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Running title: **Identification of persimmon leaf polyphenols by high resolution mass spectrometry**

Title: **POLYPHENOLIC PROFILE OF PERSIMMON LEAVES BY HIGH RESOLUTION MASS SPECTROMETRY (LC–ESI-LTQ-ORBITRAP-MS)**

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ABSTRACT

Persimmon leaves have played an important role in Chinese medicine. Persimmon extracts and formulations have been shown to possess a wide range of pharmacological activities, including antioxidant, hypolipidemic and antidiabetic, and they have been used to treat cardiovascular disease, improve homeostasis, as antibacterial and anti-inflammatory agents, and as a beauty treatment. In this work, liquid chromatography coupled to hybrid linear ion trap quadrupole Orbitrap mass spectrometry was used to accurately identify persimmon leaf polyphenols. Forty two phenolic compounds, including simple phenolic acids, hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavonols, flavanones, flavone-chalcones, tyrosols and their conjugated derivatives, were identified and quantified using high mass accuracy data and confirmed by MS^2 experiments. To the best of our knowledge, this is the most extensive study of persimmon leaf polyphenols performed so far, since 33 phenolic compounds are reported for the first time.

Keywords: Persimmon leaves; polyphenols; identification; Orbitrap-MS; liquid chromatography; flavonoids; cinnamic acid; hydroxybenzoic acids; hydroxytyrosol,

1. INTRODUCTION

Persimmon (Diospyros kaki L.) leaves, known as Shi Ye in Chinese, have a long history in Chinese traditional medicine for the treatment of ischemia stroke, angina, internal hemorrhage, hypertension, atherosclerosis and some infectious diseases. (Kotani et al., 2000 & Matsumoto et al., 2002; Tanaka et al., 2003; Sakanaka et al., 2005). Additionally, persimmon leaves are increasingly popular as constituents of health and cosmetic products in Asian countries, such as Japan, Korea and China. The leaves have been used as health-promoting beverages for centuries and are one of the most popular infusions in China and Japan.

Persimmon leaves contain a high amount of vitamin C (Mizuo, 1995; Hiromichi, 2002), flavonoids, which are the main constituents and are considered to be the active compounds (Liu et al., 2012), terpenoids, which also show certain pharmacological activities (Chen et al., 1999), and other compounds such as resins, polysaccharides, chlorophylls (Hospital, 1973), carotenes, kryptoxanthin, cellulose, hemicelluloses, lignins (Hu et al., 2002), amino acids and trace elements (Zu & Lei, 1989). Flavonoids isolated from persimmon leaves present antioxidant activity (Sun et al., 2014), hypotensive (Kameda et al., 1987) and anti-allergic effects (Kotani et al., 2000). The major polyphenols in persimmon leaves are proanthocyanidins, which have anti-hypertensive and vasorelaxant effects (Kawakami et al., 2011). Some *in vitro* studies have suggested they may also have beneficial effects on diabetes (Kawakami et al., 2010; Wang et al., 2011). Therefore persimmon leaf may be used as a functional drink or as functional ingredient to add healthy and therapeutic properties in certain foods. However, although some phenolic compounds in persimmon leaves have been reported, including kaempferol and quercetin (Chou, 1984), isoquercetin (Nakatani et al. 1989), myricitrin (Guo & Dong, 1999) and many flavonol glucosides (Chou, 1984; Chen et al., 2002), a comprehensive phenolic profiling by high resolution mass spectrometry is lacking.

LTQ-Orbitrap mass spectrometry has been used in previous studies to identify polyphenols in different food matrices such as tomato (Vallverdú-Queralt et al., 2011), walnut (Vallverdú-Queratl, 2014) beer (Quifer-Rada et al., 2015), wine (Vallverdú-Queralt et al., 2015) and culinary herbs (Vallverdú-Queralt et al., 2014) and it has proven to be a reliable tool for

structural elucidation of unknown polyphenols in complex samples. The aim of this work was to identify the full range of polyphenols found in persimmon leaves have not yet been described.

2. MATERIALS AND METHODS

2.1 Standards, reagents and materials

All solvents were HPLC grade and all chemicals were analytical reagent grade. Vanillic acid, catechin, 3-hydroxybenzoic acid, apigenin, kaempferol-3-*O*-glucoside, myricetin, isorharmnetin and isoquercetin were purchased from Fluka (St. Louis, MO, USA). Gallic acid, caffeic acid, 3,5-dimethoxybenzoic acid, 2,5-dihydroxybenzoic acid, *p*-coumaric acid, sinapic acid, naringenin, quercetin, chlorogenic acid and 3,5-dhydroxybenzoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxytyrosol, kaempferol, procyanidin B1 and naringenin chalcone were purchased from Extrasynthese (Lyon, France). Ethanol, methanol and HPLC-grade formic acid were obtained from Scharlau (Barcelona, Spain) and ultrapure water (Milli-Q) from Millipore (Billerica, MA, USA). Samples were stored in the shade and protected from light until analysis.

2.2 Extraction and analysis of polyphenols

2.2.1 Samples

Persimmon leaves (Diospyros kaki, Rojo Brillante var.) were picked from trees in an orchard in Valencia (Spain), blanched at 100 ºC for 5 min and dried at 100 ºC for 30 min in a convective drier. This drying method was chosen because in a previous study it allowed a good preservation of the antioxidant properties of persimmon leaves (Martínez-Las Heras, Heredia, Castelló & Andrés, 2014).

2.2.2 Extraction of polyphenols

Samples were treated in a darkened room with a red safety light to avoid oxidation of the analytes. The extraction of polyphenols was done following the procedure of Rodríguez-Pérez et al., 2013 with some modifications. First, 0.2 g of persimmon leaves was extracted using 8 mL of methanol/water (80:20, v/v) in an ultrasound bath (Sonorex, Bandelin) for 15 min at room temperature. Then, the samples were centrifuged for 15 min at 2700 g at 4 ºC to remove solids. After centrifugation, the pellets were again extracted with fresh solvent under the same

conditions. The supernatants were combined and evaporated under nitrogen flow, and the residue was reconstituted with water 0.2% formic acid up to 3 mL and filtered through a 0.22 µm PTFE filter into an amber vial for HPLC analysis. Samples were stored at -20 ºC until analysis.

2.2.3 LC-ESI-LTQ-Orbitrap-MS

An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative mode was used for accurate mass measurements. Mass spectra were acquired in profile mode with a resolution setting of 30,000 at m/z 400. Operation parameters were as follows: source voltage, 4 kV; sheath gas, 20 (arbitrary units); auxiliary gas, 10 (arbitrary units); sweep gas, 2 (arbitrary units); and capillary temperature, 275 °C. Default values were used for most other acquisition parameters (FT Automatic gain control (AGC) target 5·105 for MS mode and 5·104 for MS^n mode). Persimmon leaf samples were analysed in full scan mode at a resolving power of 30,000 at m/z 400 and data-dependent MS/MS events acquired at a resolving power of 15,000. The most intense ions detected during full scan MS triggered data-dependent scanning. Data-dependent scanning was carried out without the use of a parent ion list. Ions that were not intense enough for a data-dependent scan were analysed in MS^2 mode with the Orbitrap resolution also set at 15,000 at m/z 400. An isolation width of 100 amu was used and precursors were fragmented by a collision-induced dissociation C-trap (CID) with normalised collision energy of 35 V and an activation time of 10 ms. The mass range in FTMS mode was from m/z 100 to 1000. The data analysis was achieved using XCalibur software v2.0.7 (Thermo Fisher Scientific, Hemel Hempstead, UK).

Liquid chromatography analysis was performed using an Accela chromatograph (Thermo Scientific) equipped with a quaternary pump, a photodiode array detector (PDA) and a thermostated autosampler. Chromatographic separation was accomplished with an Atlantis T3 column 2.1x100 mm, 3µm (Waters, Milford, MA, USA). Gradient elution of analytes was carried out with water/0.1% formic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 350 µL/min, and the injection volume was 10 µL. A non-linear gradient was

applied: 0 min, 2% B; 0–2 min, 2% B; 2–5 min, 8% B; 5–14 min, 20% B; 14–18 min, 30% B; 18–22 min, 100% B; 22–24 min, 100% B; 24-25, 2%B and the column was equilibrated for 5 min to initial conditions.

Samples were quantified using pure standards when available. Some analytes, such as glycosylated forms or dimers, were quantified using the aglycon form or the monomer.

3. RESULTS AND DISCUSSION

3.1 General

Persimmon leaves are interesting for their bioactive compounds that may exert beneficial effects on human health (Xie et al., 2015). Table 1 shows a list of the 41 phenolic compounds identified by LC-ESI-LTQ-Orbitrap-MS along with their retention times, accurate mass measurements, molecular formula, error in ppm of the experimental mass compared to the theoretical mass of each polyphenol, the product ions used for identification and the concentration they are found in persimmon leaf in mg/kg. Phenolic compounds were identified by generating the molecular formula using accurate mass with some restrictions (C=30, H=100), O=15), and matching with the isotopic pattern. This molecular formula was then identified using the polyphenol database (http://phenol-explorer.eu/).

24 phenolic compounds were further confirmed by comparing the retention times, exact mass and fragmentation patterns with pure standards. Identification of the remaining 17 compounds without available standards was based on accurate mass measurements of the $[M-H]^-$ ion and the fragmentation pattern, which was compared with the literature, since the MS^2 spectra of many of these compounds have previously been reported in other studies (Quifer-Rada et al., 2015; Dou et al., 2007; Callemien et al., 2008; Jiang et al., 2015).

In total, 41 polyphenols were identified and quantified, including 9 benzoic acids, 5 hydroxycinnamic acids, 11 flavanols, 13 flavonols, 1 flavanones, 1 flavone and 1 tyrosol (Table 1).

Figure 1 shows an FTMS chromatogram of a persimmon leaf sample.

3.2 Phenolic acids

3.2.1 Benzoic acids and derivatives

Hydroxybenzoic acids have a C6–C1 chemical structure and show a characteristic loss of CO_2 [M-H-44]− in MS^2 experiments (Quifer-Rada et al., 2015; Kammerer et al., 2004; Schütz et al., 2005). The data-dependent scan and the examination of the chromatograms in FTMS mode revealed the presence of gallic acid (m/z 169.0139, 1.01 ppm), gallic acid 4-*O*-glucoside (m/z 331.0663, 2.17 ppm), 3,5-dihydroxybenzoic acid (m/z 153.0191, 1.77 ppm), 2-hydroxybenzoic acid (m/z 137.0240, 2.82 ppm), 2,5-dihydroxybenzoic acid (m/z 153.0191, 1.71 ppm), 4-hydroxybenzoic acid (m/z 137.0240, 1.87 ppm), 3-hydroxybenzoic acid (m/z 137.0240, 4.74 ppm), vanillic acid (m/z 167.0347, 1.6 ppm) and 3,5-dimethoxybenzoic acid (m/z 181.0502, 0.36 ppm). All ions showed a loss of 44 u in the MS^2 spectra. Moreover, 2,5-dihydroxybenzoic acid showed an extra fragmentation due to the oxygen loss from the carboxylic group [M-H-16]−; vanillic acid and 3,5-dimethoxybenzoic acid presented an extra [M-H-15]− loss due to the methyl group, and gallic acid *O*-glucoside had an [M-H-163]− loss due to the glucoside group. All benzoic acids, except for gallic acid 4-*O*-glucoside, were confirmed by comparing the retention time and the MS^2 spectrum with pure standards.

Gallic acid, 2-hydroxybenzoic acid and 4-hydroxybenzoic acid have previously been reported in leaves used for infusions such as anise, fennel and camomile (Proestos et al., 2006; Khalil et al., 2007). Moreover, gallic acid, *p*-hydroxybenzoic acid and vanillic acid were also found in persimmon fruit. Results show that persimmon leaves contain twice as much gallic acid as the persimmon fruit itself. In the case of vanillic acid, persimmon fruit has slightly higher levels than persimmon leaves. Finally, both contain almost the same quantity of *p*-hydroxybenzoic acid (Lee et al., 2012; Jiménez-Sánchez et al., 2015). To the best of our knowledge, this is the first time that benzoic acids have been identified in persimmon leaves.

3.2.2 Hydroxycinnamic acids and derivatives

Hydroxycinnamic acids have a C6-C3 structure with a double bond in the side chain in *cis* or *trans* configuration. A few hydroxycinnamic acids were identified after examination of the chromatograms: chlorogenic acid (m/z 353.0872, 1.77 ppm), coumaric-*O*-hexoside (m/z 325.0924, 0.3 ppm), caffeic acid (m/z 179.0345, 0.2 ppm), *p*-coumaric acid (m/z 163.0397, 2.13

ppm) and sinapic acid (m/z 223.0607, 2.09 ppm). The typical loss of CO_2 [M-H-44] was observed for all hydroxycinnamic acids except for the conjugated derivatives of chlorogenic acid and coumaric-*O*-hexoside, which showed a loss of the quinic acid m/z 191.0552 with a 0.3 ppm of error and the sugar moiety [M-H-162]-, respectively. Moreover, chlorogenic, *p*-coumaric and sinapic acids were confirmed by comparing the retention time and the MS^2 spectra with pure standards.

To the best of our knowledge, hydroxycinnamic acids have not been reported previously in persimmon leaves, however they have been previously found in persimmon fruit. Among them, we can find chlorogenic acid, caffeic acid, *p*-coumaric acid and ferulic acid, almost all in similar concentration as in persimmon leaves (Lee et al., 2012). Some hydroxycinnamic acids such as caffeic, ferulic, *o*-coumaric, *p*-coumaric and 5-caffeoylquinic acids have been found in other leaves used for infusions, such as Greek aromatic plants, and green and black tea (Proestos et al., 2006; Khalil et al., 2007; Stewart et al., 2005).

3.3 Flavonoids

Flavonoids are a large family of compounds with a common chemical structure: a diphenylpropane skeleton bearing two benzene rings (A and B) connected by a pyran ring attached to the A ring (Williams, Spencer, & Rice-Evans, 2004).

3.3.1 Flavanols and derivatives

Epigallocatechin (m/z 305.0664, 0.87 ppm), gallocatechin (m/z 305.0661, 1.98 ppm), catechin-*O*-hexoside I (451.1244, 0.49 ppm), catechin-*O*-hexoside II (m/z 451.1243, 0.56 ppm), catechin-*O*-hexoside III (m/z 451.1243, 0.58 ppm), procyanidin B1 (m/z 577.1351, 0.12 ppm), procyanidin dimer I (m/z 577.1349, 0.34 ppm), procyanidin dimer II (m/z 577.1352, 0.001 ppm), catechin (m/z 289.0712, 1.88 ppm), epicatechin 3-*O*-gallate (m/z 441.0822, 1.42 ppm) and prodelphinidin dimer B3 (m/z 609.1242, 1.44 ppm) were identified in persimmon leaves by analysing the chromatograms in FTMS. Product ions with the fragmentation pattern of epigallocatechin, gallocatechin and prodelphinidin dimer B3 have been described previously (Dou et al., 2007 & Callemien et al., 2008). Derivatives of catechin (catechin-O-hexoside I, II and III) showed the loss of the sugar moiety in MS^2 spectra. Procyanidin B1, catechin and

207 epicatechin 3-*O*-gallate were confirmed with pure standards. Figure 2 shows the MS^2 spectra of
208 procyanindin B1.
209 Many of these flavanols have been reported previously in other plant leaves used to prepare
210 infusions such as green and black tea (Dou et al., 2007; Wang et al., 2008), *Byrsonima* species
211 (Rinaldo et al., 2010), and *Styphnolobium* japonicum (Kite et al., 2007). Epigallocatechin,
212 catechin and epicatechin gallate were also found in persimmon fruit which coincide with the
213 flavanols found in leaves. The amount of catechin in persimmon leaves is 20 times higher than
214 in persimmon fruit. Nevertheless, the quantity of epigallocatechin and epicatechin gallate is
215 lower in persimmon leaves (Jiménez-Sánchez et al., 2015).
216 *3.3.2 Flavonols and derivatives*
217 Among the flavonols identified, quercetin, kaempferol, and myricetin are present in high
218 concentrations in a variety of plant-based foods and beverages (Jiang et al., 2015).
219 Myricetin-*O*-hexoside I (m/z 479.0826, 0.65 ppm), myricetin-*O*-hexoside II (m/z 479.0828,
220 0.65 ppm), isoquercetin (m/z 463.0877, 1.46 ppm), quercetin-*O*-hexoside (m/z 463.0897, 1.7
221 ppm), quercetin-*O*-pentoside I (m/z 433.077, 1.39 ppm), quercetin-*O*-pentoside II (m/z 433.077,
222 1.39 ppm), kaempferol-3-*O*-glucoside (m/z 447.0923, 2.15 ppm), kaempferol-*O*-hexoside II
223 (m/z 447.093, 0.68 ppm), myricetin (m/z 317.0296, 2.3 ppm), kaempferol-*O*-rhamnoside (m/z
224 430.0905, 0.15 ppm), quercetin (m/z 301.0347, 2.17 ppm), kaempferol (m/z 285.040, 1.65 ppm)
225 and isorhamnetin (m/z 315.0505, 1.79 ppm) were identified by analyzing the chromatograms in
226 FTMS.
227 Isoquercetin, kaempferol-3-*O*-glucoside, myricetin, quercetin, kaempferol and isorhamnetin
228 were further confirmed by comparing the chromatograms with pure standards. Myricetin-*O*-
229 hexosides, quercetin-*O*-hexoside, and kaempferol-*O*-hexoside showed the typical loss of 162 u
230 due to the loss of the sugar moiety.
231 Quercetin-*O*-pentoside showed the loss of the pentoside moiety (132 u), and kaempferol-*O*-
232 rhamnoside presented a loss of 146 u due to the loss of the rhamnoside group.
233 Flavonols were the main flavonoids in persimmon leaves, as reported previously. Kaempferol,
234 quercetin, isoquercetin and myricitrin have been described (Chou, 1984, Nakatani et al., 1989,

Guo and Dong, 1999). Conjugated flavonols such as quercetin-3-*O*-β-D-glucopyranoside, quercetin-3-*O*-β-D-galactopyranoside, quercetin-3-*O*-β-L-arabinopyranoside, kaempferol-3-O-α-L-rhamnopyranoside, kaempfetol-3-O-β-D-galactopyranoside, kaempferol-3-β-D-xylopyranoside and kaempefrol-3-*O*–L-arabinopyranoside have also been reported (Cai & Yang, 2001).

In this study, two quercetin-*O*-hexosides were found, one being isoquercetin and the other isomer possibly corresponding to the quercetin-3-*O*-β-D-galactopyranoside reported by Cai and Yang (2001). Two quercetin-*O*-pentosides were also identified, one of them tentatively as quercetin-3-*O*-β-L-arabinopyranoside, which was previously described by Cai and Yang (2001). Two kaempferol-*O*-hexosides were identified: kaempferol-3-O-glucoside and another hexoside isomer that may be kaempferol-3-*O*-β-D-galactopyranoside, reported by Cai and Yang (2001). Kaempferol-*O*-rhamnoside was also found, in agreement with the Cai and Yang study (2001), where kaempferol-3-O-α-L-rhamnopyranoside is described in persimmon leaf samples.

However, to our knowledge, kaempferol-3-*O*-glucoside, isorhamnetin and myricetin-*O*-hexoside are reported for the first time in persimmon leaves. These compounds have been reported in other herbs such as *Drosera peltata* (Braunberger et al., 2013) and *Carduus acanthoides* (a traditional Tibetan herbal medicine) (Li et al., 2014), as well as extracts of apples (Schieber et al., 2002) and citrus species (Brito et al., 2014). In persimmon fruit there were reported others flavonols: quercetin-*O*-hexoside-gallate, quercetin-3-*O*-glucoside, quercetin acetyl hexoside, kaempferol-*O*-hexoside-gallate, kaempferol-3-*O*-glucoside and kaempferol acetyl hexoside (Jiménez-Sánchez et al., 2015).

3.3.3 Flavanones and derivatives

Flavanones are usually glycosylated with a disaccharide at the C7 position, forming flavanonglycosides. The LTQ-Orbitrap analysis confirmed the presence of naringenin by giving the characteristic ion of the deprotonated molecule [M– H]- (m/z 271.0604, 2.75 ppm) and the ions corresponding to Retro Diels Alder fragmentation in the C-ring involving 1,3-scission (m/z 151.0034). Naringenin was further identified and confirmed by comparing its retention time with the pure standard.

3.3.4 Flavones

Flavones are a class of flavonoids based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one). The examination of the chromatograms in FTMS mode and dependent scan led to the identification of apigenin (m/z 269.0449, 2.32 ppm), which was confirmed by comparing its retention time with the pure standard.

Apigenin and apigenin derivatives such as apigenin 7-*O*-apiosyl-glucoside and apigenin 7-*O*-(6"-malonyl-apiosyl-glucoside) were also identified in the persimmon leaf samples. These compounds have also been reported in leaves of celery and oregano (Lin et al., 2007; Zheng et al., 2001).

3.4 Tyrosols

Another group of compounds found in persimmon leaves were characterised as phenylethanol-related compounds. Among them, hydroxytyrosol (m/z 153.0555, 1.74 ppm), characteristic of virgin olive oil, was identified by comparing the MS^2 spectrum with literature data (Michel et al., 2015) and with the pure commercial standard.

4. CONCLUSIONS

In conclusion, high-resolution mass spectrometry provided a powerful tool for the identification of polyphenol diversity in persimmon leaves, even in the absence of standards. We were able to identify and quantify 41 phenolic compounds, most of them, as far as we know, for the first time. The majority of these polyphenols were hydroxybenzoic acids, hydroxycinnamic acids, flavanols and flavonols, and their respective derivatives, as well as flavanones, and hydroxytyrosol. Our results show that persimmon leaves have a complex phenolic profile that may help to explain the beneficial effects of their traditional use as a medicinal herb.

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FIGURE CAPTIONS

Figure 1. Persimmon leaf FTMS chromatogram.

Figure 2. MS^2 spectra of procyanindin B1.

TABLES

Table 1. Phenolic compounds tentatively identified in persimmon leaves.