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Analysis of condensed and hydrolysable tannins from commercial plant extracts

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Abstract

High performance liquid chromatography (HPLC)/DAD and MS qualitative and quantitative analyses of polyphenols, hydrolysable and condensed tannins from *Pinus maritima* L. and tannic acid (TA) extracts were performed using normal and reverse phase.

Normal-phase HPLC was more suitable for pine bark (PBE) and tannic acid extracts analysis. The chromatographic profile revealed that *P. maritima* L. extract was mainly composed by polymeric flavanols (containing from two to seven units) and tannic acid (characterized by a mixture of glucose gallates containing from three to seven units of gallic acid).

Concerning their antimycotic properties, *P. maritima* L. extract exhibited a broad activity towards yeast strains of the genera *Candida*, *Cryptococcus*, *Filobasidiella*, *Issatchenkia*, *Saccharomyces*: MICs from 200 to 4000 μg/ml (corresponding to 140–2800 μg/ml of active polyphenols) were determined. Conversely, no activity of tannic acid was observed over the same target microorganisms. Taken into consideration the above results of HPLC analysis and on the basis of the current literature, we may conclude that only 70.2% of polyphenols (recognized as condensed tannins) occurring in *P. maritima* L. extract can be apparently considered responsible for its antimycotic activity.

Keywords: HPLC/DAD/MS; Normal phase; Pine bark extract; Tannic acid; Yeasts; MICs

1. Introduction

Among all known natural drugs, those originating from plant tissues have been celebrated since antiquity as an apparently limitless source of novel antimicrobial molecules [1–14]. Among them, catechins (as well as their galloyl-derivatives) are the well-known class of compounds exhibiting antimicrobial activity worthy of note [7,8,15–20]. In particular, a recent study [21] pointed out that epicatechin-3-*O*-gallate and epigallocatechin-3-*O*-gallate (occurring in leaf extracts of *Camellia sinensis* L.) demonstrated to possess a widespread antimycotic activity towards yeast and yeast-like microorganisms.

Condensed tannins (otherwise labeled as proanthocyanidins) are oligomers and polymers of flavan-3-ol units, which are most frequently linked either via C4–C6 or C4–C8 bonds (B-type proanthocyanidins). The most common condensed tannins occurring in plant tissues are procyanidins, which are derived from catechin or epicatechin and may contain gallic acid esters [22]. Condensed tannins are known to be able to interact with biological systems through the induction of some physiological effects, such as antioxidant, anti-allergy, anti-hypertensive, as well as antimicrobial activities [23]. Accordingly, a few plant extracts enriched in these compounds, in particular pine bark (PBE) (Pycnogenol®) and grape seed extracts (Leucoselect™ Phytosome®), have recently entered into commercial use for their antioxidant properties.

Tannic acid (TA) is a typical hydrolyzable tannin which consist of a mixture of different gallic acid esters of glucose. Similarly to condensed tannins, also tannic acid (commercial extracts enriched in hydrolysable tannins) is known for its ability to induce beneficial effects on human health through the expression of some biological activities, including antimutagenic, anticancer and antioxidant properties [24]. Recent studies revealed that its antioxidant activity seems to be correlated with its copper chelating ability [25]. In addition, its ability to reduce serum cholesterol and triglycerides, and to suppress lipogenesis

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by insulin has been documented [26–29]. On the other hand, some toxic effects related to its in vivo administration have been reported. In particular, a barium enemas containing tannic acid was found to induce fatal liver damage [30], whereas, when administrated by intra-abomasal dosage, it is considered able to damage the abomasums, liver and kidney in sheep's [31].

Notwithstanding all the above reported bioactivities, to the authors' knowledge only a few studies have been so far carried out on condensed and hydrolysable tannins as possible antimycotic agents towards eukaryotic microorganisms [32,33]. In addition, only a few detailed studies aimed at establishing the existence of correlations between the qualitative–quantitative composition of tannins in commercial plant extracts and their ability to induce physiological effects on microbiological systems have been hitherto carried out [34,35].

In order to assess both classes of compounds for their antimycotic activity towards yeast and yeast-like microorganisms, the present paper compared commercial extracts of pine bark (*Pinus maritima* L.) (containing condensed tannins as the main polyphenol constituents) with commercial tannic acid (rich in hydrolysable tannins).

2. Experimental

2.1. Materials

Methanol, acetonitrile (high performance liquid chromatography (HPLC grade) and formic acid (ACS reagent) were purchased from Aldrich Company Inc. (Milwaukee, Wisconsin, USA); methylene chloride was purchased from Riedel de Haën (Seelze, Germany). The pure standard of (+) catechin, gallic acid, protocatechuic acid and taxifolin were purchased from Extrasynthèse (Lyon, Nord-Genay, France).

Commercial extracts from pine bark (*P. maritima* L.), purchased from Farmacotecnica (Maringà-Paranà, Brazil) and of tannic acid, purchased from Fluka (Buchs SG, Switzerland), were also tested.

2.2. Sample preparation

Three milligrams of PBE and TA were separately dissolved in 1 ml of ethanol/water (pH 2 with formic acid) 70:30. Both samples were directly analyzed by HPLC/DAD and HPLC/MS.

2.3. HPLC/DAD and HPLC/MS analysis

PBE and TA were analyzed by using reverse-phase and normal-phase high performance liquid chromatography. The analysis was carried out by 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 negative ionisation mode under the following conditions: gas temperature 350 °C, nitrogen flow rate 10.0 l min^{-1}, nebulizer pressure 40 psi, quadrupole temperature 40 °C, and capillary voltage 3500 V. Fragmentors operated in the range 50–180 V, in particular 50 V for normal-phase method and 120 V for reverse-phase method.

Normal-phase HPLC: A Supelcosil LC-Si column (4.6 mm × 250 mm; 5 μm) (Supelco Inc., Supelco Park, Bellefonte, PA) was used. The mobile phase was (A) MeOH–HCOOH–H_2O 97:2:1 and (B) CH_2Cl_2–MeOH–HCOOH–H_2O 83:14:2:1. The elution conditions were 0.75 ml/min and a linear gradient from 0 to 60% A in 50 min according to Kennedy and Waterhouse [36].

Quantification of individual compounds was performed using a five-point regression curve, each point in duplicate, developed through the use of authentic standards operating in the range 0–10 μg (amount in peak area). Calibration curves with $r^2 \geq 0.998$ were considered. The quantification was performed at 280 nm, using (+) catechin, gallic acid, protocatechuic acid and taxifolin. The PBE reported values, expressed as mg/g of powder, are the means of three determinations and were obtained by applying the correction for molecular weight.

Reversed-phase HPLC: A LiChrosorb RP18 column (4.6 mm × 250 mm; 5 μm) (Merck Darmstadt, Germany) was used. The eluents were H_2O (pH 3.2 by H_3PO_4) and CH_3CN. A multi-step linear solvent gradient was used, starting from 100% H_2O up to 100% CH_3CN, over a 106 min period, at a flow rate of 1 ml/min [37].

Identification of condensed and hydrolysable tannins was carried out on the basis of their retention times, spectroscopic and spectrometric data, using authentic standards, isolated and synthesized compounds [38].

2.4. Microorganisms and media

Twenty-four yeast (belonging to 13 species of nine genera) and three yeast-like (*Prototheca* spp.) strains, belonging to either well-known or emerging pathogenic species [39–42] were used as target microorganisms. All strains are conserved in the Industrial Yeast Collection DBVPG, University of Perugia, Italy, www.agr.unipg.it/dbvpg.

2.5. Determination of the antimycotic activity spectrum

The antimycotic activity spectrum of both PBE and TA was evaluated by using the agar diffusion well bioassay (ADWB) [21,42]. Amphotericin B (AmB) and ketoconazole (Keto) (Calbiochem Inc., USA) were also tested as antimycotic control agents. All tests were carried out in triplicate.

2.6. Determination of minimal inhibitory concentration (MIC)

MICs of PBE, AmB and Keto were determined in 96-well microplates (Corning Inc., USA), in agreement with the NCCLS recommendations [43].

2.7. Assessment of fungistatic/fungicidal activity of PBE

Cells of *Candida glabrata* DBVPG 3828, obtained as reported [21], were inoculated (10^6 cells/ml) in test medium [Yeast Nitrogen Base broth (YNB) (Difco) +2 g/l glucose] aliquots containing increasing concentrations (range

1.75–3.25 mg/ml) of PBE. The viable cell number was monitored at 4 h intervals on YEPG agar dishes, over a period of 24 h, and compared with a control test (PBE free).

3. Results and discussion

3.1. Qualitative HPLC/DAD and MS analysis

HPLC/DAD and HPLC/MS qualitative analysis of condensed and hydrolysable tannins of commercial extract of pine bark (*P. maritima* L.) and commercial tannic acids were carried out in normal- and reverse phase. Reverse-phase chromatographic profiles for the two extracts analyzed were much more complicated and not able to separate hydrolysable tannins and oligomers of condensed tannins higher than trimers. Consequently, normal-phase HPLC is a useful technique for separating tannins by their molecular weights and Figs. 1 and 2 show the chromatographic profiles of PBE and TA, respectively.

The main constituents of PBE are known to be flavan-3-ols momomers, (+) catechin and (−) epicatechin, polymeric flavanols (procyanidins) and taxifolin. Normal-phase HPLC is more suitable for analysis of PBE as it is rich in procyanidin oligomers [22]. In the chromatogram, the degree of polymerisation of procyanidins was determined by mass data. With the increasing degree of polymerisation, procyanidins form multiple charges, so the diagnostic ions detected were mainly $[M-H]^-$, $[M-H]^{2-}$ (Table 1). The chromatographic profile shows that

Table 1
Degree of polymerisation and galloylation (DP and DG) and the observed $[M-H]^-$, $[M-H]^{2-}$ of commercial pine bark extract (PBE) and tannic acid (TA) in normal-phase analysis at 50 V of fragmentor

Extracts			*m/z* of ions	
	DP[a]	DG[b]	$[M-H]^-$	$[M-H]^{2-}$
PBE	1		289	
	2		577, (593)[c]	
	3		865, (881)[c]	
	4		1153	576
	5		1441	720
	6		1729	864
	7			1008
TA		1G[d]	169, 331	
		2G	483	
		3G	635	
		4G	787	
		5G	939	
		6G		545
		7G		621
		8G		697

[a] DP, degree of polymerisation.
[b] DG, degree of galloylation.
[c] Traces of gallocatechin dimers and trimers.
[d] G, galloyl group.

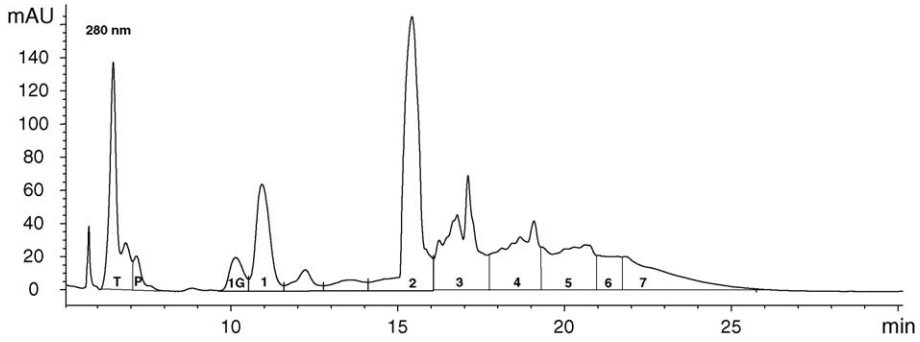

Fig. 1. Normal-phase HPLC-UV trace (280 nm) of commercial pine bark extract (PBE). The labels 1–10 indicate the degree of polymerisation of procyanidins. (T) taxifolin, (P) protocatechuic acid, (1G) gallic acid, (1) (+)catechin, (−)epicatechin and traces of gallocatechins, (2) dimers and trace of gallocatechin dimers, (3) trimers and trace of gallocatechin trimers, (4) tetramers, (5) pentamers, (6) hexamers and (7) heptamers.

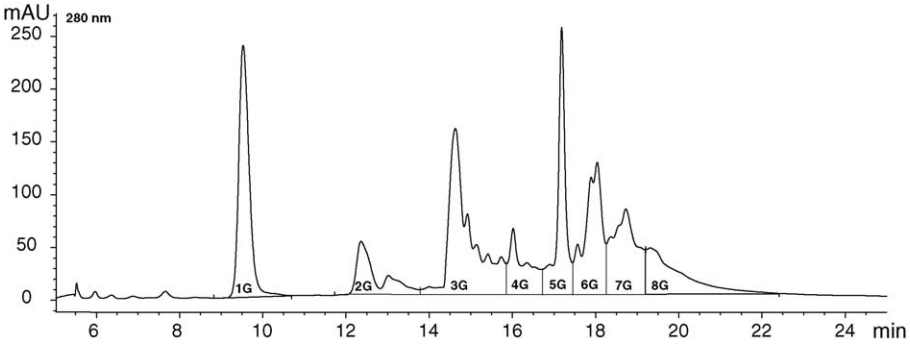

Fig. 2. Normal-phase HPLC-UV trace (280 nm) of commercial tannic acid (TA). (1G) Gallic acid and monogalloyl-glucose, (2G) digalloyl-glucose, (3G) trigalloyl-glucose, (4G) tetragalloyl-glucose, (5G) pentagalloyl-glucose, (6G) hexagalloyl-glucose, (7G) heptagalloyl-glucose and (8G) octagalloyl-glucose.

PBE is mainly composed of oligomers with two to seven units.

The constituents of TA are gallic acid esters of glucose. Also for this extract reverse and normal-phase HPLC, coupled with DAD and MS detector, were applied. The best results were again obtained in normal-phases HPLC and the chromatographic profile reported in Fig. 2 shows that TA is composed of a mixture of glucose gallates containing three to seven units of gallic acid. The main ions identified for TA are shown in Table 1.

3.2. Antimycotic activity spectrum

PBE exhibited a widespread antimycotic activity spectrum towards strains of the species *C. glabrata*, *Candida parapsilosis*, *Candida zeylanoides*, *Issatchenkia orientalis*, *Saccharomyces cerevisiae*, *Cryptococcus laurentii* and *Filobasidiella neoformans* (Table 2). On the other hand, no activity of TA was observed against the same target microorganism panel. Accordingly, we used TA as negative control in order to demonstrate that its hydrolysable tannins were unable to express such activity. Forty-eight percent strains exhibited no susceptibility to 100 μg/ml amphotericin B, whereas 37% to 100 μg/ml ketoconazole, respectively. In some cases, PBE was active towards amphotericin B- or ketoconazole-insensitive strains (Table 2).

Table 3
Quantitative polyphenols composition of commercial pine bark extract (PBE) obtained in normal phase (expressed in mg/g of powder)

Compound	mg/g
Taxifolin	33.1
Protocatechuic acid	9.1
Gallic acid	24.4
Catechin, epicatechin and gallocatechins	74.2
Procyanidin and gallocatechin dimers	285.3
Procyanidin and gallocatechin trimers	177.7
Procyanidin tetramers	124.0
Procyanidin pentamers	90.9
Procyanidin hexamers	8.8
Procyanidin heptamers	15.7
Total contents	843.0

Data reported are the means of three determinations (S.E. <10%).

3.3. Quantitative analysis of PBE

The quantitative analysis of PBE carried out in normal phase is reported in Table 3: 84.3% of its composition is constituted by polyphenols.

Since preliminary tests, carried out by using the pure polyphenols occurring in PBE, indicated that a detectable antimycotic activity can be observed only in epicatechin-3-*O*-gallate and

Table 2
Antimycotic activity of commercial pine bark extract (PBE), amphotericin B (AmB), and ketoconazole (Keto) towards yeast and yeast-like strains

Species	DBVPG accession number	Diameter of inhibition halos (mm)		
		PBE 3000 μg/ml[a]	AmB 100 μg/ml[b]	Keto 100 μg/ml[c]
Candida albicans	6133	n.a.	22.5	60.8
Candida albicans	6157	n.a.	16.4	73.6
Candida glabrata	7212	21.9	17.0	n.a.
Candida glabrata	3828	20.3	18.7	n.a.
Pichia guilliermondii	6140	n.a.	n.a.	64.5
Candida parapsilosis	6150	18.2	n.a.	65.6
Candida tropicalis	3982	n.a.	n.a.	n.a.
Candida zeylanoides	6163	16.3	18.0	58.8
Clavispora lusitaniae	6142	n.a.	n.a.	67.7
Clavispora lusitaniae	6148	n.a.	n.a.	64.6
Issatchenkia orientalis	6782	13.8	n.a.	31.1
Kluyveromyces marxianus	6141	n.a.	n.a.	72.4
Saccharomyces cerevisiae	6173	20.7	n.a.	n.a.
Saccharomyces cerevisiae	6497	17.5	n.a.	n.a.
Saccharomyces cerevisiae	6500	14.3	n.a.	n.a.
Yarrowia lipolitica	6053	n.a.	n.a.	46.0
Cryptococcus laurentii	3883	14.2	14.2	45.2
Cryptococcus laurentii	4272	16.3	27.7	n.a.
Cryptococcus laurentii	6265	18.1	15.7	47.9
Filobasidiella neoformans	3428	15.1	20.5	56.6
Filobasidiella neoformans	6010	18.5	15.9	50.4
Filobasidiella neoformans	6225	17.7	13.1	50.8
Filobasidiella neoformans	6981	19.3	16.2	43.5
Filobasidiella neoformans	6982	17.4	19.0	53.4
Prototheca wickerhamii	8879	n.a.	16.6	n.a.
Prototheca zopfii	8880	n.a.	n.a.	n.a.
Prototheca zopfii	8830	n.a.	n.a.	n.a.

n.a. = no activity.
[a] Commercial extracts from bark of pine (*Pinus maritima* L.).
[b] Amphotericin B.
[c] Ketoconazole.

Table 4
Minimal inhibitory concentration (MIC) of commercial pine bark extract (PBE), amphotericin B (AmB), and ketoconazole (Keto) towards yeast strains

Species	DBVPG accession number	MICs		
		PBE μg/ml[a]	AmB μg/ml[b]	Keto μg/ml[c]
Candida glabrata	3828	1800	1.6	2.5
Candida zeylanoides	6163	200	0.9	2.5
Candida parapsilosis	6150	1000	>100	5.0
Cryptococcus laurentii	6265	600	2.0	48.2
Filobasidiella neoformans	6010	1800	1.5	48.1
Issatchenkia orientalis	6782	4000	>100	5.0
Saccharomyces cerevisiae	6173	400	>100	>100

[a] Commercial extracts from bark of pine (*Pinus maritima* L.).
[b] Amphotericin B.
[c] Ketoconazole.

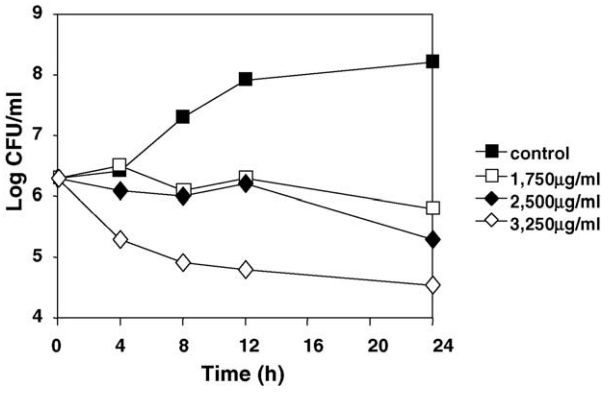

Fig. 3. Assessment of fungistatic/fungicidal activity of commercial pine bark extract (PBE) towards *Candida glabrata* DBVPG 3828.

epigallocatechin-3-*O*-gallate, but not in protocatechuic acid, gallic acid, (+) catechin, (−) epicatechin or taxifolin [21], we may conclude that only 70.2% of polyphenols occurring in PBE (3 mg/ml) can be apparently considered responsible for its observed antimycotic activity.

3.4. Determination of minimal inhibitory concentration and assessment of fungistatic/fungicidal activity of PBE

MICs of PBE from 200 to 4000 μg/ml were observed. On the other hand, control antibiotics exhibited MIC values from 0.9 to >100 μg/ml and from 2.5 to >100 μg/ml, for AmB and Keto, respectively (Table 4).

The effect of PBE on yeast cells was apparently dose-dependent (Fig. 3). Concentrations of PBE ≥3250 μg/ml caused a rapid decrease of viable cells of *C. glabrata* DBVPG 3828 (about two logarithmic numbers) whereas concentrations within the range 1750–2500 μg/ml appeared to be only fungistatic (Fig. 3).

4. Conclusions

On the basis of above results, we may conclude that extracts from pine bark were found to possess a broad antimycotic activity towards some yeast species of biomedical interest. To the author's knowledge, this represents the first evidence on the existence of a relationship between the occurrence of condensed tannins (different oligomers) in *P. maritima* L. extracts and the expression of an antimycotic activity. In a few cases, PBE even exhibited an antimycotic activity against strains insensitive to concentrations (>100 μg/ml) of amphotericin B or ketoconazole.

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