Antifungal Activity of Coumarins

Cristina Montagner[a], Simone M. de Souza[a], Cláudia Groposo[a], Franco Delle Monache[b], Elza F. A. Smânia[a], and Artur Smânia Jr.[a,*]

[a] Departamento de Microbiologia e Parasitologia, Centro de Ciências Biológicas da Universidade Federal de Santa Catarina, Campus Universitário, Trindade, Florianópolis-SC 88040-900, Brazil. E-mail: smania@mbox1.ufsc.br

[b] Centro Chimica dei Recettori (CNR), Università Cattolica del Sacro Cuore, Largo Francesco Vito, 1, Roma 00168, Italy

* Author for correspondence and reprint requests

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The antifungal activity of 40 coumarins was tested against the fungal strains: *Candida albicans* (ATCC 14053), *Aspergillus fumigatus* (ATCC 16913) and *Fusarium solani* (ATCC 36031), using the broth microdilution method. Osthenol showed the most effective antifungal activity among all the compounds tested, with a MIC value of 125 μg/ml for *Fusarium solani* and 250 μg/ml for *Candida albicans* and *Aspergillus fumigatus*. The antifungal potential of this prenylated coumarin can be related to the presence of an alkyl group at C-8 position.

Key words: Coumarins, Antifungal Activity, Osthenol

Introduction

Coumarin (a derivative name from the plant *Coumarouna odorata*) is a member of a class of compounds called benzopyrones which consist of fused benzene and α-pyrone rings. Nearly one thousand coumarins have been described (Bruneton, 1995) and they are widely distributed in the vegetable kingdom, particularly in angiosperms. Most of these are secondary metabolites of green plants but some are produced in fungi and bacteria (Murray, 1978).

In 1822, Vogel isolated and purified coumarin from the tonka bean (*Dipteryx odorata*) (Bruneton, 1995). Coumarin is used in the cosmetic, perfumery and household products industry because of its pleasant bitter-sweet odour (Egan *et al*., 1990). In 1954, the Food and Drugs Administration, USA (FDA) banned its use in food because of reports on coumarin producing hepatotoxicity in rats. Although hepatotoxic in rats, coumarin is not hepatotoxic in other species, including mice, hamsters and gerbils, because coumarin-induced rat liver toxicity is metabolism-dependent (Lake and Grasso, 1996).

In view of the established low toxicity, relative cheapness, and presence in the diet, coumarins and their derivatives have been found to exhibit a wide range of biological and pharmacological activities (Hoult and Payá, 1996). Warfarin is a particular well-known coumarin which is used as an oral anticoagulant (Pineo and Hull, 2003), but the coumarins also have long been recognized to possess antioxidant (Tyagy *et al*., 2005), anti-inflammatory (Kontogiorgis and Hadjipavlou-Litina, 2003), antifilarial (Tripathy *et al*., 2000), antiulcerogenic (Bighetti *et al*., 2005), trypanocidal (Alvim *et al*., 2005), antibacterial (Kayser and Kolodziej, 1999), antitumour (Kempen *et al*., 2003) and anti-HIV activities (Uchiumi *et al*., 2003).

In the present study the antifungal activity of a series of naturally occurring and synthetic coumarins was evaluated against three fungal species: *Candida albicans*, *Aspergillus fumigatus* and *Fusarium solani* using the broth microdilution method.

Materials and Methods

Test compounds

Thirty five natural coumarins, three of a commercial source and two obtained by simple modification were assayed in the present work. The identity of natural and semi-synthetic compounds was proved by comparison of their spectroscopic data (^{1}H and ^{13}C NMR) with literature data (Table I).

Antifungal susceptibility testing

The antifungal activity of coumarins was investigated by estimating the minimal inhibitory concentration (MIC) using broth microdilution tech-

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Table I. Chemical structure of coumarins studied in this work.

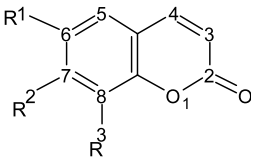
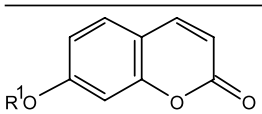
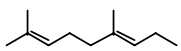
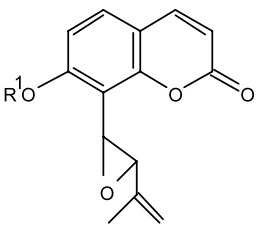
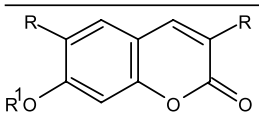
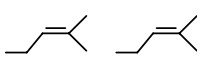
I. Simple coumarins	R^1	R^2	R^3	Source
				
Monosubstituted				
Coumarin (**1**)	H	H	H	Gottlieb *et al.*, 1979
6-Methylcoumarin (**2**)	CH_3	H	H	Gottlieb *et al.*, 1979
6-Hydroxycoumarin (**3**)	OH	H	H	Gottlieb *et al.*, 1979
6-*O*-Acetylcoumarin (**4**)	$O-C_2H_3O$	H	H	Gottlieb *et al.*, 1979
6-Methoxycoumarin (**5**)	$O-CH_3$	H	H	Gottlieb *et al.*, 1979
6-Chlorocoumarin (**6**)	Cl	H	H	Gottlieb *et al.*, 1979
6-Iodocoumarin (**7**)	I	H	H	Gottlieb *et al.*, 1979
6-Aminocoumarin (**8**)	NH_2	H	H	Gottlieb *et al.*, 1979
6-Carboxycoumarin (**9**)	COOH	H	H	Gottlieb *et al.*, 1979
6-Cyanocoumarin (**10**)	CN	H	H	Gottlieb *et al.*, 1979
6-Aldehydocoumarin (**11**)	CHO	H	H	Gottlieb *et al.*, 1979
6-Nitrocoumarin (**12**)	NO_2	H	H	Gottlieb *et al.*, 1979
7-Methoxycoumarin (herniarin) (**13**)	H	$O-CH_3$	H	Sarsynthese, Genay, France
7-Methylcoumarin (**14**)	H	CH_3	H	Gottlieb *et al.*, 1979
7-*O*-Acetylcoumarin (**15**)	H	$O-C_2H_3O$	H	Gottlieb *et al.*, 1979
7-Chlorocoumarin (**16**)	H	Cl	H	Gottlieb *et al.*, 1979
7-Nitrocoumarin (**17**)	H	NO_2	H	Gottlieb *et al.*, 1979
Disubstituted				
6-Methoxy-7-hydroxycoumarin (scopoletin) (**18**)	$O-CH_3$	OH	H	Torres *et al.*, 1979
6,7-Di-hydroxycoumarin (esculetin) (**19**)	OH	OH	H	Fluka, Buchs, Switzerland
Di-*O*-methyl-esculetin (**20**)	$O-CH_3$	$O-CH_3$	H	Esculetin methylation
Di-*O*-methyl-daphnetin (**21**)	H	$O-CH_3$	$O-CH_3$	Daphnetin methylation
Trisubtituted				
Fraxetin (**22**)	$O-CH_3$	OH	OH	Sarsynthese, Genay, France
II. Prenylated coumarins				
				
Auraptene (**23**)				Delle Monache *et al.*, 1995
				
Phebalosin (**24**)	CH_3			Cuca-Suarez and Delle Monache, 1991
				
Balsamiferone (**25**)	H			Cuca-Suarez, personal communication

Table I (continued).

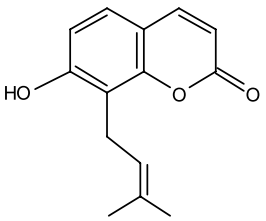
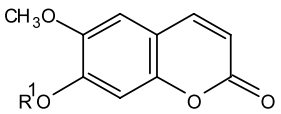
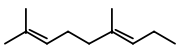
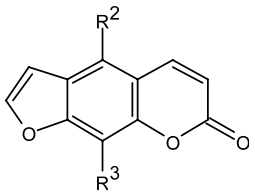
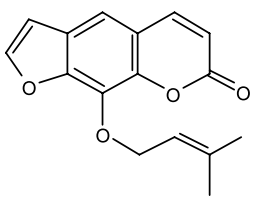
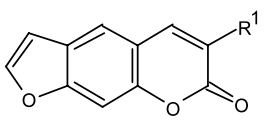
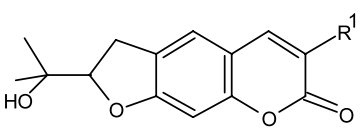
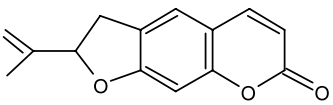
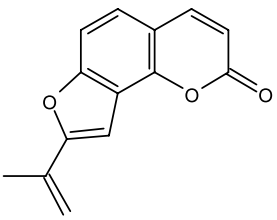
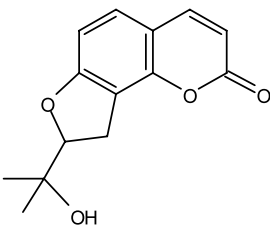
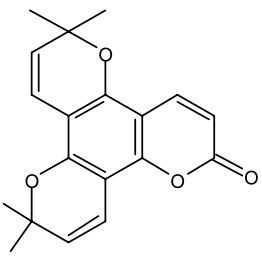
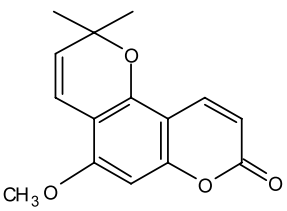
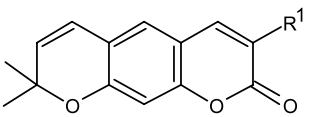
	R^1	R^2	R^3	Source
				
Osthenol (**26**)				Cuca-Suarez *et al.*, 1998
				
7-*O*-Geranyl-esculetin (**27**)				Torres *et al.*, 1979
III. Furanocoumarins				
				
Bergaptene (**28**)		$O-CH_3$	H	Compagnone *et al.*, 1993
Xanthotoxin (**29**)		H	$O-CH_3$	Compagnone *et al.*, 1993
Isopimpinellin (**30**)		$O-CH_3$	$O-CH_3$	Trani *et al.*, 2004
				
Imperatorin (**31**)				Trani *et al.*, 1997
				
Dimethyl allyl psoralene (**32**)				Delle Monache *et al.*, 1976
				
Marmesin (**33**)	H			Delle Monache *et al.*, 1989

Table I (continued).

	R^1	R^2	R^3	Source
 Isoangenomalin (**34**)				Delle Monache *et al.*, 1977
 Oroselone (**35**)				Murray, 1978
 Columbianetin (**36**)				Cuca-Suarez *et al.*, 1998
IV. Pyranocoumarins				
 Hortiline (**37**)				Delle Monache *et al.*, 1976
 Alloxanthoxyletin (**38**)				Cuca-Suarez *et al.*, 2002
 Xanthyletin (**39**)	H			Delle Monache *et al.*, 1976
Dimethyl allyl xanthyletin (**40**)				Delle Monache *et al.*, 1976

niques as described by the National Committee for Clinical Laboratory Standards for yeasts (NCCLS, 2002a) as well as for filamentous fungi (NCCLS, 2002b) in microtiters of 96 wells (Kartell, Milano, Italy).

The assay was carried out with three fungal strains, *Candida albicans* (ATCC 14053), *Aspergillus fumigatus* (ATCC 16913) and *Fusarium solani* (ATCC 36031) provided by the National Institute of Quality Control in Health of the Fundação Oswaldo Cruz (Rio de Janeiro, Brazil).

To induce conidium and sporangiospore formation the fungi were grown on potato dextrose agar (Oxoid, Hampshire, UK). MIC values were determined in RPMI-1640 medium, with glutamine and without sodium bicarbonate (Cultilab, São Paulo, Brazil), buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS) (Fisher Scientific, Pittsburgh, PA, USA). The starting inoculum for filamentous fungi corresponded to approx. $0.4 \cdot 10^4$ to $5 \cdot 10^5$ CFU/ml, and for the yeast to $0.5 \cdot 10^3$ to $2.5 \cdot 10^3$ CFU/ml. The coumarins were dissolved in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and diluted with RPMI-1640 medium to a concentration of 2000 μg/ml. Further 1:2 serial dilutions were performed by addition of RPMI-1640 medium, and 100 μl of each dilution were distributed in 96-well plates; each test and growth control well was inoculated with 100 μl of inoculum suspension to reach a final concentration range of 1000 μg/ml to 15.6 μg/ml. The growth control contained RPMI-1640 medium, DMSO without test substance; the sterility control contained RPMI-1640 medium, the test substance without fungal inoculum. The standard drugs amphotericin B and fluconazole were used as positive controls. Microtiter trays were incubated at 35 °C for 46–50 h for filamentous fungi and 24 h for the yeast *Candida albicans*.

All experiments were performed in duplicate. The MIC was defined as the lowest concentration of the coumarin which resulted in total inhibition of the fungal growth, detected visually. The results are expressed in μg/ml and mM.

Results and Discussion

Coumarins display wide variations in the benzopyrone nucleus. Therefore, their structural modifications were evaluated for antifungal activity in order to obtain insight into structure-activity relationships. Fungal susceptibility to coumarins was evaluated by determining the minimal inhibitory concentration (MIC). In Table II the antifungal activity of different coumarin derivatives is shown.

Coumarin *per se* (**1**) was low active against *Candida albicans, Aspergillus fumigatus* and *Fusarium solani*. Sardari *et al*. (1999) also observed similar results against *Candida albicans*. The addition of a methyl group at position C-6 to the aromatic nucleus of the coumarin core structure (compound **2**) maintained the same antifungal activity against fungal species, but a methyl group at position C-7 (compound **14**) resulted in diminished antifungal activity against *Candida albicans*.

6-Hydroxycoumarin (**3**) showed MIC values higher than 500 μg/ml against *A. fumigatus*. A similar inexpressive antifungal effect for 6-hydroxycoumarin was observed by Jurd *et al*. (1971) against *Candida tropicalis*, *Saccharomyces cerevisiae*, *Aspergillus flavus*, *Aspergillus niger* and *Alternaria* spp.

Varied substitution patterns as 6- and 7-*O*-acetyl groups (compounds **4** and **15**), 6- and 7-*O*-methyl groups (compounds **5** and **13**), a 6-amino group (compound **8**), a 6-carboxy function (compound **9**), and a 6-cyano group (compound **10**) resulted in low activity against *Candida albicans, Aspergillus fumigatus* and *Fusarium solani*. Also the addition of halogen groups at positions C-6 and C-7 [6-chlorocoumarin (**6**), 7-chlorocoumarin (**16**) and 6-iodocoumarin (**7**)] did not show significant antifungal activity.

Among the monosubstituted coumarins, 6-nitrocoumarin (**12**) presented the best antifungal activity, but only against *F. solani*, the MIC was 125 μg/ml (0.65 mM).

The antifungal activity of the monosubstituted coumarins studied did not depend on the substitution pattern in the coumarin nucleus, not even on the characteristics of the substituting groups. So, if some structure-activity relationship might be discussed, the hypothesis about a possible species-specific activity cannot be discarded (Godoy *et al*., 2005).

The di- and trisubstituted coumarins (**18**–**22**) did not show relevant antifungal activity. According to Jurd *et al*. (1971) 6,7-di-hydroxycoumarin (esculetin, **19**) did not show antifungal activity.

Within the group of prenylated coumarins, osthenol (**26**) showed the most prominent activity against fungal species, with a MIC of 125 μg/ml (0.54 mM) for *F. solani* and 250 μg/ml (1.08 mM) for *C. albicans* and *A. fumigatus*. Earlier studies, in

Table II. Antifungal activity, expressed as MIC [μg/ml (mM)], of coumarins against three fungal strains.

Coumarin	*Candida albicans*	*Aspergillus fumigatus*	*Fusarium solani*
Monosubstituted coumarins			
Coumarin (**1**)	500 (3.42)	1000 (6.84)	500 (3.42)
6-Methylcoumarin (**2**)	500 (3.12)	1000 (6.24)	500 (3.12)
6-Hydroxycoumarin (**3**)	500 (3.08)	>1000 (6.16)	500 (3.08)
6-*O*-Acetylcoumarin (**4**)	500 (2.44)	500 (2.44)	1000 (4.89)
6-Methoxycoumarin (**5**)	500 (2.83)	>1000 (5.67)	500 (2.83)
6-Chlorocoumarin (**6**)	500 (2.76)	1000 (5.53)	500 (2.76)
6-Iodocoumarin (**7**)	500 (1.83)	1000 (3.67)	500 (1.83)
6-Aminocoumarin (**8**)	500 (3.10)	1000 (6.20)	1000 (6.20)
6-Carboxycoumarin (**9**)	1000 (5.25)	> 1000 (5.25)	1000 (5.25)
6-Cyanocoumarin (**10**)	500 (2.92)	1000 (5.84)	250 (1.46)
6-Aldehydocoumarin (**11**)	500 (2.87)	1000 (5.74)	250 (1.43)
6-Nitrocoumarin (**12**)	500 (2.61)	500 (2.61)	125 (0.65)
7-Methoxycoumarin (**13**)	500 (2.83)	1000 (5.67)	1000 (5.67)
7-Methylcoumarin (**14**)	1000 (6.24)	1000 (6.24)	500 (3.12)
7-*O*-Acetylcoumarin (**15**)	250 (1.22)	500 (2.44)	250 (1.22)
7-Chlorocoumarin (**16**)	500 (2.76)	1000 (5.53)	500 (2.76)
7-Nitrocoumarin (**17**)	250 (1.30)	250 (1.30)	250 (1.30)
Disubstituted coumarins			
Scopoletin (**18**)	500 (2.60)	N.T.[a]	N.T.
Esculetin (**19**)	500 (2.80)	1000 (5.60)	1000 (5.60)
Di-*O*-methyl-esculetin (**20**)	1000 (4.84)	1000 (4.84)	1000 (4.84)
Di-*O*-methyl-daphnetin (**21**)	500 (2.42)	1000 (4.84)	1000 (4.84)
Trisubstituted coumarins			
Fraxetin (**22**)	500 (2.40)	1000 (4.80)	1000 (4.80)
Prenylated coumarins			
Auraptene (**23**)	1000 (3.35)	>1000 (3.35)	1000 (3.35)
Phebalosin (**24**)	500 (1.93)	1000 (3.87)	>1000 (3.87)
Balsamiferone (**25**)	500 (1.67)	1000 (3.35)	>1000 (3.35)
Osthenol (**26**)	250 (1.08)	250 (1.08)	125 (0.54)
7-*O*-Geranyl-esculetin (**27**)	500 (1.52)	1000 (3.04)	1000 (3.04)
Furanocoumarins			
Bergaptene (**28**)	250 (1.24)	1000 (4.99)	N.T.
Xanthotoxin (**29**)	>1000 (4.99)	1000 (4.99)	1000 (4.99)
Isopimpinellin (**30**)	500 (2.17)	>1000 (4.34)	1000 (4.34)
Imperatorin (**31**)	1000 (3.70)	1000 (3.70)	1000 (3.70)
Dimethyl allyl psoralene (**32**)	500 (1.96)	>1000 (3.93)	1000 (3.93)
Marmesin (**33**)	500 (2.03)	1000 (4.06)	1000 (4.06)
Isoangenomalin (**34**)	500 (2.19)	1000 (4.38)	500 (2.19)
Oroselone (**35**)	500 (2.21)	1000 (4.42)	N.T.
Columbianetin (**36**)	1000 (4.06)	1000 (4.06)	1000 (4.06)
Pyranocoumarins			
Hortiline (**37**)	500 (1.61)	>1000 (3.22)	>1000 (3.22)
Alloxanthoxyletin (**38**)	500 (1.93)	1000 (3.87)	1000 (3.87)
Xanthyletin (**39**)	500 (2.19)	1000 (4.38)	1000 (4.38)
Dimethyl allyl xanthyletin (**40**)	1000 (3.37)	1000 (3.37)	1000 (3.37)
Fluconazole	0.24 (0.00078)		
Amphotericin B		0.48 (0.00051)	0.90 (0.00097)

[a] N.T., not tested.
For all compounds, deviation from the mean $d = \pm 0\,\mu$g/ml.

our laboratory, related the antibacterial activity of osthenol to Gram-positive bacteria like *Staphylococcus aureus* and *Bacillus cereus* (Souza *et al.*, 2005).

Osthenol is a compound with prenylation at C-8, and this could be related to the relative lipophilicity of osthenol, which favours its permeation more efficiently through the lipid layer of the

fungi (Rehman *et al.*, 2005). In the same way the free hydroxy group at position C-7 suggests that those groups are required for good antifungal activity (Sardari *et al.*, 1999).

However, the coumarin balsamiferone (**25**) also has a free hydroxy group at position C-7, but did not show good antifungal activity. It is possible that the two prenyl chains at C-3/C-6 reduced the antifungal activity like the presence of OMe at C-7 and an α,β-epoxidation of the C-8 prenyl group (phebalosin, **24**) and the prenyl chain at C-7 of the coumarin 7-*O*-geranyl-esculetin (**27**). Hence, in prenylated coumarins, the pattern of substitution and the characteristics of the substituting groups are important for their antifungal activity.

The furanocoumarins **28**–**36** were ineffective against *A. fumigatus* and *F. solani*. Only the coumarin bergaptene (**28**) showed a MIC value of 250 μg/ml (1.24 mM) against *C. albicans*. Nevertheless, Ojala *et al.* (2000) did not find antifungal activity of bergaptene against *C. albicans*.

In the group of the pyranocoumarins **37**–**40** no relevant results were observed, suggesting that the pyrano ring is not required for antifungal activity. The antifungal activity of pyranocoumarins, although weak, was more intense against *C. albicans* (MIC = 500 μg/ml) than against the filamentous fungi (MIC = 1000 μg/ml).

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