NPC Natural Product Communications

2017
Vol. 12
No. 3
409 - 412

Phenolic Isomers from *Plantago catharinea* Leaves: Isolation, Identification, Quantification and *in vitro* Antioxidant Activity

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Received: November 30th, 2016; Accepted: December 20th, 2016

In this study we isolated two polyphenolic acids of *m/z* 639, called catharinol A and catharinol B, from *Plantago catharinea* L. (Plantaginaceae) leaves. Although presenting very similar structures, catharinol A showed higher antioxidant activity when compared with gallic acid and quercetin standards. These compounds are position isomers and present in their chemical structure the rare sugar D-allose. Molecules with similar constitution are known to have important biological activities such as antitumor and immunosuppressive. These compounds were isolated by high-performance liquid chromatography (HPLC) and characterized by mass spectrometry (FIA-ESI-IT-MS/MS) and nuclear magnetic resonance (NMR). This work is the first study on the chemical composition of *P. catharinea* and encourages the production of *Plantago* species as a good source of bioactive molecules.

Keywords: *Plantago*, Antioxidant activity, D-allose, Polyphenolic acids.

The family Plantaginaceae has pharmacological importance [1], and the genus *Plantago* has been an excellent source for the production of medicines. *Plantago* species are known worldwide for treating various symptoms, acute and chronic, and so have been a target of the pharmaceutical industry [2]. *P. catharinea* L. grows in coastal salt marsh ecosystems, specifically in the zones of beaches and dunes [3]. *P. catharinea* is classified as medicinal, as well as other species of the genus, but little is known about the biological activity of its secondary metabolites to prove their efficacy [3]. Salt marshes are also exposed to high air temperatures and soil stress, interfering with the production of particular metabolites [4]. Therefore, *P. catharinea* is a great choice for cultivation as a biologically active species of great interest for the pharmaceutical industry. This study aimed at the isolation, identification and quantification of secondary metabolites from *P. catharinea*. Also, the antioxidant activity of the extract and the isolated molecules was evaluated.

We assessed the chemical profile of the polyphenolic acids from *P. catharinea*. No chemical investigation of this species could be found in the literature, even though *Plantago* genus has been studied for centuries [2]. In order to obtain a preliminary fingerprint of the chemical composition of *P. catharinea*, different mass spectrometric conditions were tested. The negative ionization mode was selected and other optimized conditions are presented in the experimental section. LC-MS analysis showed the presence of two compounds of *m/z* 639 with retention times (r_t) of 3.32 min and 3.61 min (Figure 1A and 1B). However, this method does not characterize the structural differences between these molecules. Therefore, we concentrated our efforts on the isolation of the compounds for further structural elucidation by NMR. In addition, these compounds were the only ones with enough mass to continue the analyses and probably are the major compounds.

Two molecules with *m/z* 639 were isolated by gel permeation chromatography using Sephadex (LH-20), followed by preparative high performance chromatography coupled with a refractive index detector (HPLC-RI) and detected by LC-MS. The first one was isolated from fraction 31 of the separation column and named catharinol A (26.2 mg), and the second one, catharinol B, from fractions 33 and 34 (26.0 mg). Purification of the compounds was achieved after the fractions were subjected to preparative HPLC-RI (refractive index). Differential characterization of the isomers was perceptible only by NMR analysis.

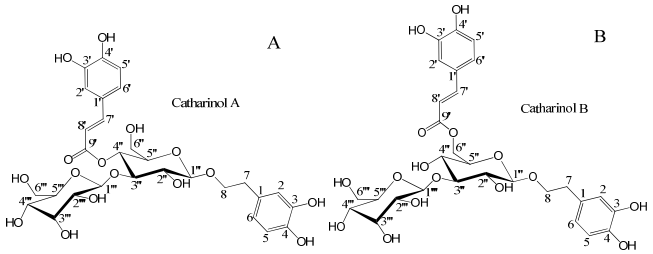

Figure 1: Structural differences of the isomers. **(A)** catharinol A, **(B)** catharinol B. Note that the caffeoyl group is inserted at different positions in the central ring of glucose

Analysis by direct injection FIA-ESI-IT-MS indicated a major precursor ion at *m/z* 639 $[M-H]^-$. MS^2 fragmentation of the precursor ion at *m/z* 639 led to a product ion at *m/z* 477 $[M-162-H]^-$ (loss of a hexose unit). MS^3 fragmentation of the precursor ion of *m/z* 477 led to the product ion at *m/z* 315 $[M-162-162-H]^-$ (loss of hexose unit + caffeic acid unit). MS^4 fragmentation of the precursor ion of *m/z* 315 led to the product ions at *m/z* 135 $[M-162-162-180-H]^-$ (loss of hexose + caffeic acid unit + loss of hexose) (Supplementary Material – Figures S1-S3). These fragmentation patterns and ^1H NMR, ^{13}C NMR, HSQC, HMBC, TOCSY data correspond to 2-(3,4-dihydroxyphenyl)-ethyl-3-*O*-β-D-allopyranosyl-4-*O*-caffeoyl-β-D-glucopyranoside (catharinol A; Figure 1A) and 2-(3,4-dihydroxyphenyl) ethyl-3-*O*-β-D-allopyranosyl-6-*O*-caffeoyl-β-D-glucopyranoside (catharinol B;

Figure 1B). The difference between these isomers is the position of the phenolic acid linked to the central ring of the glucose molecule. These compounds have the rare monosaccharide D-allose, which is considered a rare sugar and is defined as a monosaccharide in nature of which there is little or limited amount [5]. The biological effects of these rare monosaccharides are not well described. Some of the physiological effects of D-allose sugar derivatives are described by Yamaguchi *et al.* [6] and Hirata *et al*. [7], which report the importance of these monosaccharides in inhibiting the proliferation of malignant tumor cells through inactivation of cell cycle progression. In addition, suppression of hypertension development [8], immunosuppressive effect [9], antioxidant [10], treatment of amyotropical lateral sclerosis (ALS) and possible protection of neurons [11] have been reported.

The lower limit of detection (LLOD) of the MS analysis was defined as the analyte concentration in the *P. catharinea* sample clean-up method that corresponds to three times the baseline noise (S/N ≥ 3). The lower limit of quantitation (LLOQ) of the assay was assessed as the lowest concentration of the calibration curve that could be quantitatively determined with an acceptable precision less than 20% and an accuracy within ±20%, which was established based on five replicates independent of the QC samples. The limit of quantification (LQD) for the assay was determined to be 3.5 ng/mL of catharinol A in *P. catharinea*, with a signal/noise ratio of 20:1. The calibration curve obtained for the assay of catharinol A showed a correlation coefficient (r^2) of 0.973 using a/x weighting linear regression (calibration curve: 82.3144x + 756.871). (%) The percentage of catharinol A in the extract (4.53%) suggests that it is a chemical marker of *P. catharinea*.

The total phenolic content of *P. catharinea* leaves extract was 9.2 mg GAE/g. These compounds have an important role in growth and reproduction, providing protection against pathogens and predators [12]. The flavonoid content of the leaves extract was 2.1 mg QE/g. Despite having flavonoids reported in the genus *Plantago*, these are not the main compounds [2]. The results of the radical scavenging activity of the extract obtained with the DPPH test with quercetin and gallic acid standards are combined in Figure 2. The antioxidant activity of the phenolic compounds is due primarily to their reducing properties and chemical structure [13]. These characteristics play an important role in the neutralization or sequestration of free radicals and the chelation of transition metals, acting both in the initiation and propagation step of the oxidative process [14].

Thus, studies using the DPPH test revealed that the antioxidant activity of catharinol A was highly significant compared with the standards (gallic acid and quercetin) (Figure 2). However, the extract of *P. catharinea* and catharinol B did not show pronounced activity (Figure 2). Although catharinol A and B are isomers, it is known in the literature that the antiradical activity is related to catechol groups [14]. Catharinol A has, as well as catharinol B, two catechol groups, but inserted in different positions in the core structure of the molecules. Probably, the difference in ability to scavenge free radicals occurs because catechol groups in catharinol B interact with each other, reducing the antioxidant activity of the molecule. This does not occur in catharinol A, evidenced by its pronounced antioxidant activity.

This work acts as an incentive to the production of *Plantago* species as sources of bioactive molecules, as the two isolated substances have not been commercially exploited. In addition, catharinol A showed intense antioxidant activity.

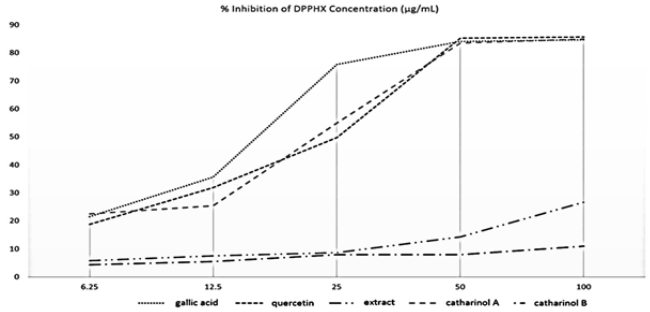

Figure 2: Antioxidant activity of catharinol A, catharinol B and total extract compared with gallic acid and quercetin standards.

Experimental

Plant material and preparation of extract: *P. catharinea* was collected at the reproductive stage in the Garden of Medicinal Plants of Botucatu (UNESP-Brazil). Mature leaves from 10 plants were used for the analysis of chemical constituents. The voucher was deposited and registered in the herbarium ESA at ESALQ/USP- *P. catharinea* (voucher: 120487). Leaves were dried in a circulated air incubator at 45°C for 7 days. Then 200 g of dry leaves were powdered and stored in amber glass bottle. The plant powder was then added to 500 mL of 70% EtOH/H_2O (v/v), left for 2 h and then poured into a percolator (1000 mL). The eluate was collected at a flow rate of 1.0 - 2.0 mL/min/kg) and the solvent dried under reduced pressure at 40°C in a rotary evaporator. The extracts were transferred to tared glass and left in a fume hood until complete solvent removal. After this process, the yield of leaf extract was determined (14.03 g = 7%).

Chemicals and reagents: Gallic acid (GA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and quercetin (QE) were purchased from Sigma–Aldrich (Saint Louis, MO, USA). Sodium carbonate, Folin–Ciocalteu's phenol reagent, ferric chloride and aluminum chloride were provided by Applichem, GmbH, Darmstadt, Germany.

Fractionation by gel permeation chromatography (Sephadex LH-20): Fractionation by gel permeation chromatography was performed using Sephadex LH-20. Briefly, 3.0 g of the crude extract was dissolved in 8 mL of MeOH: H_2O (8:2), centrifuged and injected into the column, which was eluted with MeOH:H_2O (8:2). A total of 38 fractions of 15 mL were collected, dried and stored in labeled vials. Samples were analyzed by TLC, eluted with chloroform: methanol: *n*-propanol: water (5:6:1:4) and those that showed similar profiles were combined.

Isolation of compounds by preparative high performance liquid chromatography: Fractions from gel permeation fractionation with more than 10 mg were subjected to preparative HPLC-RI using a C18 reversed-phase column (Synergi Hydro, 10 µm, 250 mm × 10.00 mm; 80 Å; Phenomenex®, Torrance, CA, USA), with water + TFA (0.05%) and methanol + TFA (0.05%) as mobile phases, at a flow rate of 2.0 mL/min, and isocratic elution with water: methanol (75:25) for 30 min with an injection volume of 100 µL.

Catharinol A: 2-(3,4-dihydroxyphenyl)ethyl-3-*O*-β-D-allopyranosyl-4-*O*-caffeoyl-β-D- glucopyranoside (1)

Amorphous brown solid.

$[\alpha]_D^{18.2}$: -16.7 (*c* 0.1 MeOH).

^{1}H NMR (MeOH–d_4, 600 MHz) δ: 6.74 (1H, d, *J*=2.0 Hz, H-2), 6.69 (1H, d, *J*=8.0 Hz, H-5), 6.61 (1H, dd, *J*=2.0, 8.0 Hz, H-6), 2.83 (1H, m, H-7), 3.77, 4.10 (1H, m, H-8), 7.10 (1H, d, *J*=2.0 Hz, H-2), 6.82 (1H, d, *J*=8.0 Hz, H-5'), 7.02 (1H, dd, *J*=2.0, 8.0 Hz, H-

6'), 7.62 (1H, d, *J*=16.0 Hz, H-7'), 6.35 (1H, d, *J*=16.0 Hz, H-8'), 4.46 (1H, d, *J*=7.5 Hz, H-1''), 3.53 (1H, dd, *J*=7.9, 9.0 Hz, H-2''), 3.95 (1H, dd, *J*=9.0, 9.0 Hz, H-3'') 4.97 (1H, dd, *J*= 9.0, 9.0 Hz, 4''), 3.53 (1H, m, H-5''), 3.6 (1H, dd, *J*=5.0, 11.4 Hz, H-6''), 3.8 (dd, *J*=2.5, 11.4 Hz, H-6''), 4.90 (1H, d,*J*=7.4 Hz, H-1'''), 3.30 (1H, dd, *J*=2.8, 7.4 Hz, H-2'''), 4.05 (1H, dd, *J*=2.8, 2.8 Hz, H-3'''), 3.50 (1H, dd, *J*= 2.8, 9.2, H-4'''), 3.61 (1H, m, H-5'''), 3.52 (1H, dd, *J*= 5.0, 12.0, H-6'''), 3.90 (1H, dd, *J*= 3.0, 12.0, H-6''').

^{13}C NMR (MeOH–d_4, 150 MHz) δ: 129.2 (C-1), 116.6 (C-2), 143.2 (C-3), 144.7 (C-4) 116.3 (C-5), 121.0 (C-6), 36.5 (C-7), 72.3 (C-8), 125.1 (C-1'), 115.2 (C-2'), 145.2 (C-3'), 148.1 (C-4'), 116.3 (C-5'), 123.1 (C-6'), 147.5 (C-7'), 114.9 (C-8'), 166.9 (C-9'), 104.3 (C-1''), 68.5 (C-2''), 84.4 (C-3''), 71.1 (C-4''), 74.8 (C-5''), 62.3 (C-6''), 103.7 (C-1'''), 73.4 (C-2'''), 72.7 (C-3'''), 68.0 (C-4'''), 75.4 (C-5'''), 62.7 (C-6''').

ESI MS/MS (Negative mode): *m/z* 639.33 [M-H]$^-$, 477.25, 315.08, 134.92.

Catharinol B: 2-(3,4-dihydroxyphenyl)ethyl 3-*O*-β-D-allopyranosyl-6-*O*-caffeoyl-β-D-glucopyranoside (2)

Amorphous white solid.

$[\alpha]_D^{18.2}$: -98.3 (*c* 0.1 MeOH).

^{1}H NMR (MeOH–d_4, 600 MHz) δ 6.80 (1H, d, *J*=2.0 Hz, H-2), 6.68 (1H, d, *J*=8.0 Hz, H-5), 6.59 (1H, dd, *J*= 2.0, 8.0 Hz, H-6), 2.80 (1H, m, H-7), 3.74, 3.94 (1H, m, H-8), 7.06 (d, *J*=2.0 Hz, H-2'), 6.90 (1H, d, *J*=8.0 Hz, H-5'), 7.00 (1H, d, *J*=2.0, 8.0 Hz, H-6'), 7.58 (1H, d, *J*=16.0 Hz, H-7'), 6.30 (1H, d, *J*=16.0 Hz, H-8'), 4.41 (1H, d, *J*=7.5 Hz, H-1''), 3.45 (dd, *J*= 7.9, 9.0Hz, H-2''), 3.57 (1H, dd, *J*= 9.0, 9.0 Hz, H3''), 3.54 (dd, *J*= 9.0, 9.0 Hz, H-4''), 3.60 (1H, m, H-5''), 4.30 (1H, dd, *J*=5.0, 11.4 Hz, H-6''), 4.55 (1H, dd, *J*=2.5, 11.4 Hz, H-6''), 4.90 (1H, d, *J*=7.4 Hz, H-1'''), 3.43 (1H, dd, *J*=2.8, 7.4 Hz, H-2'''), 4.10 (1H, dd, *J*= 2.8, 2.8 Hz, H-3'''), 3.51 (1H, dd, *J*=2.8, 9.2 Hz, H-4'''), 3.84 (1H, m, H-5'''), 3.67 (1H, dd, *J*=5.0, 12.0 Hz, 6'''), 3.90 (1H, dd, *J*=3.0, 12.0 Hz, H-6''').

^{13}C NMR (MeOH–d_4, 150 MHz) δ: 129.7 (C-1), 114.9 (C-2), 144.2 (C-3), 143.2 (C-4), 115.2 (C-5), 116.7 (C-6), 34.9 (C-7), 70.7 (C-8), 126.2 (C-1'), 113.4 (C-2'), 145.2 (C-3'), 147.8 (C-4'), 121.7 (C-5'), 121.5 (C-6'), 145.9 (C-7'), 113.3 (C-8'), 167.7 (C-9'), 103.9 (C-1''), 72.0 (C2''), 87.7 (C-3''), 68.4 (C-4''), 74.8 (C-5''), 64.3 (C-6''), 102.8 (C-1'''), 73.7 (C-2'''), 72.3 (C-3'''), 67.0 (C-4'''), 75.4 (C-5'''), 62.2 (C-6''').

ESI MS/MS (Negative mode): *m/z* 639.33 [M-H]$^-$, 477.25, 315.08, 134.92.

Mass spectrometric analysis: MS analysis were carried out in a Thermo Finnigan (San Jose, CA, USA) LCQ mass spectrometer equipped with an electro-spray ionization source, ion-trap analyzer and Xcalibur software for data processing. The capillary voltage was set at −32 V, the spray voltage at 5 kV, and the tube lens off set at 30 V. The capillary temperature was 280°C. Data were acquired in MS^1 and MS^n scanning modes using a syringe pump (flow rate 5 μL/min). Each pure compound dissolved in methanol was infused in the ESI source.

Nuclear magnetic resonance spectroscopy: NMR spectra were acquired on a Bruker DRX 600 (UX software NMR) spectrometer operating at 600 MHz to 150 MHz for ^{1}H and ^{13}C, respectively. The bi-dimensional spectra were measured in CD_3OD. In the ^{1}H NMR spectrum, CD_3OD was used as the standard signal relating to CHD_2OD at δ 3.34 ppm. The ^{1}H NMR values are reported in ppm, multiplicity, together with the values of *J* and functions. For ^{13}C NMR spectra, the signal at δ 49.0 for CD_3OD was used as the reference signal. The delay in the transfer of polarization was corrected to an average CH coupling of 135 Hz. Homonuclear direct correlation (DQF - COSY, double quantum filtered COSY) spectra were obtained using the conventional pulse sequence. DQF - COSY spectra obtained using a data set (t1 x t2) of 1024 x 1024 points for a spectral width of 1165 Hz were processed in data array using a sine function, and the digital resolution obtained in the F1 dimension and F2 was 1.13 Hz/Pt. The direct correlation ^{1}H-^{13}C HSQC heteronuclear was conducted in a matrix of 512x1024 using a CH coupling constant of 135 Hz and a delay of 1.5 sec. The data were processed using UX-NMR software. The spectra acquired are shown in Figures S4-S13 in the Supplementary Material.

Polarimetry measurement: The optical rotations were measured in methanol on a JASCO polarimeter in a 1 cm tube at 18.2°C.

Quantification by UPLC-MS/MS. Instrument and conditions: Determination was performed on a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA), consisting of a quaternary solvent delivery system and an auto-sampler. A Waters Acquity Xbridge TM BEH C18 2.5 μm, 2.1 x 100 mm column XP was used for all the analyses. The mobile phase consisted of A (0.1% formic acid, v/v) and B (acetonitrile), and was used in gradient elution. UPLC linear gradient conditions were: 0–2 min, 60–100% A, with a flow rate of 0.3 mL/min. The column was equilibrated for 2 min before each injection. The column temperature and injection volume were set at 30°C and 10 μL, respectively. All the analyses were performed using MassLynxTM XS Software. Mass spectrometry detection was performed using a Xevo TM Triple Quadrupole MS (Waters) equipped with an electrospray ionization (ESI) source. The parameters in the source were set as follows: the desolvation gas flow rate set to 600 L/h at a temperature of 550°C, the cone gas flow rate set at 50 L/h and the source temperature at 300°C. The capillary voltage was set to 3,000 V. Analyte detection was performed using multiple reaction monitoring (MRM). The cone voltage was set depending upon each specific MRM for each compound. Data were collected in MRM mode by screening parent and daughter ions simultaneously. The software automatically set dwell time. In order to obtain a calibration curve, stock solution of 2-(3,4-dihydroxyphenyl)ethyl-3-*O*-β-D-allopyranosyl-6-*O*-caffeoyl-β-D-glucopyranoside was prepared in methanol at a concentration of 1.0 mg/mL and stored at 4ºC. A series of working standard solutions of 2-(3,4-dihydroxyphenyl)ethyl-3-*O*-β-D-allopyranosyl-6-O-caffeoyl-β-D-glucopyranoside ranging from 5 to 60 ng/mL was prepared by diluting the stock solution with MeOH. All the solutions were kept at 4°C and were brought to room temperature before analysis. The calibration curve was acquired by plotting the ratio of sum of peak area of 2-(3,4-dihydroxyphenyl)ethyl-3-*O*-β-D-allopyranosyl-6-*O*-caffeoyl-β-D-glucopyranoside. The standard curve was fitted to linear regression (y = ax + b) using 1/x as the weighting factor. Blank sample was analyzed to confirm the absence of interferences but was not used to construct the calibration function. The concentrations of analyte in test samples were calculated by the regression parameters from the calibration curves. The LLOD and LLOQ of the assay were established based on 5 replicates independent of the QC samples.

Determination of total phenolic and flavonoid contents: Total phenolic content was estimated using Folin–Ciocalteu reagent as previously reported by Singleton *et al*. [15]. Total flavonoid content was assessed by the $AlCl_3$ method, as described by Quettier-Deleu *et al*. [16].

***Free radical scavenging activity (DPPH assay)*:** The DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was performed as described in the methodology of Mensor (2001) [17]. The solvent extracts of the sample were taken in the following concentration range 6.25, 12.5, 25.0, 50.0, 100.0, 200.0 μg/mL in

each test tube and the volume was made up to 1 mL with the solvent and 2 mL of 0.3 mM DPPH was added to all the tubes. The mixture was shaken well and incubated at room temperature for 30 min. Absorbance was measured at 517 nm using a spectrophotometer (BIOTEK 360, Winooski, VT, USA). The decrease in absorbance was converted to percentage of antioxidant activity using the Equation: AA%=100–{[(Abs sample–Abs blank) x100]/Abs DPPH}.

Acknowledgements - We thank the "Fundação de Amparo a Pesquisa do Estado de São Paulo- FAPESP" for fundings (2009/52237-9) and fellowships (2011/23113-0 and 2013/07681-3).

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