MJ, Fairlie DP 1992. Flavones are inhibitors of HIV-1 proteinase. *Biochem Biophys Res Commun* 30: 631-637.
- Cushinie TTP, Lamb AJ 2005. Antimicrobial activity of flavonoids. *Int J Antimicrob Ag* 26: 343-356.
- Espinel Ingroff A, Dawson K, Pfaller M, Anaissie E, Breslin B, Dixon D, Fothergill A, Paetznick V, Petter J, Rinaldi M, Walsh T 1995. Comparative and collaborative evolution of standardization of antifungal susceptibility for filamentous fungi. *Antimicrob Agents Chemother* 39: 314-319.
- Fang X, Qiu F, Yan B, Wang H, Mort AJ, Stark RE 2001. NMR studies of molecular structure in fruit cuticle polyester. *Phytochemistry* 57: 1035-1042.
- Hamed MA, Hetta MH 2005. Efficacy of *Citrus reticulata* and Mirazid in treatment of *Schistosoma mansoni*. *Mem Inst Oswaldo Cruz* 100: 771-778.
- Iwasse Y, Takemura Y, Ju-ichi M, Yano M, Ito C, Furukawa H, Murukainaka T, Kuchide M, Tokuda H, Ivishino H 2001. Cancer chemopreventive activity of 3,5,6,7,8',3',4'-heptamethoxyflavone from the pulp of *Citrus* plant. *Cancer Lett* 163: 7-9.
- Johann S 2003. *Avaliação da Atividade Antimicrobiana de Flavonóides isolados de Citrus spp.*, MSc Thesis, Departamento de Microbiologia e Parasitologia, UFSC, Florianópolis, 83 pp.
- Johann S, Smania-Jr A, Pizzolatti MG, Schripsema I, Braz-Filho R, Branco A 2007. Complete ¹H and ¹³C NMR assignments and antifungal activity of two 8-hydroxy flavonoids in mixture. *An Acad Bras Cienc* 79: 215-222.
- Mizuno M, Linumo M, Ohara M, Tanoka T, Iwasa M 1991. Chemotaxonomy of the genus *Citrus* based on polymethoxyflavones. *Chem Pharm Bull* 39: 945-949.
- Nostro A, Germano MP, Ângelo V, Marino A, Cannatelli MA 2000. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Let Appl Microbiol* 30: 379-384.
- Ortunõ AM, Arcas MC, Garcia BO, Del Río JA 1999. Evolution of polymethoxy flavones during development of tangelo Nova fruits. *Food Chem* 66: 217-20.
- Rahalison L, Hamburger M, Mono M, Frenk E, Hostettmann K 1994. Antifungal test in phytochemical investigations comparison of bioautographic methods using phytopathogenic and human pathogenic fungi. *Planta Med* 60: 41-44.
- Robards K, Li X, Antalovich M, Boyd S 1997. Characterization of *Citrus* by chromatographic analysis of flavonoids. *J Sci Food Agric* 75: 87-101.
- Robbins RC 1976. Regulatory action of phenylbenzo-gamma-pyrone (BPB) derivatives on blood-constituents affecting rheology in patients with coronary heart-disease (CHD). *Int J Vitam Nutr Res* 46: 338-347.

- Rossetti V, Muller GW, Costa AS 1993. Doenças dos citros causadas por algas, fungos, bactérias e vírus, Fundação Cargill, 84 pp.
- Souza SM, Delle Monache F, Smania-Jr A 2005. Antibacterial activity of coumarins. *Z Naturforsch C Biosci* 60: 693-700.
- Tatum JH, Berry RE 1997. 6,7-dimethoxycoumarin in the peel of *Citrus*. *Phytochemistry* 16: 1091-1092.
- Tripoli E, Guardia MA, Giammanco S, Majo DD, Giammanco M 2007. *Citrus* flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chem* 104: 466-479.
- Young J, Nielsen SE, Haraldsdóttir J, Daneshvar B, Lauridsen ST, Knuthsen P, Crozier A, Sandströen B, Dragsted LO 1999. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. *Am J Clin Nutr* 69: 87-94.
- Yousef RT, Tawil GG 1980. Antimicrobial activity of volatile oils. *Pharmazie* 35: 698-701.
- Wang YC, Chuang YC, Hsu HW 2007. The flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. *Food Chem*, in press.
- Wedge DE, Nagle DG 2000. A new 2D-TLC bioautography method for the discovery of novel antifungal agents to control plant pathogens. *J Nat Prod* 63: 1050-1054.

