# IDENTIFICATION AND QUANTITATION OF POLYPHENOLS IN LEAVES OF MYRTUS COMMUNIS L.

# Identification and Quantitation of Polyphenols in Leaves of *Myrtus communis* L.

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## Key Words

Column liquid chromatography
DAD and MS detection
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Catechin derivatives
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## Summary

A liquid-solid extraction and purification procedure (LSE) was developed to identify and quantify polyphenols in the leaf tissue of *Myrtus communis* L. Identification and quantitation of individual compounds was performed using HPTLC, HPLC-DAD and HPLC-MS analysis. Leaves of *Myrtus communis* L. contain small amounts of phenolic acids (caffeic, ellagic and gallic acids) and quercetin derivatives (quercetin 3-O-galactoside and quercetin 3-O-rhamnoside), whereas catechin derivatives (epigallocatechin, epigallocatechin 3-O-gallate, epicatechin 3-O-gallate) and myricetin derivatives (myricetin 3-O-galactoside, myricetin 3-O-rhamnoside) are present in large amounts. This is the first report on the occurrence of galloyl-derivatives of catechin and gallo-catechin in *Myrtus communis* L. leaves.

## Introduction

*Myrtus communis* L. is a species typically evolved in the Mediterranean basin [1, 2], but the presence of this shrub has also been reported in areas of the Middle East and Asia [3, 4]. *Myrtus communis* L. usually faces water deficit, high temperatures and excess of UV-B radiation during the summer season [5, 6]. Other schlerophyllous shrubs typically evolved under similar environments, namely *Olea europaea* L. and *Phillyrea* spp., present an interesting spectrum of leaf polyphenolic compounds [7, 8, 9]. It has been shown that polyphenols protect leaf cell metabolism of plants exposed to high temperatures and excess of UV-B radiation [10].

*Myrtus communis* L. tissues are generally used in the preparation of extracts with important pharmacological and antimicrobial activity that is usually ascribed to essential oils, polyphenols and hydrolizable tannins [3, 4, 11, 12]. Thus identification and quantitation of polyphenolic compounds in leaves of *Myrtus communis* L. appears interesting from both a biological and ecophysiological point of view.

Various polyphenols have been previously detected in myrtle tissues, but in most cases the identity of the compounds was not conclusive and quantitation was not carried out [1, 2, 13, 14). In the present work we report data on the extraction and purification procedures, identification and quantitation of polyphenolic compounds in leaves of *Myrtus communis* L. To the best of our knowledge this is the first report on the quantitation of individual polyphenols in *Myrtus communis* L. tissues.

## Experimental

### Sample Preparation

Fully-expanded leaves were collected from one-year-old plants grown in containers and supplied twice a week during September with a half strength Hoagland solution [15]. The main vein was excised from the leaf lamina and the tissue was rapidly quenched in liquid nitrogen and stored at –80 °C before proceeding with the analysis. The leaf material was then ground in a mortar with a pestle under liquid nitrogen.

### Extraction and Purification Procedures

*Extraction*

The leaf tissue (1–2 g) was extracted with 4 × 80 mL of 70 % EtOH. The raw ethanolic extract was then concentrated under vacuum (Rotavapor 144R, Büchi, Switzerland) and rinsed with ultrapure water (Milli Q System, Waters Co., Milford, MA, USA) to reach a final volume of 100 mL. The solution was then extracted with 4 × 50 mL n-hexane to completely remove lipophilic compounds. The ethanolic extract was concentrated under reduced pressure and rinsed with water (pH 2 by HCOOH) to 10 mL final volume, similarly to the protocol previously reported by Romani et al. [9].



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*Purification*

Purification was carried out by using a liquid-solid extraction (LSE) procedure. 10 mL of the aqueous solution were deposited on a 20 mL Extrelut® cartridge (Merck, Darmstat, Germany) and after 20 min, eluted using: **1)** 200 mL of n-hexane to collect a colourless eluate; **2)** 250 mL of EtOAc; **3)** 250 mL of acidic MeOH (pH 2 by HCOOH).

Finally, the EtOAc and MeOH fractions were evaporated to dryness, then diluted with 1 mL of acid water (pH 2 by HCOOH) /MeOH /$CH_3CN$ 20:60:20 (v/v) and used for polyphenol analysis by HPTLC, HPLC-DAD and HPLC-MS.

## Identification and Quantitation of Individual Polyphenols

Identification of individual polyphenols was carried out by using chromatographic (HPTLC), spectroscopic or spectrometric techniques. UV-vis data were recorded by using a Diode Array Detector coupled to an HPLC system, and compared to those of authentic standards. The pure standards were purchased from Extrasynthese S.A. (Lyon, Nord-Genay, France), except for myricetin-3-O-galactoside which was isolated by centrifugal preparative chromatography similar to that previously reported for other polyphenols [9]. Characterization of polyphenols was also carried out by HPLC/API-electrospray MS.

Quantitation of individual polyphenols was performed by using a four-point regression curves ($r^2 \geq 0.99$) through the use of both authentic standards and isolated compounds. The calibration was directly performed by HPLC-DAD at maximum absorbance wavelength for each polyphenol.

## Analytical Techniques and Equipment

*HPLC-DAD Analysis*

All the analyses were carried out using an HP 1090L liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (Hewlett & Packard, Palo Alto, CA, USA). The column was a 250 × 4.6 mm (5 µm) LiChrosorb RP18 (Merck) maintained at 26 °C equipped with a 10 × 4 mm LiChrosorb RP18 pre-column. The eluent was $H_2O$ (pH 3.2 by $H_3PO_4$) /$CH_3CN$. A four-step linear solvent gradient was used, starting from 100 % $H_2O$ up to 100 % $CH_3CN$, during a 106 min period, at a flow rate of 1 mL $min^{-1}$, similar to that previously reported by Romani et al. [9]. UV-vis spectra were recorded in the range 190–450 nm, and chromatograms were acquired at 254, 280, 310, 330 and 360 nm

*HPLC-MS Analysis*

The HPLC-MS analyses were performed using an HP 1090L liquid chromatograph equipped with a DAD detector. The interface was an HP 1100 MSD API-electrospray (Hewlett & Packard). The interface geometry, with the orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of HPLC-DAD analysis. A four-step linear solvent gradient starting from 100 % $H_2O$ up to 100 % $CH_3CN$ was performed at a flow rate of 0.8 mL $min^{-1}$ during a 67 min period (Table I). The column was a 200 × 4.6 mm (5 µm) Hypersil ODS, (Phase Separations) operating at 26 °C. The eluent was $H_2O$ (adjusted to pH 3.2 by HCOOH)/$CH_3CN$ (with the addition of 0.8 % HCOOH). 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.

**Table I.** The linear solvent gradient system used in HPLC-MS analysis of polyphenols. Analysis was carried out during a 67-min period at a flow rate of 0.8 mL $min^{-1}$. Solvent was $H_2O$ (pH 3.2 by HCOOH)/$CH_3CN$ (with 0.8 % HCOOH).

| Time (min) | $H_2O$ % | $CH_3CN$ % |
|---|---|---|
| 0.1 | 100 | 0 |
| 20 | 85 | 15 |
| 25 | 85 | 15 |
| 35 | 75 | 25 |
| 43 | 75 | 25 |
| 53 | 55 | 45 |
| 57 | 55 | 45 |
| 62 | 0 | 100 |
| 67 | 0 | 100 |

*HPTLC Analysis*

Two-dimensional thin layer chromatography was performed using a Desaga horizontal-separating chamber equipped with a tight-fitting glass lid (Carlo Erba, Milano, Italy). The HPTLC plates were 5 × 5 cm Silica gel 60 $F_{254}$, ex 10 × 10 cm pre-scored (Merck), eluted with EtOAc:MeOH:$H_2O$ (77:13:4) during the first run. The plates were then dried at room temperature before the second elution with $CHCl_3$: MeOH:$H_2O$ (7:3:0.5) was performed. Finally the plates were dried and sprayed with 1 % methanolic diphenylboryloxyethylamine (Aldrich Chemicals), followed by 5 % ethanolic polyethylene glycol-4000. Spots were identified by their fluorescence at 365 nm.

# Results and Discussion

## Extraction and Purification Method

The liquid-solid fractioning procedure (LSE) allowed isolation of the polyphenols listed in Table II. The EtOAc fraction contained more than 95 % of the total polyphenols detected in leaves of *Myrtus communis* L. and can be reasonably used for quantitative analysis of several polyphenols. Analysis of the MeOH fraction was crucial in properly identifying and quantifying phe-

**Table II.** List and relative amounts of polyphenols detected in leaf mesophyll of *Myrtus communis* L. collected during September. Quantitation was performed for each polyphenol using a 4-point regression curve ($r^2 \geq 0.99$) through the use of both authentic standards and isolated compounds. The calibration was directly performed by HPLC-DAD at maximum absorbance wavelength for each polyphenol. Data are the mean of three replicated samples.

| Polyphenol | μg $g^{-1}$ dry weight |
|---|---|
| Gallic acid | 630 |
| Caffeic acid | 890 |
| Ellagic acid | 170 |
| (+) Catechin | 210 |
| (–) Epicatechin 3-O-gallate | 175 |
| (–) Epigallocatechin | 19200 |
| (–) Epigallocatechin 3-O-gallate | 2710 |
| Myricetin 3-O-galactoside | 2980 |
| Myricetin 3-O-rhamnoside | 11250 |
| Myricetin derivatives | 2610 |
| Quercetin 3-O-galactoside | 110 |
| Quercetin 3-O-rhamnoside | 300 |

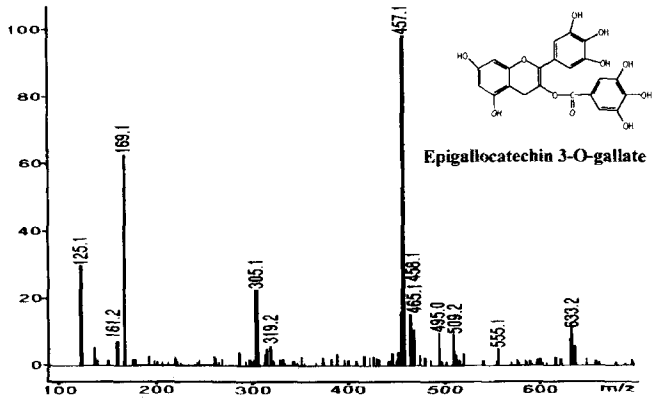


**Figure 1**

Negative ion mass spectrum of epigallocatechin 3-O-gallate acquired during the API-electrospray HPLC-MS analysis. Operating conditions: 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. Mass spectra were recorded in the range 200–700 AMU.

nolic acids, namely caffeic acid and ellagic acid. However, HPLC analysis of the latter fraction, that contains large quantities of tannins [3, 4], cannot be used for routinary analysis of polyphenols in myrtle tissues.

## Identification and Quantitation of Individual Polyphenols

On the whole, the identity of polyphenols was ascertained using data from HPLC-DAD and HPLC-MS analysis, by comparison and combination of their retention time, and UV-vis and mass spectra.

Caffeic, gallic and ellagic acids were identified by comparison of retention times and UV-vis spectra of leaf extracts with those of authentic standards, using HPLC-DAD.

Catechins and catechin derivatives were identified by comparison of their retention times and UV-vis spectra with those of authentic standards and by HPLC-MS. In Figure 1 we show the mass spectrum of (–) epigallocatechin 3-O-gallate. Three peaks were recorded at 457, 305 and 169 m/z, corresponding to the molecular ion, the fragment relative to epigallocatechin, and the fragment corresponding to gallic acid, respectively. Galloyl derivatives of both catechin and gallo-catechin have been previously detected in leaf extracts of green tea [16], but this is the first report on the identification and quantitation of these compounds in leaves of *Myrtus communis* L.

We also identified and quantified two derivatives of myricetin in myrtle leaves, namely myricetin 3-O-galactoside and myricetin 3-O-rhamnoside, and the chemical structure was fully ascertained by HPLC-MS. Figure 2 shows the mass spectrum of myricetin 3-O-galactoside, with peaks at 479 and 317 m/z, corresponding to the molecular ion and to the fragment after the loss of galactose. We also detected two additional myricetin-derivatives (Table II) but the chemical structure was not ascertained in this experiment. A myricetin derivative, namely myricetin-3-β-D [6"-O-galloyl--galactoside], has been isolated and characterized using both mass spectrometry and $^{13}$C-NMR by Pichon et al. [14]. Furthermore it has been suggested previously that myricetin 3-O-glucoside, myricetin 3-O-rhamnoglucoside, and myricetin 3,3′-O-digalactoside occur in myrtle leaves, even if their chemical structure was not fully clarified [1, 2, 17].

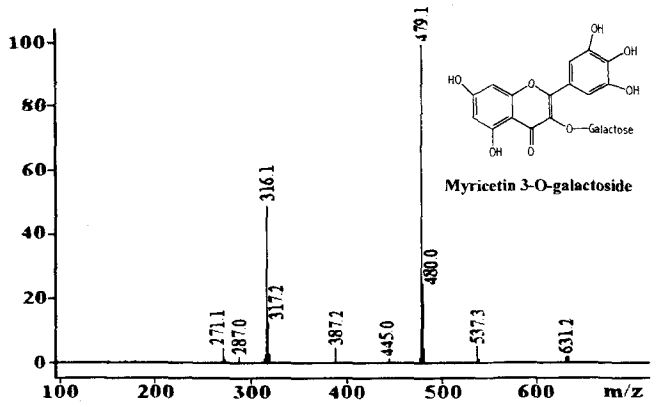


**Figure 2**

Negative ion mass spectrum of myricetin 3-O-galactoside acquired during the API-electrospray HPLC-MS analysis. Operating conditions as in Figure 1.

The presence of quercetin 3-O-rhamnoside in leaf extracts *of Myrtus communis* L. was ascertained by HPLC-MS. The fragmentation pattern reported in Figure 3 shows two signals at 447 and 301 m/z, corresponding to the molecular ion and to the fragment after the loss of rhamnose. The identification of quercetin 3-O-galactoside was carried out by HPTLC analysis, since neither HPLC-DAD (retention times of quercetin 3-O-galactoside and quercetin 3-O-glucoside differed by less than 0.6 min) nor HPLC-MS were conclusive for the

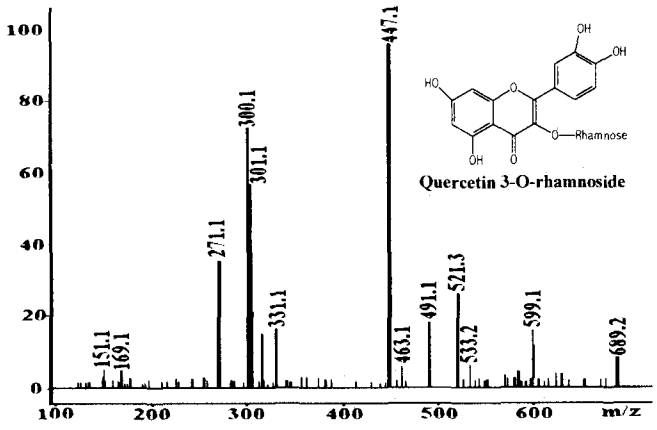


**Figure 3**

Negative ion mass spectrum of quercetin 3-O-rhamnoside acquired during the API-electrospray HPLC-MS analysis. Operating conditions as in Figure 1.

molecular characterization. Fluorescence characteristics (at 365 nm) and $R_F$ of the myrtle extract were compared with those of authentic standards of both quercetin 3-O-glucoside and quercetin 3-O-galactoside. Our findings partially agree with previous results that showed the occurrence of both quercetin 3-O-glucoside and quercetin 3-O-galactoside in *Myrtus communis* L. [12, 17]. Quercetin derivatives, with different sugar substituents, have also been previously identified in *Olea europaea* L. [8] and *Phillyrea* spp. [9].

### Polyphenol Composition of *Myrtus communis* L. Leaves

In Table II the relative amounts of polyphenols of leaf mesophyll of *Myrtus communis* L. are reported. On the whole, polyphenol content is high, representing 4 % of the dry matter, but our data do not completely agree with previous findings, probably due to differences in both analytical methods and tissue drying techniques [1].

Among polyphenols, more than 50 % is represented by galloyl derivatives of catechin and gallo-catechin. The anticargenogenic activity of these catechin derivatives have previously been demonstrated using leaf extracts of green tea [16, 18]. Recently, an interesting suggestion by Northlup et al. [19] links the evolution of tannin-rich plant communities throughout the world to their adaptation to infertile soils with inorganic nitrogen limitation, that are typical conditions in which *Myrtus communis* L. has evolved [5, 6]. Myricetin derivatives represent more than 40 % of the total polyphenols, but we don't have any reference in the literature to their relative distribution in analogous myrtle tissues. Phenolic acids and quercetin-derivatives, as previously found in leaves *of Olea europaea* and *Phillyrea* spp. [8, 9] are in relatively lower amounts than other polyphenols.

The polyphenol composition of *Myrtus communis* L. leaves does not have significant affinity with those of *Phillyrea* spp. and *Olea europaea* L., indicating different strategies to cope with similar environmental constraints [5, 6] at the level of both primary [1, 9, 20] and secondary metabolism products.

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