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Phenolic profiles and antioxidant properties of *Physalis angulata* L. as quality indicators

[Perfiles fenólicos y propiedades antioxidantes de extractos de *Physalis angulata* L. como indicadores de calidad]

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Abstract

Context: Previous reports informed about the species-specific condition of plant phenolic profiles and the association between phenolic composition and plant antioxidant properties. The species-specific condition of phenolic patterns offers the opportunity to develop chemical quality markers for plants used in traditional medicine. However, changes in phenolic composition can occur during plant growth. *Physalis angulata* is an important medicinal plant for which the age-related variation of phenolic composition and antioxidant properties is unknown.

Aims: To assess the phenolic composition and antioxidant properties of above- and under-ground parts of *P. angulata* at four growth stages and evaluate their potential as chemical quality indicators.

Methods: A method of HPLC-DAD was used to assess the phenolic profiles. Anti-radical potential, total antioxidant capacity and iron reducing power were evaluated by spectrometric methods. Sample distinctiveness was determined by cluster analyses.

Results: The HPLC-DAD analysis revealed 21 phenolic compounds, which were accumulated in a tissue- and age-specific manner. On the contrary, a clear distinctiveness between phenolic contents and antioxidant properties of samples was not observed. Mature flowers highlighted for its total phenolic content (6.50 mg/g fresh tissue) and total antioxidant capacity (416.1 µg/mL). Young leaves were outstanding for its flavonoid content (37.86 µg/g fresh tissue) and reducing power (A_{700nm} = 4.04). Immature calyces were worthy for its phenolic acid concentration (1.28 µg/g fresh tissue) and its free radical scavenging activity (EC_{50} = 0.12 mg/mL).

Conclusions: Phenolic profiles represent reliable chemical quality indicators for determining plant age, tissular origin, authenticity, and adulteration of herbal preparations of *P. angulata*.

Keywords: flavonols; growth stages; kaempferol glycosides; quality chemical markers.

Resumen

Contexto: Reportes previos han informado sobre la especificidad de los perfiles fenólicos de las plantas y sobre la asociación entre éstos y las propiedades antioxidantes. La especificidad de los perfiles fenólicos permitiría desarrollar marcadores químicos de calidad para plantas medicinales. Sin embargo, la composición fenólica puede cambiar durante el crecimiento. Para *Physalis angulata* se desconoce la variación asociada al desarrollo de la composición fenólica y de las propiedades antioxidantes.

Objetivos: Determinar la composición fenólica y propiedades antioxidantes de diferentes partes de *P. angulata* en cuatro estados de desarrollo y evaluar su potencial como indicadores químicos de calidad.

Métodos: Los perfiles fenólicos se determinaron por HPLC-DAD, las propiedades antioxidantes por métodos espectrométricos, y la discriminación entre muestras por un análisis de agrupamiento.

Resultados: Se encontraron 21 compuestos fenólicos, los cuales se acumularon de manera específica por tejido y edad de la planta. No se observó una clara diferencia entre los contenidos fenólicos y las propiedades antioxidantes de las muestras. Las flores maduras sobresalieron por su contenido fenólico (6,50 mg/g de tejido fresco) y capacidad antioxidante total (416,1 µg/mL). Las hojas jóvenes por su contenido de flavonoides (37,86 µg/g tejido fresco) y poder reductor (A_{700nm} = 4,04). Los cálices inmaduros por su concentración de ácidos fenólicos (1,28 µg/g de tejido fresco) y actividad bloqueadora de radicales libres (EC_{50} = 0,12 mg/mL).

Conclusiones: Los perfiles fenólicos representan fiables marcadores químicos de calidad para determinar la edad de la planta, el origen tisular, la autenticidad y la adulteración de preparaciones herbales de *P. angulata*.

Palabras Clave: canferol glicósidos; estados de crecimiento; flavonoles; marcadores químicos de calidad.

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INTRODUCTION

Physalis angulata L. is an edible and medicinal species of Solanaceae family. It is native of the American continent (Vargas-Ponce et al., 2015) but, at present, it grows as an introduced plant in several Asian and African countries (Hseu et al., 2011). Currently, *P. angulata* is a wild species, for which recent plantations have been established in Mexico (Vargas-Ponce et al., 2015) and Brazil (Freitas et al., 2006). The species is considered as an alternative crop for its edible fruits and for its diversity of secondary metabolites, which have an important potential in the bio-products industry (Vargas-Ponce et al., 2015). In Mesoamerica, this species is used in traditional medicine since pre-Columbian times (Santiaguillo and Blas, 2009) and presently, forms part of the Chinese traditional medicine, recognizing for it an important capacity as inhibitor of tumor metastasis and angiogenesis (Hseu et al., 2011), and antinociceptive properties (Lima et al., 2014), among others. Different medicinal properties have been associated with different parts of *P. angulata*. For instance, antinociceptive properties were first recognized for roots (Bastos et al., 2006) and more recently for stems (Lima et al., 2014), whereas cytotoxicity against murine leukemia cell line P-388, epidermoid carcinoma of the nasopharynx KB-16 cells, and lung adenocarcinoma A-549 has been determined for leaves (Ismail and Alam, 2001). For this reason, tools to authenticate herbal preparations of this species become an important issue.

Phenolic compounds are plant secondary metabolites with a wide spectrum of biological activities. The antioxidant activity is one the most relevant because oxidation is related to the development of neurodegenerative diseases and cancer (López-Laredo et al., 2012). Plant phenolic composition is the result of the interaction of genetic and environmental factors and has been reported as species-specific (Medina-Medrano et al., 2015). However, changes associated with growth stages, seasons of growing and eco-geographical growth conditions have been reported for some species, like *Rosmarinus officinales* (Del Baño et al., 2003), *Silene littorea* (Del Valle et al., 2015), and several species of *Betula* (Raal et al., 2015). These changes can modify the flavor and biological properties of edible and medicinal plants and reveal that neither the concentration nor the relevance of different phenolic compounds in plants are always the same (Jiang et al., 2013). The characterization of those variations may contribute to ensure the reliability and quality of edible and medicinal plant species to make a more efficient use of their phytochemicals for nutraceutical and pharmaceutical purposes. Besides, chemical characterization may support the development of quality control tools of plant-based medicinal preparations, as adulteration is a global latent risk for traditional herbal preparations, as documented by Ahmad et al. (2009).

Few studies about the phenolic composition of *P. angulata* have been carried out, one of them is that of Medina-Medrano et al. (2015), who compared the phenolic profiles of five species of *Physalis*, included *P. angulata*. To the best of our knowledge, except for the fruits of *P. peruviana* (Licodiedoff et al., 2013), studies on the changes of phenolic composition during development of *Physalis* species have not been carried out. The aim of the present paper was to determine the growth-related variation of phenolic composition and antioxidant properties of above- and under-ground parts of *P. angulata*, during four growth stages to evaluate their potential as chemical quality indicators for herbal preparations of this edible and medicinal plant.

MATERIAL AND METHODS

Plant material

Physalis angulata seeds were collected in Durango, Mexico (24° 08′ 15.4″ N, 104 °31′ 58.5″ W) in August 2012. Voucher specimen was deposited at Herbarium CIIDIR (curatorial number: 42854). Seeds were germinated (May 2013) in a greenhouse located in Durango, Mexico (24° 3’ 5” N, 104° 36’ 34” W). The germination pots contained moss peat and perlite (5:1). After 30 days, seedlings were individually transplanted into plastic bags (5 L capacity), containing compost, sand and soil (1:1:1). Plants were watered weekly. Minimum temperature in the greenhouse varied between 11 and 14.3°C, whereas maximum temperature fluctuated between 34 and 37.5°C all over study.

Samples were collected from plants at four different growth stages (GS), according to the Biolo-

gische Bundesanstalt Bundessortenamt and Chemical (BBCH)-based phenological scale described for *P. peruviana* L. (Ramírez et al., 2013): GS1: 25-day-old plants with first couple of leaves (GS-102 according to BBCH scale), GS2: 42-day-old plants in early flowering (GS between 602 and 608 according to BBCH scale), GS3: 57-day-old plants with much foliage, late flowering and unripe fruits (GS-701 according to BBCH scale), and GS4: 115-day-old plants in decline, with ripe fruits (GS between 806 and 809 according to BBCH scale). Roots, stems, leaves, flowers, calyces, and fruits were separately collected, ground in liquid nitrogen at excision moment, and individually analyzed.

Instrumentation and reagents

Ethanol (analytical grade) was purchased from J. T. Baker (Xalostoc, Estado de México, Mexico). Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the standards gallic acid, quercetin, epichatechin, chlorogenic acid, caffeic acid, *p*-coumaric acid, and quercitrin (quercetin-3-*O*-rhamnoside) came from Sigma-Aldrich (St. Louis, Missouri, USA). A Boeco U-32R centrifuge was used. All spectrometric measurements were registered using a Thermo Scientific Genesys 10S UV-Vis spectrophotometer. The phenolic profiles were obtained from a Perkin Elmer Series 200 HPLC system with diode array detector (DAD), and a Perkin Elmer Brownlee Analytical C18 column (4.6 x 250 mm, 5 µm).

Preparation of extracts

Ground samples (10 g) were combined with 80 mL of 80% ethanol (v/v), sonicated for 1 hour at 25°C, and then, macerated for 24 hours (100 rpm, in darkness, at room temperature). Extracts were centrifuged at 7600 rpm for 10 minutes at room temperature. Supernatants were collected, concentrated to dryness under reduced pressure in a rotary evaporator, and then dissolved in 80% ethanol (v/v) to a final concentration of 100 mg/mL. Aliquots were taken for the further analysis.

Total phenolic contents

The concentrations of total phenolics were determined with the Folin-Ciocalteu reagent, according to Nurmi et al. (1996). Extract (1 mL) was combined with 1 mL of Folin-Ciocalteu reagent. After 5 min, 2 mL of 20% Na_2CO_3 were added. Samples were incubated for 10 min at room temperature and darkness before being centrifuged (2000 rpm for 10 min). Absorbances were registered at 760 nm. Total phenolic contents were estimated from a standard curve of gallic acid (Abs_{760nm} = 0.003[Gallic acid] - 0.0241, r = 0.9986), constructed with four concentrations of this compound between 8 and 92 µg/mL. Total phenolics were expressed as milligram of gallic acid equivalents per gram of fresh weight (mg GAE/g fw).

Antioxidant properties

The antioxidant properties were evaluated by the total antioxidant capacity (TAC), free radical scavenging capacity (DPPH assay) and iron reducing power (RP).

Total antioxidant capacity

TAC was assessed according to Prieto et al. (1999). In this method, the reduction of Mo (VI) to Mo (V) is carried out by the antioxidant, resulting a green phosphate/Mo (V) complex at acid pH. Extract (100 µL) was combined with 1 mL of a solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. Then samples were incubated at 95°C for 90 min. After samples got room temperature, absorbance was registered at 695 nm against a blank prepared in the same manner but adding ethanol instead of sample. Quercetin (0.1 mg/mL) and epicatechin (0.1 mg/mL) were analyzed as references. TAC was calculated according to the following standard curve: A_{695} = 4.2133 [AA] - 0.2365, r = 0.9987, constructed with ascorbic acid between 0.1 and 1 mg/mL. TAC values were expressed as milligrams of ascorbic acid equivalents per milliliter (mg AAE/mL).

DPPH scavenging capacity

The DPPH assay reported by Lue et al. (2010) was used to evaluate the free radical scavenging activity. Five concentrations of each extract (0.5 to 5 mg/mL) were prepared and individually analyzed. Extract (1 mL) was added to 3 mL of 0.1 mM DPPH (prepared in ethanol) and allowed to stand for 30

min at room temperature and darkness. Then absorbance was registered at 523 nm. The DPPH concentrations in the reaction medium against the extract concentrations were plotted to determine the efficient concentration at 50%, defined as the amount of extract needed to decrease by 50% the initial DPPH concentration (EC_{50}). The following calibration curve, made with DPPH between 2 and 60 µg/mL, was used to calculate the DPPH concentration (µg/mL) in the reaction medium: A_{523} = 0.0019 [DPPH] + 0.0309, r = 0.9998. Anti-radical activities were expressed in terms of EC_{50} in mg/mL. Quercetin and epicatechin were analyzed as references.

Iron reducing power

The RP was evaluated according to Lue et al. (2010). Aliquots (100 µL) of each extract (0.1 mg/mL) were mixed with 250 µL of 0.2 M phosphate buffer (pH 6.6) and 250 µL of 30 mM $K_3Fe(CN)_6$ and incubated for 20 min at 50°C and darkness. Then 250 µL of 0.6 M trichloroacetic acid were added. Samples were centrifuged (10 min, 2000 rpm) and 200 µL of upper layer were removed, which were mixed with 200 µL of double-distilled water and 40 µL of 0.1% $FeCl_3$ (w/v). Samples were incubated for 10 min. The formation of ferrous ions (Fe^{2+}) was registered by the absorbance at 700 nm. The highest absorbance values the greatest capacity of reducing ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. Quercetin (0.1 mg/mL) was analyzed as reference.

HPLC-DAD analysis

The gradient method described by Campos and Markham (2007) was used. Injection volume was 50 µL and flow rate was 0.8 mL/min. The mobile phase was formed of acidified water (pH 2.5 with orthophosphoric acid) like solvent A and acetonitrile like solvent B. The solvent gradient started with 100% of solvent A, then decreasing to 91% over the next 12 min, to 87% over the next 8 min, to 67% over the next 12 min, to 57% over the next 10 min, and held at this level until the end of the 60 min analysis. Chromatograms were registered at 265 and 340 nm. UV spectra for all peaks were obtained between 220 and 400 nm, using diode array detection. Structural information of each phenolic compound resolved in the chromatograms was obtained by comparison of its retention time (RT) and UV spectrum with those of the following reference compounds: chlorogenic acid (RT: 27.76 min; λ_{max}: 243sh, 296sh, 326), caffeic acid (RT: 53.13 min; λ_{max}: 239sh, 295sh, 318), *p*-coumaric acid (RT: 37.2 min; λ_{max}: 294sh, 308), quercetin (RT: 47.05 min; λ_{max}: 255, 268sh, 299sh, 370), and quercitrin (quercetin-3-*O*-rhamnoside; RT: 38.54 min; λ_{max}: 255, 264sh, 295sh, 348). Structural information was also obtained from the UV spectra according to the UV theory of flavonoids and phenolic acids developed by Campos and Markham (2007). All compounds resolved in the respective chromatograms formed the phenolic profile of each extract. The contents of the individual phenolic acids and flavonoids were determined by area measurements, using standard curves of quercetin for flavonols (Area = 1.08 x 10^7 [quercetin] + 3.19 x 10^6; r = 0.9985) and of gallic acid for phenolic acids (Area = 2.54 x 10^8 [gallic acid] + 3.02 x 10^6; r = 0.996). The sum of individual flavonoid concentrations in a single sample represented the total flavonoid concentration, expressed as micrograms per gram of fresh weight (µg/g fw), whereas the sum of individual phenolic acids corresponded to the total phenolic acid concentration, expressed as micrograms per gram of fresh weight (µg/g fw).

Statistical analysis

Each analysis was carried out for three independent pools of each sample. Data were subjected to an analysis of variance ($p \leq 0.05$) and means were separated by Tukey test by using Statistica 7. Correlations between the antioxidant properties and the concentration of phenolic compounds were carried out by Spearman test, using SPSS 17.0. The comparison of phenolic contents as well as antioxidant activities among the different extracts was carried out by a cluster analysis (based on the Neighbour Joining algorithm and the Jaccard similarity measure) by using Past 3.10. The comparison among the phenolic profiles was carried out by constructing a binary matrix, coded by 1 (presence) or 0 (absence), considering the phenolic profiles of each sample and subjecting the matrix to a cluster analysis.

RESULTS AND DISCUSSION

Total phenolics

Table 1 shows the contents of total phenolics of different parts of *P. angulata*. Some significant tissue- and age-dependent differences were found. Flowers at GS3 and GS2 (6.50 and 5.25 mg/g fw, respectively), young leaves at GS1 (4.98 mg/g fw) and immature calyces at GS3 (4.87 mg/g fw) accumulated the highest amounts of total phenolics. The current results indicated that the mature flowers of *P. angulata* accumulated similar amounts of total phenolics to those of flowers of other medicinal plants, as *Castilleja tenuiflora* Benth. (7.95 mg/g dry weight), used in traditional cancer treatment in Mexico (López-Laredo et al., 2012). The contents of total phenolics found for the young leaves of *P. angulata* were 14-fold higher than the contents reported for the young leaves of the medicinal species *Barringtonia racemosa* Roxb. (0.34 mg/g dry weight) and 26-fold higher than those of *B. spicata* Blume (0.19 mg/g dry weight) reported by Dalila et al. (2015).

A clear increasing tendency of total phenolic concentration in accordance with age was found for roots (r = 0.97), whereas a clear decreasing tendency in accordance with age was observed for leaves (r = 0.99) (Table 1). Decreasing trends of total phenolic concentrations associated to plant age have also been reported for the leaves of *Rosmarinus officinalis* (Del Baño et al., 2003), *B. racemosa* and *B. spicata* (Dalila et al., 2015).

Considering the concentration of total phenolics of all plant parts at a single growth stage (values estimated from data on Table 1), *P. angulata* accumulated the highest levels (16.70 mg/g fw,) at GS3 (57 days after germination). At GS3, flowers (6.50 mg/g fw) and calyces (4.87mg/g fw) were the major contributors. At GS3, *P. angulata* accumulated 2.4-fold more total phenolics than at GS1 (6.95 mg/g fw), when plants were 25 days old.

Flavonoid contents

In fruits and mature calyces no flavonoids were found (Table 1). The current results are contrasting with those reported for the calyces of other species of *Physalis*, like *P. ixocarpa*, whose calyces accumulate four flavonoids (Matlawska et al., 1994), and *P. solanaceus*, in whose calyces, the flavonoid rutin was found (Pérez-Castorena et al., 2013). Roots accumulated only low levels at GS2; the current results partially agreed with those reported for the roots of other species of *Physalis*, like *P. solanaceus*, for which Pérez-Castorena et al. (2013) reported no flavonoids. Young leaves (at GS1) accumulated the highest flavonoid concentration (37.86 µg/g fw). This value was a little higher than the value reported for the leaves of *C. tenuiflora* (35.78 µg/g dry weight) by López-Laredo et al. (2012). At any growth stage, leaves accumulated higher flavonoid concentrations than any other plant part. Clear decreasing trends in flavonoid concentrations in accordance with plant age were observed for stems and leaves (r = 0.85 and 0.83, respectively). Mature flowers accumulated higher flavonoid concentrations (9.44 µg/g fw) than immature ones (4.11 µg/g fw).

Taking in account the flavonoid contents of all plant parts at a single growth stage, *P. angulata* accumulated the highest levels at GS1 and GS3 (48.96 and 47.83 µg/g fw, respectively, values estimated from data of Table 1); at these two stages, leaves were the major contributors (37.86 and 23.17 µg/g fw at GS1 and GS3, respectively). As plants at GS3 have more biomass (given mainly by the number and size of leaves) than at GS1, and fruits are commonly collected immature to prolong storage, GS3 could be the best growth stage to obtain flavonoids. This can be relevant because the remaining leaves after the fruit collection could be retrieved in an easy and inexpensive way, to extract flavonoids, as a manner of an integral exploitation of this resource.

The high flavonoid levels found in the young leaves and steams (at GS1) of *P. angulata* (Table 1) may be associated with both the cellular differentiation processes required for development and the photoprotection and defence that plants need at the most vulnerable stages (early growth stages), as these compounds have been reported to play those roles (Santiago et al., 2008).

Phenolic acid contents

The roots of *P. angulata* practically accumulated no phenolic acids (Table 1). Except for fruits, the above-ground parts accumulated lower phenolic acid concentrations than flavonoid concentrations, being the young calyces, at GS3, the structures with

Table 1. Contents of total phenolics, flavonoids, phenolic acids, and antioxidant evaluations of different structures of *P. angulata* collected in Durango, Mexico, at four growth stages.

Sample	Total phenolics (mg/g fw)	Flavonoids (µg/g fw)	Phenolic acid (µg/g fw)	TAC (µg/mL)	EC_{50} (mg/mL)	RP (A_{700nm})
Root GS1	0.38 ± 0.01 [f]	Not found	Not found	43.44 ± 3.11 [f]	1.02 ± 0.09 [b]	0.05 ± 0.00 [g]
Root GS2	0.68 ± 0.08 [f]	<0.05	<0.05	35.28 ± 1.08 [f]	1.22 ± 0.03 [a]	0.10 ± 0.00 [fg]
Root GS3	0.76 ± 0.01 [ef]	Not found	Not found	37.94 ± 1.65 [f]	0.68 ± 0.04 [c]	0.18 ± 0.02 [fg]
Root GS4	0.81 ± 0.01 [ef]	Not found	Not found	42.53 ± 2.17 [df]	0.55 ± 0.01 [cd]	0.22 ± 0.01 [fg]
Stem GS1	1.59 ± 0.01 [ef]	11.10 ± 1.55 [d]	0.20 ± 0.00 [bc]	107.5 ± 4.11 [adf]	0.55 ± 0.01 [cd]	0.68 ± 0.03 [def]
Stem GS2	1.18 ± 0.00 [ef]	3.53 ± 0.24 [ef]	<0.05	75.75 ± 3.98 [bdf]	1.02 ± 0.02 [b]	0.35 ± 0.02 [fg]
Stem GS3	0.50 ± 0.04 [f]	0.89 ± 0.08 [f]	0.09 ± 0.02 [c]	23.69 ± 2.96 [f]	1.08 ± 0.09 [ab]	0.13 ± 0.03 [fg]
Stem GS4	0.89 ± 0.02 [ef]	0.92 ± 0.08 [f]	<0.05	48.35 ± 5.53 [cdf]	0.49 ± 0.03 [cd]	0.37 ± 0.02 [fg]
Leaf GS1	4.98 ± 0.23 [abc]	37.86 ± 2.90 [a]	1.12 ± 0.17 [a]	222.3 ± 26.12 [bc]	0.15 ± 0.00 [ef]	3.04 ± 0.36 [a]
Leaf GS2	3.92 ± 0.10 [bcd]	21.25 ± 3.35 [bc]	0.20 ± 0.03 [b]	132.1 ± 5.87 [cd]	0.24 ± 0.00 [ef]	1.30 ± 0.41 [cd]
Leaf GS3	2.85 ± 0.06 [cde]	23.17 ± 1.28 [b]	0.99 ± 0.03 [a]	151.8 ± 2.96 [cd]	0.29 ± 0.01 [e]	0.45 ± 0.01 [fg]
Leaf GS4	1.79 ± 0.28 [def]	15.42 ± 5.31 [cd]	0.31 ± 0.10 [b]	178.8 ± 14.94 [bc]	0.25 ± 0.01 [ef]	1.35 ± 0.37 [c]
Flower GS2	5.25 ± 0.26 [ab]	4.11 ± 0.44 [ef]	0.19 ± 0.00 [bc]	306.1 ± 13.57 [b]	0.17 ± 0.06 [ef]	0.43 ± 0.01 [fg]
Flower GS3	6.50 ± 1.26 [a]	9.44 ± 1.16 [de]	0.59 ± 0.07 [b]	416.1 ± 23.78 [a]	0.19 ± 0.02 [ef]	1.25 ± 0.31 [cde]
Calyx GS3	4.87 ± 0.09 [abc]	14.33 ± 1.48 [d]	1.28 ± 0.05 [a]	217.8 ± 32.01 [c]	0.12 ± 0.01 [f]	2.57 ± 0.14 [b]
Calyx GS4	2.42 ± 0.31 [de]	Not found	0.05 ± 0.01 [c]	177.2 ± 17.11 [c]	0.62 ± 0.06 [cd]	0.47 ± 0.03 [fg]
Fruit GS3	1.22 ± 0.01 [ef]	Not found	0.06 ± 0.01 [c]	83.03 ± 7.25 [df]	0.56 ± 0.02 [cd]	0.67 ± 0.03 [f]
Fruit G4	1.10 ± 0.17 [ef]	Not found	<0.05	106.81± 17.84 [df]	0.63 ± 0.05 [cd]	0.32 ± 0.07 [fg]
Quercetin				80.00 ± 2.00 [df]	$5.29E^{-3}$±$0.39E^{-3}$ [g]	0.78±0.004 [d]
Epicatechin				110.00± 5.00 [df]	$14.33E^{-3}$±$0.29E^{-3}$ [g]	Not evaluated

The values represent the mean and standard deviation of three independent analyses. Different letters in the same column mean significant differences ($p<0.05$) according to the Tukey test.

TAC: Total antioxidant capacity; EC_{50}: Efficient concentration at 50% for the free radical scavenging activity; RP: Iron reducing power; GS1 to GS4: Growth stages 1 to 4.

the highest levels of this class of acids (1.28 µg/g fw). However, still this value was lower than the reported for other plant species, like sugarcane leaves (around 16 µg/g fw) by Santiago et al. (2008).

Phenolic composition

According to the UV theory developed by Campos and Markham (2007), on the base of the number of absorption bands, intensity and shape of bands, as well as the number, position and shape of shoulders in the UV spectra, it is possible to determine the types of phenolic compounds and some OH- and glycoside-substitutions in their structures. Under our experimental conditions, the HPLC-DAD analysis revealed a total of 21 compounds in the different parts of *P. angulata*: 15 phenolic acids, two quercetin-3-*O*-glycosides, two kaempferol-3-*O*-glycosides, and two kaempferol-3,7-di-*O*-glycosides. Table 2 displays the retention time and spectral data for each compound present in a single tissue at a given growth stage.

The retention times and spectral data of compound **1** (RT: 27.948 min; λ_{max}: 243sh, 296sh, 326) and compound **1R** (RT: 37.565 min; λ_{max}: 204sh, 308) corresponded to those of the references chlorogenic

acid (RT: 27.76 min; λ_{max}: 243sh, 296sh, 326) and *p*-coumaric acid (RT: 37.2 min; λ_{max}: 294sh, 308) and were assumed as those compounds, respectively. Qualitative plant part- and age-dependent variations were found in the phenolic composition of *P. angulata*. Chromatograms and UV spectra of some of the major compounds are shown in Figs. 1-3.

Table 2. Retention time, spectrometric data, and type of phenolic compound found in different parts of *Physalis angulata* at four stages of development.

Sample	Retention time (min)	λ_{max}	Code	Type of phenolic compound
Leaf GS1	27.948 ± 0.647	243, 296sh, 326	**1**	Phenolic acid (Chlorogenic acid)
	31.329 ± 0.219	256, 266sh, 357	**2**	Quercetin-3-*O*-glycoside
	32.255 ± 0.659	227, 302sh, 314	**3**	Phenolic acid
	32.990 ± 0.388	227sh, 248sh, 268, 351	**4**	Kaempferol-3,7-di-*O*-glycoside
	33.961 ± 0.374	229sh, 248sh, 269, 348	**5**	Kaempferol-3,7-di-*O*-glycoside
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
	41.903 ± 0.298	267, 302sh, 351	**7**	Kaempferol-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Leaf GS2	31.580 ± 0.359	256, 266sh, 357	**2**	Quercetin-3-*O*-glycoside
	32.255 ± 0.659	227, 302sh, 314	**3**	Phenolic acid
	32.990 ± 0.388	227sh, 248sh, 268, 351	**4**	Kaempferol-3,7-di-*O*-glycoside
	33.961 ± 0.374	229sh, 248sh, 269, 348	**5**	Kaempferol-3,7-di-*O*-glycoside
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
	41.903 ± 0.298	267, 302sh, 351	**7**	Kaempferol-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Leaf GS3	32.255 ± 0.659	299sh, 311	**1L**	Phenolic acid
	32.990 ± 0.388	227sh, 248sh, 268, 351	**4**	Kaempferol-3,7-di-*O*-glycoside
	33.961 ± 0.374	229sh, 248sh, 269, 348	**5**	Kaempferol-3,7-di-*O*-glycoside
	35.503 ± 0.111	309	**2L**	Phenolic acid
	35.867 ± 0.111	230, 303sh, 315	**3L**	Phenolic acid
	41.903 ± 0.298	267, 302sh, 351	**7**	Kaempferol-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Leaf GS4	27.948 ± 0.647	242, 302sh, 327	**1**	Phenolic acid (Chlorogenic acid)
	32.990 ± 0.388	227sh, 248sh, 268, 351	**4**	Kaempferol-3,7-di-*O*-glycoside
	33.961 ± 0.374	229sh, 248sh, 269, 348	**5**	Kaempferol-3,7-di-*O*-glycoside
	35.503 ± 0.111	309	**2L**	Phenolic acid
	35.867 ± 0.111	230, 303sh, 315	**3L**	Phenolic acid
	38.220 ± 0.019	237, 301sh, 329	**4L**	Phenolic acid
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
	41.903 ± 0.298	267, 302sh, 351	**7**	Kaempferol-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Stem GS1	31.830 ± 0.359	256, 266sh, 357	**2**	Quercetin-3-*O*-glycoside
	32.255 ± 0.659	227, 302sh, 314	**3**	Phenolic acid
	32.990 ± 0.388	227sh, 248sh, 268, 351	**4**	Kaempferol-3,7-di-*O*-glycoside
	33.961 ± 0.374	229sh, 248sh, 269, 348	**5**	Kaempferol-3,7-di-*O*-glycoside

Table 2.continued...

	33.981 ± 0.308	238, 300sh, 325	**2S**	Phenolic acid
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Stem GS2	32.990 ± 0.388	227sh, 248sh, 268, 351	**4**	Kaempferol-3,7-di-*O*-glycoside
	33.961 ± 0.374	229sh, 248sh, 269, 348	**5**	Kaempferol-3,7-di-*O*-glycoside
	33.981 ± 0.308	238, 300sh, 325	**2S**	Phenolic acid
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
	41.903 ± 0.298	267, 302sh, 351	**7**	Kaempferol-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Stem GS3	32.669 ± 0.422	235, 297sh, 323	**1S**	Phenolic acid
	32.990 ± 0.388	227sh, 248sh, 268, 351	**4**	Kaempferol-3,7-di-*O*-glycoside
	33.961 ± 0.374	229sh, 248sh, 269, 348	**5**	Kaempferol-3,7-di-*O*-glycoside
	38.675 ± 0.159	238, 300sh, 325	**3S**	Phenolic acid
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
	41.903 ± 0.298	267, 302sh, 351	**7**	Kaempferol-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Stem GS4	32.669 ± 0.422	242, 302sh, 327	**1S**	Phenolic acid
	38.675 ± 0.159	238, 300sh, 325	**3S**	Phenolic acid
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
	41.903 ± 0.298	267, 302sh, 351	**7**	Kaempferol-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Root GS2	37.565 ± 0.000	294sh, 308	**1R**	Phenolic acid (*p*-coumaric acid)
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
Flower GS2	32.255 ± 0.659	227, 302sh, 314	**3**	Phenolic acid
	33.961 ± 0.374	229sh, 248sh, 269, 348	**5**	Kaempferol-3,7-di-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Flower GS3	32.255 ± 0.659	227, 302sh, 314	**3**	Phenolic acid
	33.961 ± 0.374	229sh, 248sh, 269, 348	**5**	Kaempferol-3,7-di-*O*-glycoside
	34.493 ± 0.037	305sh, 327	**1W**	Phenolic acid
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Fruit GS3	34.947 ± 0.094	311sh, 332	**1F**	Phenolic acid
	35.980 ± 0.112	300sh, 329	**2F**	Phenolic acid
Fruit GS4	34.947 ± 0.094	311sh, 332	**1F**	Phenolic acid
Calyx GS3	27.948 ± 0.647	242, 302sh, 327	**1**	Phenolic acid (Chlorogenic acid)
	32.255 ± 0.659	227, 302sh, 314	**3**	Phenolic acid
	33.346 ± 0.621	219, 242sh, 305sh, 326	**1C**	Phenolic acid
	33.659 ± 0.655	235sh, 302sh, 324	**2C**	Phenolic acid
	40.343 ± 0.353	255, 264sh, 294sh, 355	**6**	Quercetin-3-*O*-glycoside
	42.841 ± 0.271	267, 301sh, 352	**8**	Kaempferol-3-*O*-glycoside
Calyx GS4	33.346 ± 0.621	219, 242sh, 305sh, 326	**1C**	Phenolic acid

The retention time represent the mean and standard deviation of minimum three independent analyses, depending on the times a compound was found in the different samples.

λ_{max}: Wavelength of maximum absorption; GS1 to GS4: Growth stages 1 to 4.

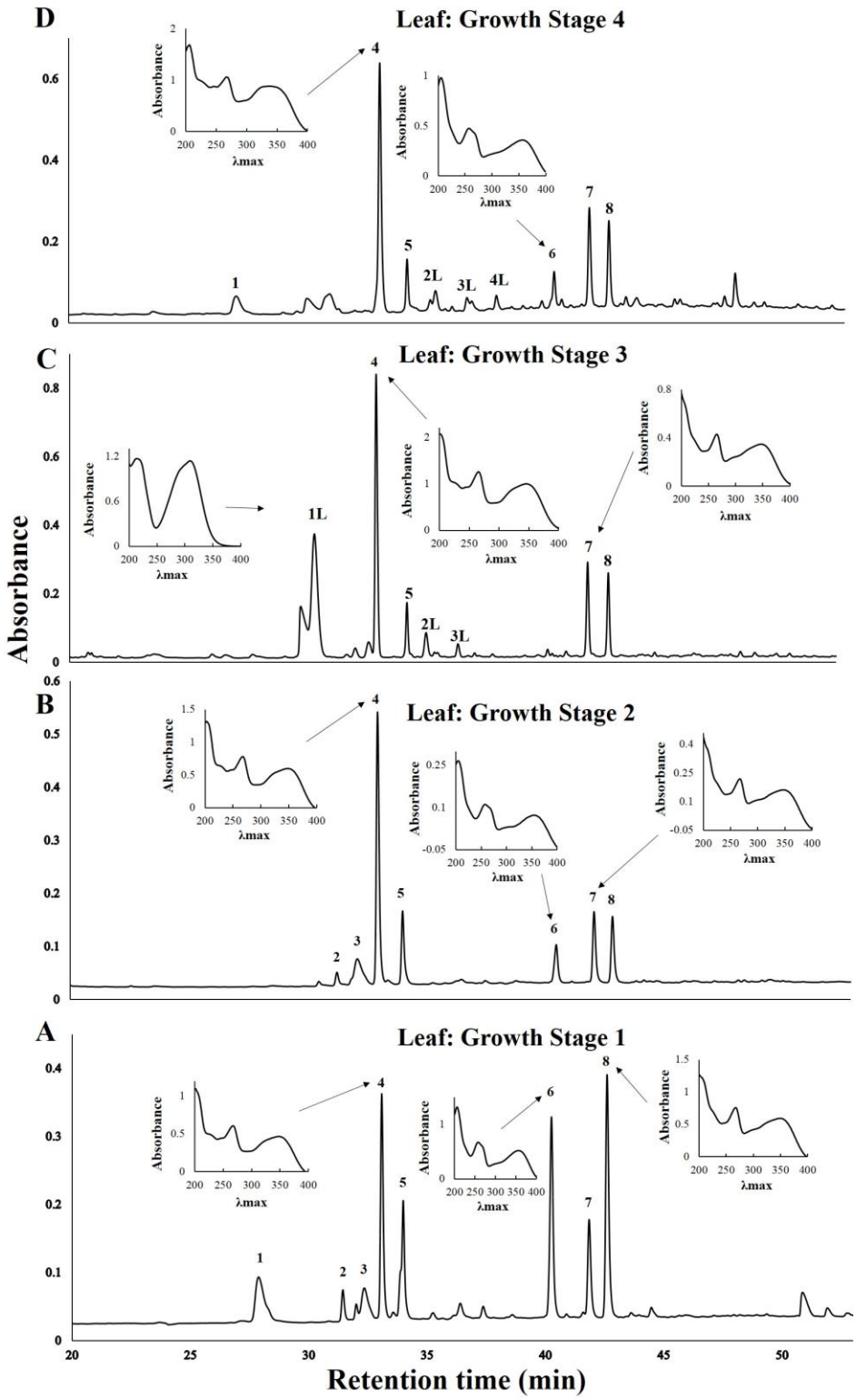

Figure 1. The foliar phenolic profiles of *Physalis angulata* showed qualitative and quantitative differences through growth.

A. At growth stage 1, the HPLC chromatograms of *Physalis angulata* leaves revealed eight phenolic compounds (**1** to **8**), being **4**, **6** and **8** the mayor ones. **B.** At growth stage 2, compound **1** was not found and the concentrations of compounds **2** to **8** increased compared to growth stage 1, particularly compounds **6** and **8**. **C.** At growth stage 3, the most relevant event was the occurrence of new compounds (**1L**, **2L** and **3L**) and the disappearance of **2** and **3**. **D.** At growth stage 4, the recurrence of compounds **1** and **6** and the occurrence of **4L** were relevant.

The DAD-obtained UV spectra of some compounds are displayed for each growth stage. The number of compounds corresponds to those of Table 2.

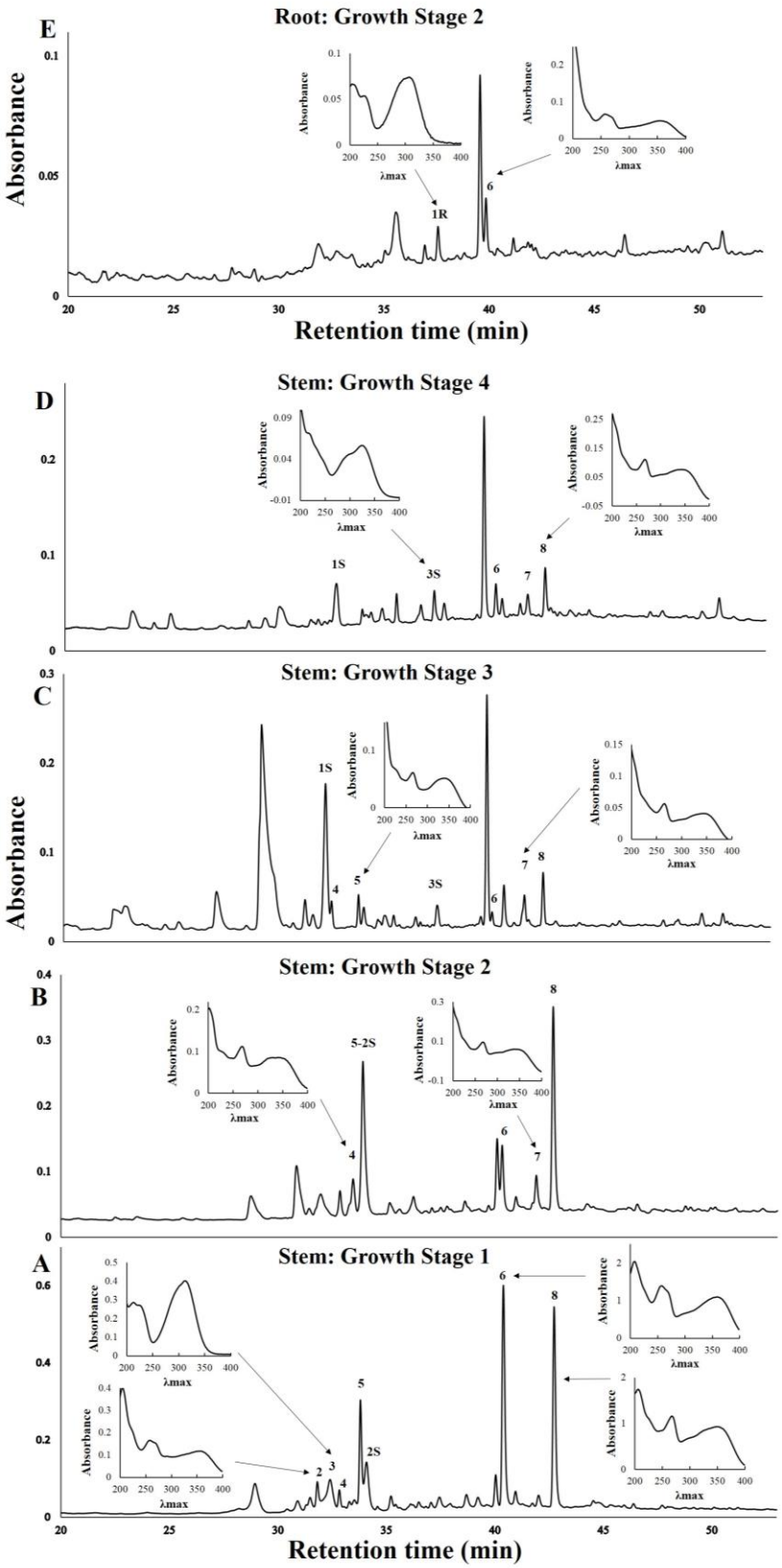

Figure 2. The phenolic profiles of *Physalis angulata* stem were variable at different growth stages. The profile of root at growth stage 2 is also shown.

A. At growth stage 1, the HPLC chromatograms of *Physalis angulata* stem was formed by seven phenolic compounds (**2**, **3**, **4**, **5**, **2S**, **6**, and **8**), from which only 2S was exclusive of stems. Like for leaves, compounds **6** and **8** were predominant. **B.** Growth stage 2 was characterized by the disappearance of compounds **2**, **3**, and **7** and the drastic concentration decrease of compound **6**. **C.** At growth stage 3 occurred two new compounds, **1S** and **3S**, whereas **2S** disappeared. The concentrations of **4**, **5**, **6**, and **8** diminished. **D.** At growth stage 3, compounds **4** and **5** disappeared, the concentration of **1S** diminished, whereas those of **3S**, **6**, **7**, and **8** kept low. **E.** The phenolic composition of roots was one of the most simple. The HPLC chromatogram at growth stage 2 revealed only two phenolic compounds (**1R** and **6**), both at low concentrations.

The DAD-obtained UV spectra of some compounds are displayed for each growth stage. The number of compounds corresponds to those of Table 2.

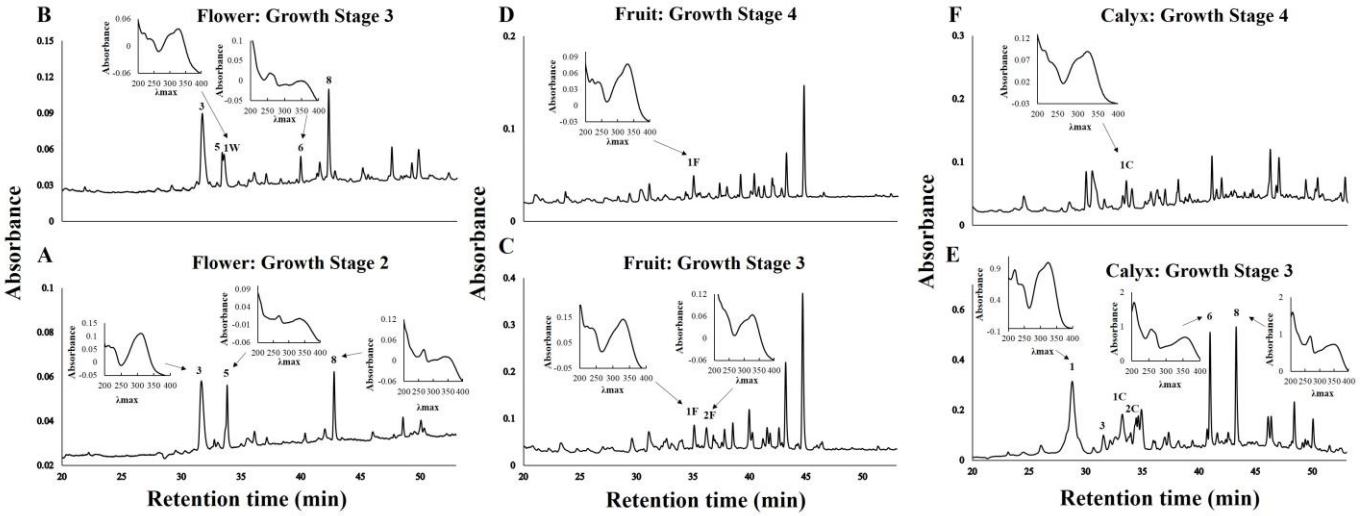

Figure 3. The phenolic profiles of flower, fruit and calyces of *Physalis angulata* varied according to the growth stage.

A. When immature (growth stage 2), flowers accumulated three phenolic compounds (**3**, **5**, and **8**), as revealed by the respective HPLC chromatogram. **B.** Mature flowers (growth stage 3) accumulated two new compounds (**1W** and **6**) besides the already found at growth stage 2. The HPLC chromatogram indicated that **3** and **8** increased in concentration. **C.** At growth stage 3, the HPLC chromatogram of unripe fruits (growth stage 3) revealed only two phenolic acids, compounds **1F** and **2F**. **D.** The HPLC chromatogram of ripe fruits (growth stage 4) revealed a very simple phenolic composition, formed only by one phenolic acid (compound **1F**). **E.** The HPLC chromatogram of immature calyces (growth stage 3) revealed six compounds (**1**, **3**, **1C**, **2C**, **6**, and **8**). **F.** The HPLC chromatogram of mature calyces (growth stage 4) was contrasting with that of immature calyces, revealing only one phenolic acid (**1C**).

The DAD-obtained UV spectra of some compounds are displayed for each growth stage. The number of compounds corresponds to those of Table 2.

The cluster analysis based on the phenolic profiles gave a dendrogram, which revealed the tissue- and age-specific condition of phenolic profiles (Fig. 4). The differences allowed associating a specific phenolic profile with each plant part at a single growth stage. Phenolic profiles of leaves at GS1 and GS2 were the most similar (Jaccard similarity index of 0.86. A value of 1 means equality). The current results suggest that the HPLC-DAD phenolic profiles besides being significant specific markers (Medina-Medrano et al., 2015) are also worthy chemical markers, which distinguish the tissular origin and the age of *P. angulata* extracts. Thus, phenolic profiles can be used as a quality control tool for identifying the tissular origin and the plant age used for herbal preparations of this species. This chemical typification could also reveal adulteration of herbal preparations of the species.

Depending on the growth stage, leaves accumulated between 7 and 9 different phenolic compounds, stem between 5 and 7, flowers between 3 and 5, calyces between 1 and 6, fruits between 1 and 2, whereas roots at GS2, only 2 (Table 2). Leaves, steams, and flowers accumulated majorly flavonols (kaempferol-3,7-di-*O*-glycosides, kaempferol-3-*O*-glycosides and quercetin-3-O-glycosides), whereas fruits and calyces accumulated mainly phenolic acids, although at low concentrations.

Tissue- and age-dependent variations in the concentrations of individual compounds were found (Fig. 5). Compound **4**, a kaempferol-3,7-di-*O*-glycoside, reached the highest concentration in leaves at GS3 (12.99 µg/g fw). Compound **8**, a kaempferol-3-*O*-glycoside, was found in all parts, except in roots, reaching the highest concentration (10.51 µg/g fw) in young leaves (at GS1). Recently, kaempferol glycosides have been reported to have important anti-obesity and anti-diabetic activities (Zang et al., 2015). According to the current results, leaves of *P. angulata* can be a relevant source of kaempferol derivatives. In young stems (at GS1) and immature calyces, compounds **6** and **8** were the most abundant, whereas compound **8** was the major one in mature flowers (Fig. 5).

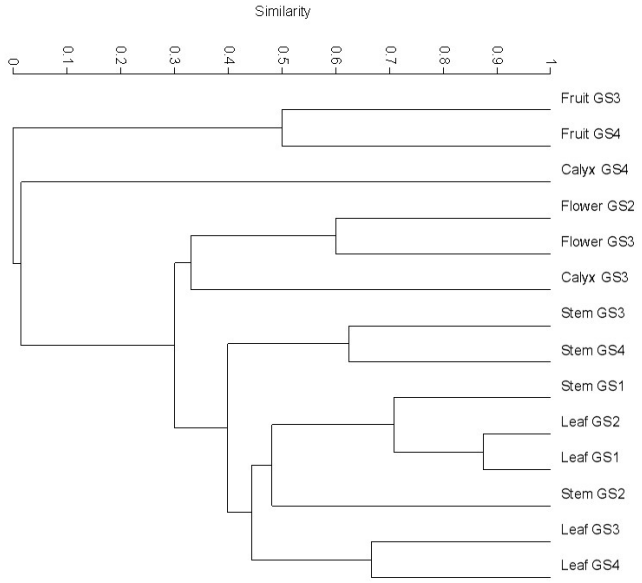

Figure 4. HPLC-DAD obtained phenolic profiles clearly discriminated every plant part at a given growth stage of *Physalis angulata*.

The results of a cluster analysis (Neighbour Joining Algorithm and Jaccard similarity measure) comparing phenolic profiles of five different plant parts of *Physalis angulata* at four growth stages (GS1 to GS4) showed a high discriminating power for distinguishing every plant part at a particular growth stage.

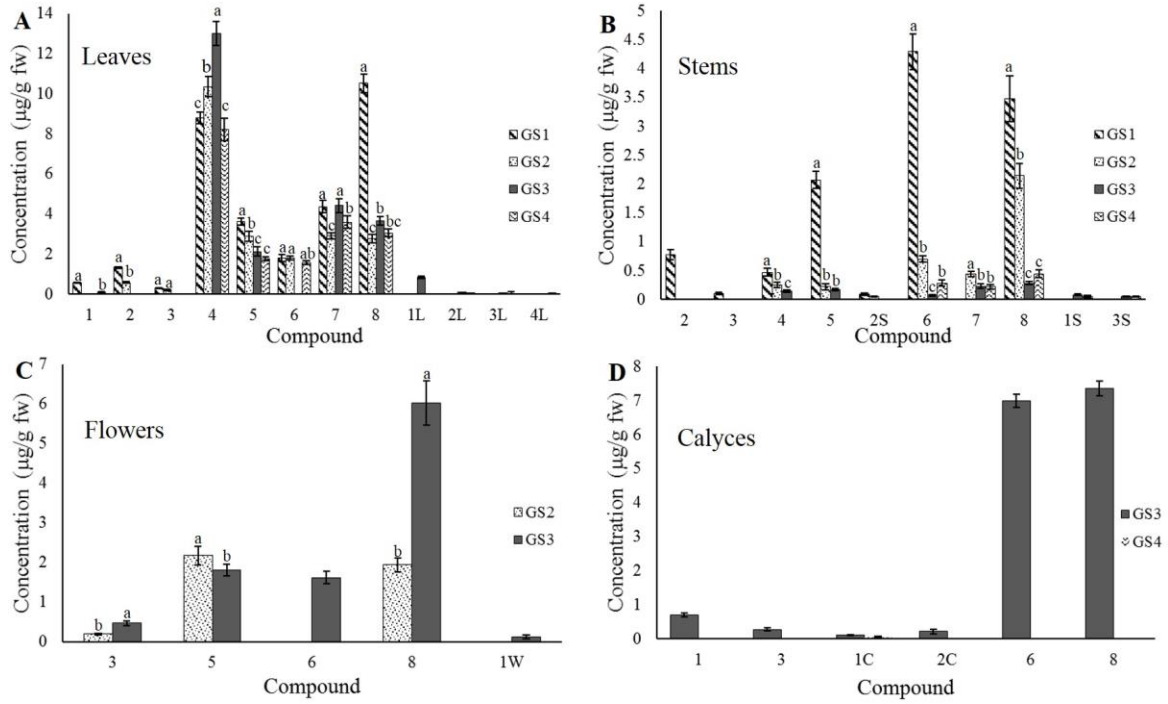

Figure 5. The qualitative phenolic composition and concentration of individual compounds changed according to the age in different plant parts of *Physalis angulata*.

A. In leaves, compound **4** (a kaempferol-3,7-di-*O*-glycoside) was the most abundant in every growth stage (GS1 to GS4), reaching 12.99 µg/g fw at GS3. **B.** Compound **6** (a quercetin-3-*O*-glycoside) was the most abundant in stems at GS1, reaching 4.29 µg/g fw. **C.** In flowers, compound **8** (a kaempferol-3-*O*-glycoside) was predominant in mature flowers (6.02 µg/g fw), this concentration being about 3-fold higher than that found for the same compound in immature flowers (1.94 µg/g fw). **D.** Compounds **6** and **8** were dominants in immature calyces, in which reached 6.98 and 7.35 µg/g fw, respectively.

Thin lines in bars represent the standard deviation of three independent analysis. Different letters for each compound mean significant differences ($p<0.05$) among different growth stages (according to the Tukey test). The absence of a letter means that only one compound was found in a given growth stage or that compounds were found at low concentrations.

Del Baño et al. (2003) have suggested that the concentration variations of compounds, which are common to different parts of growing plants, could be due to *in situ* biosynthesis in the earlier growth stages, and in the last stages, to the contribution of increasing of the transport processes. The current results suggests that the biosynthesis, degradation, and transport of phenolic compounds, and therefore its tissue-dependent distribution are regulated by the plant age.

Antioxidant properties

Mature flowers (at GS3) displayed the most relevant TAC (416.1 µg/mL). That capacity was 3.7-fold higher than that of epicatechin and 5.2-fold higher than that of quercetin, both evaluated as references (Table 1) and reported as potent antioxidants (Jiménez-Aliaga et al., 2011; Meng et al., 2015). As DPPH free radical scavengers, the unripe calyces (GS3) were the most important structures (EC_{50} = 0.12 mg/mL); these, along with leaves and flowers at any stage of development showed higher scavenging activities than the fruits of *P. peruviana*, which required 300 µg extract/mL to scavenge 52.72% of DPPH (Chang et al., 2008). RP was highlighting in the youngest leaves (A_{700nm} = 3.04). The RP of all extracts of *P. angulata* were higher than those found for rutin and its acylated derivatives (A_{700nm} between 0.02 and 0.5), which were reported as important antioxidant flavonoids (Lue et al., 2010).

High correlations ($p<0.05$) were found between total phenolic concentration and TAC, anti-radical capacity and RP (r = 0.885, 0.993, 0.746, respectively). High correlations ($p<0.05$) were also found between total flavonoid concentrations and RP (r = 0.754). These results suggest that the differences here registered for the antioxidant properties of the different parts of *P. angulata,* at the same and at different stage of development, can be the consequence of the particular expression patterns of genes involved in the biosynthesis of phenolic compounds. The current results reveal the importance of the levels of phenolics and flavonoids to define the antioxidant properties of *P. angulata*.

Significant correlations were also revealed between the concentrations of some individual compounds and antioxidant activities. The significant correlations were found between compound **5** and anti-radical activity and RP (r = 0.781 and 0.762, respectively), compound **6** and anti-radical activity (r = 0.858) and RP (r = 0.978), compound **8** and TAC (r = 0.671), compound **4** and TAC (r = 0.766), compound **6** and TAC and RP (r = 0.958 and 0.811, respectively), and compound **8** and TAC and RP (r = 0.944 and 0.748, respectively). These results suggest an important participation of the flavonols **4**, **5**, **6** and **8** to determine the antioxidant properties of *P. angulata*.

Contrary to the cluster analysis based on the phenolic profiles, that based on the contents of total phenolics, flavonoids and phenolic acids, as well as the antioxidant activities revealed a non-clear tissue- and age-specific condition for the *P. angulata* extracts (Fig. 6), in such a way that is not possible to typify each extract considering these parameters.

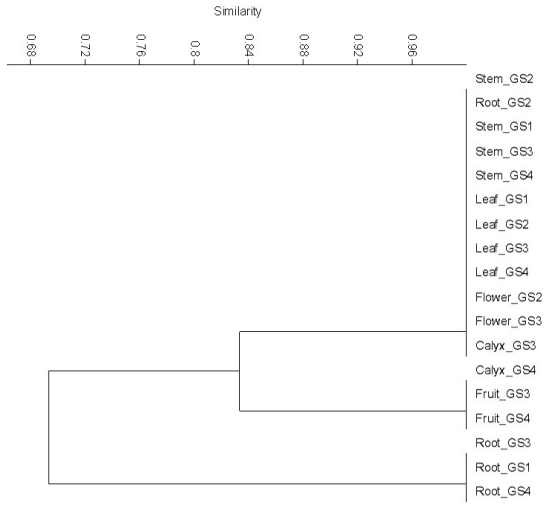

Figure 6. The different plant parts at a given growth stage of *Physalis angulata* could not be discriminated by the levels of total phenolics, flavonoids, phenolic acids, and the antioxidant properties.

Cluster analysis results (Neighbour Joining Algorith and Jaccard similarity measure) comparing total phenolics, flavonoids and phenolic acid levels, along with antioxidant properties (DPPH scavenging capacity, total antioxidant capacity, and iron reducing power) were undiscriminating indicators concerning the growth stage (GS1 to GS4) of *Physalis angulata*. Concerning the plant structures, these indicators only discriminated fruits and partially roots.

CONCLUSIONS

Leaves, stems and flowers of *P. angulata* are important sources of kaempferol glycosides. Flowers and calyces at GS3 (57-day-old-plants) were the best source of total phenolics, whereas at this same growth stage, leaves were the best source of flavonoids. Roots and fruits accumulated non relevant amounts nor diversity of phenolic compounds. The levels of total phenolics, flavonoids and the concentrations of some of the individual flavonols were significantly correlated to the antioxidant properties. The different parts of *P. angulata* at a single growth stage accumulated no particular levels of total phenolics, flavonoids and phenolic acids, neither displayed distinctive antioxidant properties, in such a way that a clear tissue- and age-specificity was not found. Contrary, important tissue- and age-dependent variations in the qualitative phenolic composition were found. A typical HPLC phenolic profile for each plant part at a single growth stage was clearly observed. Thus, HPLC-obtained phenolic profiles can be useful as reliable chemical fingerprints and quality indicators for determining the plant age, tissular origin, authenticity, and adulteration of herbal preparations of this species. Knowing the variations of distribution and abundance of phenolic compounds through plant development is an essential issue, and needs to be examined to do a rational and integral exploitation of natural resources. The collected data contribute to develop a kind of passport-ID of *P. angulata* for commercial, safety, and integral use of this medicinal and edible plant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contribution:

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Concepts or Ideas			X			X	
Design	X		X				
Definition of intellectual content	X	X	X				
Literature search	X	X			X		
Experimental studies	X	X	X	X	X	X	
Data acquisition	X	X					
Data analysis	X	X	X		X		X
Statistical analysis	X		X				X
Manuscript preparation	X		X	X			X
Manuscript editing			X	X	X	X	X
Manuscript review			X		X	X	X

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