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Phenological variations of polyphenols in *Smilax campestris* (Smilacaceae)

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Abstract: Polyphenol profiles can suffer quali-quantitative modifications as the plant modifies its phenological condition. The objective of this work was to determine if there is a rhythm of production in the synthesis of polyphenols according to the phenological condition in the leaves, roots, and rhizomes of *Smilax campestris* Griseb. The plant material analysed corresponded to individuals of a colony of *S. campestris* collected in different phenological conditions. Standard methodology was used for the chromatographic profiles of flavonols (kaempferol, quercetin, isorhamnetin, and their glycosides) and proanthocyanidins (procyanidin and propelargonidin) and quantification of total phenol, condensed tannins, and flavonols. Appearance of metabolic changes was established in the studied organs of *S. campestris* according to the phenological condition.

Key words: *Smilax campestris*, phenological condition, polyphenols, proanthocyanidins, flavonoids

1. Introduction

Smilax campestris Griseb. (Smilacaceae) is a dioecious rhizomatous creeper with winter blooming; it is present from the north and east of South America to the Platense coast. In Argentina *S. campestris* is the most abundant and the most geographically distributed species of the gender; it is found in the northern provinces and it reaches the Plate Delta (Guaglianone & Gattuso, 1991; Mandrile & Bongiorno de Pfirter, 1991). It is a characteristic species from the forest, abundant in bushes and scrubland, close to rivers and streams, as well as in hills and high fields. It blooms early (winter in the southern hemisphere) from July to September, and it bears fruit from October to April (spring and summer) (Guaglianone & Gattuso, 1991). The leaves and rhizomes of the *S. campestris* samples are extremely variable, not only between individuals but also within the same individual. This phenotypic plasticity is due to ecological factors (Andreati, 1997).

The roots and rhizomes are used for therapeutic purposes such as antirheumatic, diuretic, and diaphoretic; the leaves and tender branches are used as a bitter tonic, refreshing and digestive (Mandrile & Bongiorno de Pfirter, 1991). In previous studies, antioxidant (Rugna et al., 2003) and antifungal (Battista et al., 2007) activities were determined.

The polyphenols constitute a group of compounds that have an important function in practically every

interaction that a plant establishes with its environment (Haslam, 1988, 1989; Rezanejad, 2012). They present a wide range of biological activities, among which the antibiotic and antioxidant could be mentioned (Middleton & Kandaswami, 1994; Yanagida et al., 1999).

In previous studies, the presence of quercetin, kaempferol, and free isorhamnetin, and the glycosides of 3-O-glucoside of quercetin and kaempferol and 3-O-rutinoside of quercetin, kaempferol, and isorhamnetin were detected in the leaves. Moreover, free quercetin and the glycosides 3-O-glucoside and 3-O-rutinoside were only detected in the roots and rhizomes (Rugna et al., 1999, 2002, 2005). Proanthocyanidins (procyanidin and propelargonidin) were found in all organs (Rugna et al., 2005). On the other hand, it could be proved that flavonoid production is qualitatively stable in female samples as well as in male ones in the different phenological stages (Rugna et al., 2002).

Furthermore, it could be determined that *S. campestris* is affected by biotic and abiotic environmental conditions. Solar radiation, as well as the attack of certain herbivores, water stress, and the altitude and developmental condition of the plant are susceptible to quantitative changes, and, occasionally, qualitative ones in the production of flavonols. Solar radiation produces a significant quantitative increase in flavonols as well as total phenols (Rugna et al., 2007). Such quantitative increases occur more in the young leaves

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when compared to adult ones (Rugna et al., 2008) and in those samples that grow in unfavourable water conditions or high lands (Rugna, 2006). On the other hand, samples that are attacked by herbivores show both quantitative and qualitative changes in their polyphenol production, such as the increase in the degree of glycosidation of the present flavonols (Rugna et al., 2011). It is known that flavonols can be affected by the phenological condition, increasing their content qualitatively or quantitatively, which is of importance when the plants are collected for therapeutic aims or to perform chemotaxonomic studies (Nikolova et al., 2007; Toker, 2009).

The objective of this work was to determine if the production of polyphenols in *S. campestris* is modified according to the phenological condition.

2. Materials and methods

2.1. Plant material

Leaves, roots, and rhizomes of *S. campestris* populations (6 to 8 samples of each organ in the analysed months) were used; they came from Puerto Gaboto (32°26'0"S, 60°49'0"W), San Jerónimo District in Santa Fe Province (Argentina). Samples were collected throughout 1 year. Blooming plants (July and September), fructification plants (November, January, and March), and plants in the stable stage (May) were used. The plant material was collected and identified by Susana Gattuso and it is stored in the Pharmacobotanics Chair in the Faculty of Pharmacy and Biochemistry (Buenos Aires University, Argentina).

2.2. Methods

2.2.1. Sample preparation

The sample preparation was done at room temperature, over 24 h, with 10 mL of methanol 80%, on 1 g of dried and ground plant material.

2.2.2. Polyphenol fingerprint

This was performed through bidimensional chromatographies in a thin cellulose layer (TLC), according to standard methodology (Mabry et al., 1970; Markham, 1982). The solvents systems were TBA (tertiary butanol/ acid acetic/water, 3:1:1) for the first dimension and acetic acid 15% for the second dimension. It was performed in duplicate.

A series of chromatograms were observed under UV 254 nm and 365 nm light, before and after exposition to ammoniac vapours and developed with the natural product reactive (NP 1% in absolute ethanol). Spraying with vanillin reactive/HCl cc. (4:1) was performed in the other series.

2.2.3. Total phenol quantification

This was performed through the Folin-Ciocalteu method according to Makkar et al. (1993). Aliquots (50 µL) of the samples were transferred to test tubes and the volume was

made up to 500 µL with deionised water. Following this, 250 µL of Folin-Ciocalteu reactive was added and 1.25 mL of watery sodium carbonate solution to the 20%. After 40 min the absorbance was measured to 725 nm.

A calibration curve was prepared with tannic acid. The total phenol content was expressed as tannic acid mg/dry material g. All the measurements were obtained in triplicate.

2.2.4. Quantification of condensed tannins (proanthocyanidins)

Condensed tannins were determined through the proanthocyanidin reaction (Porter et al., 1986). For this, 0.50-mL aliquots of the samples were transferred to test tubes and 3 mL of butanol-HCl reactive (butanol:HCl, 95:5 V/V) was added and 0.1 mL of ferric reactive to 2% (2% ferric-ammoniac sulphate in HCl 2 M). The test tubes were shaken and then boiled in a water bath for 60 min. After cooling, the absorbances to 550 nm were measured against a target (Waterman & Mole, 1994).

Proanthocyanidins were expressed as optical density to 550 nm. All measurements were obtained in triplicate.

2.2.5. Quantification of total flavonols

Quantification of total flavonols, from the original methanolic sample, was performed according to methodology developed previously (Rugna et al., 2005). From each sample 2 mL was taken and hydrolysis was performed with a HCl 2 N solution. Extraction was conducted with ethyl acetate. It was done to dryness, followed by resuspension in 3 mL of methanol. Absorbance of the solution was read at 370 nm (Markham, 1982).

A calibration curve was created with quercetin solutions from 10 µg/mL to 1 mg/mL concentrations. The total flavonols' content was expressed as micrograms of quercetin per gram of dry material. All measurements were performed in triplicate.

2.2.6. Statistical analysis

The results were expressed as mean ± standard deviation. Significant differences with $P < 0.05$ were considered. Data were analysed by ANOVA and significant differences in the mean values were analysed using the Graph Pad Prism software.

3. Results and discussion

The results obtained show that there are quali-quantitative differences in the production of secondary metabolites between the leaves and the underground organs in different phenological conditions.

The leaf produces quercetin, kaempferol, and isorhamnetin (free and glycosidated), whereas the underground organs only produce free and glycosidated quercetin (Table 1). Concentration of flavonols in the leaves is higher than that in the underground organs, especially if compared with the root (Table 2). Moreover,

Table 1. Detected flavonoids in different organs analysed in *Smilax campestris*.

Plant parts	Flavonoids									
	Q	I	K	QG	KG	QRG	IRG	KRG	ProCyn	ProPg
Leaves	+	+	+	+	+	+	+	+	+	+
Rhizomes	+	-	-	+	-	+	-	-	+	+
Roots	+	-	-	+	-	+	-	-	+	+

Abbreviations: Q = quercetin; I = isorhamnetin; K = kaempferol; QG = quercetin-3-O-glucoside; QRG = quercetin-3-O-rutinoside; KG = kaempferol-3-O-glucoside; IRG = isorhamnetin-3-O-rutinoside; KGR = kaempferol-3-O-rutinoside; ProCyn = procyanidin; ProPg = propelargonidin

Table 2. Quantification of total flavonoids in *Smilax campestris*.

Month	Leaves*	Rhizomes*	Roots*
January	215.5 ± 6.29	98.04 ± 4.90	75.22 ± 3.76
March	218.3 ± 5.98	201.30 ± 10.06	97.58 ± 4.88
May	263.8 ± 7.32	151.25 ± 7.56	65.91 ± 1.98
July	326.8 ± 10.07	58.73 ± 1.76	35.27 ± 1.06
September	308.5 ± 9.98	71.90 ± 3.59	30.50 ± 0.92
November	231.0 ± 8.76	251.70 ± 2.58	70.57 ± 3.53

*µg quercetin/g dry material, measured to 370 nm (P < 0.05)

synthesis of flavonols in the leaves increases in winter, when the blooming of *S. campestris* occurs in this season in Puerto Gaboto; in contrast, a decrease in the production of flavonols occurs in the rhizomes and roots.

Total phenol concentration also follows an equivalent distribution to the total flavonols. The leaves present higher values than the underground organs at any time of the year (Table 3). An increase is produced in the leaves in winter, whereas there is a noticeable decrease in rhizomes and roots.

The 3 organs studied produce proanthocyanidins (procyanidins and propelargonidin) all year round. The concentrations vary in an equivalent way to the modification of the total phenols and total flavonols (Table 4). The leaf is the organ that produces a higher concentration of condensed tannins and its maximum production peak appears in the winter months; conversely, the roots and rhizomes produce smaller quantities of proanthocyanidins and the values decrease in a very noticeable way during the winter months.

Table 3. Quantification of total phenol in *Smilax campestris*.

Month	Leaves*	Rhizomes*	Roots*
January	10.44 ± 0.31	5.60 ± 0.21	2.77 ± 0.14
March	9.18 ± 0.27	4.75 ± 0.33	3.33 ± 0.16
May	14.08 ± 0.70	6.36 ± 0.43	4.08 ± 0.20
July	21.43 ± 1.07	1.37 ± 0.17	1.87 ± 0.09
September	16.76 ± 0.84	1.85 ± 0.47	1.54 ± 0.07
November	11.26 ± 0.34	4.45 ± 0.28	2.45 ± 0.12

*mg tannic acid/g dry material, measured to 720 nm (P < 0.05)

Table 4. Quantification of proanthocyanidins in *Smilax campestris*.

Month	Leaves*	Rhizomes*	Roots*
January	0.5912 ± 0.0373	0.4676 ± 0.0231	0.0639 ± 0.0028
March	0.7485 ± 0.615	0.7575 ± 0.0433	0.1854 ± 0.0102
May	1.4522 ± 0.534	0.7046 ± 0.0342	0.2855 ± 0.0118
July	1.8029 ± 0.637	0.0519 ± 0.0166	0.0152 ± 0.0039
September	1.2293 ± 0.542	0.0752 ± 0.0139	0.0045 ± 0.0009
November	0.8443 ± 0.591	0.3617 ± 0.0157	0.0781 ± 0.0023

*Optical density to 550 nm (P < 0.05)

The results obtained show that phenol production in *S. campestris* is related to the time of the year and to the phenological condition of the plant. In this way, a rhythm of production can be established: the leaf gradually increases phenol production throughout the year; its maximum peak occurs in winter during the blooming period and then it gradually decreases until the minimum values during the vegetative condition. These results are concordant with phenological studies performed in other plant species. For instance, in different species of the genera *Artemisia* L. and *Hypericum* L., flavonols showed the same qualitative composition in all the phenological stages even though they were quantitatively higher during the blooming stage (Çirak et al., 2007; Nikolova et al., 2007; Toker, 2009).

In contrast, the underground organs have behaviour opposite to that of the leaves, i.e. their metabolism is kept stable during the vegetative period but it decreases during the blooming period. This is plausible since the air organs in *S. campestris* are more exposed to climate change than the underground organs. Therefore, as the blooming stage coincides with low temperatures, an increase in the production of the secondary metabolites occurs in the leaves in detriment of the flavonols in the underground organs.

It is important to stress that the phenological cycle of the plant can be altered by extreme temperature

conditions. It was observed in *S. campestris* that during 2007, when strong frosts occurred, the blooming was delayed. In this way, the existence of a relationship between the phenological condition and the production of polyphenols could be established, given that during 2007 the quantitative results in winter months were similar to those found in the vegetative state (autumn) (Rugna et al., 2009).

4. Conclusion

The production of polyphenols is affected by the phenological condition, since the leaves increase phenol and polyphenol production in the blooming period while the underground organs decrease it.

According to the observations made in this study as in others performed by the authors (Rugna et al., 1999, 2002, 2004), free quercetin and rutin could be considered as chemosystematic markers for the species, owing to the fact that they are produced in every organ and all year round.

From the point of view of its application in therapeutics, the quantitative differences constitute an important factor to be considered at the moment of collecting this plant for such purposes.

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