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In vitro Antimycotic Activity of Some Plant Extracts Towards Yeast and Yeast-like Strains

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As part of screening aimed at the selection of novel antimycotic compounds of vegetable origin, leaf extracts of *Camellia sinensis* L., *Cupressus sempervirens* L. and *Pistacia lentiscus* L. and the seed extract of *Glycine soja* Sieb. *et* Zucc. were tested against yeast and yeast-like species implicated in human mycoses. Of the extracts only those of *C. sinensis* (obtained from a commercial preparation of green tea) exhibited broad activity towards *Candida glabrata*, *Clavispora lusitatiae*, *Cryptococcus laurentii*, *Filobasidiella neoformans*, *Issatchenkia orientalis*, *Saccharomyces cerevisiae* and *Prototheca wickerhamii* strains. MICs ranging from 300 to 4800 μg extract/mL (corresponding to 130–2010 μg/mL total polyphenols) were observed. Concentrations of the *C. sinensis* extract over 25 000 μg/mL caused a rapid decrease of viable cells of *Fil. neoformans* and its activity was dose-dependent. Tests carried out using the pure polyphenols present in *C. sinensis* extract composition, showed that only epicatechin-3-O-gallate (ECG) and epigallocatechin-3-O-gallate (EGCG) possess antimycotic activity. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: antimycotic activity; *Camellia sinensis* L.; yeast and yeast-like strains; ECG; EGCG.

INTRODUCTION

The antimicrobial properties of drugs from medicinal and other edible plants have been recognized since antiquity (Cowan, 1999). Notwithstanding the wide literature concerning the beneficial effects of plant polyphenols in human health (Cowan, 1999; Krauze-Baranowska *et al.*, 1999; Lin *et al.*, 1999; Cassidy *et al.*, 2000; Wang, 2000), to our knowledge little information is available on their antimycotic properties towards yeast and yeast-like microorganisms, which are implicated in many human pathologies (Hazen, 1995; Rex *et al.*, 1995; Warren and Hazen, 1998). In view of the increased number of fungal infections, especially in immunodepressed subjects (Kalowky *et al.*, 1997), and of the development of resistance towards antifungal agents (Hazen, 1995; Rex *et al.*, 1995; Warren and Hazen, 1998; Sanglard and Odds, 2002), there is a considerable scientific and commercial interest in the discovery of novel classes of antimycotic compounds.

Plants represent a good source of novel antimicrobial molecules (Rusia and Srivastava, 1988; Ayoub, 1989; Mahajan *et al.*, 1991; Etkin, 1996; Arora and Ohlan, 1997; Chapman *et al.*, 1997; Johns, 1999; Pieroni, 2000; Buzzini and Pieroni, 2003). Therefore the antimycotic activity was investigated of an extract of *Camellia sinensis* L. leaves and *Glycine soja* Sieb. *et* Zucc seeds, plants of special importance in the oriental diet which is very rich in polyphenols. In particular, the former is rich in galloyl-catechins (Riemersma *et al.*, 2001) while the latter is very rich in isoflavones such as genistein and daidzein glycosides (Romani *et al.*, 2003). In order to investigate whether other polyphenol classes may have an antimycotic activity another edible plant of the Mediterranean region was selected, *Pistacia lentiscus*, which contains galloyl-quinic esters and myricetin glycosides as the main compounds (Romani *et al.*, 2002b). Moreover, *Cupressus sempervirens* extract has been investigated because it is rich in biflavonoids, quercetin and kaempferol glycosides (Romani *et al.*, 2002a). The present paper compared the activity of different plant extracts in order to identify what class of polyphenols is able to express antimycotic activity towards yeast and yeast-like microorganisms implicated in many human pathologies (Hazen, 1995; Rex *et al.*, 1995; Warren and Hazen, 1998).

MATERIALS AND METHODS

Plant material. The leaves of the mastic tree and cypress tree were collected from fully developed plants growing in Tuscany (Italy) at the end of June and September (mastic tree) and October (cypress tree) 2002. In both cases the leaves were processed as previously described (Romani *et al.*, 2002a; Romani *et al.*, 2002b). Soy seeds, collected from plants grown under natural conditions, were ground and extracted as previously reported (Romani *et al.*, 2003). Green tea extract (from *Camellia sinensis* leaves) was obtained from Indena S.p.A., Milan, Italy. The extracts were

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stored at –20 °C until use and were stable for at least 12 months.

HPLC/DAD and HPLC/MS analysis. The analysis was carried out using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent-Technologies, Palo Alto, USA) operating in positive and negative ionization mode. Analytical conditions were described previously (Romani *et al.*, 2003). Identification of individual polyphenols was carried out on the basis of their retention times, spectroscopic and spectrometric data. Quantification of individual polyphenols was directly performed by HPLC-DAD by using four-point regression curves built with the available standards or isolated compounds. The following standards were used for the identification and characterization of plant extracts: phenolic acid (ellagic acid, gallic acid, caffeic acid, chlorogenic acid and cynarin); secoiridoid (oleuropein); flavones (apigenin, cosmetin, homoorientin, vitexin, luteolin, luteolin-4′-glucoside); flavonols (quercetin, quercetagetin, quercetin-3-arabinoside, quercitrin, isoquercitrin, fisetin, kaempferol, kaempferol-7-neohesperidoside, myricetin, myricitrin, rhamnetin, isorhamnetin and robinin); flavanones (eriodictyol, hesperidin, naringenin, naringenin-7-glucoside and pinocembrin-7-methylther); isoflavones (daidzein and genistein); chalcones (phloridzin) and catechins, epicatechin-3-O-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-O-gallate (EGCG). Calibration curves with $r^2 \geq 0.9998$ were considered. The quantification was performed at the maximum wavelength of UV-Vis absorbance by applying the correction for molecular weight.

In particular, in *G. soja* seed extract genistein and glycitein derivatives were determined at 260 nm using genistein as a reference compound, while daidzein derivatives were determined at 305 nm with daidzein as a reference; in *P. lentiscus* leaf extract the galloyl-glycosides and galloyl-quinic acid amounts were calculated at 280 nm using gallic acid as a reference, while myricetin and quercetin glycosides were calibrated at 350 nm with myricitrin and rutin, respectively, as a reference; in *C. sempervirens* leaf extract the flavonol content was determined at 350 nm employing the authentic standard quercitrin and kaempferol, in the same way biflavonoids and methylbiflavonoids were quantified with the authentic standard hinokiflavone and amentoflavone; finally, the commercial extract of green tea was calibrated at 280 nm using the authentic standards of catechin and gallo-catechins, while its flavonol content was calculated at 350 nm using rutin as a reference compound.

Microorganisms. Twenty-four yeast (belonging to 13 species of nine genera) and three yeast-like (*Prototheca* spp.) strains of different origin (conserved in the DBVPG Collection of Industrial Yeasts at the Dipartimento di Biologia Vegetale e Biotecnologie Agroambientali, University of Perugia, Italy, www.agr.unipg.it/dbvpg) (Buzzini and Martini, 2000) were used as target microorganisms (Table 1). Several of these, although belonging to species commonly regarded as species of agro-industrial interest, have gained the status of emerging pathogens in recent years because they are implicated in opportunistic infections (Hazen, 1995; Rex *et al.*, 1995; Warren and Hazen, 1998). Working cultures were maintained on YEPG (g/L: yeast

Table 1. Salient information on yeasts and yeast-like microorganisms used in the present study

Species	Strain	Status	Source of isolation	Locality of isolation
Candida albicans	DBVPG 6133	T	Skin mycosis	Uruguay
Candida albicans	DBVPG 6157	NT of *Candida stellatoidea*	Unknown	Unknown
Candida glabrata	DBVPG 7212		Unknown	Unknown
Candida glabrata	DBVPG 3828	T	Faeces	Unknown
Pichia guilliermondii	DBVPG 6140	T of *Candida guilliermondii*	Sputum	Unknown
Candida parapsilosis	DBVPG 6150	T	Ex case of sprue	Puerto Rico
Candida tropicalis	DBVPG 3982	T	Bronchitic patient	Unknown
Candida zeylanoides	DBVPG 6163	NT	Blastomycotic macroglossia	Unknown
Clavispora lusitaniae	DBVPG 6142	T of *Candida lusitaniae*	Caecum of pig	Portugal
Clavispora lusitaniae	DBVPG 6148		Sputum	Norway
Issatchenkia orientalis	DBVPG 6782		Fruit juice	Unknown
Kluyveromyces marxianus	DBVPG 6141	T of *Candida kefir*	Kefyr grain	The Netherlands
Saccharomyces cerevisiae	DBVPG 6173	T	Brewer's top yeast	The Netherlands
Saccharomyces cerevisiae	DBVPG 6497		Unknown	Unknown
Saccharomyces cerevisiae	DBVPG 6500		Unknown	Unknown
Yarrowia lipolitica	DBVPG 6053	T	Maize-processing plant	USA
Cryptococcus laurentii	DBVPG 3883		Phlegm of a tuberculosis patient	Unknown
Cryptococcus laurentii	DBVPG 4272		Human oral cavity	Italy
Cryptococcus laurentii	DBVPG 6265	T	Palm wine	Congo
Filobasidiella neoformans	DBVPG 3428		Unknown	Unknown
Filobasidiella neoformans	DBVPG 6010	T of *Cryptococcus neoformans*	Fermenting fruit juice	Unknown
Filobasidiella neoformans	DBVPG 6225		Spinal fluid	USA
Filobasidiella neoformans	DBVPG 6981		Cerebrospinal fluid	USA
Filobasidiella neoformans	DBVPG 6982		Droppings of cuckoo	Thailand
Prototheca wickerhamii	DBVPG 8879		Unknown	Unknown
Prototheca zopfii	DBVPG 8880		Unknown	Unknown
Prototheca zopfii	DBVPG 8830		Milk from a mastiytic cow	Italy

T, Type strain; NT, Neotype.

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extract 10, peptone 10, glucose 20, agar 15) or potato dextrose agar (PDA) (Atlas and Parks, 1993) slants at 4 °C until use.

Antimycotic activity test. Antimycotic activity of the crude extracts was evaluated by using the agar diffusion well bioassay (ADWB) (Buzzini and Martini, 2001) with YEPG or yeast nitrogen base (YNB) agar (Difco, Detroit, Michigan, USA) + 2% glucose as substrates.

One mL of 24 h cell suspensions (10^5 cells/mL corresponding to an A_{580} = 0.1 – 0.2) of strains were mixed thoroughly with 19 mL molten medium (45 °C) and poured into a Petri dish. After the agar had solidified, six wells (diameter 8 mm) were cut equidistant from the edge of the inoculated dish. An aliquot (100 μL) of extract or standard stock solution was placed in each well. Dishes were checked for the presence of inhibition zones (halos) every 12 h. In this phase of the work, antimicrobial activity was assessed by measuring the diameter of the clear area of inhibition.

Ketoconazole (as control antibiotic) (Calbiochem Inc., USA) and standard compounds, as listed above, were also tested for their antimycotic activity by ADWB. All tests were performed in triplicate.

Determination of minimal inhibitory concentration (MIC). MIC determination was carried out in 96-well microplates (Corning Inc., USA) by a broth microdilution method in agreement with the NCCLS recommendations (NCCLS, 1997). The extracts were diluted in YNB broth + 2% glucose in order to obtain concentrations ranging from 5000 to 100 μg/mL. Stock solutions (100 μg/mL) of ketoconazole (as control antibiotic) in 10% dimethyl sulphoxide (DMSO) (Carlo Erba, Italy) was prepared before use and concentrations ranging from 100 to 0.1 μg/mL were used.

Assessment of fungicidal activity of *C. sinensis* extract. Cells of *Fil. neoformans* DBVPG 6010, grown for 24 h at 25 °C on YEPG agar slants, were suspended in sterile distilled water (10^6 cells/mL). A quantity of 50 μL of cell suspension was used as the inoculum of 450 μL of YNB broth + 2% glucose. Aliquots of extract were added to obtain increasing concentrations: 1250, 2500, 6250, 12 500, 25 000 and 50 000 μg/mL of extract. Over a period of 32 h, aliquots of cultures were collected at 4 h intervals and viable cells were plated on YEPG agar dishes. Colonies were counted after 48–72 h. A control test (extract free) was also included.

RESULTS AND DISCUSSION

C. sinensis extract was particularly rich in galloyl-derivatives of catechins with epigallocatechin-3-O-gallate, epigallocatechin and epicatechin being the lead compounds (Table 2). Indeed in *P. lentiscus* extract, the most prominent compounds were galloyl-derivatives (galloyl quinic acid, galloyl glycosides), gallic acid and flavonol glycosides (Table 2). *G. soja* extracts were very rich in isoflavones such as daidzein and genistein (Table 2), whereas the flavonoids and biflavonoids were the major compounds in the leaf extract of *C. sempervirens* (Table 2). The polyphenol composition of extracts was in line with previously reported data (Romani *et al.*, 2002a; 2002b; 2003).

The extracts from the cypress tree, mastic tree and soybean did not show any antimycotic activity against yeast and yeast-like strains (data not shown). Thus, galloyl quinic acid, galloyl glycosides, gallic acid, myricetin glycosides present in *P. lentiscus*, isoflavones of *G. soja* and the flavonoids and biflavonoids of *C. sempervirens* had no antimycotic activity.

Table 2. Quantitative analysis of polyphenols (expressed as μg/mL) present in the extracts from leaves of *C. sempervirens*, *C. sinensis* and *P. lentiscus*, and seeds of *G. soja*

	Extract composition (μg/mL)			
Compound	*C. sempervirens*	*C. sinensis*	*G. soja*	*P. lentiscus*
Genistein	n.d.	n.d.	5.3	n.d.
Genistein-glycosides	n.d.	n.d.	303	n.d.
Daidzein	n.d.	n.d.	13	n.d.
6 OH-Daidzein	n.d.	n.d.	2.7	n.d.
Daidzein glycosides	n.d.	n.d.	490	n.d.
Glycithin glycosides	n.d.	n.d.	28	n.d.
Caffeic acid derivatives	n.d.	n.d.	16	n.d.
Gallic acid	n.d.	0.9	n.d.	25
Galloyl glycosides	n.d.	n.d.	n.d.	19
Galloyl quinic acid	n.d.	n.d.	n.d.	310
Myricetin glycosides	n.d.	n.d.	n.d.	102
Quercetin glycosides	174	n.d.	n.d.	25
Kaemferol glycosides	16	n.d.	n.d.	n.d.
Flavonols (as equivalent rutin)	n.d.	35	n.d.	n.d.
Biflavonoids	1460	n.d.	n.d.	n.d.
Methylbiflavonoids	4	n.d.	n.d.	n.d.
Epigallocatechin-3-O-gallate (EGCG)	n.d.	211	n.d.	n.d.
Epigallocatechin (EGC)	n.d.	105	n.d.	n.d.
Epicatechin (EC)	n.d.	27	n.d.	n.d.
Epicatechin-3-O-gallate (ECG)	n.d.	36	n.d.	n.d.
Catechin	n.d.	4	n.d.	n.d.

n.d., not detected.

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Table 3. Antimycotic activity of the *C. sinensis* extract and of ketoconazole towards yeast and yeast-like strains

		Diameter of inhibition area (mm)			
		Camellia sinensis extract (2500 μg/mL)		Ketoconazole (100 μg/mL)	
Species	Strain	A	B	A	B
Candida albicans	DBVPG 6133			56.2	60.8
Candida albicans	DBVPG 6157			51.6	73.6
Candida glabrata	DBVPG 7212	17.7	38.9		
Candida glabrata	DBVPG 3828	17.3	25.5		
Pichia guilliermondii	DBVPG 6140			54.6	64.5
Candida parapsilosis	DBVPG 6150			60.3	65.6
Candida tropicalis	DBVPG 3982				
Candida zeylanoides	DBVPG 6163			49.1	58.8
Clavispora lusitaniae	DBVPG 6142	12.9	12.0	54.2	67.7
Clavispora lusitaniae	DBVPG 6148	13.2	22.3	56.7	64.6
Issatchenkia orientalis	DBVPG 6782	16.5	17.5	29.6	31.1
Kluyveromyces marxianus	DBVPG 6141			56.2	72.4
Saccharomyces cerevisiae	DBVPG 6173	16.8	23.5		
Saccharomyces cerevisiae	DBVPG 6497	16.9	16.1		
Saccharomyces cerevisiae	DBVPG 6500	19.3	26.0		
Yarrowia lipolitica	DBVPG 6053			35.6	46.0
Cryptococcus laurentii	DBVPG 3883	17.8	17.3	39.7	45.2
Cryptococcus laurentii	DBVPG 4272	28.1	26.0		
Cryptococcus laurentii	DBVPG 6265	19.1	18.8	42.6	47.9
Filobasidiella neoformans	DBVPG 3428	18.8	22.0	44.8	56.6
Filobasidiella neoformans	DBVPG 6010	15.9	27.9	46.7	50.4
Filobasidiella neoformans	DBVPG 6225	16.0	17.4	36.8	50.8
Filobasidiella neoformans	DBVPG 6981	16.0	26.3	37.4	43.5
Filobasidiella neoformans	DBVPG 6982	17.4	28.0	38.4	53.4
Prototheca wickerhamii	DBVPG 8879	22.0	23.7		
Prototheca zopfii	DBVPG 8880				
Prototheca zopfii	DBVPG 8830				

A, test carried out on YNB + 2% glucose agar dishes; B, test carried out on YEPG agar dishes.

Table 4. Minimal inhibitory concentration (MIC) of the *C. sinensis* extract and of ketoconazole towards yeasts and yeast-like strains

		MIC (μg/mL)		
		Camellia sinensis		
Species	Strain	Extract	Total polyphenols	Ketoconazole
Candida glabrata	DBVPG 3828	1200	502	r
Clavispora lusitaniae	DBVPG 6148	700	293	7.5
Issatchenkia orientalis	DBVPG 6782	2400	1006	98.1
Saccharomyces cerevisiae	DBVPG 6173	2400	1006	r
Cryptococcus laurentii	DBVPG 6265	600	251	48.2
Filobasidiella neoformans	DBVPG 6010	4800	2011	48.1
Prototheca wickerhamii	DBVPG 8879	300	126	r

r, resistant to concentration of 100 μg/mL.

The growth of *Clavispora lusitaniae, Issatchenkia orientalis*, *Cryptococcus laurentii* and *Filobasidiella neoformans* strains was inhibited by *C. sinensis* extract and ketoconazole. In addition, some strains of *Prototheca wickerhamii*, *Saccharomyces cerevisiae*, *Cr. laurentii* and *Candida glabrata*, which were resistant to ketoconazole, were susceptible to *C. sinensis* extract while *Candida tropicalis* and *Prototheca zopfii* strains which are resistant to ketoconazole were also resistant to *C. sinensis* extract (Table 3).

The MICs of *C. sinensis* extract ranged from 300 to 4800 μg/mL (corresponding to 130–2010 μg/mL of total polyphenols) (Table 4). This study shows that the leaf extract of *C. sinensis* possesses a broad antimycotic activity and, more importantly, is still active against yeast and yeast-like strains resistant to ketoconazole. As already mentioned, drug resistance is a real problem in the clinical, setting in agreement with recent literature reporting the occurrence of yeast strains able to grow in the presence of azole-derivatives (Marichal *et al.*, 1997; De Resende and De Resende, 1999; Bouchara *et al.*, 2000).

At very high concentration (25 000 μg/mL), *C. sinensis* extract caused a rapid decrease of viable cells of *Fil. neoformans* DBVPG 6010, whereas lower concentrations appeared to have an initially fungistatic phase

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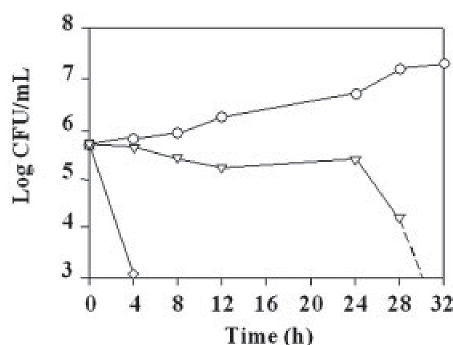

Figure 1. Assessment of fungicidal activity of the *C. sinensis* extracts towards *Fil. neoformans* DBVPG 6010. ○, control; ◇, 25 000 μg/mL; ▽, 12 500 μg/mL.

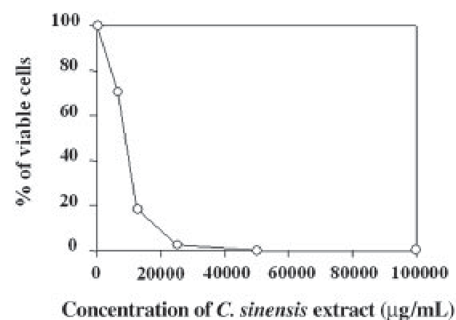

Figure 2. Dose-response effect of the *C. sinensis* extract towards *Fil. neoformans* DBVPG 6010.

(24 h), rapidly followed by cell death (Fig. 1), the effect of the extract being dose-dependent (Fig. 2).

In order to identify the molecules involved in the antimycotic activity, the pure polyphenols present in *C. sinensis* extract composition (Table 2) were tested by ADWB. Among them only epicatechin-3-O-gallate (ECG) and epigallocatechin-3-O-gallate (EGCG) were able to inhibit the growth of *Fil. neoformans* DBVPG 6010 (data not shown).

Catechins and galloyl-derivatives of catechins are a well known class of compounds exhibiting a considerable antibacterial activity (Nisiyama and Kozaki, 1974; Ryu, 1982; Cheng-Chun *et al.*, 1999; Johns, 1999; Pieroni, 2000). Chou and Lin (1987) fractionated tea flush and found that the fraction exhibiting the strongest antimicrobial activity contained mainly EGC and ECG, whereas Hara *et al.* (1995) demonstrated that EGC, ECG and EGCG inhibit the growth of *Clostridium botulinum*. Interestingly, the green tea catechins were also able to inhibit adenovirus activity (Weber *et al.*, 2003). On the other hand, the antimycotic activity of these compounds, to our knowledge, has been very little investigated (Hirasawa and Takada, 2004). On the basis of the results of the present investigation, it is possible to point out that the broad activity of *C. sinensis* extract towards yeast and yeast-like strains may be ascribed apparently to the presence of ECG and EGCG.

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