Industrial Crops and Products 44 (2013) 104–110

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Characterization of phenolic compounds in wild medicinal flowers from Portugal by HPLC–DAD–ESI/MS and evaluation of antifungal properties

Lillian Barros[a,b], Carlos Tiago Alves[c], Montserrat Dueñas[b], Sónia Silva[c], Rosário Oliveira[c], Ana Maria Carvalho[a], Mariana Henriques[c,**], Celestino Santos-Buelga[b], Isabel C.F.R. Ferreira[a,*]

[a] CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal
[b] Grupo de Investigación en Polifenoles (GIP-USAL), Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain
[c] IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal

ARTICLE INFO

Article history:
Received 26 June 2012
Received in revised form 11 October 2012
Accepted 2 November 2012

Keywords:
Medicinal flowers
Phenolic compounds
HPLC–DAD–ESI/MS
Candida species
Antifungal effect

ABSTRACT

In the present work, the phenolic compounds of *Castanea sativa*, *Filipendula ulmaria* and *Rosa micrantha* flowers from Northeastern Portugal were characterized by HPLC–DAD–ESI/MS. Furthermore, it was performed a screening of their antifungal potential against *Candida* species (*Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*).

C. sativa sample gave the highest amount of phenolic compounds (18973 ± 40 μg/g, fw) and hydrolysable tannins (14873 ± 110 μg/g). The highest amounts of phenolic acids (569 ± 20 μg/g) and flavonoids (6090 ± 253 μg/g) were obtained in *F. ulmaria* and *R. micrantha* samples, respectively. Hydrolysable tannins (e.g. tri and digalloyl HHDP glucose) were the main group of phenolic compounds in *C. sativa* and *F. ulmaria* samples, while flavonoids (e.g. (+)-catechin and procyanidin dimers and trimers) were the most abundant group in *R. micrantha*. Thus, the stronger effect showed by this latter against all the *Candida* species (MIC ≤ 0.155 mg/mL) and, particularly its fungicide effects in *C. glabrata*, might be related to the mentioned flavonoids that were inexistence in the other samples.

1. Introduction

In healthy individuals, many species of *Candida* are endogenous commensals of the gastrointestinal and urogenital tracts (Soll, 2002). However, the prevalence of opportunistic fungal infections has been increasing dramatically over the recent decades and this is particularly evident in immunocompromised individuals, where these species become frequently opportunistic pathogens (Pfaller and Diekema, 2007). Although, *Candida albicans* has been regarded as the most common causative agent of fungal infection in humans, nowadays other non-*C. albicans Candida* (NCAC) species such as *Candida glabrata*, *Candida tropicalis*, and *Candida parapsilosis*, are emerging as significant nosocomial pathogens (Silva et al., 2010). Moreover, NCAC species tend to be inherently less susceptible to the available antifungal drugs like the azole drugs and their derivatives, which continue to dominate as the antifungal agents of choice against *Candida*-related infections (Redding et al., 2002; Hajjeh et al., 2004; Ruhnke, 2006). Furthermore this difficulty highlights the necessity to develop new alternative antifungal agents, in order to increase the spectrum of activity against *Candida* species.

In the recent years the interest in natural compounds has raised, specifically some phenolic compounds including phenolic acids and flavonoids have been reported to inhibit various pathogenic bacteria and fungi (Rauha et al., 2000; Erasto et al., 2004; Tepe et al., 2004). Previous work conducted by our research group has highlighted the importance of wild plants as sources of phenolic compounds, such as phenolic acids, flavonoids and anthocyanins (Barros et al., 2011a). Moreover, flowers from semi-wild and wild species such as *Castanea sativa*, *Filipendula ulmaria* and *Rosa micrantha* have been traditionally used for several folk medicinal applications. Decoctions of *C. sativa* flowers are used for colds, cough, diarrhea and cholesterol; infusions of *F. ulmaria* are used for pneumonia and flu, urinary tract infections, rheumatism and headache; and rosewater and decoctions of *R. micrantha* are used for acne, skin condition and injuries and eye inflammations (Camejo-Rodrigues et al., 2003; Novais et al., 2004; Neves et al., 2009; Carvalho, 2010). Their antioxidant potential was already reported by us (Barros et al., 2010, 2011b; Guimarães et al., 2010).

As far as we know there are no reports neither on anti-NCAC activity of the mentioned wild flowers, nor in phenolic composition of *R. micrantha*. The available studies in literature described phenolic composition in *C. sativa* leaves (Calliste et al., 2001) and heartwood (Sanz et al., 2010), but not in its flowers.

* Corresponding author. Tel.: +351 273303219; fax: +351 273325405.
** Corresponding author. Tel.: +351 253604408; fax: +351 253604429.
E-mail addresses: mcrh@deb.uminho.pt (M. Henriques), iferreira@ipb.pt (I.C.F.R. Ferreira).

0926-6690/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.indcrop.2012.11.003

The phenolic composition of *F. ulmaria* flowers was studied, but with plant material from other European countries, i.e. not growing under Mediterranean particular climatic and soil conditions (Krasnov et al., 2006, 2009; Shilova et al., 2006; Pemp et al., 2007; Fecka, 2009; Harbourne et al., 2009). These flowers revealed the presence of a number of polyphenolic constituents including salicylates (e.g. spiraein, salicylic acid and methyl salicylate), flavonols (e.g. spiraeoside, hyperoside, rutin, kaempferol 4′-*O*-glucosides, quercetin-4′-*O*-β-D-galactopyranoside and quercetin-3-*O*-β-glucopyranoside) and ellagitannins (tellimagrandins I and II, and rugosin D) (Krasnov et al., 2006, 2009; Shilova et al., 2006; Pemp et al., 2007; Fecka, 2009; Harbourne et al., 2009).

It has been indicated that the antifungal activity of plant extracts is related to the different compounds present (with diverse functional groups and chemical substituents) and to possible synergistic interactions between them (Dorman and Deans, 2000). Therefore, in the present work, an exhaustive characterization of the phenolic compounds present in the extracts of *C. sativa*, *F. ulmaria* and *R. micrantha* flowers was carried out by HPLC–DAD–ESI/MS. Furthermore, a screening of the antifungal potential of those extracts against *Candida* species was also performed.

2. Materials and methods

2.1. Samples

Several ethnobotanical surveys conducted in Portugal for the last 10 years (Camejo-Rodrigues et al., 2003; Novais et al., 2004; Neves et al., 2009; Carvalho, 2010) highlighted the importance of folk medicine founded on traditional uses of plants. Considering our group previous research (Barros et al., 2010, 2011a, 2011b), several species were chosen to be screened for antifungal activity and further characterization, but only three of them highlight as interesting species: *C. sativa* Mill, *F. ulmaria* (L.) Max and *R. micrantha* Borrer ex Sm.

Flowers and inflorescences (the parts most cited for folk medicinal purposes by key-informants) of the selected species were collected in the Natural Park of Montesinho territory (Trás-os-Montes, Northeastern Portugal), in 2009, according to local medicinal criteria of use and each plant growth pattern. *C. sativa*, the upright catkins during anthesis (flower fully opened and functional) in late summer; *F. ulmaria*, the inflorescences with flowers fully open and functional in early summer; *R. micrantha*, the petals removed from floral buds and also from flowers after anthesis (anthers already opened, stamens becoming dry) in early spring.

Voucher specimens are kept at the Herbário da Escola Superior Agrária de Bragança (BRESA). Each sample was lyophilized (Ly-8-FM-ULE, Snijders, Netherlands) and stored in the deep-freezer at −20 °C for subsequent analysis.

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, France). The phenolic compounds standards were from Extrasynthese (Genay, France). RPMI 1640 medium was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and purchased from chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Preparation of the extracts

Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (v/v) at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman no. 4 paper. The residue was then re-extracted twice with additional 30 mL portions of methanol:water 80:20 (v/v). The combined extracts were evaporated at 35 °C (rotary evaporator Büchi R-210) to remove methanol. The aqueous phase was lyophilized and re-dissolved in (a) 20% aqueous methanol at 5 mg/mL and filtered through a 0.22-μm disposable LC filter disk for high performance liquid chromatography (HPLC) analysis or (b) distilled water at 200 mg/mL for antifungal assays.

2.4. Phenolic compounds identification and quantification

The extracts were analyzed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water and (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in a API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at −4500 V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to record full scan spectra so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) −450 V, entrance potential (EP) −6 V, collision energy (CE) −10 V. Spectra were recorded in negative ion mode between m/z 100 and 1000. Analysis in EPI mode was further performed in order to obtain the fragmentation pattern of the parent ion(s) detected in the previous experiment using the following parameters: DP −50 V, EP −6 V, CE −25 V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the samples were characterized according to their UV and mass spectra and retention times compared with commercial standards when available. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (2.5–100 μg/mL) of different standards compounds: catechin ($y = 132.76x - 59.658$; $R^2 = 0.9997$); caffeic acid ($y = 617.91x - 691.51$; $R^2 = 0.9991$); gallic acid ($y = 556.94x - 738.37$; $R^2 = 0.9988$); isorhamnetin ($y = 629.14x - 2323.4$; $R^2 = 0.9967$); isorhamnetin-3-*O*-glucoside ($y = 262.31x - 9.8958$; $R^2 = 1.000$); kaempferol-3-*O*-glucoside ($y = 190.75x - 36.158$; $R^2 = 1.000$); kaempferol-3-*O*-rutinoside ($y = 175.02x - 43.877$; $R^2 = 0.9999$); myricetin ($y = 778x - 1454.3$; $R^2 = 0.9990$); quercetin-3-*O*-glucoside ($y = 316.48x - 2.9142$; $R^2 = 1.000$); and quercetin-3-*O*-rutinoside ($y = 222.79x - 243.11$; $R^2 = 0.9998$); The results were expressed in μg/g of fresh weight (fw), as mean ± standard deviation of three independent analyses.

2.5. Antifungal activity

Four reference strains from the American Type Culture Collection (ATCC) namely *C. albicans* (ATCC 90028), *C. tropicalis* (ATCC 750), *C. glabrata* (ATCC 2001) and *C. parapsilosis* (ATCC 22019) were used in the course of this study. Before the experiments, all strains

were grown on Sabouraud Dextrose Agar (SDA; Merck, Germany) for 24 h at 37 °C. Yeast cells from at least five colonies (1 mm diameter) were suspended in 5 mL of sterile saline solution (0.85% NaCl). The resulting yeast suspension was mixed for 15 s with a vortex. Then, the suspension was adjusted by spectrophotometric method, adding saline solution to reach the value of the 0.5 McFarland scale. The process makes final inocula of $3.0 \pm 2.0 \times 10^6$ cells/mL.

Minimal inhibitory concentration (MIC) was determined according with the guidelines from the CLSI M27-A2 document (NCCLS, 2002) with some modifications. Thus, serial dilutions of the three plant extracts (0.05, 0.155, 0.625, 1.25, 2.5 and 5 mg/mL) were prepared in RPMI 1640 medium at pH 7. Aliquots of each plant extract (100 μL), at a twofold final concentration, were dispensed into the 96-well plates (Orange Scientific, Braine-l' Alleud, Belgium). Furthermore, the plates were also incubated with aliquots (100 μL) at a twofold concentration of the four *Candida* species. Drug-free and yeast controls were also included.

The 96-well plates were incubated at 37 °C for 48 h. After visualization of the resultant plate the MIC value was correspondent to the antifungal concentration where there was no growth, by comparison with the control (cells grown without extract). Moreover, the number of viable cells was assessed by the determination of number of colony forming units (CFUs). CFUs were enumerated by plating 10 μL of each cell suspension (from each well) onto SDA. After 24 h of incubation at 37 °C, the number of colonies formed was counted. These experiments were performed three times and, at least, in quadruplicate.

3. Results and discussion

3.1. Identification and quantification of phenolic compounds present in the extracts from wild flowers

The extraction yields for *R. micrantha* and *C. sativa* samples were similar (33.16% ± 1.22% and 35.80% ± 1.87%, respectively), being slightly higher than the one obtained for *F. ulmaria* (27.56% ± 2.01%). Fig. 1 shows the phenolic compounds profile of *C. sativa*, *F. ulmaria* and *R. micrantha* flowers extracts. The obtained profiles included phenolic acids, flavonoids including procyanidins, and hydrolysable tannins. Data (retention time, λ_{max} in the visible region, molecular ion and main fragment ions observed in MS^2) obtained by HPLC–DAD–ESI/MS analysis, identification of compounds and individual quantification are presented in Table 1.

Total amounts of the different phenolics groups in the three plant samples are shown in Fig. 2. Hydrolysable tannins were the main group in *C. sativa* and *F. ulmaria* samples, while flavonoids including procyanidins were the most abundant group in *R. micrantha*. *C. sativa* sample gave the highest amount of phenolic compounds (18973 ± 40 μg/g, fw) and hydrolysable tannins (14873 ± 110 μg/g). The highest amount of phenolic acids (569 ± 20 μg/g) was determined in *F. ulmaria* sample, while the highest amount of flavonoids (6090 ± 253 μg/g) was found in *R. micrantha* sample.

3.2. Hydroxybenzoic and hydroxycinnamic derivatives

Gallic acid (a hydroxybenzoic acid), found in *C. sativa* and *F. ulmaria*, and 5-*O*-caffeoylquinic acid (a hydroxycinnamic acid derivative), found in *R. micrantha*, were identified by comparison of their UV spectra and retention time with commercial standards. In the *F. ulmaria* sample, caffeic acid derivatives were also detected.

3.3. Flavonols

Flavonols were the only flavonoids found in the studied samples, being quercetin derivatives particularly abundant.

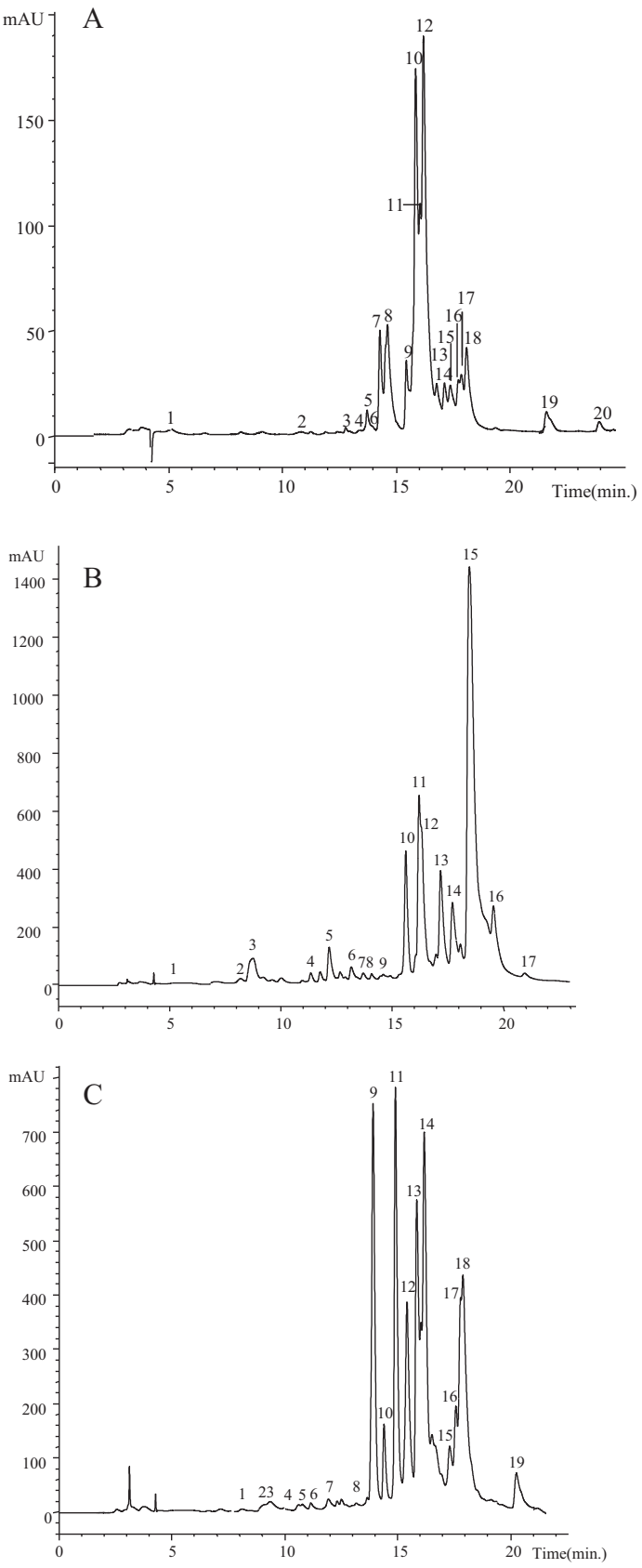

Fig. 1. Individual chromatograms of the studied wild flowers: (A) *Castanea sativa*; (B) *Filipendula ulmaria* and (C) *Rosa micrantha* all recorded at 370 nm.

Table 1
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, relative abundances of fragment ions, identification and quantification of the phenolic compounds in the studied wild flowers.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Identification	Quantification (μg/g, fw)
Castanea sativa						
1	5.0	270	169	125(100)	Gallic acid	16.71 ± 0.62
2	10.3	280	289	245(35), 203(14), 137(21)	(+)-Catechin	379.94 ± 17.18
3	13.4	276	635	465(100), 313(15), 169(6)	Trigalloyl glucose	2067.68 ± 17.22
4	13.7	276	937	937(100), 767(4), 635(4), 465(26), 301(3)	Trigalloyl HHDP glucose	1508.57 ± 23.32
5	13.9	274	937	937(100), 637(8), 465(67), 301(10)	Trigalloyl HHDP glucose	9854.25 ± 73.64
6	14.0	356	493	317(100)	Myricetin 3-*O*-glucuronide	359.41 ± 0.09
7	14.1	358	479	317(100)	Myricetin 3-*O*-glucoside	380.92 ± 4.98
8	15.3	274	937	937(100), 767(2), 637(4), 467(81), 301(25)	Trigalloyl HHDP glucose	992.26 ± 65.69
9	15.6	354	609	301(100)	Quercetin 3-*O*-rutinoside	65.68 ± 7.90
10	15.8	354	477	301(100)	Quercetin 3-*O*-glucuronide	672.55 ± 51.58
11	15.9	276	937	937(100), 767(12), 637(12), 467(20), 301(30)	Trigalloyl HHDP glucose	450.54 ± 35.57
12	16.0	356	449	317(100)	Myricetin 3-O-pentoside	386.14 ± 2.94
13	16.1	356	463	301(100)	Quercetin 3-*O*-glucoside	899.07 ± 0.88
14	16.8	342	593	285(100)	Kaempferol 3-*O*-rutinoside	191.58 ± 18.99
15	17.1	356	623	315(100)	Isorhamnetin 3-*O*-rutinoside	135.73 ± 11.90
16	17.3	356	433	301(100)	Quercetin *O*-pentoside	138.75 ± 7.35
17	17.7	348	447	301(100)	Quercetin 3-*O*-rhamnoside	100.75 ± 7.50
18	18.0	356	477	315(100)	Isorhamnetin 3-*O*-glucoside	249.33 ± 6.70
19	21.6	354	609	463(100), 301(93)	Quercetin-*O*-rhamnoside-*O*-hexoside	53.05 ± 3.65
20	23.9	356	593	447(8), 285(100)	Kaempferol-*O*-rhamnoside-*O*-hexoside	71.00 ± 5.10
Filipendula ulmaria						
1	4.9	274	169	125(100)	Gallic acid	58.97 ± 2.37
2	8.7	330	297	179(9), 161(12), 135(100)	Caffeic acid derivative	164.04 ± 11.96
3	9.0	276	785	785(100), 633(5), 483(7), 301(38)	Digalloyl-HHDP-glucose	712.87 ± 20.30
4	11.5	276	785	785(100), 633(5), 483(17), 301(56)	Digalloyl-HHDP-glucose	1462.02 ± 86.23
5	12.2	322	297	179(36), 161(27), 135(100)	Caffeic acid derivative	360.40 ± 6.19
6	13.9	276	785	785(100), 633(4), 483(6), 301(31)	Digalloyl-HHDP-glucose	529.48 ± 38.54
7	14.4	273	937	937(100), 767 (4), 635(10), 465(7), 301(11)	Trigalloyl HHDP glucose	464.39 ± 69.03
8	14.5	276	937	937(100), 767(8), 635(4), 465(8), 301(21)	Trigalloyl HHDP glucose	2091.98 ± 108.72
9	15.3	280	937	937(100), 767(6), 635(2), 465(4), 301(12)	Trigalloyl HHDP glucose	1031.90 ± 26.27
10	15.6	354	609	301(100)	Quercetin-3-*O*- rutinoside	484.56 ± 75.39
11	16.2	354	463	301(100)	Quercetin-3-*O*-glucoside	444.69 ± 72.78
12	16.3	356	477	315(100)	Isorhamnetin *O*-hexoside	542.06 ± 11.11
13	17.1	356	433	301(100)	Quercetin *O*-pentoside	444.85 ± 24.87
14	17.7	354	433	301(100)	Quercetin *O*-pentoside	376.16 ± 28.71
15	18.5	356	463	301(100)	Quercetin 4′-*O*-glucoside	2365.70 ± 34.64
16	19.5	348	519	315(100)	Isorhamnetin acetylhexoside	573.97 ± 17.87
17	20.9	354	585	433(27), 301(100)	Quercetin pentoside derivative	98.46 ± 10.48
Rosa micrantha						
1	8.9	276	577	425(36), 289(68)	Procyanidin dimer	420.18 ± 36.50
2	9.4	280	577	425(60), 289(65)	Procyanidin dimer	475.74 ± 39.28
3	9.9	280	865	865(100), 577(48), 287(17)	Procyanidin trimer	289.80 ± 12.03
4	10.2	280	289	245(55), 203(30), 137(14)	(+)-Catechin	1354.14 ± 79.43
5	10.7	326	353	191(100), 179(13), 173(2), 135(3)	5-*O*-caffeoylquinic acid	54.98 ± 6.99
6	11.4	274	785	483(38), 301(100)	Digalloyl-HHDP-glucose	397.10 ± 62.59
7	12.3	280	577	425(72), 289(55)	Procyanidin dimer	120.25 ± 18.71
8	13.4	278	865	865(100), 577(27), 287(31)	Procyanidin trimer	327.23 ± 44.85
9	14.1	354	625	463(6), 301(100)	Quercetin-*O*-hexoside-*O*-hexoside	439.65 ± 15.44
10	14.6	355	639	315(100)	Isorhamnetin-*O*-hexoside-*O*-hexoside	109.97 ± 0.97
11	15.1	346	609	447(8), 285(100)	Kaempferol-*O*-hexoside-*O*-hexoside	617.66 ± 46.54
12	15.6	356	433	301(100)	Quercetin *O*-pentoside	274.69 ± 22.12
13	16.0	354	477	301(100)	Quercetin-3-*O*-glucuronide	291.06 ± 31.59
14	16.3	354	463	301(100)	Quercetin-3-*O*-glucoside	442.39 ± 18.44
15	17.5	347	447	285(100)	Kaempferol *O*-hexoside	152.60 ± 24.39
16	17.7	348	461	285(100)	Kaempferol-3-*O*-glucuronide	162.49 ± 16.23
17	17.9	346	447	285(100)	Kaempferol-3-*O*-glucoside	280.80 ± 24.22
18	18.0	348	447	301(100)	Quercetin-3-*O*-rhamnoside	270.00 ± 17.32
19	20.4	344	431	285(100)	Kaempferol-3-*O*-rhamnoside	60.98 ± 10.94

Quercetin-3-*O*-rutinoside was found in *C. sativa* and *F. ulmaria*, quercetin-3-*O*-glucuronide and quercetin-3-*O*-rhamnoside were found in *C. sativa* and *R. micrantha*, quercetin-4′-*O*-glucoside was in found *F. ulmaria*, while quercetin-3-*O*-glucoside was found in all the analyzed samples. They showed similar UV spectra with λ_{max} at 348–356 nm, and presented pseudomolecular ions $[M-H]^-$ at m/z 609, 477, 447 and 463, respectively, all of them releasing a unique MS^2 fragment at m/z 301, corresponding to quercetin. All those compounds were positively identified according to their mass and UV characteristics and comparison with commercial standards.

Different peaks that could be associated to quercetin pentosides according to their UV and mass spectral characteristics were also found in the samples. Peaks 16 in *C. sativa*, 13 and 14 in *F. ulmaria* and 12 in *R. micrantha* showed a pseudomolecular ion $[M-H]^-$ at m/z 433, releasing a unique MS^2 fragment at m/z 301, which allowed their identification as quercetin monopentosides. Peak 17 in *F. ulmaria* was also associated to an unknown a quercetin pentoside derivative owing its fragment ion at m/z 433. Other unknown quercetin glycosides were detected in *R. micrantha* (peak 9, $[M-H]^-$ at m/z 625) and *C. sativa* (peak 19, $[M-H]^-$ at

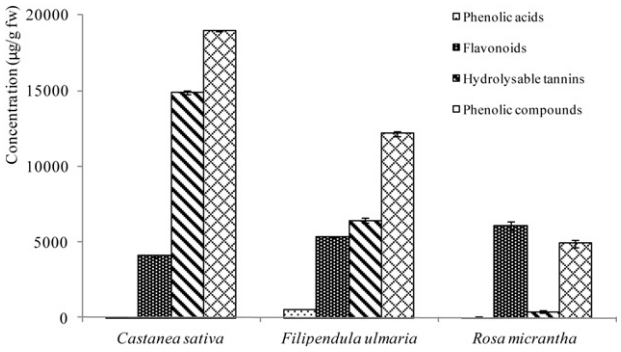

Fig. 2. Total amounts of the different classes of phenolic compounds, determined by HPLC analysis, in the studied wild flowers.

m/*z* 609) and identified as a quercetin *O*-hexoside-*O*-hexoside and a quercetin-*O*-rhamnoside-*O*-hexoside, respectively, according to their pseudomolecular ions and fragmentation patterns.

Isorhamnetin derivatives were also found in all the studied samples, presenting UV spectra with λ_{max} at 348–356 nm, and different pseudomolecular ions that release a unique MS^2 fragment at *m*/*z* 315, coherent with isorhamnetin. Peaks 15 and 18 in *C. sativa* were positively identified as isorhamnetin-3-*O*-rutinoside and isorhamnetin-3-*O*-glucoside by comparison with commercial standards, whilst the identity of the other peaks was established according to their pseudomolecular ions and fragmentation patterns and identified as isorhamnetin-*O*-hexoside (peak 12 in *F. ulmaria*, $[M-H]^-$ at *m*/*z* 477), isorhamnetin acetylhexoside (peak 16 in *F. ulmaria*, $[M-H]^-$ at *m*/*z* 519), isorhamnetin-*O*-hexoside-*O*-hexoside (peak 10 in *R. micrantha*, $[M-H]^-$ at *m*/*z* 639).

Kaempferol derivatives were found in *C. sativa* and *R. micrantha*. Peak 14 in *C. sativa* (kaempferol-3-*O*-rutinoside) and peaks 16 (kaempferol-3-*O*-glucuronide), 17 (kaempferol-3-*O*-glucoside) and 19 (kaempferol-3-*O*-rhamnoside) in *R. micrantha* were positively identified according to their UV and mass spectra and comparison with standards. Peak 20 in *C. sativa* (kaempferol-*O*-rhamnoside-*O*-hexoside, $[M-H]^-$ at *m*/*z* 593) and peak 15 in *R. micrantha* (kaempferol-*O*-hexoside, $[M-H]^-$ at *m*/*z* 447) were assigned according to their pseudomolecular ions and fragmentation patterns.

C. sativa was the only sample that presented myricetin derivatives, which could be identified as myricetin-3-*O*-glucuronide (peak 6), myricetin-3-*O*-glucoside (peak 7) and myricetin 3-*O*-pentoside (peak 12) by comparison of their UV and mass spectra with authentic standards.

3.4. Procyanidins

Peaks 2 and 4 in *C. sativa* and *R. micrantha* samples were identified as (+)-catechin by comparison of UV spectrum and retention time with a commercial standard. Procyanidin oligomers were also detected in *R. micrantha* sample. Peaks 1, 2, 3, 7 and 8 showed UV spectra with λ_{max} 276–280 nm, characteristic of proanthocyanidins. Peaks 1, 2 and 7 presented a pseudomolecular ions corresponding to procyanidin dimers ($[M-H]^-$ at *m*/*z* 577), and peaks 3 and 8 to procyanidin trimers ($[M-H]^-$ at *m*/*z* 865).

3.5. Hydrolyzable tannins

All the studied plant extracts presented compounds that showed UV spectral coherent with galloyl and hexahydroxydiphenol (HHDP) derivatives (Cantos et al., 2003), and in accordance with their mass spectra data, they were associated to different isomers of trigalloyl glucopyranose and di, trigalloyl-hexahydroxydiphenoyl glucopyranose (Table 1). According to the literature, the main characteristic in the mass spectra of these compounds was the deprotonated molecula $[M-H]^-$ (*m*/*z* 483, 635, 787, 939, 785, 937) and the loss of one or more galloyl groups (152 μm) and/or gallic acid (170 μm) (Salminen et al., 1999; Zywicki et al., 2002). Thus, peak 3 in *C. sativa* with a pseudomolecular ion $[M-H]^-$ at *m*/*z* 635 and MS^2 fragment ions at *m*/*z* 465 (loss of gallic acid residue), *m*/*z* 313 (loss of a galloyl residue) and *m*/*z* 169 (gallic acid) was identified as trigalloyl glucose.

Peaks 4, 5, 8 and 11 in *C. sativa* and 7, 8 and 9 in *F. ulmaria* presented a pseudomolecular ion $[M-H]^-$ at *m*/*z* 937 and they were identified as different isomers of trigalloyl-HHDP-glucopyranose. The $[M-H]^-$ ion suffered the loss of gallic acid (*m*/*z* 797) and the fragments *m*/*z* 635, 465 were due to the loss of hexahydroxydiphenoyl moieties from the $[M-H]^-$ and $[M-H-\text{gallic acid}]^-$ ions respectively. Similar ellagitannins have already been reported in *C. sativa* heartwood (Sanz et al., 2010).

Peaks 3, 4 and 6 in *F. ulmaria* and 6 in *R. micrantha* with an *m*/*z* 785 were identified as isomers of digalloyl-HHDP-glucopyranose. Their fragmentation pattern involved the loss of galloyl and hexahydroxydiphenoyl moieties (*m*/*z* 633 and 483, respectively). The fragment at *m*/*z* 301 besides the $[M-H-302]^-$ ion represented an evidence of the presence of an HHDP group $[\text{ellagic}-H]^-$ in the molecule.

As far as we know, there was no information on the phenolic composition of *C. sativa* and *R. micrantha* flowers, but a few previous studies have been published dealing with the phenolic composition of *F. ulmaria* flowers from Russia (Krasnov et al., 2006, 2009; Shilova et al., 2006), Poland (Fecka, 2009) and Austria (Pemp et al., 2007). Some differences were found in the *F. ulmaria* sample (flowers from Portugal) studied herein, including the presence of different compounds such as isorhamnetin glucoside derivatives and caffeic acid derivatives. Krasnov et al. (2006, 2009) identified and isolated two quercetin glucosides (filimarin and isoquercitrin) from *F. ulmaria* aerial parts during flowering, and Fecka (2009) reported different ellagitannins such as rugosin A, B, D and E, which were not found in the sample herein studied. Pemp et al. (2007) only described flavonols *in F. ulmaria* sample and they did not identify any ellagitannin. Samples from Poland (132 ± 18 mg/g dw; Fecka, 2009) presented a higher concentration of phenolic compounds than the sample studied here (60 ± 0.8 mg/g dw; data converted in dry weight basis excluding the moisture content). It was not possible to compare to the quantity found in the sample from Austria, because the results were expressed in percentage (Pemp et al., 2007), neither with those of Shilova et al. (2006) that used thin-layer chromatography for the analysis of phenolic compounds.

3.6. Antifungal activity of the extracts from wild flowers

In the last years, the number of *Candida* species resistant to the common antifungal agents has been increasing (Bonjar, 2004). In order to overcome this problem it is of major importance to identify new compounds, especially natural ones, that are active against the most broaden spectrum of these species. In this study, the principal aim was to determine, for the first time, the antifungal effect of *C. sativa*, *F. ulmaria* and *R. micrantha* flowers extracts against four *Candida* species.

The minimum inhibitory concentration (MIC) values (Table 2) ranged from concentrations under 0.05 to 0.625 mg/mL. Furthermore, it is important to highlight that the extracts presented different activity against the different *Candida* species under study. According to the classification established by Aligiannis et al. (2001) and with the values present in Table 2 it is possible to assume that the extract of *F. ulmaria* presented a moderate activity against *C. albicans*, but a strong effect against the other three species. *C. sativa* extract presented similar behavior, with the exception of

Table 2
Minimum inhibitory concentrations (MIC; mg/mL) of the wild flowers extracts against *Candida* species.

	C. albicans	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*
Castanea sativa	0.625	<0.05	<0.05	0.625
Filipendula ulmaria	0.625	<0.05	<0.05	0.155
Rosa micrantha	0.05	<0.05	<0.05	0.155

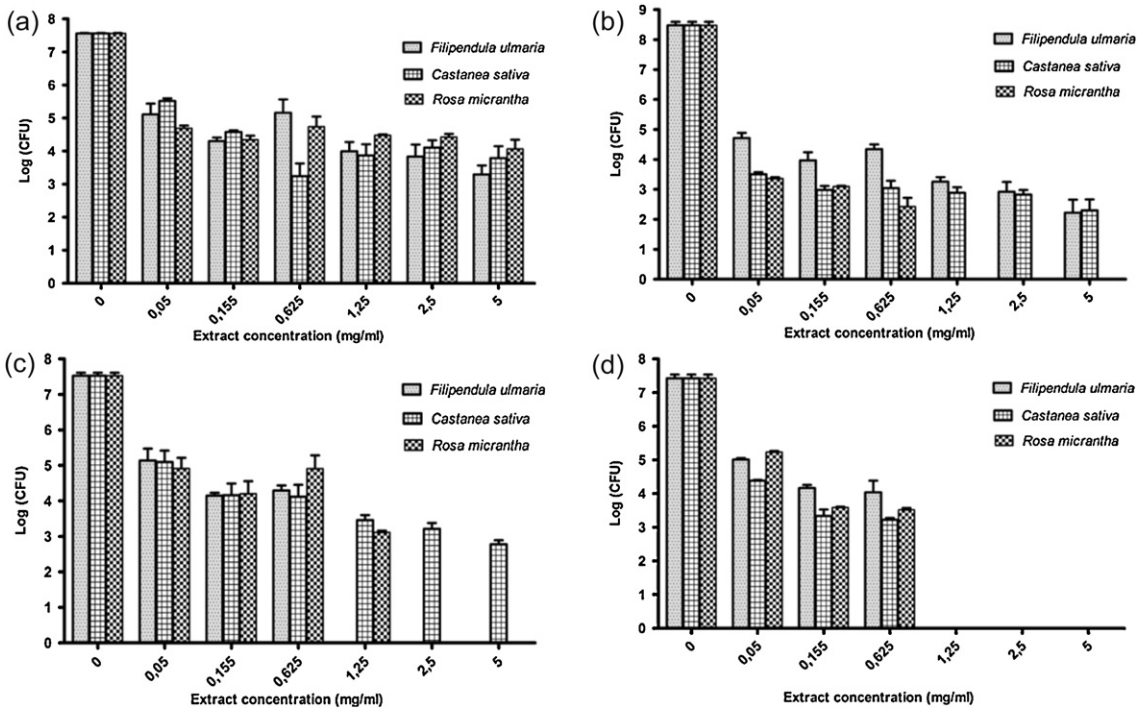

Fig. 3. Logarithm of number cells of *C. albicans* (a), *C. glabrata* (b), *C. parapsilosis* (c) and *C. tropicalis* (d) cultured in different concentrations of three plant extracts, formed in SDB after 48 h. Error bars represent standard deviation.

C. tropicalis, against which it also presented a moderate activity. Regarding *R. micrantha* extract, a different profile was found, since it showed a strong effect against the four strains assayed. This higher activity of *R. micrantha* extract could explain the traditional use of this plant to treat acne and skin conditions closely related with *Candida* species (Jain et al., 2010). Additionally, the effect of the extracts on the viability of the *Candida* species was assessed by CFU counts (Fig. 3). This evaluation is of utmost importance to distinguish between the fungicidal and fungistatic effects.

The results showed that all the samples presented a strong activity against the four *Candida* species studied. It should be noticed that all the extracts caused at least 2 log of reduction for all strains, at the lowest concentration tested (0.05 mg/mL). *C. glabrata* (Fig. 3) presented the highest initial inhibition for all the extracts tested, with more than 3 log of reduction. However, only *R. micrantha* extract was able to completely eliminate all *C. glabrata* cells, in concentrations 1.25 mg/mL. These results confirm the fungicidal effect of this plant against *C. glabrata*, in detriment of the fungistatic effect of the other two extracts. *C. sativa*, *F. ulmaria* and *R. micrantha* extracts were able to cause a similar effect in *C. parapsilosis* and *C. tropicalis* at low concentrations. However, *C. sativa* sample was not able to cause a total inhibition of the former species, even in the highest concentrations tested, and *R. micrantha* sample was only able to cause a significant CFUs reduction at concentrations above than 2.5 mg/mL.

C. tropicalis demonstrated a strong susceptibility to all the samples, and more important at concentrations above 1.25 mg/mL, all the assayed extracts were able to cause total inhibition of the growth of this fungi species (Fig. 3).

Although being *C. tropicalis* often associated with urinary tract infections (Silva et al., 2011) and *F. ulmaria* traditionally used on these infections, it was not observed any distinct effect of its extract. Interestingly, despite being *C. tropicalis* the NCAC species with the highest genetic similarity to *C. albicans* (Butler et al., 2009), no similarities were observed in terms of their sensitivity to the assayed extracts. On the contrary, *C. albicans* was the species with the lowest rate of inhibition and none of the flowers extracts tested was able to cause a total inhibition effect.

Overall, extracts of *C. sativa*, *F. ulmaria* and *R. micrantha* revealed promising antifungal effects against *Candida* species. Hydrolysable tannins (e.g. tri and digalloyl HHDP glucose) were the main group of phenolic compounds in *C. sativa* and *F. ulmaria* samples, while flavonoids (e.g. (+)-catechin and procyanidin dimers and trimers) were the most abundant group in *R. micrantha*. Thus, the stronger effect showed by this latter against all the *Candida* species and, particularly its fungicide effects in *C. glabrata*, might be related to the mentioned flavonoids that were inexistence in the other samples. Furthermore, the present manuscript highlights the importance of natural products in discovery of new antifungal compounds.

Acknowledgements

L. Barros, C. Alves and S. Silva thank to FCT, POPH-QREN and FSE for their grants (SFRH/BPD/4609/2008, SFRH/BD/72742/2010 and BPD/71076/2010, respectively). M. Dueñas thanks to the *Programa Ramón y Cajal* for a contract. The GIP-USAL is financially supported by the Spanish *Ministerio de Ciencia e Innovación* through the *Consolider-Ingenio 2010* Programme (FUN-C-FOOD,

CSD2007-00063), and *Junta de Castilla y León* (Grupo de Investigación de Excelencia, GR133).

References

Aligiannis, N., Kalpotzakis, E., Mitaku, S., Chinou, I.B., 2001. Composition and antimicrobial activity of the essential oils of two *Origanum* species. J. Agric. Food Chem. 40, 4168–4170.

Barros, L., Cabrita, L., Vilas-Boas, M., Carvalho, A.M., Ferreira, I.C.F.R., 2011a. Chemical biochemical and electrochemical assays to evaluate phytochemicals and antioxidant activity of wild plants. Food Chem. 127, 1600–1608.

Barros, L., Dueñas, M., Ferreira, I.C.F.R., Carvalho, A.M., Santos-Buelga, C., 2011b. Use of HPLC–DAD–ESI/MS to profile phenolic compounds in edible wild greens from Portugal. Food Chem. 127, 169–173.

Barros, L., Oliveira, S., Carvalho, A.M., Ferreira, I.C.F.R., 2010. In vitro antioxidant properties and characterization in nutrients and phytochemicals of six medicinal plants from the Portuguese folk medicine. Ind. Crops Prod. 32, 572–579.

Bonjar, S., 2004. Evaluation of antibacterial properties of some medicinal plants used in Iran. J. Ethnopharmacol. 94, 301–305.

Butler, G., Rasmussen, M.D., Lin, M.F., Santos, M.A.S., Sakthikumar, S., et al., 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature 459, 657–662.

Calliste, C.-A., Trouillas, P., Allais, D.-P., Simon, A., Duroux, J.-L., 2001. Free radical scavenging activities measured by electron spin resonance spectroscopy and B16 cell antiproliferative behaviors of seven plants. J. Agric. Food Chem. 49, 3321–3327.

Camejo-Rodrigues, J., Ascensão, L., Bonet, M.À., Vallès, J., 2003. An ethnobotanical study of medicinal and aromatic plants in the Natural Park of "Serra de São Mamede" (Portugal). J. Ethnopharmacol. 89, 199–209.

Cantos, E., Espin, J.C., Lopez-Bote, C., De la Hoz, L., Ordonez, J.A., Tomás-Barberán, F.A., 2003. Phenolic compounds and fatty acids from acorns (*Quercus spp.*) the main dietary constituent of free-ranged Iberian pigs. J. Agric. Food Chem. 51, 6248–6255.

Carvalho, A.M., 2010. Plantas y Sabiduría Popular del Parque Natural de Montesinho Un Estudio Etnobotánico en Portugal. CSIC, Biblioteca de Ciencias 35, Madrid.

Dorman, H.J.D., Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial of plant volatile oils. J. Appl. Microbiol. 88, 308–316.

Erasto, P., Bojase-Moleta, G., Majinda, R.R., 2004. Antimicrobial and antioxidant flavonoids from the root wood of *Bolusanthus speciosus*. Phytochemistry 65, 875–880.

Fecka, I., 2009. Qualitative and quantitative determination of hydrolysable tannins and other polyphenols in herbal products from meadowsweet and dog rose. Phytochem. Anal. 20, 177–190.

Guimarães, R., Barros, L., Carvalho, A.M., Ferreira, I.C.F.R., 2010. Studies on chemical constituents and bioactivity of *Rosa micrantha*: an alternative antioxidants source for food, pharmaceutical, or cosmetic applications. J. Agric. Food Chem. 58, 6277–6284.

Hajjeh, R.A., Sofair, A.N., Harrison, L.H., Lyon, G.M., Arthington-Skaggs, B.A., Mirza, S.A., Phelan, M., Morgan, J., Lee-Yang, W., Ciblak, M.A., Benjamin, L.E., Sanza, L.T., Huie, S., Yeo, S.F., Brandt, M.E., Warnock, D.W., 2004. Incidence of bloodstream infections due to *Candida* species and *in vitro* susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. J. Clin. Microbiol. 42, 1519–1527.

Harbourne, N., Marete, E., Jacquier, J.C., O'Riordan, D., 2009. Effect of drying methods on the phenolic constituents of meadowsweet (*Filipendula ulmaria*) and willow (*Salix alba*). LWT 42, 1468–1473.

Jain, A., Jain, S., Rawat, S., 2010. Emerging fungal infections among children: a review on its clinical manifestations, diagnosis, and prevention. J. Pharm. Bioallied Sci. 2, 314–320.

Krasnov, E.A., Raldugin, V.A., Avdeeva, E.Y., 2009. Filimarin a new flavanol glycoside from *Filipendula ulmaria* and its antioxidant activity. Pharm. Chem. J. 43, 613–614.

Krasnov, E.A., Raldugin, V.A., Shilova, I.V., Avdeeva, E.Y., 2006. Phenolic compounds from *Filipendula ulmaria*. Chem. Nat. Prod. 42, 148–151.

National Committee For Clinical Laboratory Standards (NCCLS), 2002. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast. Approved Standard. NCCLS Document M27-A2. National Committee for Clinical Laboratory Standards, Wayne, PA.

Neves, J.M., Matosa, C., Moutinho, C., Queiroz, G., Gomes, L.R., 2009. Ethnopharmacological notes about ancient uses of medicinal plants in Trás-os-Montes (northern of Portugal). J. Ethnopharmacol. 124, 270–283.

Novais, M.H., Santos, I., Mendes, S., Pinto-Gomes, C., 2004. Studies on pharmaceutical ethnobotany in Arrabida Natural Park (Portugal). J. Ethnopharmacol. 93, 183–195.

Pemp, E., Reznicek, G., Krenn, L., 2007. Fast quantification of flavonoids in *Filipendulae ulmariae* flos by HPLC/ESI-MS using a nonporous stationary phase. J. Anal. Chem. 62, 669–673.

Pfaller, M.A., Diekema, D.J., 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. 20, 133–163.

Rauha, J.-P., Remes, S., Heinonen, M., Hopia, A., Kahkonen, M., Kujalac, Y., Pihlajac, K., Vuorela, H., Vuorela, P., 2000. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. Int. J. Food Microbiol. 56, 3–12.

Redding, S., Kirkpatrick, W., Coco, B., Sadkowski, L., Fothergill, A., Rinaldi, M., Eng, T., Patterson, T., 2002. *Candida glabrata* oropharyngeal candidiasis in patients receiving radiation treatment for head and neck cancer. J. Clin. Microbiol. 40, 1879–1881.

Ruhnke, M., 2006. Epidemiology of *Candida albicans* infections and role of non-*Candida-albicans* yeasts. Curr. Drug Target. 7, 495–504.

Salminen, J.P., Ossipov, V., Loponen, J., Haukioja, E., Pihlaja, K., 1999. Characterisation of hydrolysable tannins from leaves of *Betula pubescens* by high performance liquid chromatography mass spectrometry. J. Chromatogr. A 864, 283–294.

Sanz, M., Cadahia, E., Esteruelas, E., Muñoz, A.M., Simon, B.F., Hernandez, T., Estrella, I., 2010. Phenolic compounds in chestnut (*Castanea sativa* Mill) heartwood. Effect of toasting at cooperage. J. Agric. Food Chem. 58, 9631–9640.

Shilova, I.V., Krasnov, E.A., Korotkova, E.I., Nagaev, M.G., Lukina, A.N., 2006. Antioxidant properties of extracts from the above-ground parts of *Filipendula ulmaria*. Pharm. Chem. J. 40, 660–662.

Silva, S., Henriques, M., Martins, A., Oliveira, R., Williams, D., Azeredo, J., 2010. Silicone colonization by non-*Candida albicans Candida* species in the presence of urine. J. Med. Microbiol. 59, 747–754.

Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D., Azeredo, J., 2011. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology epidemiology, pathogenicity and antifungal resistance. *FEMS* Microbiol. Rev. 36, 288–305.

Soll, D.R., 2002. Candida commensalism and virulence: the evolution of phenotypic plasticity. Acta Trop. 81, 101–110.

Tepe, B., Daferera, D., Sokmen, M., Polissiou, M., Sokmen, A., 2004. In vitro antimicrobial and antioxidant activities of the essential oils and various extracts of *Thymus eigii*. J. Agric. Food Chem. 52, 1132–1137.

Zywicki, B., Reemtsma, T., Jekel, M., 2002. Analysis of commercial vegetable tanning agents by reverse-phase liquid chromatography-electrospray ionization-tandem mass spectrometry and its application to wastewater. J. Chromatogr. A 970, 191–200.