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Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 415–420
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Analysis of condensed and hydrolysable tannins from commercial plant extracts

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Received 11 August 2005; received in revised form 25 November 2005; accepted 29 November 2005
Available online 10 January 2006

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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doi:10.1016/j.jpba.2005.11.031

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