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Seasonal analysis of polyphenolic compounds in a wild population of *Baccharis spicata* (Lam.) Baill

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Abstract
Baccharis spicata is a dioecious shrub native to the south of South America, and is used as an adulterant of medicinal species of the genus. An analysis of the seasonal variation of total phenols and hydroxycinnamic derivatives was performed in a wild population from an ecological reserve in the city of Buenos Aires, Argentina, in order to know the best moment to collect material for therapeutic purposes. Besides, the ambient temperatures were registered in a range of 5 days before and after the samples were collected. In order to known the statistical significance of the differences, statistical analyses were made. The higher contents of these metabolites were registered in spring and autumn; these seasons had mild ambient temperatures.

Keywords: Wild Population, Polyphenolic Compounds, adulterant

Introduction
Baccharis is a genus native to South and North America with over 400 species, of which 120 grow in Argentina. It belongs to the Asteraceae family and its botany, phytochemistry and ethnobotany have been widely studied [1, 2, 3, 4]. *Baccharis articulata*, *B. trimera* and *B. crispa* are known as "carqueja" and are used species in the folk medicine of Argentina as infusions for its digestive properties [5]. All of these three plants have winged stems.
Its phytochemistry is well known and a wide number of compounds have been characterized, both of terpenic and polyphenolic origin. Among these last ones, the flavonoids are mainly methoxylated flavones and the hydroxycinnamic derivatives are represented by chlorogenic acid and its isomers [6, 7].
Baccharis spicata is a dioecious shrub with serrated, opposite leaves and striated, straight stems. It is native of the wetlands of the south of South America, though it has been recently reported its presence in Europe [8]. It is also known as "carqueja" just like the medicinal species above mentioned, in spite of the morphological differences in the stems and the presence of fully grown leaves. Previous phytochemical studies report its content of clerodane diterpenes [9] and essential oil composition [10]. Studies from our research group report the presence of the flavonoid rutin and the caffeoylquinic derivatives chlorogenic acid, and 3, 5; 3, 4 and 4, 5 dichlorogenic acids [11]. Barboza [12] and the above mentioned article of Retta report that this species is used as an adulterant of the medicinal species of this genus and its use as a digestive.
There are no studies in the seasonal variation of the polyphenolic compounds of this species. The fact that it is already being used turns imperative the realization of assays to know the optimal moment to harvest. The aim of this work is to characterize the dynamic of polyphenolic compounds through the seasons of a year in order to choose the best moment for the harvest of the plant for its use in pharmacological assays.

Materials and Methods
Plant Material
Leaves, stems, and aerial parts of female and male samples of *Baccharis spicata* (Lam) Baill were collected in four dates (01/10/2012, spring, sterile individuals; 15/2/2013, summer, pre flowering individuals; 01/4/2013 autumn, flowering individuals and 10/7/2013 winter, sterile individuals) at the Reserva Ecológica Costanera Sur, Ciudad de Buenos Aires and dried at room temperature. The coordinates of the sampling location are latitude: -34.598145 and longitude: -58.361738; it is detailed in Figure 1. Plant material was identified by taxonomical keys [13] and voucher specimens are placed in the herbarium of the Chair of Pharmacobotany, Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

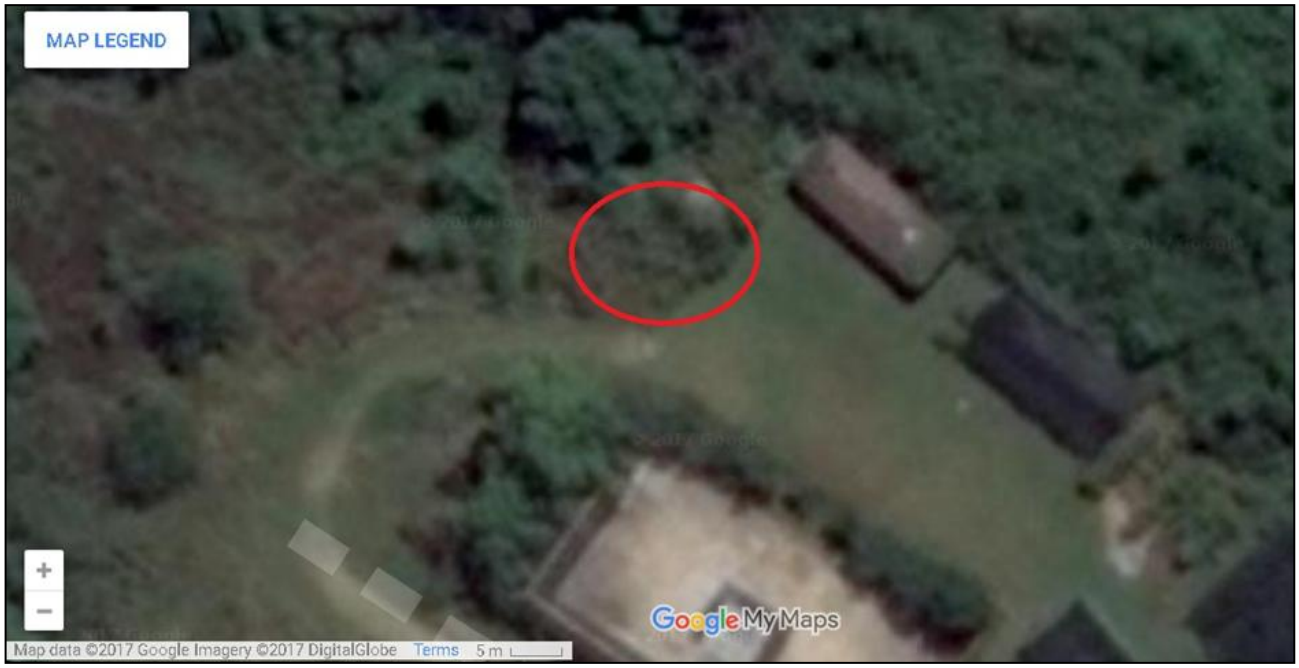

Fig 1: Location of the sampling place

Extraction of methanolic extracts

Extraction was carried out at room temperature, for 24 h, with 10 mL of methanol 50% (methanol-water, 1:1), over 200 mg dried ground plant material (leaves, stems, and flowers). It was later filtered and the frame discarded.

In addition, a methanolic extract elaborated with a mix of equal parts of leaves of female and male subjects was made.

Total phenol quantification

Determined by Folin– Ciocalteu method [14]. Aliquots (50 µL) of extracts were transferred to test tubes and the volume was taken to 500 µL with deionized water. Then, 250 µL of Folin-Ciocalteu reactive and 1.25 mL of aqueous solution of sodium carbonate 20% were added. After 40 minutes, absorbance was measured at 725 mm. A calibration curve with tannic acid was performed. Total phenol content was expressed as mg gallic acid / g dry material. All measurements were performed in triplicate.

Total hydroxycinnamic derivatives quantification

It was determined by a modification of the methodology described by Dao and Friedman [15]. Aliquots of 50 µL of each extract were taken to volume (2 mL) with absolute ethanol.

Absorbance was determined at 328 nm. A calibration curve was performed with chlorogenic acid. Values were expressed as mg chlorogenic acid / g dry material. Assays were performed in triplicate.

Meteorological conditions

Data of maximal and minimal ambient temperature were obtained for this area as a courtesy of the National Meteorological Service. Average temperature was calculated in a range of five days before and after the collection date.

Statistical analysis

Differences in organs and individuals were analyzed using Tukey´s test embedded software in the webpage http://astatsa.com/OneWay_Anova_with_TukeyHSD/.

Statistical significance was set in $p<0.05$.

Results

An analysis of total polyphenolic compounds and total hydroxycinnamic derivatives was performed over extracts of aerial parts, leaves and stems of *B. spicata*; the results of these assays can be seen in Table 1. The same assays were performed over extracts of the leaves of the 8 individuals of this population; relation between sexes was 1:1. An average of each season was calculated. Results can be seen in Table 2.The statistical analysis of the contest of total phenols and total hydroxycinnamic derivatives per season by Tukey´s test can be seen in Table 3.

The average temperature was calculated in the temporal range descripted above, and a statistical analysis with Tukey´s test was made; these results can be seen in Table 4 and 5.

Table 1: Variation in the content of total phenols and hydroxycinnamic derivatives in extracts of aerial parts, leaves and stems

	Total phenols as mg gallic acid/g dry material		Total hydroxycinnamic derivatives as mg chlorogenic acid/g dry material	
	Average	SD	Average	SD
Aerial parts	14.51	0.20	9.98	0.01
Leaf	15.97	0.08	11.35	0.19
Stem	5.15	0.19	5.05	0.04

Table 2: Seasonal variation in the content of total phenols and hydroxycinnamic derivatives

	Total phenols as mg gallic acid/g dry material		Total hydroxycinnamic derivatives as mg chlorogenic acid/g dry material	
	Average	SD	Average	SD
Spring	11.28	1.75	13.57	2.19
Summer	6.30	1.65	11.89	3.01
Autumn	10.97	1.95	12.27	2.17
Winter	8.72	2.29	7.33	2.82

Table 3: Seasonal comparison of in the content of total phenols and hydroxycinnamic derivatives and its statistical significance

Seasonal comparison	Statistical significance for the content of total phenols	Statistical significance for the content of total hydroxycinnamic derivatives
Spring vs Summer	p<0.01	There are no significative differences
Spring vs Autumn	There are no significative differences	There are no significative differences
Spring vs Winter	p<0.05	p<0.01
Summer Vs Autumn	p<0.01	There are no significative differences
Summer vs Winter	There are no significative differences	p<0.01
Autumn vs Winter	There are no significative differences	p<0.01

Table 4: Average temperatures in a 10 day range from the moment of sampling

	Temperature	
	Average	SD
Spring	18.0	3.6
Summer	25.0	4.8
Autumn	20.6	4.4
Winter	9.7	6.5

Table 5: Seasonal comparison of average temperatures and its statistical significance

Seasonal comparison	Statistical significance
Spring vs Summer	p<0.01
Spring vs Autumn	There are no significative differences
Spring vs Winter	p<0.01
Summer Vs Autumn	p<0.05
Summer vs Winter	p<0.01
Autumn vs Winter	p<0.01

Discussion

A wild population of 8 individuals (4 male, 4 females) of *B. spicata* located in an ecological reserve in the Autonomous City of Buenos Aires, Argentina was studied. This population was selected based in the proximity of all its individuals in a defined space within a well preserved area of native landscape; this fact gave us the certainty that all of them grew under the same conditions of solar exposure, weather and pressure of herbivores. Only four dates of collection were chosen for the seasonal comparison because a higher sampling frequency could have affected the plants, inducing a bias by wounding.

A pool of aerial parts, leaves and stems of all the individuals in all the seasons of this population was performed in order to know the content of polyphenolic metabolites per part. The highest content of total phenols and hydroxycinnamic derivatives was in the extracts of leaves, followed by the aerial parts and stems. A reason for this result should be the highest metabolism of this organ and the action of predators such as herbivorous arthropods and vertebrates.

In the seasonal comparison, the highest content of total phenols is observed in spring and autumn; unsurprisingly, the comparison of ambiental temperatures between these seasons do not report a significative difference (p>0.05). The phenolic compounds have a protective role against abiotic factors such as UV radiation [16-18] and biotic factors such as herbivory [19-21]. Furthermore, there is not a significative difference in the content of hydroxycinnamic derivatives between spring and autumn either (p>0.05).

When comparing the remaining pairs, there were significative differences in the content of total phenols between spring-summer, spring-winter and summer-autumn. There was not a significative difference between autumn-winter and summer-winter, besides the pair spring-autumn above mentioned. This analysis allow us to infer that the difference in the content of total phenols is related to mild temperatures between 18-20 °C and not with the highest and lowest temperatures registered for the metropolitan region of Buenos Aires.

This fact partially matches with the results of a study performed in wild and cultivated populations of *B. trimera* from Brazil [22]; the higher contents of phenolic compounds were found in the months with mild temperatures, while the warmest seasons had the lowest content. It must be noted that winters in Brazil have higher temperatures than the ones in Argentina. Overall, vegetative growth correlates with a low content of allelochemical substances, and the highest contents of chemical defenses correlate with a diminution in plant vigor and growth [23].

As for the differences in the content of hydroxycinnamic derivatives, the pairs summer-spring, summer-autumn and the above mentioned spring-autumn did not have a significative difference (p>0.05); the content of these metabolites in spring, summer and autumn was similar, in the range of 12-14 mg/g dry material. Winter had the lowest content, almost half of the content registered in the spring. There was a significative difference between the pairs spring-winter; summer-winter and autumn-winter.

The hydroxycinnamic derivatives were selected based in previous investigations of our team; these compounds might be at least partially responsible of the cholagogue activity [24, 25]; this activity is the same as the species used in folk medicine. The chlorogenic acid is the head of this family of metabolites, and has an ecological role as a resistance factor against insect plagues [26-28] and fungal infections [29-31].

This study has a main limitation: the differences between sexes are not analyzed, and we don´t know its statistical significance. However, the individuals studied have grown in a well-defined place, under the same growth conditions and protected from anthropic influences, so there is homogeneity in the results that could not be achieved if the study was performed over plants from different places. A cultivated population with a highest number of individuals of both sexes is currently being grown under controlled conditions in order to get a higher statistical power in future studies. As for the sampling points, a higher frequency of collection should be desirable, but it could have affected the plants; we considered that 3 months is a good period of time for the plants to recover from the wounding caused by the collection of the samples.

In conclusion, the best part to get an optimal content of polyphenolic metabolites is the leaf, and the best seasons to collect them are autumn and spring.

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