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RESEARCH PAPER

## Bioactive compounds of loquat (*Eriobotrya japonica* Lindl.) cv. Golden Nugget and analysis of *in vitro* functionality for hyperglycemia management

Jaime Ahumada<sup>1</sup>, Claudia Fuentealba<sup>2</sup>, José A. Olaeta<sup>1</sup>, Pedro Undurraga<sup>1</sup>, Romina Pedreschi<sup>1</sup>, Kalidas Shetty<sup>3</sup>, Rosana Chirinos<sup>4</sup>, David Campos<sup>4</sup>, and Lena Gálvez Ranilla<sup>2,4</sup>

<sup>1</sup>Pontificia Universidad Católica de Valparaíso, Facultad de Ciencias Agronómicas y de los Alimentos, Escuela de Agronomía. Calle San Francisco s/n La Palma, Quillota. Chile

<sup>2</sup>Pontificia Universidad Católica de Valparaíso, Facultad de Ciencias Agronómicas y de los Alimentos, Escuela de Alimentos, Laboratorio de Química y Bioquímica de Alimentos. Avenida Waddington 716, Valparaíso, Chile.

<sup>3</sup>North Dakota State University, Department of Plant Sciences. Fargo, ND 58108, USA.

<sup>4</sup>Universidad Nacional Agraria La Molina, Instituto de Biotecnología. Av. La Molina s/n, Lima, Perú.

### Abstract

**J. Ahumada, C. Fuentealba, J.A. Olaeta, P. Undurraga, R. Pedreschi, K. Shetty, R. Chirinos, D. Campos, and L. Gálvez Ranilla. 2017. Bioactive compounds of loquat (*Eriobotrya japonica* Lindl.) cv. Golden Nugget and analysis of *in vitro* functionality for hyperglycemia management. Cien. Inv. Agr. 44(3): 272-284.** The loquat cultivar Golden Nugget (leaves, flowers and fruits), which has potential commercial importance in Chile, was analyzed for targeted bioactive composition (total phenolic contents: TPC, phenolic profiles and triterpenic acids) and *in vitro* bioactivity such as antioxidant capacity (AC) and inhibitory activity against enzymes relevant for hyperglycemia ( $\alpha$ -amylase and  $\alpha$ -glucosidase) and hypertension (angiotensin I-converting enzyme, ACE-I) management. Leaves and flowers had higher TPC and AC than fruits and hydroxycinnamic acid derivatives were found in all structures by HPLC. Chlorogenic acid and quercetin derivatives were detected only in leaves and flowers, which also inhibited the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes, but flowers showed the highest anti-hyperglycemia activity. No inhibition of ACE-I and hyperglycemia-relevant enzymes were found in loquat fruits; additionally, TPC and AC were high at the green stage. Loquat leaves and flowers are sources of phenolic bioactives with potential for diet-based hyperglycemia management. No triterpenic acids were found in any of the loquat parts.

**Keywords:** *Eriobotrya japonica*, phenolic compounds, antioxidants, anti-hyperglycemia, loquat.

### Introduction

Loquat (*Eriobotrya japonica* Lindl.) is a small evergreen plant that is native to southern China

and later naturalized in Japan, India and many other areas of Asia. Loquat is grown mainly to produce fruits; however, its leaves have also been used since ancient times in the Chinese traditional medicine and have been suggested to prevent skin abnormalities, diabetes, chronic bronchitis, coughs, phlegm, ulcers and cancer (Ito *et al.*, 2000).

Different bioactive compounds likely associated with loquat health-relevant properties have been reported by several studies. Phenolic compounds linked to a significant antioxidant capacity have been found in fruits and leaves, with leaves showing the highest total phenolic contents and antioxidant capacity (Ferrerres *et al.*, 2009). Numerous epidemiological studies have indicated that regular consumption of phenolic compounds through the diet may play a role in the prevention of oxidation-linked chronic diseases, such as cardiovascular diseases, cancer, diabetes and neurodegenerative dysfunctions, and that these effects may be related to their potent antioxidant properties (Gil *et al.*, 2011). Triterpenoid compounds such as ursolic and oleanolic acids have been detected in leaves and flowers of loquat with variable contents depending on the developmental stage and type of cultivar (Xu and Chen, 2011). These compounds have been associated with antioxidant and anti-inflammatory properties (Tsai and Yin, 2008).

In Chile, two loquat cultivars have been well adapted to the climate of the Central region: the Golden Nugget and the Tanaka cultivars. Although the production is still small in surface area, there is a growing commercial interest in such cultivars as potential new sources of bioactive compounds, with the possibility of using other plant parts such as leaves and flowers, which also have shown great functional potential as mentioned previously. No data have been reported to date about the potential health-relevant bioactivity of the Golden Nugget cultivar grown in Chile. Therefore, the study of functional potential of loquat grown in Chile would give critical scientific information for further studies about functional food and nutraceutical applications likely promoting loquat production in Chile on a long-term basis as a value-added crop. The latter would be an additional advantage for Chilean producers especially in seasons when Asian countries have low production due to differences in climate zones.

Obesity, which stems mainly from changes in traditional dietary habits toward diets high in

sugars and fats, is increasing in Chile according to recent studies conducted by the Ministry of Education (Ministerio de Educación, 2012). This condition contributes to the prevalence of metabolic syndrome components such as hyperglycemia, dyslipidemia and hypertension, which in turn increases the risk of chronic diseases, such as type 2 diabetes and cardiovascular complications. Therefore, the study and characterization of new bioactive dietary sources such as loquat with potential impact on metabolic syndrome modulation would contribute to current prevention strategies for counteracting chronic diseases, and the use of the biodiversity of local and regional resources is essential.

With regard to hyperglycemia and its complications, it has been shown that some bioactive compounds such as certain phenolic compounds from plants are capable of inhibiting the digestive enzymes involved in breakdown of carbohydrates like  $\alpha$ -amylase and  $\alpha$ -glucosidase due to their ability to bind to the active site of the protein (McDougall and Stewart, 2005), whereas other studies have shown that phenolic compounds may play a role in the inhibition of angiotensin I-converting enzyme relevant for hypertension modulation (Kwon *et al.*, 2006). Therefore, the consumption of natural metabolic inhibitors from the diet could be an effective therapy for managing postprandial hyperglycemia and is likely to prevent other complications such as hypertension with minimal side effects, in contrast with current medications (Picot *et al.*, 2014).

Based on the above rationale, the objective of this study was to determine the composition of bioactive compounds (phenolic metabolites and triterpenic acids) of leaves (new and old), flowers and fruits (at three maturity stages) of loquat plant cv. Golden Nugget cultivated in the central region of Chile. The antioxidant capacity (DPPH, ABTS and ORAC methods) and the inhibitory potential against key enzymes relevant for hyperglycemia ( $\alpha$ -amylase and  $\alpha$ -glucosidase) and hypertension (angiotensin I-converting enzyme, ACE-I)

modulation were further investigated in extracts from all plant parts using *in vitro* models. The information resulting from this study provides preliminary key information for future cell culture and *in vivo* studies on the relevance of extraction of bioactives from loquat plant grown in Chile and their potential applications in the nutraceutical and functional food design.

## Materials and methods

### Plant material

Loquat trees grown at the same conditions were selected from the Experimental Station La Palma located at the Faculty of Agronomic and Food Sciences of the Pontificia Universidad Católica de Valparaíso, in Quillota in the central region of Chile, latitude 32°52'S and longitude 71°15'W. Each tree corresponded to a biological replicate and samples of the different plant parts were randomly collected from each tree obtaining a final composite sample (100 g for leaves and flowers, and 2 kg for fruits). Samples were collected across the year 2013 as follows: old leaves were collected on May 15<sup>th</sup>, new leaves on October 3<sup>rd</sup>, flowers (fully open) on August 3<sup>rd</sup>, and fruits at three maturity stages on November 10<sup>th</sup> (stage 1, green color), November 30<sup>th</sup> (stage 2, yellow color) and December 20<sup>th</sup> (stage 3, orange color at commercial maturity). Photographs of collected loquat fruits are shown in Figure 1. Samples were freeze-dried and powdered to a size of 500 µm. Milled samples were stored at -20 °C until analysis.

### Chemical and reagents

All chemicals and solvents employed were of high-performance liquid chromatography (HPLC) or analytical grade. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), the 2,2'-azino-di-[3-ethylbenzotiazolin sulfonate] (ABTS), the 2,2'-aziobis (2-amidino-propane) dihydrochloride (AAPH) and fluorescein sodium salt were purchased from Sigma Chemical Co. (St Louis, MO, USA). The Folin-Ciocalteu reagent, methanol, and ethanol were of analytical grade and purchased from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was purchased from Fisher Scientific (Waltham, MA, USA). Standards of caffeic acid, *p*-coumaric acid, chlorogenic acid and quercetin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Porcine pancreatic  $\alpha$ -amylase (EC 3.2.1.1), baker's yeast  $\alpha$ -glucosidase (EC 3.2.1.20), and lung rabbit angiotensin I-converting enzyme (EC 3.4.15.1) were purchased from Sigma Chemical Co. (St. Louis, MO).

### Fruit physicochemical characterization

Each maturity stage was characterized according to Martínez-Calvo *et al.* (1999) for the scale of fruit development, and the Munsell color charts for vegetative tissues (Munsell Color, New York, USA) were used for characterizing the fruit color. Additionally, the following assessments were made on fruits at each maturity stage: soluble solids at 20 °C (Thermo compensated



Figure 1. Fruits of loquat cv. Golden Nugget at three maturity stages.

refractometer Atago, 0-32°Brix), titratable acidity expressed in g malic acid/100 mL juice and moisture content according to AOAC (1984), soluble solids:acidity ratio, and pH, measured in 20 mL of filtered juice at 20 °C.

#### *Extract preparation for analyses in leaves, flowers and fruits*

Different protocols are published for extracting phenolics together with triterpenic acids from loquat parts. Therefore, two extraction methods were selected and compared according to the total phenolic contents in obtained extracts. A method proposed by Zhou *et al.* (2011a), who used sonication combined with extraction with 60% ethanol, was compared with a method that used 95% methanol, followed by a second extraction with 80% methanol (Ding *et al.*, 2001). The selected method was that proposed by Ding *et al.* (2001), which allowed the extraction of higher contents of phenolic bioactives.

Freeze-dried samples (0.5 g) were homogenized in cold methanol (95%) for 2 min, and centrifuged for 5 min. The pellet was extracted twice with 80% cold methanol, and the combined extracts were evaporated under vacuum until all methanol was removed. The sample was finally reconstituted in 25 mL of distilled water and stored at -20 °C until analysis.

#### *Total phenolic content*

The total phenolic content (TPC) was determined according to the Folin-Ciocalteu method modified by González-Muñoz *et al.* (2013). Briefly, loquat extract was mixed with 95% ethanol and distilled water. To each sample, Folin-Ciocalteu reagent (1 N) was added and mixed. After 5 min, 5% Na<sub>2</sub>CO<sub>3</sub> was added to the reaction mixture and allowed to stand for 60 min in a dark place. The absorbance was read at 725 nm. The standard curve was established using various concentrations

of gallic acid in distilled water, and the results were expressed as mg of gallic acid equivalents GAE g<sup>-1</sup> sample dried weight (DW).

#### *Antioxidant capacity by the 2,2-diphenyl-2-picrylhydrazyl radical (DPPH•) inhibition assay*

The DPPH scavenging capacity of all sample extracts was determined by an assay adapted to a microplate and as reported by Duarte-Almeida *et al.* (2006) with modifications. A DPPH methanolic solution was prepared at a concentration of 20 g mL<sup>-1</sup> to achieve a 0.6 to 0.7 absorbance at 517 nm using a microplate reader (Thermo scientific Multiscan GO, Vantaa, Finland). In each microplate cell, 40 µL of sample extract (or standard or control:water) were added followed by the addition of 250 µL of DPPH methanolic solution. Absorbance was measured after 25 min of incubation at 25°C. The inhibition percentage was calculated, and the results were expressed as µmol Trolox equivalents/g of sample DW using a standard curve of Trolox built with different concentrations (from 20 to 160 µM).

#### *Antioxidant capacity by the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS•+) inhibition assay*

The ABTS antioxidant capacity assay was developed according to González-Muñoz *et al.* (2013). Briefly, a sample aliquot was allowed to react with ABTS reagent. The mixture was incubated at room temperature for 6 min. The absorbance was recorded at 734 nm measured with a microplate reader, and a control was also run under the same conditions. The percentage of inhibition was calculated, and the antioxidant capacity was expressed as µmol Trolox equivalents/g sample DW using a standard curve of a Trolox solution at different concentrations (from 20 to 160 µM).

### *Antioxidant capacity by the oxygen radical absorbance capacity (ORAC) assay*

The ORAC assay was performed according to Ou *et al.* (2001) in a 96-well microplate fluorometer (BioTek Instruments Sinergy 2, Winooski, VT, USA). Peroxyl radicals were generated using AAPH, and fluorescein was used as the substrate. Twenty-five  $\mu\text{L}$  of 75 mM phosphate buffer (pH 7.4) (blank), and Trolox standard and extract dilutions were mixed with 250  $\mu\text{L}$  of 55 nM sodium fluorescein solution and were incubated for 10 min at 37 °C. Reactions were initiated by the addition of 25  $\mu\text{L}$  of a 153 nM AAPH solution. Fluorescence filters were used at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, and measurements were monitored at 60-s intervals for 55 min. The antioxidant capacity was calculated using the net area under the decay curve and was expressed as  $\mu\text{mol}$  Trolox equivalents TE  $\text{g}^{-1}$  of sample DW using a standard curve of Trolox solution at different concentrations (from 0.5 to 4  $\mu\text{M}$ ).

### *Phenolic profiles by high-performance liquid chromatography (HPLC) analysis*

Loquat sample extracts and mobile phases were filtered through a 0.22- $\mu\text{m}$  Millipore filter, type GV (Millipore, Bedford, MA), and the HPLC analysis was performed according to Chirinos *et al.* (2008) with modifications. An HPLC system composed of a Waters 2695 separation module (Waters, Milford, MA) equipped with an auto-injector, a 996-photodiode array detector (PDA) and the Empower software was used. An X-terra RP<sub>18</sub> (5  $\mu\text{m}$ , 250 x 4.6 mm) column (Waters, Milford, MA) and a 4.6 mm x 2.0 mm guard column were used. The phenolic separation was performed at 30°C, the injection volume was 20  $\mu\text{L}$ , the flow rate was 0.5  $\text{mL min}^{-1}$ , and the eluates were monitored from 200 to 700 nm. The mobile phase was composed of solvent (A) water: formic acid (95:5 v/v, pH 2) and solvent (B) acetonitrile. Initial conditions were 100% A, and

a gradient of solvent B was used: 0-15% B in 40 min, 15-45% B in 40 min, 45-100% B in 5 min, 100% B by 5 min, and then returned to initial conditions by 10 min. Phenolic compounds were identified and quantified by comparison of their retention times and UV-visible spectral data to known previously injected standards. The results were expressed as  $\text{mg g}^{-1}$  sample DW.

### *Ursolic and oleanolic triterpenoid acids by HPLC analysis*

The HPLC analysis for detection of ursolic (UA) and oleanolic (OA) acids was performed according to Zhou *et al.* (2007). An HPLC system series 200 with a UV/vis detector (Perkin-Elmer Inc., Shelton, CT, USA) equipped with a binary pump, an autosampler, and controlled by the TotalChrom software (Perkin-Elmer Inc., Shelton, CT, USA) was used. The analytical column was a HibarLi-Chrospher 100 RP-18 (5  $\mu\text{m}$ ) (Merck, Darmstadt, Germany). Previously, filtered sample extracts (pore size 0.2  $\mu\text{m}$ ) were injected (volume 25  $\mu\text{L}$ ), and an eluent flow rate of 1.0  $\text{mL min}^{-1}$  was used. Both compounds were detected at 210 nm at 25°C. The mobile phase consisted of methanol (A) and 0.03 mol/L phosphate buffer (pH 2.8) (B) with an isocratic ratio of 88:12 (A:B, v/v) for simultaneous detection of UA and OA, (total run time 15 min). Quantification was performed using the corresponding calibration curves ( $r=0.9990$ ) of pure standards (UA and OA) diluted in methanol, and results were expressed as  $\text{mg/g}$  sample DW.

### *$\alpha$ -Amylase inhibition assay*

The  $\alpha$ -amylase inhibitory activity was determined by an assay based on the Worthington Enzyme Manual (1993a) and modified by González-Muñoz *et al.* (2013). Briefly, each loquat structure extract and 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing  $\alpha$ -amylase solution were incubated at 25 °C for 10 min. After pre-incubation, 1% starch solution in sodium phosphate buffer

was added to each tube at timed intervals. The reaction mixtures were then incubated at 25 °C for 10 min. Then, dinitrosalicylic acid color reagent was added, and the test tubes were incubated in a boiling water bath for 10 min and cooled to room temperature. The absorbance was measured at 540 nm and the  $\alpha$ -amylase inhibitory activity was calculated as percentage of inhibition.

#### *$\alpha$ -Glucosidase inhibition assay*

The  $\alpha$ -glucosidase inhibitory activity was determined by an assay based on the Worthington Enzyme Manual (1993b) and modified by González-Muñoz *et al.* (2013). A sample extract diluted and 0.1 M potassium phosphate buffer (pH 6.9) containing  $\alpha$ -glucosidase solution (1.0 U mL<sup>-1</sup>) was incubated in 96-well plates at 25 °C for 10 min. After pre-incubation, 5 mM *p*-nitro-phenyl- $\alpha$ -D-glucopyranoside solution in potassium phosphate buffer was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min, and the absorbance were recorded at 405 nm. The  $\alpha$ -glucosidase inhibitory activity was expressed as a percentage of inhibition.

#### *Angiotensin I-converting enzyme (ACE) inhibition assay*

ACE inhibitory activity was performed based on the method developed by Kwon *et al.* (2006) and modified by González-Muñoz *et al.* (2013). A sample extract was incubated with 0.1 M NaCl-borate buffer (0.3 M NaCl, pH 8.3) containing 2 mU of ACE solution at 25 °C for 10 min. After pre-incubation, 5.0 mM substrate (hippuryl-histidyl-leucine) solution was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 h, and the reaction was stopped with 0.5 N HCl. The hippuric acid formed was detected by a high-performance liquid chromatography (HPLC) method. Sample were injected using an HPLC system series 200 with a UV/vis detector (Perkin-Elmer Inc., Shelton, CT, USA) equipped

with a binary pump and an auto-sampler and controlled by TotalChrom software (Perkin-Elmer Inc., Shelton, CT, USA). The analytical column used was HibarLiChrospher 100 RP-18 (5  $\mu$ m) (Merck, Darmstadt, Germany) at a flow rate of 1 mL min<sup>-1</sup> at 25 °C. The absorbance was recorded at 228 nm and the ACE-I inhibitory activity was expressed as a percentage of inhibition.

#### *Statistical analysis*

Each loquat tree (four in total) corresponded to a biological replicate. The results were expressed as the mean  $\pm$  standard deviation. Data were subjected to one-way analysis of variance (ANOVA) with Tukey test for multiple comparisons or the Kruskal-Wallis test ( $\alpha=0.05$ ) when necessary. ANOVA and Pearson linear correlations ( $p<0.05$ ) were performed using the Statgraphics Centurion XVI (StatPoint Inc., Rockville, MD, USA).

## **Results and discussion**

### *Phenolic bioactives, triterpenoid acids, and antioxidant capacity in leaves and flowers*

The extraction of phenolic compounds together with triterpenoid acids from loquat plant has been carried out under different parameters according to the existing literature to date. Two extraction methods were selected and compared with respect to the total phenolic content (TPC) in the final extracts in order to define the best protocol suited for the “Golden Nugget” cultivar. Old leaf samples were used for these preliminary analyses. The method of Zhou *et al.* (2011a), which uses sonication combined with extraction with 60% ethanol, allowed us to extract on average 28.83  $\pm$  3.48 mg TPC GAE g<sup>-1</sup> DW. In comparison, the results when using the method described by Ding *et al.* (2001), which combines orbital shaking with homogenization together with a two-step extraction with 95% methanol and 80% methanol, respectively, averaged 45.71

$\pm 1.87$  mg TPC GAE  $g^{-1}$  DW. Therefore, the second extraction method was selected for the analysis of all loquat parts.

Table 1 shows the TPC and the phenolic profiles measured by HPLC in old leaves, new leaves, and flowers together with results of the antioxidant capacity by the DPPH, ABTS and ORAC methods.

TPC in leaves and flowers ranged from  $40.57 \pm 5.27$  to  $44.04 \pm 3.36$  mg GAE  $g^{-1}$  DW and no significant differences were found between these three loquat parts ( $P > 0.05$ ). These results are higher than those reported by Song *et al.* (2010), who obtained  $31.47 \pm 0.48$  mg GAE  $g^{-1}$  DW in leaves of unspecified age from an indeterminate cultivar, and lower than those found by Hong *et al.* (2008), who obtained values of  $54.9 \pm 2.40$  mg GAE  $g^{-1}$  DW in leaves from different cultivars. Furthermore, higher TPC was found in loquat flowers ( $40.57 \pm 5.27$  mg GAE  $g^{-1}$  DW) when compared to results reported by Zhou *et al.* (2011b) ( $13.53 \pm 0.38$  mg/g DW) in fully open flower samples from the Ruantioabaisha cultivar grown in China; however, these results were expressed as chlorogenic acid equivalents/g DW.

No triterpenoid acids were detected by HPLC in all evaluated loquat structures under conducted experimental conditions; however, different phenolic compounds were found in leaves and flower extracts when analyzed by HPLC. Hydroxycinnamic acid derivatives such as chlorogenic acid together with other caffeic and *p*-coumaric acid derivatives (UV spectra characteristics similar to those of caffeic and *p*-coumaric acid standards) were detected in all loquat structures. Further, flavonols such as quercetin derivatives, likely rutin or similar quercetin glycoside ( $\lambda_{max}$ : 257.1, 357.0 nm for detected peaks and  $\lambda_{max}$ : 257.1, 354.6 for pure rutin standard), were found in all loquat samples. Main differences were based on the phenolic contents (Table 1). Major phenolic compound in all evaluated structures was the chlorogenic acid. Flowers had higher chlorogenic acid ( $17.53 \pm 2.49$  mg  $g^{-1}$  DW,  $P < 0.05$ ) and quercetin derivatives contents ( $4.95 \pm 0.59$  mg  $g^{-1}$  DW) than old and new leaves. However, new leaves had higher amounts of caffeic and *p*-coumaric acid derivatives ( $3.95 \pm 0.34$  and  $2.43 \pm 0.20$  mg  $g^{-1}$  DW, respectively,  $P < 0.05$ ) than old leaves and flowers. Flower extracts showed the highest total phenolic contents (by calculating the sum of all detected phenolic compounds by

**Table 1.** Total phenolic contents, phenolic profiles by HPLC-PDA and antioxidant capacity by the DPPH, ABTS and ORAC methods of loquat leaves and flowers cv. Golden Nugget.

Assay	Loquat Structure		
	Old leaves	New leaves	Flowers
Total phenolic contents (mg GAE/g DW)	$41.39 \pm 8.12^a$	$44.04 \pm 3.36^a$	$40.57 \pm 5.27^a$
Phenolic acids (mg/g DW)			
Caffeic acid derivatives <sup>a</sup>	$1.44 \pm 0.15^b$	$3.95 \pm 0.34^a$	$1.46 \pm 0.19^b$
<i>p</i> -coumaric acid derivatives <sup>b</sup>	$0.65 \pm 0.01^c$	$2.43 \pm 0.20^a$	$1.43 \pm 0.18^b$
Chlorogenic acid	$4.61 \pm 0.33^b$	$7.39 \pm 0.49^b$	$17.53 \pm 2.49^a$
Flavonoids (mg/g DW)			
Quercetin derivatives <sup>c</sup>	$0.26 \pm 0.02^b$	$0.99 \pm 0.09^b$	$4.95 \pm 0.59^a$
Total phenolic contents HPLC (mg/g DW)	$6.96 \pm 0.49^c$	$14.77 \pm 1.12^b$	$25.37 \pm 3.43^a$
Antioxidant capacity ( $\mu$ mol Trolox equivalents/g DW)			
DPPH inhibition assay	$259.37 \pm 46.46^{ab}$	$241.61 \pm 4.54^b$	$318.63 \pm 40.18^a$
ABTS inhibition assay	$278.26 \pm 42.18^a$	$283.73 \pm 12.03^a$	$345.83 \pm 60.80^a$
ORAC assay	$1084.89 \pm 65.55^b$	$1325.06 \pm 47.86^a$	$1023.97 \pm 47.05^b$

<sup>a</sup> Quantified as caffeic acid. <sup>b</sup> Quantified as *p*-coumaric acid. <sup>c</sup> Quantified as quercetin aglycon. Different letters within a row indicate significant differences ( $P < 0.05$ ).

HPLC) ( $25.37 \pm 3.43 \text{ mg g}^{-1} \text{ DW}$ ) among evaluated loquat structures. This is the first time that chlorogenic acid and quercetin derivatives are reported in flowers of loquat cv. Golden Nugget and were found at higher levels. New leaves exhibited higher total phenolic contents measured by HPLC ( $14.77 \pm 1.12 \text{ mg g}^{-1} \text{ DW}$ ) than old leaves ( $6.96 \pm 0.49 \text{ mg g}^{-1} \text{ DW}$ ). Additionally, Mondolot *et al.* (2006) found that new mature leaves from *Coffea canephora* plant contained higher caffeoylquinic acids (chlorogenic acid and dicaffeoylquinic acids) than 1-year-old leaves, indicating that such phenolic compounds may undergo degradation or transportation to other plant organs where these metabolites would be required in the lignification process.

Several hydroxycinnamic acid derivatives, mainly esters of *trans* cinnamic acids (caffeic, *p*-coumaric and ferulic acids) and quinic acid together with flavonoid glycosides such as quercetin and kaempferol glycosides were reported by Ferreres *et al.* (2009) in leaves from 6 loquat cultivars grown in Brazil. In this study, the high levels of phenolic acid detected in leaves were chlorogenic acid, and levels varied from 0.0048 to 0.0347  $\text{mg g}^{-1} \text{ DW}$ . These values were significantly lower than contents reported in current study (4.61 and 7.39  $\text{mg g}^{-1} \text{ DW}$ , for old and new leaves, respectively). In addition, the same authors reported lower total phenolic contents measured by HPLC-DAD (0.0219–0.0865  $\text{mg g}^{-1} \text{ DW}$ ) than ranges obtained in current work. Little information exists to date on the phenolic composition of loquat flowers. Zhou *et al.* (2011b) reported the total flavonoid content ( $3.01 \pm 0.13 \text{ mg}$  of rutin equivalents/ $\text{g DW}$ ) measured by a spectrophotometric method in flowers from a loquat cultivar from China; however, this value was lower than the flavonoid content (as quercetin derivatives) reported in this study ( $4.95 \pm 0.59 \text{ mg g}^{-1} \text{ DW}$ ).

These differences may be significantly influenced by the cultivar type and environmental factors, since this is the first time that pheno-

lic profiles in leaves and flowers are reported for the Golden Nugget cultivar grown in the southern latitude. Phenolic compounds play an important role in the adaptation of plants to chilling stress and UV-light radiation, among other factors, increasing its biosynthesis under stress conditions (Oh *et al.*, 2009). Loquat has generally been grown in regions of warmer sub-tropical climates like China, Japan, Korea or Brazil; therefore, its cultivation in colder Chilean conditions (monitored minimum environmental temperatures were close to zero degrees between June and August in the current study) could likely explain the higher contents of hydroxycinnamic acid derivatives such as chlorogenic acid and quercetin derivatives found in leaves and flowers of the analyzed cultivar.

According to results shown in Table 1, flower extracts showed the highest antioxidant capacity when measured by the DPPH ( $318.63 \pm 40.18 \text{ } \mu\text{mol TE g}^{-1} \text{ DW}$ ) and the ABTS ( $345.83 \pm 60.80 \text{ } \mu\text{mol TE g}^{-1} \text{ DW}$ ) free radical inhibition assays, whereas no significant differences ( $P > 0.05$ ) were found between values from old and new leaves. However, new leaves exhibited higher antioxidant capacity ( $P < 0.05$ ) than old leaves when evaluated by the ORAC method.

The results in the case of leaves are similar to those obtained by Song *et al.* (2010), who reported values of  $326.87 \pm 7.17 \text{ } \mu\text{mol TE g}^{-1} \text{ DW}$  using the ABTS method in a different loquat cultivar. Pearson coefficients revealed a significant correlation ( $P < 0.05$ ) between the chlorogenic acid and quercetin derivatives contents with the antioxidant capacity determined by the DPPH ( $r = 0.7310$  and  $r = 0.7197$ , respectively) and the ABTS methods ( $r = 0.7067$  and  $r = 0.6758$ , respectively), suggesting that the higher antioxidant capacities observed in flowers are likely related to their high chlorogenic acid and quercetin derivatives contents. In addition, higher contents of caffeic acid derivatives were directly correlated ( $r = 0.8789$ ,  $P < 0.05$ ) to higher ORAC values.



*$\alpha$ -Amylase,  $\alpha$ -glucosidase and ACE-I inhibitory activities in leaves and flowers*

No ACE-I inhibitory activity was found in all evaluated loquat parts. However, all leaves (new and old) and flower extracts significantly inhibited the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes in a dose-dependent trend, indicating a potential anti-hyperglycemia activity (Table 2).

Flowers extracts showed the highest  $\alpha$ -amylase inhibitory activities ( $86.57 \pm 6.04\%$  at a dose of 10 mg of dried sample), whereas no significant differences between extracts from old and new leaves ( $P > 0.05$ ) were found ( $54.22 \pm 17.60\%$  and  $53.94 \pm 14.53\%$ , respectively at 10 mg of dried sample). Flowers had high  $\alpha$ -amylase inhibitory activity even at the lowest dose ( $73.45 \pm 9.21\%$ , 1 mg of dried sample). Further, a high correlation ( $P < 0.05$ ) was found between this *in vitro* property with the antioxidant capacity evaluated by the DPPH ( $r = 0.8340$ ) and ABTS ( $0.7552$ ) methods (dose of 10 mg of dried sample), and with the chlorogenic acid ( $r = 0.7895$ ) and quercetin derivatives contents ( $r = 0.7907$ ) at the same sample dose. This would indicate that chlorogenic acid and flavonoids such as quercetin derivatives found in higher levels in flowers would be related not only to their high antioxidant capacity, but also to its high  $\alpha$ -amylase inhibitory activity.

With regard to the  $\alpha$ -glucosidase inhibitory activities, flowers also showed the highest inhibitory potential at all sample doses. Flowers had an average of  $97.86 \pm 2.23\%$  inhibitory activity at 100  $\mu\text{g}$  of dried sample, followed by leaves (old and new), which showed no significant differences ( $p > 0.05$ ) at the same dose ( $88.67 \pm 7.92\%$  and  $80.47 \pm 12.03\%$  for old and new leaves, respectively). There was a high correlation between the antioxidant capacity when measured by the DPPH and ABTS methods and the  $\alpha$ -glucosidase inhibitory activity ( $r = 0.8110$  and  $0.6883$ , respectively, for a dose of 50  $\mu\text{g}$  of dried sample). This activity was also correlated with the chlorogenic acid and quercetin derivatives contents ( $0.6675$  and  $0.6982$ ,  $p < 0.05$ , respectively, at the same dose).

Toshima *et al.* (2010) found that a product made from fermented tea and loquat leaves had a better inhibitory effect on the  $\alpha$ -glucosidase enzyme than the product made only with fermented tea leaves. Other reports have shown a significant role of certain phenolic compounds such as chlorogenic acid and flavonoids such as quercetin or its glycoside derivatives on the inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Jo *et al.*, 2009; Oboh *et al.*, 2015). Therefore, the high inhibitory activity against evaluated key carbohydrate digestion enzymes observed in flower extracts

**Table 2.** *In vitro*  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activity of old leaves, new leaves and flowers from loquat cv. Golden Nugget evaluated at three sample doses.

Assay	Loquat structure		
	Old leaves	New leaves	Flowers
$\alpha$ -amylase inhibitory activity (%)			
10 mg	$54.22 \pm 17.60^b$	$53.94 \pm 14.53^b$	$86.57 \pm 6.04^a$
5 mg	$29.60 \pm 11.14^b$	$27.62 \pm 8.45^b$	$82.23 \pm 5.60^a$
1 mg	$20.98 \pm 13.51^b$	$14.02 \pm 4.27^c$	$73.45 \pm 9.21^a$
$\alpha$ -glucosidase inhibitory activity (%)			
100 $\mu\text{g}$	$88.67 \pm 7.92^{ab}$	$80.47 \pm 12.03^b$	$97.86 \pm 2.23^a$
50 $\mu\text{g}$	$79.57 \pm 8.62^{ab}$	$63.93 \pm 12.65^b$	$97.18 \pm 2.89^a$
20 $\mu\text{g}$	$54.87 \pm 15.93^b$	$31.58 \pm 12.63^b$	$90.60 \pm 7.80^a$

Different letters within a row indicate significant differences ( $P < 0.05$ ).

may be partly due to their high chlorogenic acid and quercetin derivatives contents, which in turn also significantly contribute to their *in vitro* antioxidant capacity.

*Bioactive compounds, antioxidant capacity and in vitro inhibitory activity against  $\alpha$ -amylase,  $\alpha$ -glucosidase and ACE-I enzymes in loquat fruits*

Total phenolic contents (TPC), the phenolic profiles and antioxidant capacity in fruits at different maturity stages are shown in Table 3. Further, some physicochemical parameters were measured in fruits to better characterize each maturity stage (Supplementary Table 1).

Fruits showed lower TPC and antioxidant capacities compared to the other evaluated loquat structures (leaves and flowers), and important variations were observed according to their maturity stage. TPC significantly decreased from  $5.99 \pm 0.15$  to  $3.24 \pm 0.08$  mg GAE g<sup>-1</sup> DW when fruits changed from stage 1 to 2, remaining constant in the last maturity stage ( $3.41 \pm 0.07$  mg GAE g<sup>-1</sup> DW,  $P > 0.05$ ). Ding *et al.* (2001) found that total phe-

nolic contents decreased by approximately 60% in loquat fruits in other cultivars when it passed from green to a colored state. Higher TPC values were found in mature fruit (0.46 mg GAE g<sup>-1</sup> FW) compared to the results reported by Ercisli *et al.* (2012) (0.14–0.25 mg GAE g<sup>-1</sup> FW). Nevertheless, current results were lower than those reported by Xu *et al.* (2014), who obtained TPC in the range of 0.66–0.96 mg GAE g<sup>-1</sup> FW in loquat fruit from another cultivar from China.

Caffeic acid and *p*-coumaric acid derivatives were detected by HPLC (Table 3). Caffeic acid derivatives were the most abundant phenolic compounds ( $3.69 \pm 0.01$  mg g<sup>-1</sup> DW at green stage) and significantly decreased to  $2.30 \pm 0.03$  mg g<sup>-1</sup> DW in the last maturity stage. Xu *et al.* (2014) identified certain caffeic acid derivatives such as chlorogenic acid, neochlorogenic acid and 4-*o*-caffeoylquinic acid, together with protocatechuic acid, 4-hydroxybenzoic acid, caffeic acid, ferulic acid, ellagic acid, and *o*-coumaric acid in 6 cultivars of mature loquat fruits grown in China. Similar results were obtained by Ding *et al.* (2001) and Ferreres *et al.* (2009) who detected chlorogenic, and neochlorogenic acids among others phenolics

**Table 3.** Total phenolic contents, phenolic profiles by HPLC-PAD and antioxidant capacity by the DPPH, ABTS and ORAC methods of loquat fruits cv. Golden Nugget at three different maturity stages.

Assay	Stage of maturity		
	Stage 1	Stage 2	Stage 3
Total phenolic contents (mg GAE/g DW)	$5.99 \pm 0.15^a$	$3.24 \pm 0.08^b$	$3.41 \pm 0.07^b$
Phenolic acids (mg/g DW)			
Caffeic acid derivatives <sup>a</sup>	$3.69 \pm 0.01^a$	$2.43 \pm 0.22^b$	$2.30 \pm 0.03^b$
<i>p</i> -coumaric acid derivatives <sup>b</sup>	$2.11 \pm 0.01^a$	$1.08 \pm 0.17^b$	$0.312 \pm 0.001^c$
Total phenolic contents HPLC (mg/g DW)	$5.80 \pm 0.02^a$	$3.50 \pm 0.39^b$	$2.61 \pm 0.03^c$
Antioxidant capacity ( $\mu$ mol Trolox equivalents/g DW)			
DPPH inhibition assay	$45.68 \pm 0.93^a$	$35.88 \pm 0.64^b$	$30.34 \pm 1.19^c$
ABTS inhibition assay	$51.96 \pm 1.09^a$	$36.56 \pm 1.97^b$	$30.54 \pm 1.10^c$
ORAC assay	$344.49 \pm 4.67^a$	$199.78 \pm 7.28^b$	$137.01 \pm 6.15^c$

<sup>a</sup> Quantified as caffeic acid. <sup>b</sup> Quantified as *p*-coumaric acid. Different letters within a row indicate significant differences ( $P < 0.05$ ).

in different loquat fruit cultivars from Japan and Brazil, respectively. It seems that for case of the Golden Nugget cultivar grown in Chile, chlorogenic acid would be more concentrated in leaves and flowers than in fruits; however, further research is being conducted by our research group to elucidate this.

The antioxidant capacity (DPPH, ABTS and ORAC) decreased significantly ( $p < 0.05$ ) with fruit ripening. DPPH antioxidant capacity ranged from  $45.68 \pm 0.93 \mu\text{mol TE g}^{-1} \text{DW}$  at green state to  $30.34 \pm 1.19 \mu\text{mol TE g}^{-1} \text{DW}$  at mature stage (stage 3), whereas the ABTS antioxidant capacity decreased from  $51.96 \pm 1.09$  (stage 1) to  $30.54 \pm 1.10 \mu\text{mol TE g}^{-1} \text{DW}$  at the last maturity stage. Similarly, ORAC decreased significantly from  $344.49 \pm 4.67$  to  $137.01 \pm 6.15 \mu\text{mol TE g}^{-1} \text{DW}$ . These results are in accordance with those reported by Xu *et al.* (2014) and Ercisli *et al.* (2012) at mature stage.

The Pearson analysis revealed a strong correlation between the phenolic contents and the total antioxidant capacity when the DPPH, ABTS and ORAC methods were used ( $r = 0.9006, 0.9339$  and  $0.9340$ , respectively).

No triterpenoid acids were detected by HPLC in loquat fruit extracts. Moreover, no  $\alpha$ -amylase,  $\alpha$ -glucosidase and ACE-I inhibitory activities were found in evaluated fruit samples at three maturity stages.

In conclusion, this study provided preliminary insights on the potential health functionality-linked bioactive compounds of different parts from loquat plant cv. Golden Nugget cultivated in the central region of Chile. No triterpenic

acids were found in any of the loquat parts. Leaves and flowers showed higher TPC and AC than fruits, and flowers are potentially rich sources of phenolic compounds (chlorogenic acid and quercetin derivatives) that significantly contributed to their antioxidant capacity. Both leaves and flowers inhibited the hyperglycemia-relevant  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes but flowers had the highest inhibitory activity. *In vitro* inhibitory activities of these carbohydrate digestive enzymes were directly correlated to chlorogenic acid and quercetin derivative contents and with the antioxidant capacity suggesting that leaves and flowers have *in vitro* anti-hyperglycemia activity with potential to impact on the prevention of oxidation-linked complications using diet and ingredient designs. No ACE-I,  $\alpha$ -amylase or  $\alpha$ -glucosidase inhibitory activities were detected in loquat fruits when evaluated at different maturity stages; however, TPC (mainly hydroxycinnamic acid derivatives) and antioxidant capacity were high at the green stage. Loquat leaves and flowers may be potential sources of phenolic bioactives with promising applications in the nutraceutical and functional food markets.

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## Resumen

**J. Ahumada, C. Fuentealba, J.A. Olaeta, P. Undurraga, R. Pedreschi, K. Shetty, R. Chirinos, D. Campos, y L. Gálvez Ranilla. 2017. Compuestos bioactivos de níspero (*Eriobotrya japonica* Lindl.) cv. Golden Nugget y análisis de su funcionalidad *in vitro* para el manejo de la hiperglicemia.. Cien. Inv. Agr. 44(3): 272-284.** El cultivar de níspero Golden Nugget (hojas, flores y frutos), el cual presenta un potencial comercial importante para Chile, fue analizado en su composición en compuestos bioactivos (contenido de fenólicos totales: TPC, perfil de fenólicos y ácidos triterpénicos) y bioactividad *in vitro* como capacidad antioxidante (AC) y actividad inhibitoria de las enzimas relevantes para el control de la hiperglicemia ( $\alpha$ -amilasa and  $\alpha$ -glucosidasa) y la hipertensión (enzima convertidora de la angiotensina-I, ACE-I). Las hojas y flores tuvieron mayores contenidos de TPC y AC que las frutas y se encontraron principalmente derivados de ácidos hidroxicinámicos en todas las estructuras por HPLC. Solo se detectó ácido clorogénico y derivados de quercetina en las hojas y flores, las cuales inhibieron las enzimas  $\alpha$ -amilasa and  $\alpha$ -glucosidasa, aunque las flores mostraron la mayor actividad antihiperglicémica. Las frutas no mostraron inhibición de la ACE-I ni actividad antihiperglicémica, sin embargo los TPC y la AC fueron mayores en el estado verde de madurez. Las hojas y flores del cultivar de níspero evaluado constituyen fuentes de fenólicos bioactivos con potencial para el manejo de la hiperglicemia. No se encontraron ácidos triterpénicos en todas las estructuras evaluadas.

**Palabras clave:** *Eriobotrya japonica*; compuestos fenólicos; antioxidantes; anti-hiperglicemia; níspero.

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