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Analytical Methods

# HPLC/DAD/ESI-MS analyses and anti-radical activity of hydrolyzable tannins from different vegetal species

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ABSTRACT

In the present work, several tannin extracts from myrtle and pomegranate were analyzed and characterized using HPLC/DAD/ESI-MS methods. Both aqueous and hydroalcoholic myrtle leaf extracts were found to be rich in galloyl-glucosides, galloyl-quinic acids, ellagitannins and flavonoids. In these extracts we observed a predominance of galloyl-glucosides and galloyl-quinic derivatives with respect to ellagic derivatives; 87.14% and 12.86%, respectively, average gallic and ellagic derivatives with respect to total tannins content. In pomegranate extracts, substantial differences were found in polyphenol contents between peel and seed extracts with regard to the relative abundance of gallic and ellagic acid derivatives: 28.81% and 71.19%, respectively, as average total gallic and ellagic derivatives in peel extracts; 61.30% and 38.70%, in seed extracts. The antiradical properties were evaluated and compared to those of two commercial extracts of chestnut bark and grape seeds. The collected data could suggest the use of myrtle and pomegranate extracts as nutraceuticals and functional foods for their important antioxidant properties.



## 1. Introduction

Tannins are common secondary metabolites in vascular plants and they are mainly found in leaf, bud, seed, root and stem tissues, and also in the heartwood of conifers. Thanks to their antimicrobial activity, their main functions concern plant defence from many pathogen attacks, and also from the action of herbivorous animals, making assimilation of substances contained in the plants difficult, and giving them an unpleasant taste. Tannins have been used since ancient times as mordents in textiles and for the tanning of leather. Useful tannic compounds were found in the gall of walnut-tree, in the barks of trees, such as chestnut tree and oak, or in the leaves of bramble. Other plants rich in tannins, such as pomegranate (*Punica granatum* L.), were used for their mordent compounds but also for their dyeing ability. Nowadays tannins are used for fixing dyeing substances on fabrics, in the press and dye of paper, to prepare lacquers and inks, for the production of glue and to clarify wines.

On the basis of their structural characteristics, tannins are classified into two main groups: hydrolyzable tannins (HTs) and condensed tannins (CTs). In the HTs a carbohydrate, usually D-glucose, is partially or totally esterified with phenolic groups such as gallic acid (gallotannins, GTs) or ellagic acid (ellagitannins, ETs). Tannic acid, generally obtained from chestnut (*Castanea sativa* M.) aqueous extract, is a typical product containing hydrolyzable tannins, which consists of a not yet clearly identified mixture of different gallic acid esters of glucose. Condensed tannins (CTs) are oligomeric or polymeric flavonoids, based on flavan 3-ol units (commonly catechin or epicatechin) linked via a carbon–carbon bond (Hagerman, Tannin chemistry (e-book), 2002; Romani et al., 2006a).

For this class of compounds recent studies have documented several biological properties which make them suitable not only for use in textiles/dyeing, but also for other applications in cosmetics, medicine, agronomy and phytotherapy (Bhatnagar & Minocha, 2010; Bialonska, Kasimsetty, Schrader, & Ferreira, 2009; Buzzini et al., 2008; Lee, Chen, Liang, & Wang, 2010; Lupini, Cecchinato, Scagliarini, Graziani, & Catelli, 2009; Okuda, 2005).

Tannic acid 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. Recent studies revealed that its antioxidant activity seems to be correlated with its copper chelating capacity (Andrade et al., 2005). In addition, its ability to reduce serum cholesterol and triglycerides, and to suppress lipogenesis by insulin has been documented (Ong, Khoo, & Das, 1995; Yugarani, Tan, & Das, 1993).

It is known that CTs are 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. Accordingly, a few enriched plant extracts, in particular pine bark (Pycnogenol®) and grape seed extracts (Leucoselect™, Phytosome®), have recently entered into commercial use for their

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antioxidant properties. *Vitis vinifera* L. extracts, from seed and leaves, are the main ingredients of several remedies in traditional and folk medicine. Recent studies report radical scavenger, antioxidant, antimicrobial and antiproliferative properties for these extracts (Lizarraga et al., 2007; Romani et al., 2006a).

The extracts obtained from *Myrtus communis* L. leaves are very rich in polyphenols (Romani et al., 2004). In particular, they contain galloyl-glucosides, gallic and ellagic tannins, galloyl-quinic acids and flavonol glycosides; their biological activities are mainly antioxidant and antimicrobial (Romani et al., 2006b). Myrtle is used traditionally as an herb and culinary spice, and in folk medicine it is employed as an antiseptic, anti-inflammatory agent and in the treatment of diabetes mellitus (Romani et al., 2004; Yoshimura, Amakura, Tokuhara, & Yoshida, 2008).

Radical scavenging, antimicrobial and antiviral activities for HTs contained in chestnut extracts have been investigated (Lupini et al., 2009; Živković et al., 2009).

Pomegranate extracts are very rich both in GTs and ETs. Pomegranate juice is used in cosmetics and foods for its antioxidant activity (Lansky & Newman, 2007), but other properties are documented for pomegranate extracts: they have demonstrated antitumour (Khan, Afaq, Kweon, Kim, & Mukhtar, 2007; Lansky & Newman, 2007), anti-hepatotoxicity (Kaur, Jabbar, Athar, & Sarwar Alam, 2006), anti-lipoperoxidation and antibacteria (Reddy, Gupta, Jacob, Khan, & Ferreira, 2007) activities. Pomegranate was often used as an astringent agent, for eliminating parasites and as an antipyretic (Lee et al., 2010); the traditional use of bark, root, stem, peel and seed extracts as an antielmintic, astringent, thirst quenching (seeds), anticatarrhal agent and in gingivitis and pyorrhoea is documented for Italy and Bulgaria (Leporatti & Ivancheva, 2003).

In the present work, several extracts enriched in tannins obtained from myrtle and pomegranate have been analyzed and characterized using HPLC/DAD/ESI-MS methods; their antiradical activity has also been evaluated by spectrophotometric method with DPPH test. The registered antiradical activities have also been compared with those of commercial samples of grape and chestnut extracts. The aim of this work was to select Italian areas where vegetal species are cultivated and processed for the food industry and where raw materials from pruning practices, biomass, and waste could be employed as innovative sources of biomolecules and standardized extracts.

## 2. Materials and methods

### 2.1. Plant materials

Pomegranate samples were chosen from selected geographical areas (Latina and Grosseto) to offer added value to indigenous crops and cultivated ecotypes. *Punica granatum* L. fruits were collected in Grosseto (Tuscany, Italy) in September 2009 and in Latina (Lazio, Italy), in January, February and October 2010. Myrtle leaves were collected from a protected area of the Island of Elba, where there is already liqueur production from the berries. *Myrtus communis* L. samples were collected in August 2009 and April 2010.

The extract of *C. sativa* M. bark was the commercial Saviotan® by Nuova Rivart Srl (Viadana, Mn, Italy); the *V. vinifera* L. seed extract was the commercial extract marketed by SOCHIM International SpA (Cornaredo, Mi, Italy).

### 2.2. Standard and solvents

Gallic acid, ellagic acid, myricetin 3-*O*-rhamnoside (myricitrin), rutin and (+) catechin were purchased from Extrasynthese S.A. (Lyon, Nord-Genay, France). Tannic acid and DPPH radical were of analytical grade and were purchased from Sigma (St. Louis, MO, USA). All solvents (HPLC grade) and formic acid (ACS reagent) were purchased from Aldrich Company Inc. (Milwaukee, Wisconsin, USA).

### 2.3. Extraction procedure

The weight/volume ratio of the extracted material was 15% for aqueous extract of myrtle green leaves (August 2009), 10% for other myrtle leaf extracts and ranged from 11% to 13% for pomegranate extracts (both peel and seed), except for seeds from Latina collected in February 2010 (18%).

The following aqueous extracts were prepared: pomegranate peel, pomegranate seeds, myrtle green and dried leaves. The fresh plant tissues were frozen in liquid nitrogen, crushed in a mortar, then extracted with ultra pure water (MilliQ system waters Co., Milford, MA, USA) at ebullition temperature. The extracts were then concentrated under vacuum (Rotavapor 144 R, Büchi, Switzerland) and, finally, rinsed with ultra pure water for the HPLC/DAD/ESI-MS analyses. For each sample, part of the extract was lyophilized and stored at room temperature until use; they remained stable for at least 12 months.

The hydroalcoholic extract of myrtle dried leaves was prepared using 70% EtOH. The extract was completely defatted with *n*-hexane, concentrated under vacuum, and analyzed by HPLC/DAD/ESI-MS. The remaining extract, rinsed with water, was then lyophilized and stored at room temperature.

In the case of pomegranate seed hydroalcoholic extracts, the fresh material was frozen in liquid nitrogen, crushed in a mortar, then used for extraction in 70% EtOH. Pomegranate seeds were extracted both at room temperature and under heat-reflux extraction for 1 h.

The extracts were, finally, concentrated under vacuum and analyzed by HPLC/DAD/ESI-MS. For each sample, part of the extract was rinsed with water, then lyophilized, and stored at room temperature.

### 2.4. HPLC/DAD/ESI-MS analysis

The HPLC/DAD/ESI-MS analyses were conducted using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent Technologies) operating both in negative and positive ionization mode.

For HT analysis a Luna C18 column 250 × 4.60 mm, 5 μm (Phenomenex), operating al 26 °C was used. The eluents were $H_2O$ (adjusted to pH 3.2 by HCOOH) and $CH_3CN$. A four-step linear solvent gradient starting from 100% $H_2O$ (A) up to 100% $CH_3CN$ (B) was performed with a flow rate of 0.8 mL $min^{-1}$ over a 55-min period as previously described (Arapitsas, Menichetti, Vincieri, & Romani, 2007).

For CT analysis the column was a LiChrosorb RP18 250 × 4.60 mm, 5 μm (Merk Darmstadt, Germany). The eluents were $H_2O$ adjusted to pH 3.2 by HCOOH (A) and $CH_3CN$ (B). An eight-step linear solvent gradient starting from 100% $H_2O$ (A) up to 100% $CH_3CN$ (B) was performed with a slightly modified method (Romani et al., 2004), at a flow rate of 0.8 mL $min^{-1}$ over a 106-min period.

Mass spectrometer operating conditions were: gas temperature 350 °C at a flow rate of 10.0 L $min^{-1}$, nebulizer pressure 30 psi, quadrupole temperature 30 °C and capillary voltage 3500 V. The fragmentor was 120 eV.

Identification of individual polyphenols was carried out using their retention times, and both spectroscopic and spectrometric data. Quantification of the single polyphenols was directly performed by HPLC/DAD using a five-point regression curve built with the available standards. Curves with an $r^2 > 0.9998$ were considered. Calibration was performed at the wavelength of the maxi-