Scientific Paper

Seasonal and spatial variations in the concentration of phenols in *Elaeagnus angustifolia* L.

María Pía-Beker[1,2] *https://orcid.org/0000-0001-8848-3307*, Osvaldo Alberto-Fernández[3] *https://orcid.org/0000-0003-2220-0321*, María Guadalupe-Klich[1,3] *https://orcid.org/0000-0002-9799-1853*

[1] Universidad Nacional de Río Negro, Escuela de Veterinaria. Río Negro, Argentina. [2] CIT-CONICET. Consejo Nacional de Investigaciones Científicas y Técnicas. Argentina. [3] Universidad Nacional del Sur, Departamento de Agronomía. Bahía Blanca, Argentina. Correspondencia: mpbeker@unrn.edu.ar

Abstract

Objective: To characterize the variations in the phenol content in the different edible and non-edible portions of *Elaeagnus angustifolia* L. during the growth cycle of the plant.

Materials and Methods: Plant material was collected during the plant growth cycle in the Valle Medio region, Río Negro, Argentina. The collection was carried out in five sampling moments (September, November, January, February and March). For such purpose, samples were taken from the leaves, flowers and fruits of the canopy exposed to the sun and leaves in the shaded canopy. The presence of polyphenols in the different samples collected by qualitative and quantitative essays was evaluated. The data were analyzed through variance analysis; while the significant differences among means, by Tukey's test for $p < 0,05$. The statistical program Infostat® was applied.

Results: Significant differences ($p < 0,05$) were found in the content of polyphenols between the external sunlit sunny leaves and the inner shaded ones in the September sampling. The shaded leaves contained significantly more phenolic compounds than the higher sunlit sunny leaves (19,8 and 16,4 mg GAE g^{-1} DM, respectively). This variability becomes null, when the total edible thyrse is compared with the non-consumed parts.

Conclusions: Seasonal and spatial variation was determined in the concentration of total phenols in the edible and non-edible biomass of *E. angustifolia* during its growth cycle. In addition, no limiting quantities of polyphenols were found in the shaded leaves, which could reduce the voluntary intake of feed and its digestibility.

Keywords: cattle, secondary metabolites, phenols

Introduction

Elaeagnus angustifolia L. is member of the family *Elaeagnaceae*. Its common name is Russian olive, Bohemian olive or oleaster. This species is a naturalized tree in the Valle Medio, Río Negro, Argentina, which is frequently found in riparian habitats. Detailed anatomical and morphological studies revealed heteromorphology of the leaf among the different levels of the canopy (Klich, 2000).

The literature describes the compositions and applications of *E. angustifolia* extracts. Specifically, this species is highly valued for its medical therapeutic applications (Amiri Tehranizadeh *et al*., 2016). The scientific studies about its pharmacological uses are widely documented (Farzaei *et al*., 2015, Hamidpour *et al*., 2017). In addition, *E. angustifolia* is part of the diet of ruminants in the pastureland fields, and is highly appreciated as forage resource, due to the high protein content. The observations of herbivory in the *E. angustifolia* canopy revealed high variability at spatial scale in individuals in reproductive stage (Klich *et al*., 2018).

Cattle showed preference for the proliferating thyrses of the external canopy (pendulous, fructifying branches), which are highly browsed, compared with the leaves developed on the branches of the inner canopy, which the animals do not consume.

As it was previously indicated, there are several characteristics of the leaves which could be related to the consumption pattern observed in the tree canopy (Klich, 2000), although the beneficial or deleterious effect some secondary compounds in plants have, such as polyphenols and tannins, on animal health and nutrition, is also known (Frutos *et al*., 2004). This is a complex topic, which depends mainly on the consumption rate and chemical nature of these compounds. Along with other plant secondary metabolites, like alkaloids, saponins and steroids, these compounds play an important role in the protection of plants from herbivory.

The nutritional attributes of *E. angustifolia* have been referred in previous studies (Klich *et al*., 2018). Nevertheless, there is little or no information in literature about the presence and effects of its secondary compounds, already described in ruminant nutrition.

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The differential palatability and cattle preference with regards to the spatial distribution of *E. angustifolia* forage offer could be related to the presence of these secondary compounds.

Although it has been described that the bioactive compounds present in *E. angustifolia* have possible beneficial effects for humans, they deserve to be studied in more detail for their properties on animal nutrition, when considering the importance of this forage resource in semiarid pasturelands. Ranchers in regions with frequent droughts in the summer, which damage the growth of native vegetation, can utilize the presence of such resources as *E. angustifolia*, as forage reserve in quality and quantity.

Several studies have been conducted in orden to characterize the variations in phenol content in the different edible and non-edible portions of *E. angustifolia* during the plant growth cycle.

Materials and Methods

Study area. This study was conducted between in the Veterinary School of the National University of Río Negro (UNRN), Argentina. This phytogeographical region is called bush (Cabrera, 1976), with steppe scrub dominated by microphile xerophilous shrubs as prevailing vegetation.

Climate. The region is semiarid, subject to a wide range of daily and seasonal temperature. Its average values fluctuate from 6,83 ºC in the coldest month (July) to 23,0 ºC in the warmest (January). Mean annual rainfall is 303 mm.

Collection and processing of E. angustifolia samples. The collection of plant material was carried out between September, 2017, vegetative stage, until late March, 2018. Sampling was carried out at five moments (September, November, January, February and March). For such purpose, leaf, flower and fruit samples were manually taken from the external canopy, exposed to sunlight. Material was collected up to 2,5 m, considered the maximum height that the animal can reach from the thyrses or fructifying branches. However, the rejected part was collected from the inner shaded canopy (< 1 m of height). At all collection moments, material was gathered from ten well-developed and randomly-selected mature trees (89 m high) to obtain a minimum of 5 kg of material fresh weight.

All the samples were dried at temperatures between 40 and 45 ºC until reaching constant weight, and moisture content around 3 and 4 %. Afterwards, they were put in airtight containers for their analysis. To avoid during the dehydration process the degradation of biologically active compounds, which can also exhibit antioxidant properties, low temperatures were used (Wojdyło *et al*., 2019).

Before preparing the aqueous extract, the dry material was pulverized in a grinder (with an opening sieve of 1 mm).

Extraction. The plant extracts were obtained through magnetic agitation of 1 g of pulverized dry matter with 60 mL of solvent (distilled water) during 30 minutes at 60 ºC in an Erlenmeyer flask. The obtained solutions were filtered through Whatman paper number 1 and were stored at 4 ºC until their analysis.

Qualitative analysis. The presence of polyphenols in the different collected samples was evaluated through a colorimetric essay based on the formation of a complex colored in the presence of reagent $FeCl_3$. To 5 mL of the aqueous extract five drops of 1 % ferric chloride were added to evaluate the formation of a brownish green or purplish-blue, indicator of the presence of tannins (Edeoga, 2005).

Content of total phenols. The total phenol content (TPC) of the extracts from each organ was determined with the Folin-Ciocalteau reagent (Singleton and Rossi 1965, Makkar, 2000) with some modifications. Aliquots of 20 µl of the sample were mixed with 1, 58 mL of water, 100 μl of Folin-Ciocalteau phenol reagent diluted 10 times and, finally, with 300 μl of 20 % Na_2CO_3. The reaction mixture was completely mixed and left to remain at 40 ºC in Bain Marie during 30 minutes in the dark. After the incubation, the absorbance of all the sample solutions was measured at a wavelength of 765 nm. For such purpose a spectrophotometer ("Biotraza" 722 Visible) was used, and it was compared with the calibration curve of gallic acid equivalents ($r^2 = 0,9913$). The total phenol content was expressed in mg gallic acid equivalents per gram of dry matter (mg GAE g^{-1} DM). All the samples were analyzed in triplicate.

Statistical analysis. The data were analyzed through variance analysis, with previous testing of the normality and variance homogeneity assumptions. The significant differences between the phenol content of the edible and non-edible portions were compared with Tukey's test for $p < 0,05$. The data were processed with the statistical software Infostat® (Di Rienzo *et al*., 2017). The values were expressed as means ± standard errors.

Results and Discussion

The data showed that the leaves, flowers and fruits contained phenols. The colorimetric test used

revealed that the most abundant tannins in all the samples and evaluated moments correspond to those of the condensed tannin type (greenish brown color, figure a, b and d). Hydrolysable tannins were detected in the flowers and fruits in the January sampling (pulp and seed bones, figure 1c). In this period, the fruits developed in the thyrses are not completely mature and the seeds inside are soft and digestible by the cows. In this sampling, purplish-blue color was observed. In the flowers and seeds, the color that was developed when adding the ferric chloride was grayish blue. The fruit pulp, obtained in February and March, showed a light grayish blue color (figure 1 c).

In all the images the left test tube corresponds to the crude extract without $FeCl_3$, and the right tube with 5 drops of reagent to test color development, a)-Leaves from the lower canopy, b)-Leaves from the higher canopy, c) Fruit and d) Fruits and leaves (complete thyrse)

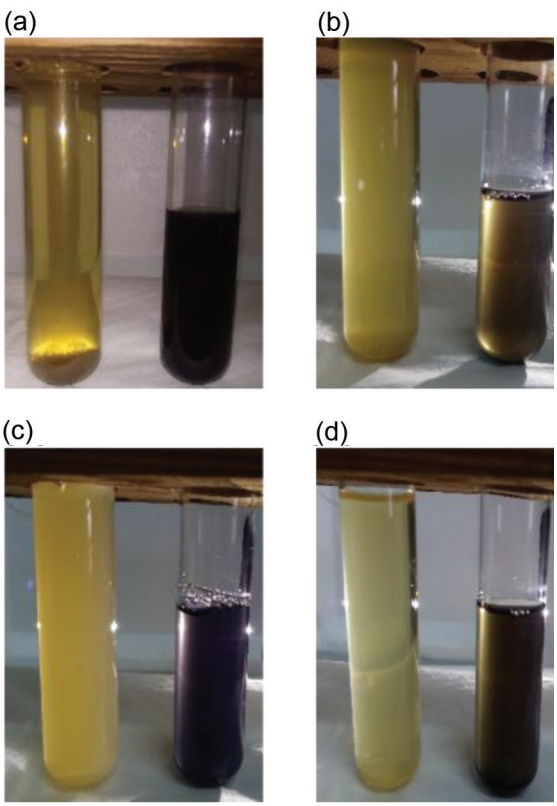

Figure 1. Colorimetric essay of total phenols in *E. angustifolia*.
The images the left test tube corresponds to the crude extract without $FeCl_3$, and the right tube with 5 drops of reagent to test color development, a)-Leaves from the lowe canopy, b)-Leaves from the higher canopy, c) Fruit and d) Fruits and leaves (complete thyrse)

Table 1 shows the phenolic content of the edible and non-edible parts of *E. angustifolia* during its development stages. Significant differences ($p < 0,05$) were found in the polyphenol content between the external sunlit leaves and inner shaded leaves in the September sampling. From the flowering to the fructification stage, the phenol content between the complete thyrse and the inner shaded leaves did not show significant differences.

Another variation source in the polyphenol content is temporary changes in the accumulation of plant secondary compounds, which comprise two types: ontogenetic and seasonal. The ontogenic stages in *E. agustifolia* include seedlings and juvenile plants (both non-edible for ruminants), and mature reproductive plants, characterized by the development of proliferating thyrses in the top branches. Each stage was characterized by distinctive anatomical, morphological and biochemical attributes (Koricheva and Barton, 2012). Environmental factors such as temperature, humidity, light and supply of water, minerals and CO_2, also influence plant growth and have direct effect on the biochemical pathways which affect the metabolism of their secondary compounds.

In general, tannins are more abundant in the plant parts susceptible of being consumed, for example, new leaves and flowers (Terril, 1992), and are considered

Table 1. Variation of the phenol content during the sampling moments of *E. angustifolia* (mg GAE g^{-1} DM).

Sampling moment	Mean ± SE
September	
Leaves inner canopy	19,8 ±1,84*
Leaves outer canopy	15,8 ±1,26*
November	
Leaves inner canopy	12,1 ± 0,80
Complete thyrse flowers + leaves	13,3 ± 2,35
January	
Leaves inner canopy	22,0 ± 2,10
Complete thyrse fruit + leaves	24,5 ± 4,77
February	
Leaves inner canopy	15,2 ± 2,01
Complete thyrse fruit + leaves	12,1 ± 1,71
March	
Leaves inner canopy	13,9 ± 0,46
Complete thyrse fruits + leaves	13,2 ± 1,44

p <0,05
GAE: gallic acid equivalents

a defense factor from herbivory. This could not be the case of *E. angustifolia*, because its more appealing parts for ruminants did not show significant differences in the phenol content with regards to the rejected parts.

The seasonal fluctuations in tannin content in the vegetative leaves of *E. angustifolia* (juvenile plants) have been approached before in literature (Zeng *et al*., 2009). A seasonal cycle of the polyphenolic content was found in the shaded leaves of *E. angustifolia* developed in the lower canopy (figure 2). The average was high in the vegetative stage (September) and the initial fruit formation stage (January), with mean values of 19,75 and 21,97 mg·GAE g^{-1} DM, respectively, with significant differences in the November, February and March sampling.

The results showed that the changes in the phenol content between the internal shaded leaves of *E. angustifolia* had a double-peak characteristic, which was in concordance with the moment of accumulation of secondary metabolites in the plant (Koricheva and Barton, 2012). This pattern was not found in the external sunlit foliage during the development stage (figure 3).

When the fruit is close to complete maturity, no significant differences were found in the hydrolysable tannin content in February and March (10,85 and 12,77 mg GAE g^{-1} DM, respectively), or in the tannins present in the seed (14,19 and 14,36 mg GAE g^{-1} DM, respectively). The development of color intensity with the ferric chloride reagent showed positive correlation with regards to the results obtained in the quantitative essay.

Conclusions

Seasonal and spatial variation was determined in the concentration of total phenols in the edible

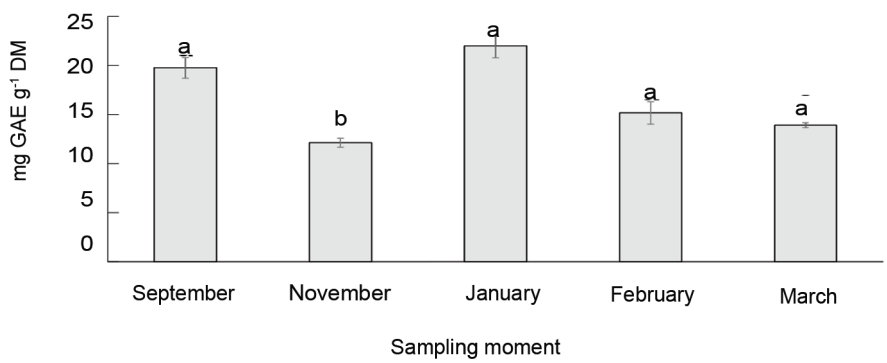

Figure 2. Seasonal variations of the phenol content in *E. angustifolia* leaves.
p < 0,05. GAE: gallic acid equivalents

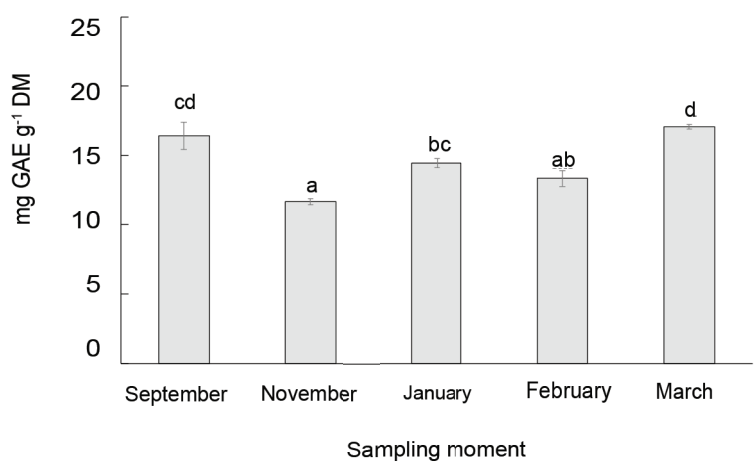

Figure 3. Seasonal variations of the phenol content in the shaded thyrses of *E. angustifolia*
p < 0,05. GAE: gallic acid equivalents

and non-edible biomass of *E. angustifolia* during its growth cycle.

No limiting quantities of polyphenols (> 55 g kg^{-1} DM) were found in the shaded leaves, which could reduce the voluntary feed intake and feed digestibility. In addition, the seasonal variation in the phenol content was in the beneficial range (20-45 g kg^{-1} DM).

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Authors' contribution

- María Pía-Beker. Experimentation, bibliographic analysis, adaptation and statistical analysis.
- Osvaldo Alberto-Fernández. Director of the research.
- María Guadalupe-Klich. Director of research, adaptation, statistical contribution and technical revision.

Conflict of interests

The authors declare that there are no conflicts of among them.

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