Month of Harvest and Leaf Age Impact the Bromatological Composition and Polyphenol Content of Gymnopodium floribundum Rolfe Leaves

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Abstract: In the present study, the effects of macro- and microclimatic conditions, month of harvest, and leaf age at harvest on the bromatological composition and polyphenol content of Gymnopodium floribundum leaves were evaluated. Leaves were harvested in December 2017 and 2018 and March, June, and September 2018. At each harvest, three composite samples of mixed-age leaves were collected from 12 trees (four trees for each sample), and the sampling was repeated on day 90 post-harvest to collect 90-day-old leaves. Fresh and dry matter, crude protein, acid and neutral detergent fibers (ADF and NDF, respectively), lignin, total tannins, condensed tannins (CT), total phenols, in vitro dry matter (IVDMD) and organic matter (IVOMD) digestibility, and metabolizable energy (ME) were estimated. Rainfall, relative humidity, and microhumidity were associated with chemical composition. IVDMD, IVOMD, and ME were highest in leaves sampled in March regardless of age (p < 0.001). Water content, ADF, NDF, and lignin were highest in the leaves sampled in September, regardless of age (p < 0.05), suggesting that leaves require more structural support in the rainy season. CT content was highest in September in the mixed-age leaves and in September and December in the 90-day-old leaves (p < 0.05). A high fiber and CT content during the period of rapid leaf growth could deter herbivory.

Keywords: polygonaceae; rainfall; harvest month; biomass; chemical composition; polyphenols; fiber content

1. Introduction

The tree species Gymnopodium floribundum Rolfe, a member of the Polygonaceae family [1], is abundant in the tropical deciduous forest (TDF) of Yucatán, México [2], where it provides several ecosystem services [3,4]. In particular, the foliage of G. floribundum is an important source of nutrients for sheep and goats in the TDF during the dry and rainy seasons [5–7]. The consumption of G. floribundum leaves permits efficient use of the protein-rich vegetation typical of the TDF, as it represents a source of foliage with a moderate crude protein content (>10% CP) and high condensed tannin (CT) content, which can block the potential excess of CP in the ruminant diet [8,9]. For small ruminants, the consumption of G. floribundum can reach up to 40% of their required dry matter (DM) under browsing [8] or pen conditions [10]. However, there is evidence that the voluntary consumption and selection of this type of forage by small ruminants varies greatly depending on the time of...
This variation in the consumption and selection of *G. floribundum* fodder could be associated with seasonal changes in its bromatological composition or its content of secondary compounds, including polyphenols. For example, recent studies suggested that the CT content of *G. floribundum* is higher in the rainy season (33.8%) and lower in the dry season (9.5%) [5,10]. Those results highlight the need to evaluate the variations in the bromatological composition and secondary compound content of *G. floribundum* leaves throughout the year.

Like other plant species, *G. floribundum* trees are exposed to a complex array of biotic and abiotic interactions, and must constantly adapt to their environment [11,12]. These adaptations may lead to qualitative and quantitative variations in macronutrients and secondary compounds in different plant tissues [13]. In addition to the influence of macro- and microclimatic factors, the bromatological composition and polyphenolic content may be affected by the age of the leaves at the moment of defoliation (harvest). For example, the age of leaves at harvest affects the total phenol content, but not the CT content of balsam fir [14].

No study has examined the associations of climatic factors, season of harvest, and leaf age at harvest with the bromatological composition or polyphenolic content of *G. floribundum* leaves. Moreover, this species of tropical tree may represent a good model to study such associations, as it can be defoliated in different months of the year. *G. floribundum* trees are exposed to extreme dry and rainy conditions that gradually change during the year, as well as a considerable difference in sunshine hours (approximately 4 h) between the shortest day (winter solstice in December) and longest day (summer solstice in June) of the year. In the present study, the effects of macro- and microclimatic conditions, harvest month, and leaf age at harvest on the bromatological composition and polyphenol content of *G. floribundum* leaves were evaluated.

2. Materials and Methods

2.1. Study Area

This study was conducted from 18 December 2017 to 21 December 2018 in an experimental area of 12,000 m² (50 m × 240 m) of deciduous forest on the Biological and Agricultural Sciences Campus of the Universidad Autónoma de Yucatán (UADY), Mérida, Yucatán, México (20°51′93″ N and 89°37′11″ W; 10 m.a.s.l.) [15]. The study area was located inside a natural reserve named Cuxtal (Figure 1). Since the creation of the reserve in 1993, *G. floribundum* trees, as well as other tree species, have been established naturally and have not been exposed to animal production activities or any other human activity. The study area was enclosed by a perimeter fence of barbed wire and electric cords to avoid the contact of ruminant herbivores with the plants. The experimental area has two types of soil: Luvisol and Cambisol [16,17]. *G. floribundum* trees growing in Luvisol soil patches were selected, as a previous study suggested that Luvisol soil promotes larger tree crowns [18]. Luvisol soil is slightly saline with a very high organic matter content (>6%), high concentrations of N (>0.20%), K (>1.2 cmol 100 g⁻¹), Ca (>12 cmol kg⁻¹), and Mg (>1.2 cmol kg⁻¹), and a high or very high cationic exchange capacity (25–29 meq 100 g⁻¹) [17].

2.2. Selection of Trees for Sampling

A botanist selected 48 growing trees with no evidence of pests or diseases. A sample of *G. floribundum* (stem, leaves, and flowers) was delivered to the UADY herbarium to confirm the botanical identification (voucher number UADY22887). The location of each *G. floribundum* tree was determined with a GPS device. In each harvest month, 12 individual trees were randomly selected and identified with ribbons of different colors tied at breast height. The height of the experimental specimens varied from 2 to 4 m. The diameter at breast height varied from 1.2 to 4.8 cm.
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2.3. Leaf Sampling

To assess the effect of the month of harvest on the leaf chemical composition, leaves were sampled during the following weeks:

(i) 18–21 December (winter solstice) 2017 and 2018,
(ii) 18–21 March (spring equinox) 2018,
(iii) 18–21 June (summer solstice) 2018, and
(iv) 18–21 September (fall equinox) 2018.

To evaluate the effect of leaf age, two types of leaves were obtained by hand pruning in each sampling month:

(a) Mixed-age leaves were sampled from the 12 non-defoliated trees randomly selected for that month. These leaves were used to prepare three composite samples (each containing all of the leaves from four trees).

(b) To obtain samples of 90-day-old leaves, the 12 trees defoliated 90 days earlier (in the previous sampling month) were defoliated again. These 90-day-old leaves were used to prepare a new set of three composite samples (each containing all of the leaves from four trees).

2.4. Environmental Data

Macroclimatic data were collected daily from the Mérida weather station in 2017 and 2018 [19] to calculate the mean, maximum and minimum relative humidity (RH, Hmax, Hmin, respectively); mean, maximum, and minimum ambient temperature (TMP, Tmax, Tmin, respectively); cumulative rainfall (RF); and cumulative sunshine hours (SH) for the 90-day period prior to the harvest date. These values were used in the statistical analyses described below. Additionally, microclimate information (MT = microtemperature, MH = microhumidity) was obtained hourly during the week of sampling (7 days) using three real-time microclimate measurement devices (Lascar© data-loggers Model EL_USB_2, Woburn, MA, USA). The devices were installed for 168 h at a height of 2 m (the level of the tree leaves) in three different locations of the experimental plot (center, south, and north). The daily mean MT and MH values were calculated.
2.5. Fresh Biomass and Dry Matter of Gymnopodium floribundum Leaves

The total quantity of fresh biomass (FB) was determined for each tree. Each of the *G. floribundum* trees was completely defoliated by hand. The total leaf biomass of each plant was immediately weighed in situ using an electronic scale (Rihno®, BAPO-10, Atizapán de Zaragoza, México). The leaves were then stored in separate identified bags for each tree and were frozen at −20 °C until further processing [20]. As mentioned above, the leaves of four trees were mixed to form a composite sample (replicate). Thus, in each of the four harvest months of the survey, three replicates of each leaf age group were prepared. Subsequently, a sample of leaves from each replicate (>30 g FB) was dried in a forced-air oven in the absence of light at 40 °C for 72 h to determine the dry matter (DM) content. The dry leaves of each replicate were ground in a mill (IKA® MF 10 basic, Wilmington, NC, USA) with a 1-mm mesh and were stored in clearly identified plastic bags until analysis.

2.6. Chemical Analysis and In Vitro Digestibility

The bromatological composition of each replicate, including CP, lignin, and ash, was determined [21]. NDF and ADF were determined using an ANKOM® fiber analyzer (Macedon, USA). The total phenol (TP) and total tannin (TT) contents in the dry ground *G. floribundum* leaves were determined using the Folin–Ciocalteu methodology described by [20] using a UV–VIS spectrophotometer (Mod. Genesys 10S, Thermo Fisher Scientific®, Madrid, España) at a wavelength of 725 nm. TP and TT were expressed as tannic acid equivalents. The CT content was quantified using the vanillin method at a wavelength of 550 nm [22] and expressed in catechin equivalents. All of the chemical analyses were carried out at the Animal Nutrition Laboratory of the FMVZ-UADY, Mérida, México.

The in vitro digestibility of the dry matter (IVDMD) and organic matter (IVOMD) in the leaf samples were estimated following the methodology described by [23] modified from [24]. In brief, 0.5 g of substrate from each replicate was incubated in a 100 mL glass bottle with 42 mL of culture medium and 18 mL of rumen fluid (culture medium/rumen fluid ratio of 70:30). A bovine donor provided the rumen fluid. Three bottles used as controls (blanks) contained only the culture medium and the rumen fluid (without substrate). To obtain true replicates of each substrate, separate tests were performed on different weeks. All of the samples were incubated at 39 °C for 48 h [23]. IVDMD was calculated as the difference between the weight before and after incubation, corrected for the weight of the bottles without a substrate (blanks). A similar procedure was used for IVOMD, but using the organic matter (OM) content of the leaves instead of the DM. The metabolizable energy (ME) in the leaf samples was calculated using the following equation:

\[
\text{ME (MJ/kg DM)} = 0.016 \times \text{digestible organic matter (%)}
\]

2.7. Statistical Analysis

Before the statistical analysis, all of the variables were tested for a normal distribution using the Shapiro–Wilk test. The Levene test was used to determine the homogeneity of variance for all of the variables. All of the variables, including the leaf biomass, chemical composition, and digestibility, were analyzed using a generalized linear model (GLM), and the main effects of the harvest month, leaf age, and the harvest month × leaf age interaction were evaluated. Means were compared using Tukey’s test with \( \alpha \leq 0.05 \). In addition, Pearson’s correlation analysis was performed between FB, DM, CP, NDF, ADF, TP, TT, and CT of the *G. floribundum* leaves and the mean values of the macroclimatic factors (mean of 90 days prior to the harvest date) and microclimatic factors (mean of 7 days prior to the harvest date). Statistical analyses were performed using Minitab 16 Statistical software [26].

3. Results

3.1. Climatic Factors during the Experimental Period

Figure 2 shows the mean values of the macroclimatic variables TMP, RH, and SH for the 90-day period prior to each harvest and the microclimatic variables MH and MT for the 7-day period prior to each harvest. Figure 2a presents the temperature results (TMP, Tmax,
Tmin, and MT). The cumulative RF of the 90-day period prior to each harvest is shown in Figure 2b. Figure 2c shows the values of RH and MH. RF and MH were the lowest in March (dry period) and the highest in September (rainy period). The mean SH was 7.75 h in December (10:49 SH on 21 December), 7.77 h in March (12:06 SH on 21 March), 8.83 h in June (13:22 SH on day 21 June), and 8.73 h in September (12:05 SH on 21 September).

![Figure 2](image-url)

**Figure 2. Cont.**
Figure 2. Macro- and microclimatic variables in the area of Yucatán, México, where the Gymnopodium floribundum plot was located. The values of the macroclimatic variables are the means for the 90-day period prior to the respective leaf harvest; the values of the microclimatic variables are the means for the 7 days prior to the respective leaf harvest. (a) TMP = mean ambient temperature, Tmin = minimum ambient temperature, Tmax = maximum ambient temperature, MT = mean temperature recorded at the level of the tree leaves (°C), (b) RF = accumulated rainfall (mm), and (c) RH = mean relative humidity.

3.2. Effects of Harvest Month, Leaf Age, and their Interaction on the Chemical Composition

Table 1 displays the FB of the G. floribundum leaves and their chemical composition at different harvest months and leaf ages. FB was highest in June and September for the mixed-age leaves and in June for the 90-day-old leaves (p < 0.05). FB was lowest in March for the mixed-age leaves, whereas FB did not differ significantly among March, September, and December for the 90-day-old leaves. Regardless of leaf age, DM was the lowest in June (p < 0.05), and the NDF, ADF, and lignin contents were the highest in September.

Table 1. Effect of month of harvest and leaf age on biomass production (g FB) and chemical composition (%) of Gymnopodium floribundum leaves, including dry matter (DM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), total phenols (TP), total tannins (TT) and condensed tannins, in vitro dry matter digestibility (IVDMD), in vitro organic matter digestibility (IVOMD), and metabolizable energy (ME in MJ/kg DM).

| Harvest Time | FB    | DM    | CP    | ADF | NDF | Lignin | TP | TT | CT | IVDMD | IVOMD | EM |
|--------------|-------|-------|-------|-----|-----|--------|----|----|----|-------|-------|----|
| March        | 84.7  | 65.6  | 15.4  | 18.3| 35.3| 8.0    | 6.7| 2.44| 23.1| 36.8  | 38.6  | 5.7 |
| June         | 1071.0| 32.3  | 14.6  | 23.1| 46.5| 10.2   | 5.7| 1.58| 22.7| 26.2  | 27.0  | 3.9 |
| September    | 903.0 | 41.46 | 14.3  | 32.3| 51.8| 18.2   | 6.3| 1.26| 41.3| 25.6  | 28.0  | 4.1 |
| December     | 545.7 | 52.96 | 13.9  | 25.4| 40.2| 12.6   | 4.6| 1.4 | 24.0| 22.9  | 26.8  | 3.9 |
| Mixed-age leaves |       |       | 90-day-old leaves |       |
| March        | 151.0 | 59.23 | 14.9  | 13.6| 33.5| 5.6    | 7.9| 1.1 | 31.3| 43.1  | 42.7  | 6.3 |
| June         | 833.6 | 33.3  | 14.4  | 24.7| 42.7| 11.5   | 4.6| 1.2 | 19.3| 29.1  | 29.4  | 4.3 |
| September    | 318.0 | 36.46 | 14.3  | 32.2| 53.1| 17.1   | 6.7| 1.4 | 44.3| 25.1  | 25.9  | 3.8 |
| December     | 127.7 | 58.9  | 13.5  | 24.8| 47.9| 11.8   | 7.4| 2.8 | 43.7| 34.2  | 34.3  | 5.1 |
| SE           | 90.17 | 1.13  | 0.68  | 1.76| 1.77| 1.23   | 0.30| 0.39| 1.15| 1.19  | 1.08  | 0.15|

(a-c) Values in the same column with different superscript letters are significantly different (p < 0.05), SE: Standard error, CT units equivalent to catechin.
The CP and TT contents of the G. floribundum leaves did not vary significantly according to harvest month, leaf age, or their interaction ($p > 0.05$). The TP content was lowest ($p < 0.05$) in December for the mixed-age leaves and June for the 90-day-old leaves. The CT content was the highest ($p < 0.05$) in September for the mixed-age leaves and September and December for the 90-day-old leaves. IVDMD and IVOMD are presented in Table 1. A significant ($p < 0.001$) effect of the harvest month × leaf age interaction on IVDMD and IVOMD was observed, and IVDMD, IVOMD, and ME were the highest in March, regardless of leaf age, although the values for the mixed-age leaves were lower than those for the 90-day-old leaves.

3.3. Relationship between Climatic Factors and the Chemical Composition

Table 2 shows the correlations between the macro- or microclimatic conditions and the chemical composition of the G. floribundum leaves. SH and TMP were positively associated with FB in the leaves of both ages ($p < 0.05$). SH, TMP, RF, and MH were negatively associated with DM, irrespective of leaf age. RH, RF, and MH were positively associated with fiber content (ADF, NDF, and lignin) ($p < 0.05$), irrespective of leaf age. SH was positively associated with ADF and lignin content in the 90-day-old leaves and with NDF in the mixed-age leaves. TMP was positively correlated with ADF, NDF, and lignin content in the 90-day-old leaves and with NDF in the mixed-age leaves. RH was positively associated with CT content, irrespective of leaf age. However, there was no clear trend in the correlations of the climatic factors with TP content. For example, MT was positively associated with TP content in the mixed-age leaves, whereas SH and TMP were negatively associated with TP in the 90-day-old leaves.

Table 2. Significant correlations of the macro- and microclimatic factors with leaf biomass (FB) and different bromatological components of Gymnopodium floribundum leaves at different harvest ages.

|      | FB     | DM     | CP     | ADF   | NDF   | Lignin | TP     | TT     | CT     |
|------|--------|--------|--------|-------|-------|--------|--------|--------|--------|
| SH   | 0.806 ** | −0.879 ** | −     | −     | 0.829 ** | −     | −     | −     | −     |
| RH   | −      | −      | −      | 0.712 ** | 0.625 * | 0.787 ** | −     | −     | 0.965 ** |
| TMP  | 0.822 ** | −0.874 ** | −     | −     | 0.885 ** | −     | −     | −     | −     |
| RF   | 0.761 ** | −0.762 ** | −     | 0.789 ** | 0.946 ** | 0.779 ** | −     | −0.587 * | 0.805 ** |
| MT   | −      | −      | −      | −     | −     | 0.655 * | −     | −     | −     |
| MH   | 0.738 ** | −0.702 * | −0.864 ** | 0.863 ** | 0.838 ** | −     | 0.753 ** | 0.707 * |

Mixed-age leaves

|      | FB     | DM     | CP     | ADF   | NDF   | Lignin | TP     | TT     | CT     |
|------|--------|--------|--------|-------|-------|--------|--------|--------|--------|
| SH   | 0.768 ** | −0.996 ** | −     | 0.643 * | −     | 0.613 * | −0.780 ** | −     | −     |
| RH   | −      | −      | −      | 0.868 * | 0.816 ** | 0.690 * | −     | −     | −0.872 ** |
| TMP  | 0.650 * | −0.992 ** | −     | 0.810 ** | 0.663 * | 0.771 ** | −0.717 * | −     | −     |
| RF   | −      | −      | −0.857 ** | −     | 0.727 ** | 0.836 ** | −     | −     | −     |
| MT   | −      | −      | −      | −     | −     | −0.705 * | −0.768 * | −     | −     |
| MH   | −      | −      | −0.704 * | −     | 0.931 ** | 0.881 ** | 0.908 ** | −     | −     |

90-day-old leaves

− = Not significant. * = $p < 0.05$. ** = $p < 0.01$. $n = 24$.

4. Discussion

The present study is the first to provide evidence of the variation in the chemical composition of G. floribundum leaves according to harvest month and leaf age under natural field conditions in a tropical forest. The climatic variables reveal strong rainfall seasonality in the study area. Only 50 mm of rainfall accumulated in the 90 days before the March harvest, whereas >260 mm and >430 mm accumulated in the 90 days before the June and September harvests, respectively. This resulted in large variations in RH and MH,
which were lowest in March and highest in September, corresponding to the rainy season (Figure 2).

The environmental seasonality was clearly reflected in a greater quantity of *G. floribundum* leaves (FB) in the June and September harvests. Thus, *G. floribundum* appears to take advantage of rainy months by increasing the leaf biomass. More leaf biomass can maximize the leaf surface area and permit the capture of more sunlight in order to optimize carbohydrate production, thereby allowing the trees to grow, reproduce, and retain nutrients to withstand the challenge of the next dry season (from December to May) [27,28]. The increase in FB is the result of cellular processes such as division and expansion [29].

The increase in FB from June to September was accompanied by a higher leaf water content, consistent with observations in tropical grasses [30]. FB growth was also associated with a higher leaf fiber content (ADF, NDF, and lignin) (Table 1). The increase in fiber content might reflect the need to increase structural components in order to allow leaves to store more water during the rainy season. Lignin and hemicellulose provide strength and rigidity to plant cell walls and other structures of the plant vascular system and the necessary hydrophilicity to transport water and solutes [31]. Moreover, the higher fiber content helps explain the low selection of *G. floribundum* leaves by goats during the rainy season in field trials [7,9]. A high fiber and lignin content reduce the rumen degradability of leaves [32], and accordingly, the digestibility, particularly IVOMD, was lower in the June and September harvests than in the March harvest, irrespective of leaf age. In contrast, the leaves harvested in March had the lowest ADF, NDF, and lignin contents and thus the highest digestibility (IVDMD and IVOMD), regardless of leaf age.

The high fiber and lignin content in *G. floribundum* leaves during the rainy season suggests that its consumption may represent a waste of time and effort for ruminants, which must select the best-quality diet in the short period of time available for daily browsing. Accordingly, goats seem to express nutritional wisdom by avoiding *G. floribundum* leaves during the rainy season, which would require more mastication for this material with a very low digestibility and a poor harvest of nutrients for absorption. The nutritional wisdom of goats is further confirmed by the results of a field study performed during the dry season (March–April), which reported that goats consumed a large quantity of *G. floribundum* leaves [5]. The present study demonstrated that leaves harvested in March have the lowest fiber content and the highest in vitro digestibility (Table 1). Although the quantity of *G. floribundum* leaves is small during the dry season, these leaves represent one of the most abundant dry season feed resources for ruminants in the TDF. The low fiber and lignin contents of the leaves of the March harvest could reflect the conditions of the dry season, when the leaves do not need structural support to preserve water. In this period, most of the leaves of *G. floribundum* are lost, and the remaining leaves have a low water content likely due to evaporation [28].

The CP content of *G. floribundum* leaves was constant throughout the harvest months, regardless of any climatic factor or leaf age (Tables 1 and 2). The CP content of *G. floribundum* makes it a better foraging option for ruminants than tropical grasses, which contain <8% CP at their best point of use [33].

The values of CT content in the present study were within the range reported previously for the TDF of Yucatán [6,10,34]. The RH maximum recorded at the September harvest corresponded with the CT content, which was highest in September, regardless of leaf age (Table 1). The higher CT content in the September harvest (mid-rainy season) could be considered a defense mechanism against insect herbivores or other consumers. Herbivorous insects (defoliants or removers) may become abundant during the rainy season in tropical forests and cause up to 75% of leaf damage [35,36]. For example, [37] reported that the interaction of TMP, RF, and an herbivorous insect (*Helopeltis theivora*) was associated with a higher phenolic content in tea leaves. Any increase in CT could also be influenced by pathogenic organisms such as fungi and bacteria [38,39]. CTs are the main phenolic fraction responsible for the astringent characteristics of plant species and are unpleasant for plant consumers [38,39]. Reduced consumption due to a high CT content has been previously
reported for herbivorous insects [40], grazing goats [9], and goats fed in cafeteria trials [6]. In some plant species, CT content is associated with protection against solar radiation, water stress [41,42], or high temperatures [42,43], but these climatic factors were not clearly associated with the CT content of G. floribundum leaves in the present study (Table 2).

Leaves harvested in June, when the maximum biomass growth (FB) was observed, were exposed to the maximum MT (Figure 2) but had the lowest CT content (Table 1). These results are consistent with the trade-off theory, which posits that plants allocate resources for vital functions (i.e., growth or reproduction) rather than synthesizing defensive compounds such as CT, which also incur a metabolic cost for the plant [28]. Finally, in mixed-age leaves, the TP content was lowest in the December harvest. In December, most deciduous trees lose their leaves and thus have a higher proportion of old leaves that might be ready to fall from the tree. Consequently, plants do not need to invest energy resources by defending these senescent leaves.

5. Conclusions

The present study confirmed the occurrence of variations in leaf production (FB) and the leaf chemical composition of G. floribundum under natural field conditions. The fiber content (ADF, NDF, and lignin) and CT content were the highest in the leaves harvested in the rainy months of June and September. These variables were positively associated with RH, RF, and MH, irrespective of leaf age. The increase in fiber content seemed to be a response of G. floribundum trees to the increase in foliage water content, which requires more structural support. Meanwhile, in the March harvest, the fiber and CT contents were the lowest, and the in vitro digestibility (IVDMD and IVOMD) was the highest. However, some leaf components, such as CP and TT, remained constant, regardless of season or leaf age. Although MT was negatively associated with the leaf CT content, there was no association of the leaf CT content with SH or TMP.

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