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Pharmacokinetics of low molecular weight phenolic compounds in gerbil plasma after the consumption of calafate berry (*Berberis microphylla*) extract

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Hydroquinone (PubChem CID: 785)
Syringic Acid (PubChem CID: 10742)
Vanillic Acid (PubChem CID: 8468)
Phenylacetic Acid (PubChem CID: 999)
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ABSTRACT

Calafate is a berry with high concentration of anthocyanins and hydroxycinnamic acids that grows in South Patagonia. To date, no metabolism studies of phenolic compounds using calafate have been carried out. A calafate extract was characterized by HPLC-DAD-ESI-MS/MS. After extract administration (300 mg/kg), a pharmacokinetic study of phenolic compounds in gerbil plasma was performed by GC–MS/MS. Sixteen phenolic acids increased after intake. Phenylacetic acid derivatives exhibit the highest concentration, while main increase of phenolic catabolites was observed 2 h post-intake. 3-hydroxyphenylacetic and phenylacetic acids increased at 4–8 h post-intake. All catabolites found in gerbil plasma exhibit concentration peaks between 0.1 and 1 μM, however no parental anthocyanins were detected. Establish *in vivo* plasmatic concentration ranges of phenolic compounds derived from polyphenol consumption following WHO recommendations, plays a key role to carry out future *in vitro* assays in order to correctly assign biological benefits of calafate berry consumption.

1. Introduction

Numerous studies have shown epidemiologic and mechanistic data supporting the beneficial effects of phenolic compounds present in fruits and vegetables (Cassidy et al., 2015; Del Rio et al., 2013), revealing the potential benefits of these compounds over diverse pathologies, such as cardiovascular diseases, diabetes, and cancer (Del Rio et al., 2013; Juurlink, Azouz, Aldalati, AlTinawi, & Ganguly, 2014). Lately, health promotion studies have been focusing on their metabolites (Bolca, Van de Wiele, & Possemiers, 2013). In order to understand their benefits, it is necessary to consider the effects of the parent compound(s) as well as their colonic metabolites generated *in vivo*, in accordance with recommendations discussed by Kay (Kay, 2010). The main metabolites produced after the intake of flavonoids and other phenolic compounds are small weight phenolics, such as benzoic, phenylacetic, and 3-phenylpropionic acids, which have also been associated with beneficial effects (De Ferrars, Cassidy, Curtis, & Kay, 2014; De Ferrars, Czank, et al., 2014; El-Seedi et al., 2012; Stalmach, Edwards, Wightman, & Crozier, 2013).

Calafate (*Berberis microphylla*) is a shrub with a dark-skinned berry that grows in Chilean and Argentinean Patagonia. It contains high levels of vitamin C and phenolic compounds, like anthocyanins, but also hydroxycinnamic acids derivatives (HCADs) and flavonols, demonstrating high antioxidant capacity in studies using several antioxidant quantification methods (Ruiz et al., 2010, 2013, 2014). Promising flavonoid and HCADs concentrations have been reported in this fruit. The anthocyanin content ranges between 14.21 and 26.13 μmol/g fresh weight (FW), while the flavonol and HCADs content vary between 0.12

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and 2.89 μmol/g FW, and 0.32 to 8.28 μmol/g FW, respectively (Ruiz et al., 2010, 2013, 2014). Comparatively, the levels of (poly)phenol concentration found in the calafate berry are higher than the concentrations reported in other widely studied berries (Rothwell et al., 2016).

According to de Ferrars et al., an *in vivo* intake study utilizing ^{13}C cyanidin-3-glucoside revealed low bioavailability of parent anthocyanin in plasma (< 0.2%), with a maximum concentration of 141 nM at 1.8 h, but its metabolites showed 42 times higher concentration at their respective maximum concentrations (De Ferrars, Cassidy, et al., 2014; De Ferrars, Czank, et al., 2014). Moreover, anthocyanins are rapidly absorbed in the stomach and small intestine, achieving peak plasma concentration within the first two hours (Lila, Burton-Freeman, Grace, & Kalt, 2016).

The absorption in the gastrointestinal tract (GIT) is dependent on distal passage and on the pH of the absorption zone. Relevant catabolites are consequently found in plasma due to the predominance and stability of absorbed anthocyanin and the increase of the microbial catabolism (De Ferrars, Cassidy, et al., 2014; De Ferrars, Czank, et al., 2014; Esposito et al., 2015; Ludwig et al., 2015; Stalmach et al., 2013). Main anthocyanin catabolites are produced from heterocyclic C-ring cleavage. B-ring products correspond to hydroxylated benzoic acid, reported as 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid, methyl-3,4-dihydroxybenzoate, benzoic acid, 3-methoxy-4-hydroxycinnamic acid, and hippuric acid, due to their origins from cyanidin-3-glucoside (a B-ring dihydroxy-anthocyanin), while A-ring conserved products include 2-(2,4,6-trihydroxyphenyl)acetic acid or phloroglucinaldehyde, and 4-hydroxybenzaldehyde, among others (Czank et al., 2013; De Ferrars, Cassidy, et al., 2014; De Ferrars, Czank, et al., 2014; Stevens & Maier, 2016). In this context, Del Río et al. discuss how catabolites may exert protective action and the need to perform *in-vivo* intervention studies (Del Rio et al., 2013).

The number of B-ring hydroxyl groups present in anthocyanidins has an effect on their bioactivity and bioavailability. A lower presence of biomarkers related to metabolic and cardiovascular disease risk were observed in subjects that consumed berries that contained high concentration of trihydroxy derivatives (such as blueberry, blackcurrant, bilberry, grape) rather than vegetables with high concentrations of dihydroxy derivatives (such as elderberry, blood orange, and purple carrot) (Lila et al., 2016). The presence of trihydroxy derivatives, such as delphinidin-3-glucoside, malvidin-3-glucoside, and petunidin-3-glucoside in Calafate arouse the interest of this berry consumption (Ruiz et al., 2010).

A study regarding the ingestion of HCAD rich foods, like coffee, demonstrated that the availability of HCADs derivatives in ileal fluid, urine, and plasma showed microbial phase I and II biotransformation through dehydroxylation, O-methylation, reduction, sulfation, glucuronization, and the addition of GSH or amino acids such as glycine (El-Seedi et al., 2012). Proposed metabolites found in plasma due to HCADs consumption are mainly hydroxycinnamic, 3-phenylpropionic, and hydroxybenzoic acid derivatives (El-Seedi et al., 2012).

Several human and animal feeding studies in berries with high anthocyanin content are focused mainly on cranberries, blueberries, raspberries, strawberries, and grape (Czank et al., 2013; De Ferrars, Cassidy, et al., 2014; De Ferrars, Czank, et al., 2014; Stevens & Maier, 2016). To the best of our knowledge, no reports of intake studies with calafate berries have been performed. The objective of this study was to determine and study the pharmacokinetics of phenolic compounds in gerbil plasma after the intake of a single dose of 300 mg/kg calafate berry extract.

2. Material and methods

2.1. Reagents, standards, and extraction materials

Commercial standards of delphinidin-3-glucoside chloride (99%), quercetin-3-glucoside (99%), and 5-caffeoylquinic acid (99%) were purchased by Phytolab (Vestenbergsreuth, Germany). Standards of acids 2-hydroxybenzoic (2-HBA), 3-hydroxybenzoic (3-HBA), 4-hydroxybenzoic (4-HBA), 3,4-dihydroxybenzoic (3,4-HBA), 3,4,5-trihydroxybenzoic (3,4,5-HBA), 4-methoxybenzoic (4-OMBA), 3-methoxy-4-hydroxybenzoic (3-OM-4-HBA), 4-hydroxy-3,5-dimethoxybenzoic (3,5-OM-4-HBA), phenylacetic (PAA), 3-hydroxyphenylacetic (3-HPAA), 4-hydroxyphenylacetic (4-HPAA), 3,4-dihydroxyphenylacetic (3,4-HPAA), 3-methoxy-4-hydroxyphenylacetic (3-OM-4-HPAA), 3-methoxyphenylacetic (3-OMPAA), 4-methoxyphenylacetic (4-OMPAA), 3,4-dimethoxyphenylacetic (3,4-OMPAA), 3-phenylpropionic (PPA), 3-(4-hydroxyphenyl)propionic (4-HPPA), 3-(3,4-dihydroxyphenyl)propionic (3,4-HPPA), 3-(3-methoxyphenyl)propionic (3-OMPPA), 3-(4-methoxyphenyl)propionic (4-OMPPA), 3-(3,4-dimethoxyphenyl)propionic (3,4-OMPPA), cinnamic (CA), 3-hydroxycinnamic (3-HCA), 4-hydroxycinnamic (4-HCA), 3,4-dihydroxycinnamic (3,4-HCA), 3-methoxy-4-hydroxycinnamic (3-OM-4-HCA), 4-methoxycinnamic (4-OMCA) and 3,4-dimethoxycinnamic (3,4-OMCA), 3-methoxy-4-hydroxybezaldehyde (3-OM-4-HBAld), 3,4-dihydroxybenzaldehyde (3,4-HBAld), 1,4 dihydroxybenzene (1,4-HBz), 1,2,3-trihydroxybenzene (1,2,3-HBz), 1,3,5-trihydroxybenzene (1,3,5-HBz), and β-glucuronidase type H-5 from *Helix pomatia* were purchased from Sigma (St. Louis, Missouri, USA). *Trans*-cinnamic acid-d_6 was procured from Toronto Research Chemicals (Brisbane Rd, Toronto, Canada). N,O-Bis(trimethylsilyl)trifluoroacetamide: trimethylchlorosilane (BSTFA:TMCS) 90:10 was provided by UCT Inc. (Levittown, USA). Sodium acetate p.a., EDTA p.a., Amicron® ultra centrifugal filters MWCO 100 KDa, methanol, acetonitrile, ethyl acetate, hexane, toluene (LiChrosolv grade), and formic and hydrochloride acid were procured from Merck (Darmstadt, Germany). Reaction vessels from Supelco (Bellefonte, Pennsylvania, USA), a nitrogen evaporator from Pierce (Waltham, Massachusetts, United States) and a centrifuge from Thermo Scientific (Waltham, Massachusetts, USA) were used, 150 mg Oasis MCX 6 mL mixed phase SPE cartridges were purchased from Waters (Milford, Connecticut, USA), and potable ethanol from Oxiquim (Concepción, Chile) was also utilized.

2.2. Instruments

An analytical balance from Denver Instrument Company (New York, New York, USA), an ultrasonic bar homogenizer Series 4710 from Cole Palmer (Chicago, Illinois, USA), a Stuart S01 mechanical shaker from Bibby Scientific LTD (Stanford, UK), an Alpha 2–4 LD plus lyophilizing system and a Sigma 3-16p centrifuge from Martin Christ (Osterode, Germany), and a rotavapor with a V-700 vacuum pump and V-850 controller system from Büchi (Flawil, Switzerland) were used for sample preparation.

HPLC-DAD-ESI-MS/MS analyses for identification and quantification of anthocyanins, flavonols and hydroxycinnamic acids in calafate extract were carried out using a Nexera UHPLC/HPLC system from Shimadzu Corporation (Kyoto, Japan) equipped with a quaternary LC-30AD pump, a DGU-20A5R degassing unit, a Prominence CTO-20 AC oven, a SIL-30AC autosampler, and an SPD-M20A UV–Vis photodiode array spectrophotometer detector coupled in tandem with a QTrap 3200 LC–MS/MS detector from MDS Sciex (Redwood City, California, USA). Instrument control and data collection were carried out using CLASS-VP DAD Chromatography Data System from Shimadzu Corporation (Kyoto, Japan) and MS/MS analysis was performed using Analyst software version 1.5.2 from AB Sciex (Framingham, Massachusetts, USA).

An Agilent 7890A GC (Palo Alto, California, USA) with a multimode injector, interfaced to an Agilent 7000 GC/MS Triple Quad detector, and fitted with an Agilent CTC PAL autosampler was used for phenolic acid quantification in plasma samples. Agilent MassHunter GC/MS acquisition software (Palo Alto, California, USA) was used for a control system. Chromatographic separations were performed using an HP-5MS