

# A simple assay for measuring tannin-protein precipitation capacity offers insights into the diet and food choice of black howler monkeys (*Alouatta pigra*)

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## Abstract

Phenolics, like tannins, are plant-specialized metabolites that play a protective role against herbivory. Tannins can reduce palatability and bind with proteins to reduce digestibility, acting as deterrents to feeding and impacting nutrient extraction by herbivores. Some assays measure tannin and total phenolics content in plants but lack determination of their biological effects, hindering the interpretation of tannin function in herbivory and its impacts on animal behavior and ecology. In this study, we successfully applied the radial diffusion assay to assess tannin protein precipitation (PP) capacity and evaluate the anti-nutritional effects of tannins in food plants ( $n = 24$ ) consumed by free-ranging black howler monkeys (*Alouatta pigra*) in Tabasco, Mexico. We found PP rings in five plant species consumed by the monkeys. The mature fruit of *Inga edulis* was the most consumed food plant, despite having a high tannin PP capacity (56.66 mg tannic acid equivalent/g dry matter). These findings highlight the presence of tannins in the black howler diet and provide insight into the primates' resilience and potential strategies for coping with anti-nutritional aspects of the diet.

## KEYWORDS

anti-nutritional, assay, consumption, herbivores, precipitation

## 1 | INTRODUCTION

Phenotypic plasticity, local adaptation, competition with nearby species, and the plant's response to light, water, and nutrient availability influence the presence and quantity of plant-specialized metabolites (PSMs; Moore et al., 2014). PSMs are a highly diverse group of plant compounds (Coley, 1983; DeGabriel et al., 2009). Tannins as PSMs, are found in most plants (Gedir et al., 2005). They are typically classified into hydrolysable (HT) and condensed tannins (CT), with CT generally being more

abundant than HT in plant species (Hassanpour et al., 2011; Min et al., 2003).

Tannins are produced by plants at an energetic cost, their role and evolutionary significance is still unclear. It was speculated that CTs acted as feeding deterrents or defense mechanisms against herbivory (Kelln et al., 2021). High CT concentrations (>50 mg/g dry matter [DM]) have been found to negatively impact DM intake, organic matter digestibility, and microbial digestion. This is due to their protein-binding properties that affect feeds, enzymes, and microbial cells, leading to anti-nutritional effects. At lower levels

**Abbreviations:** BSA, bovine serum albumin; CT, condensed tannins; DC, daily consumption; DM, dry matter; DMI, dry-matter intake; GLM, generalized linear models; HT, hydrolysable tannins; Int, intake; MF, mature fruits; ML, mature leaves; N, nitrogen; OM, organic matter; PP, protein precipitation; PSMs, plant specialized metabolites; RDA, radial diffusion assay; TP, total phenolics; TT, total tannins; WM, wet mass; YL, young leaves.

(5–10 mg/g DM), CT can have positive effects like reducing bloat risk, increasing ruminal undegradable protein, decreasing enteric methane production, and enhancing anthelmintic activity (Barry & Manley, 1984; Dschaak et al., 2011).

One of the key herbivory defenses of tannins is their astringent taste (Bernays & Cornelius, 1992), perceived when salivary proteins interact with tannins during chewing (Espinosa-Gómez et al., 2018). Tannins also have a post-ingestion effect, forming complexes with dietary proteins and reducing nitrogen (N) availability, influencing food choice. In vitro assays have shown that tannins can decrease N availability by binding to proteins in the diets of ruminants (Silanikove et al., 2001) and other herbivores such as Stephen's wood rat (*Neotoma stephensi*), the white-throated wood rat (*N. albigula*) (Sorensen et al., 2005), and the common brushtail possum (*Trichosurus vulpecula* Kerr) (DeGabriel et al., 2009). The study of the binding of tannins with proteins, facilitated by protein precipitation (PP) assays, provides insights into the biological effects of this interaction (Martin & Martin, 1983; Rickard, 1986).

Tannin PP assays have disadvantages, including the complexity of forming and isolating the precipitate and the requirements for specialized materials like radiolabeled compounds (Hagerman & Butler, 1980; Makkar et al., 1993; Martin & Martin, 1983). To overcome these limitations and make tannin research more accessible, we applied the radial diffusion assay (RDA) proposed by Hagerman (1987) to assess PP in plant foods consumed by black howler monkeys (*Alouatta pigra*). This method, known for its cost-effectiveness, simplicity, and sensitivity, facilitates the comparison of plant extracts with varying concentrations and types of tannins to a standard protein dissolved in an agarose gel. It is particularly useful for the analysis of numerous plant samples. Noteworthy is its prior application in primatology research (Mowry et al., 1996; Remis et al., 2001; Simmen et al., 2014; Wrangham et al., 1998).

Howler monkeys (genus *Alouatta*) are a radiation of 14 species ranging from Mexico, Central America, the Amazon basin, and as far south as northern Argentina and Paraguay (Crockett, 1998). Their diet is composed of leaves, fruits, and floral nectar (Dias & Rangel-Negrín, 2015). They are hindgut, colon fermenters (Espinosa-Gomez et al., 2018; Milton et al., 1980; Pavelka & Knopff, 2004) and possess trichromatic color vision, which aids in detecting ripe fruits or young leaves (YL) (Dominy & Lucas, 2001). Previous studies have examined the concentrations of total CT, HT, and phenolics in the plant parts consumed by howler monkeys (Espinosa Gómez et al., 2015; Guimarães Leitão et al., 1999; Milton, 1979; Milton et al., 1980; Righini et al., 2017; Welker et al., 2007). Our research explores the PP capacity of tannins from the plant species consumed by free-ranging black howlers using the RDA (Hagerman, 1987).

The black howler monkey (*A. pigra*) is an endangered primate species distributed from southeastern Mexico, Guatemala, and Belize (Estrada et al., 2002; IUCN, 2022). The black howler diet is reported to include 37% leaves, 58.2% fruits, and 4.7% flowers/inflorescence, based on DM intake (Righini et al., 2017). Studies on their food choice suggest that fiber (cellulose, hemicellulose), CT, TT, available protein, lipids, and N-free content may affect the consumption of plant parts

(Supporting Information S1: Tables 1 and 2; Aristizábal, 2013; Behie & Pavelka, 2012; Bridgeman, 2012; Righini et al., 2017; Silver et al., 2000). Our study examines the black howler diet, and to evaluate, based on RDAs (Hagerman, 1987), how the PP capacity of dietary tannins affects their feeding ecology. We tested the following hypotheses: H1—Black howlers avoid consuming foods with high levels of CT and total phenolics (TP). H2—Black howlers evade consuming food plants characterized by the presence of and/or high levels of tannins with PP capacity. H3—Following days in which black howlers consume foods with high levels of CT and/or TP, their consumption of these phenolic PSMs decreases dramatically.

## 2 | METHODS

### 2.1 | Diet data collection

During October 2014, fieldwork was carried out applying a focal-animal sampling method (Altmann, 1974) to record the behavior of adults and subadults in three groups of wild black howler monkeys (*A. pigra*) that inhabit a 2.2 ha forest fragment near Balancan, Mexico (17°44'05"N; 91°30'17"W) (Espinosa-Gómez et al., 2018). Focal-animal sampling was conducted at 1 h intervals, from sunrise to sunset ( $\approx 10$  h), over 8 successive days per study group. Focal animals were selected on a rotating basis, and the food consumption of adults ( $n = 11$ ) and subadults ( $n = 2$ ) was recorded. We recorded each feeding tree's species, tree identification number, and the number of discrete food units consumed on each feeding bout (mature leaves [ML]; YL; and mature fruits [MF]). Additionally, during each observation on the day that the group fed, we collected and weighed 50–100 units of each food item to estimate the average on a fresh weight basis (grams of wet mass [WM]) (Aristizabal et al., 2017). In addition, we collected 200 g of fresh material per food item and partially dried it out of direct sunlight in a cool and dark place at room temperature ( $\approx 24^\circ\text{C}$ ) for later chemical analyses.

### 2.2 | Laboratory analyses: CT and TP

During 2014, we conducted chemical analyses in the nutrition laboratory of the Faculty of Veterinary Medicine of the Universidad Autónoma of Yucatan (FMVZ-UADY). We analyzed one sample of each species and plant part consumed by the monkeys separately by group, the partial field dried plant samples were placed in a forced air oven ( $<45^\circ\text{C}$ ) until they reached a constant weight. These thoroughly dried food samples were ground and put through a 1 mm sieve using a laboratory mill (Knifetec; Foss™ FOSS Analytical). Next, the DM was calculated by drying 2 g of each sample at  $110^\circ\text{C}$  for 6 h until a constant weight was reached. To establish the TP content, we used the Folin-Ciocalteu reagent assay (Singleton & Rossi, 1965) based on the method proposed by Kaur and Kapoor (2002). Moreover, 100  $\mu\text{L}$  of crude TP extract of each plant species was combined in 3 mL of distilled water and mixed well with 0.5 mL of the Folin-Ciocalteu

reagent. After 3 min, 2 mL of sodium carbonate was added. The sample was left to stand for 60 min in the dark for the color to develop, and the absorbance at 650 nm was measured. A vanillin assay (Price et al., 1978) using catechin as a standard, was employed to analyze CT.

We recognize that the analysis of phenols in plant species is highly complex due to their high structural variability, that is, CT might be polymeric with many isomeric forms (Rautio et al., 2007; Salminen & Karonen, 2011). In addition, using commercial standards such as catechin and gallic acid instead of an internal standard can over- or under-estimate the phenolic and tannin concentrations in samples, since the commercial standards may differ from those in the plants sampled (Rothman, Dusingberre, et al., 2009). For this reason, results were measured as mg gallic acid equivalent/100 g of WM for TP and mg catechin equivalent/100 g of WM for CT. All analyses were conducted in duplicate, following the quality standards set by the Association of Official Agricultural Chemists. In case the results exhibited a coefficient of variation greater than 2%, the analyses were repeated.

### 2.3 | Tannin and phenolics intake estimations

Daily DM (g/DM) consumption of each food category was calculated first by multiplying the unit's average on a WM (g/WM) basis by the actual number of units consumed by the focal animal. Then, the consumption in WM was subtracted according to the moisture calculated in the laboratory for each food category to calculate the total g/DM consumed in each feeding event. Finally, based on the total amount consumed by a daily series of focal animals (adults/subadults), we calculated the total consumption of plant parts across the 8 study days per group.

To calculate the daily consumption (DC) of phenolics (TP) and tannins (CT), first, we calculate the intake (Int) multiplying the concentration of TP (g gallic acid equivalent/100 g DM) or CT (g catechin equivalent/100 g DM) by the dry-matter intake of each food category (i.e., ML, YL, MF) per plant species (*i*):

$$Int_i = [\text{concentration}_{TPorCT}] \times DMI$$

Second, we summed all daily feeding events:

$$DC_{TPorCT} = \sum_{i=1}^n Int_i$$

Where DC = daily consumption of TP or CT, *n* = total number of feeding events of *i* food category per plant species.

To calculate the concentration of tannins with the capacity to precipitate protein for each species/plant part, the following formula was used:

$$x = \frac{y - b}{m}$$

Where *y* represents the area of the tannin-protein ring, *b* represents the intercept, and *m* represents the slope of the

standard curve. The results are reported in mg tannic acid equivalent/g DM.

### 2.4 | Biological effect: Capacity of tannins to precipitate proteins in the plant species preferred by *A. pigra*

The RDA proposed by Hagerman (1987), was utilized to assess the PP of dietary tannins. This analysis was conducted at the Biotechno-environmental Research Laboratory of the Universidad Popular Autonoma del Estado de Puebla (UPAEP) in early 2019. For the assay, an agarose gel matrix was prepared using three specified buffers, following the methodology described by Hagerman (1987). An analytical grade standard protein (bovine serum albumin) (fatty acid-free fraction V) was dissolved in the gel. The gel solution was dispensed into Petri dishes in 9.5 mL aliquots and allowed to cool.

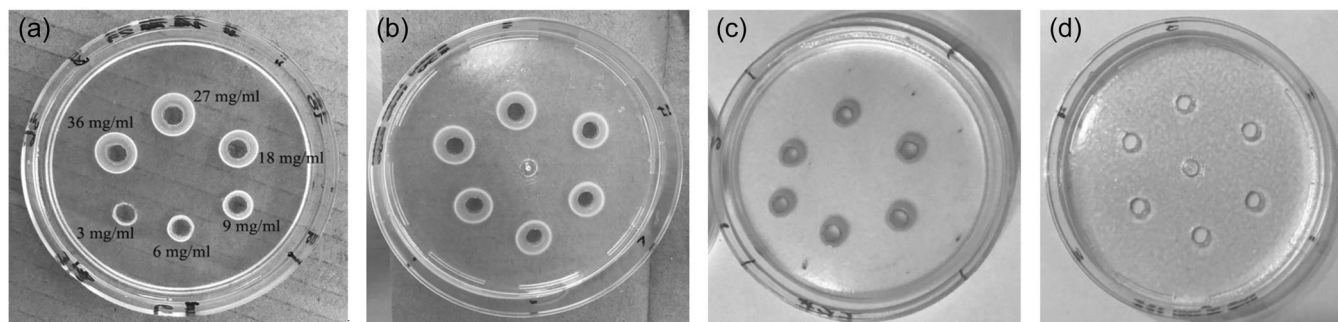
Tannins were extracted from the dried and ground plant samples with 70% acetone. Volumes of these extracts (36  $\mu$ L) were then added to premade wells in the gel. The Petri dishes were covered and sealed with Parafilm and incubated at room temperature (20–30°C) from 96 to 120 h. Each plant sample was analyzed six times, and the diameters of the rings were measured at three different angles using a digital vernier caliper to minimize errors, given that the rings were not uniform. Tannin concentration was calculated from the square of the average of the diameters using a calibration curve (Supporting Information S1: Table 3).

### 2.5 | Calibration

We dissolved 250 mg of tannic acid in 25 mL of acetone (70%) to determine the calibration curve, although inexact in composition, tannic acid represents a mixture of phenolic compounds and water-soluble tannins (Lucas et al., 2001). Following this, 2, 3, 9, 18, 27, and 36  $\mu$ L of this solution were added to different wells of the gels (Figure 1a). The calibration curve was repeated three times, and the diameter of the rings was measured at three different angles using a digital vernier caliper. Results are expressed as equivalents to the 6-point standard curve (Supporting Information S1: Table 4).

### 2.6 | Data analysis

We conducted a normality test on the residuals of the study variables using the Shapiro–Wilk normality test. To assess the effect of TP, CT, and protein-precipitating tannins on DM intake, we used the regression coefficient (or estimation) of generalized linear models (GLM) with a Gaussian link function. Statistical analyses were performed using the R 3.4.0 software (R Core Team, 2019) with the GLM function of the R Performance Analytics package (Peterson &



**FIGURE 1** (a) The radial diffusion assay (RDA) results were used to determine the ability of tannins to precipitate protein with different concentrations of tannic acid. These results were used to generate a standard curve. (b) The result of the RDA was used to determine the precipitating protein capacity of the tannins present in extracts of plants consumed by *Alouatta pigra*. The image shows the precipitation ring in response to the injection of 36  $\mu$ L of extract from the mature leaf (ML) of *Haematoxylum campechianum*. (c) Result of the RDA to determine the precipitating protein capacity of the tannins present in extracts of plants consumed by *A. pigra*. The image shows the precipitation ring generated by injecting 36  $\mu$ L from the young leaf (YL) of *Inga edulis*. (d) Result of the RDA to determine the precipitating protein capacity of tannins present in extracts of plants consumed by *A. pigra*. The image shows an example of plant extracts that did not develop a precipitation ring. These were evaluated with no detectable ability to precipitate proteins. In the test, 36  $\mu$ L extract from the young leaf (YL) of *Acaciella angustissima* was injected.

Carl, 2019) for linear mixed effects models. A significance level of 0.05 was applied for all analyses.

### 3 | RESULTS

#### 3.1 | DM intake

A total of 240 h of behavioral observations were recorded for black howlers (80 h for each study group). The diet of the howlers consisted of 49.47% MF, 26.69% YL, and 23.84% ML based on feeding time. The monkeys consumed 14 plant species corresponding to nine families (Supporting Information S1: Table 5). From a single representative of each species and plant part consumed, we obtained 18 extracts of YL, ML, and MF for analysis (Table 1). The average daily food consumption per focal animal per group was  $124.66 \pm SD 97.03$  g (32.27–393.82 g). The most consumed plant species/part over the 8-day period were the MF of *Spondias mombin* (1583.72 g), followed by the MF of *Manclura tinctoria* (270.60 g), and the YL of *Coccoloba spicata* (252.16 g) (Table 1). Overall, based on DM intake (g/DM), the diet of the howlers consisted of 63.21% MF, 23.55% YL, and 13.23% ML.

#### 3.2 | The concentration of TP and CT

During the sampling period, the monkeys consumed an average of  $12.88 \pm SD 7.54$  g gallic acid equivalent/100 g DM (TP) and  $33.76 \pm SD 11.38$  g catechin equivalent/100 g DM (CT). The average DC of TP was  $1.61 \pm SD 2.78$  g gallic acid equivalent/100 g DM and  $4.22 \pm SD 4.71$  g catechin equivalent/100 g DM of CT ( $n = 24$ ). The highest TP and CT intake was from the YL of *C. spicata*, totaling 19.04 g gallic acid equivalent/100 g DM for TP and 37.73 g catechin

equivalent/100 g DM for CT over the 8-day period. Among the food plants consumed the YL of *Inga edulis* had the highest percentage of TP (4.10%) and CT (17.86%) based on the Folin-Ciocalteu analysis and the vanillin analysis, respectively (Table 1). These leaves accounted for 35.88 g of DM consumed over the 8-day period, which was below the mean DM intake per 8 days.

#### 3.3 | Daily variation in the intake of TP and CT

The daily dietary intake of TP and CT varied significantly. TP consumption ranged from 13.80 to 0.09 g gallic acid equivalent/100 g DM per day, while CT consumption ranged from 17.74 to 0.13 g catechin equivalent/100 g DM per day. Overall, the highest TP intake (average  $5.44 \pm SD 7.28$  g gallic acid equivalent/100 g DM) occurred on Day 6 of the 8-day sampling period. While the lowest intake ( $0.56 \pm SD 0.30$  g gallic acid equivalent/100 g DM) was observed on Day 4. This suggests that black howlers consume higher amounts of TP after several days of low intake (more than double that consumed on previous days).

The pattern of CT consumption exhibited a slightly different trend. The highest average CT intake ( $10.88 \pm SD 5.95$  g catechin equivalent/100 g DM) was recorded on the first day and on the 7th day of sampling ( $8.43 \pm SD 6.50$  g catechin equivalent/100 g DM), while the lowest average daily intake ( $1.42 \pm SD 0.94$  g catechin equivalent/100 g DM) was on the 8th day. Generally, the monkeys consumed 1.35–5.44 g catechin equivalent/100 g DM of CT per day for 1–2 days and then reduced their consumption to 0.56–0.61 g catechin equivalent/100 g DM the next 1–2 days. This pattern suggests a dietary strategy aimed at exploiting a diverse range of plant parts/species with varying nutrient characteristics.

In general, the black howler groups consumed plant foods daily, with varying concentrations of TP and CT. In two out of the three study

**TABLE 1** Mean ( $\pm$ SD) daily consumption of total phenols, condensed tannins, and their protein precipitation capacity in the plant species and plant parts consumed by each study group of *Alouatta pigra*.

Group	Plant species	Plant part	DM (g/DM)	TP (%DM gallic acid equivalent)	TP (g gallic acid equivalent/100 g DM)	CT (%DM catechin equivalent)	CT (g catechin equivalent/100 g DM)	PPC (mg tannic acid equivalent/g DM)
1	<i>Maclura tinctoria</i>	MF	33.82 $\pm$ 17.50	0.96	0.33 $\pm$ 0.17	0.75	0.26 $\pm$ 0.13	ND
		YL	0.09 $\pm$ 0.25	1.19	0.001 $\pm$ 0.003	0.83	0.001 $\pm$ 0.002	ND
	<i>Pithecellobium lanceolatum</i>	YL	5.39 $\pm$ 4.05	0.97	0.05 $\pm$ 0.04	1.98	0.11 $\pm$ 0.08	ND
		ML	7.07 $\pm$ 8.85	1.41	0.10 $\pm$ 0.12	4.05	0.29 $\pm$ 0.36	ND
	<i>Psidium guajaba</i>	MF	1.32 $\pm$ 3.72	1.44	0.02 $\pm$ 0.05	5.48	0.07 $\pm$ 0.20	ND
	<i>Inga edulis</i>	YL	4.48 $\pm$ 5.66	4.10	0.18 $\pm$ 0.23	17.86	0.80 $\pm$ 1.01	71.15
		MF	14.03 $\pm$ 19.61	3.11	0.44 $\pm$ 0.61	17.70	2.48 $\pm$ 3.47	56.66
	<i>Enterolobium cyclocarpum</i>	YL	4.55 $\pm$ 7.35	0.71	0.03 $\pm$ 0.05	2.13	0.10 $\pm$ 0.16	ND
	<i>Ficus</i> sp.	MF	6.60 $\pm$ 10.41	0.73	0.05 $\pm$ 0.08	4.16	0.27 $\pm$ 0.43	ND
2	<i>Guazuma ulmifolia</i>	ML	16.43 $\pm$ 21.86	0.62	0.10 $\pm$ 0.13	1.19	0.20 $\pm$ 0.26	ND
	<i>Maclura tinctoria</i>	YL	12.16 $\pm$ 10.81	1.18	0.14 $\pm$ 0.13	1.04	0.13 $\pm$ 0.11	ND
	<i>Haematoxylum campechianum</i>	ML	12.65 $\pm$ 10.08	ND	ND	4.60	0.58 $\pm$ 0.46	205.88
	<i>Lonchocarpus guatemalensis</i>	ML	0.30 $\pm$ 0.84	3.46	0.01 $\pm$ 0.03	6.28	0.02 $\pm$ 0.05	61.95
	<i>Acaciella angustissima</i>	YL	6.29 $\pm$ 9.66	0.77	0.05 $\pm$ 0.07	0.46	0.03 $\pm$ 0.04	ND
	<i>Coccoloba spicata</i>	YL	31.52 $\pm$ 46.05	3.33	2.38 $\pm$ 4.76	15.02	4.72 $\pm$ 6.89	51.90
	<i>Tabebuia rosea</i>	ML	13.79 $\pm$ 13.20	1.12	0.15 $\pm$ 0.15	0.36	0.05 $\pm$ 0.05	ND
	<i>Pithecellobium lanceolatum</i>	YL	4.93 $\pm$ 5.58	0.97	0.05 $\pm$ 0.05	2.25	0.11 $\pm$ 0.13	ND
3	<i>Tabebuia rosea</i>	ML	2.89 $\pm$ 4.36	1.45	0.04 $\pm$ 0.06	2.96	0.09 $\pm$ 0.13	ND
	<i>Guazuma ulmifolia</i>	YL	11.39 $\pm$ 10.76	2.13	0.24 $\pm$ 0.23	8.16	0.93 $\pm$ 0.88	30.61
	<i>Albizia leucocalyx</i>	YL	0.57 $\pm$ 0.96	1.55	0.01 $\pm$ 0.01	2.63	0.02 $\pm$ 0.03	ND
	<i>Enterolobium cyclocarpum</i>	YL	0.88 $\pm$ 2.48	3.35	0.03 $\pm$ 0.08	2.63	0.02 $\pm$ 0.07	ND
	<i>Coccoloba spicata</i>	YL	8.32 $\pm$ 15.97	3.33	0.28 $\pm$ 0.53	15.02	1.25 $\pm$ 2.40	ND
	<i>Acaciella angustissima</i>	YL	3.96 $\pm$ 4.99	0.77	0.01 $\pm$ 0.01	0.46	0.02 $\pm$ 0.03	ND
	<i>Spondias mombin</i>	MF	197.96 $\pm$ 96	1.70	0.04 $\pm$ 0.06	1.74	0.09 $\pm$ 0.13	ND

Note: Samples in which total phenols, condensed tannins, and protein precipitation capacity were not recorded are shown as not detected (ND).

Abbreviations: CT, condensed tannins; DM, dry matter; MF, mature fruits; ML, mature leaves; ND, not detected; PPC, protein precipitation capacity; TP, total phenols; YL, young leaves.

groups, at least one plant species/part consumed each day contained tannins with PP capacity. Interestingly, on days when black howlers consumed the highest amounts of foods with the greatest amount of CT, they also consumed foods with the greatest amount of tannins capable of precipitating protein. For example, on days in which group members had the highest consumption of the YL of *C. spicata* (17.11 g), they also increased their consumption of the MF of *I. edulis* (6.33 g), which tested positive for the presence of tannins with PP capacity (Table 1).

### 3.4 | PP capacity

The RDA results indicated that only six plant parts from five of the 14 tree species sampled formed a tannin-protein ring (Table 1). ML of *Haematoxylum campechianum* had the highest PP capacity with 205.88 mg tannic acid equivalent/g DM. YL of *I. edulis* ranked second with 71.15 mg tannic acid equivalent/g DM, followed by the ML of *Lonchocarpus guatemalensis* with 61.95 mg tannic acid equivalent/g

DM (Table 1). Examples of precipitation rings formations can be seen in Figure 1b,c, while Figure 1d shows a negative test result.

Despite having a high PP capacity, certain plant species were consumed almost daily by the howlers. For example, the ML of *H. campechianum* had the highest PP capacity, and despite this, it was consumed in small quantities (0.06–1.15 g) during all days. Similarly, YL of *Guazuma ulmifolia* had a PP capacity of 30.61 mg tannic acid equivalent/g DM and was consumed in small amounts (0.08–2.24 g) during 7 out of the 8 consecutive days of the study period.

### 3.5 | Relationship between DM consumption and the concentration of TP, CT, and tannins with the capacity to precipitate protein

In general, the howler monkeys consumed higher quantities (g/DM) of plant species/parts with lower TP and CT concentrations (Table 1). The exceptions were the YL and MF of *I. edulis* and the YL of *C. spicata*. However, there were no statistically significant relationships between DM consumption per species/plant part and TP content ( $p = 0.735$ ), CT ( $p = 0.572$ ), or DM consumption of tannins with the ability to precipitate proteins ( $p = 0.703$ ). Similarly, there were no statistically significant relationships between TP and CT ( $p = 0.921$ ), TP and tannins with the ability to precipitate protein ( $p = 0.897$ ), CT and tannins with the ability to precipitate protein ( $p = 0.617$ ), or between the three variables ( $p = 0.756$ ). These findings suggest that the presence and concentration of TP and CT in foods consumed by black howler monkeys did not play a primary role in food choice.

## 4 | DISCUSSION

This study explored the influence of phenolic PSMs on food consumption in wild black howler monkeys. The black howlers, belonging to the genus *Alouatta*, have an expanded hindgut compared to their close relatives, the spider monkeys (*Ateles*), woolly monkeys (*Lagothrix*), and muriquis (*Brachyteles*) (Amato & Garber, 2014; Chivers & Hladik, 1980). The presence of phenolic-degrading bacteria in their gut microbiome may enable these primates to tolerate foods that contain higher concentrations of tannins (Mallott et al., 2022). Also, the acidic pH of their stomach, which is typically <4.5, may limit tannins' protein-precipitating capacity (Milton & McBee, 1983; Osborne & McNeill, 2001).

Based on data collected on three black howler groups, each studied over a consecutive 8-day period, we tested three hypotheses. Our results indicated that H1 was not supported, as there was no direct relationship between TP and CT concentrations (%DM) and the amount of consumed resources. H2 was also not supported, as five of the 14 plant species consumed by black howlers precipitated tannin-protein rings, and the PP capacity of tannins was not negatively correlated with the amount of plant tissue consumed (g/DM). However, H3 received support, as black howlers exhibited a dietary pattern of reducing CT and TP consumption in the days following increased intake.

These findings contradict previous studies on howler monkeys, which suggested a negative impact of CT and alkaloids on food choice (Glander, 1981, 1982; Milton, 1981; Welker, 2004). The role of tannins and phenols in primate food selection is still a subject of debate, with current evidence not conclusively supporting the idea that primates, in general, based their diets on PSMs (Windley et al., 2022). Some primate species choose food plants with high PSM levels, and tannins, due to their protein-binding ability, are implicated in aiding self-medication (De la Fuente et al., 2022; Huffman, 2003; Morrogh-Bernard et al., 2017), exhibiting antiparasitic effects (Rothman, Pell, et al., 2009), and promoting body weight and milk secretion (Carrai et al., 2003).

The monkeys in our study consumed high amounts of DM from plant species that contained tannins with PP capacity. For example, *H. campechianum* (205.88 mg of tannic acid equivalent/g DM), and *I. edulis* (56.66 mg of tannic acid equivalent/g DM). Moreover, every species/plant part ingested by the black howlers was found to contain CT. In the case of TP, *H. campechianum* was the only species with no detectable levels of this PSM. Some primate species tolerate high amounts of CT (Carrai et al., 2003; Chapman & Chapman, 2002; Struhsaker et al., 1997), while others select plant parts with lower levels of this compound (Ganzhorn, 1988, 1989; Thureau et al., 2021). For example, *Callithrix jacchus* and *Callimico goeldii* reject fruits when the tannic acid content reaches 4% of the fructose content (0.4 g/L: Simmen, 1994), *Microcebus murinus* has an inhibition threshold corresponding to 0.54 g/L of tannic acid (Simmen et al., 1999), and chimpanzees (*Pan troglodytes*) can consume immature fruits containing up to 5% CT (12 g/L; Wrangham & Waterman, 1983).

It is noteworthy that certain plants consumed by the monkeys exhibited higher concentrations of CT than TP, with the mentioned case of *H. campechianum* having no detectable levels of TP and a CT concentration of  $0.58 \pm 0.46$  g catechin equivalent/100 g DM. Biologically, this scenario is impossible since CT is just one category within TP. This issue is primarily due to the heterogeneity in the chemical structure of CT and the use of a single standard to estimate the quantity of this PSM in solutions containing unknown CT structures (Schofield et al., 2001). The commercial standards used in PSM analyses can contain different concentrations of the metabolite of interest. For example, quebracho, a standard commonly used in the acid butanol assay for CT, contains between 50% and 80% CT (Asquith & Butler, 1985; Robbins et al., 1991). Moreover, these standards may contain non-CT impurities and react differently than the CT from the plant species of interest (Rothman, Dusingberre, et al., 2009). The use of commercial standards in the study could have over- or underestimated the concentrations of these PSM, which is why the obtained values were not taken as absolute but as equivalents of the metabolite of interest.

Primate species show varying degrees of resistance to phenolic PSMs due to specific adaptations (Beaune et al., 2017; Remis et al., 2001). In black howler monkeys, salivary tannin-binding proteins are the first line of defense against the anti-nutritional effects of tannins, precipitating them and protecting valuable dietary proteins (Bennick, 2002; Espinosa-Gómez et al., 2018). The next line

of defense is the gut microbiome. Studies by Amato et al. (2015) indicate that the presence of bacteria such as *Papillibacter*, *Prevotella*, and *Butyrivibrio* in the gut of black howlers is likely to play an essential role in detoxification. The liver and kidneys serve as the third line of defense (McArthur et al., 1993). In species such as prairie voles (*Microtus ochrogaster*) (Lindroth & Batzli, 1983) and the snowshoe hare (*Lepus americanus*) (Bryant et al., 1985), enzyme systems present in the liver and kidneys eliminate phenolic PSMs by binding with glucuronic acid (Freeland & Janzen, 1974).

To avoid the anti-nutritional effects of phenolic PSMs, animals also may selectively bite plant parts with lower phenolic levels or consume alternative individuals of the same or different species in the same area (Bailey et al., 1996). For example, herbivorous mammals like koalas (*Phascolarctos cinereus*; Moore & Foley, 2005) and red deer (*Cervus elephus*; Duncan et al., 1994) exhibit individual tree preference over stand-level preference, spending less time foraging and eating from trees (of the same species) with higher phenolic PSMs concentrations. The variation in PSMs among individual trees and plant parts may have caused the missing of some of the effects of phenolic PSMs in our study.

One important finding of our research is that black howler monkeys exhibited a dietary pattern characterized by increased consumption of CT and TP, followed by a period of limited intake. These foraging strategies are likely influenced by phenolic PSMs pre- and post-ingestive mechanisms. Pre-ingestive mechanisms act quickly (e.g., via trigeminal nerve stimulation), while post-ingestive mechanisms (e.g., tannin PP) have a delayed effect on foraging behavior (Iason, 2005). Similar deterrent effects of phenolics have been observed in primate species like *Lemur catta* and *Propithecus verreauxi verreauxi* (Yamashita, 2008). Also, there is evidence that after periods of high phenolic PSMs consumption, animals go through periods of low intake to allow plasma phenolic levels to decrease below a threshold level (Pfister et al., 1997; Stapley et al., 2000).

It is important to note that the diet of herbivores is influenced by both nutrient and PSMs content of available foods (Villalba et al., 2002). Protein and soluble carbohydrates have been found to play an important role in food selection by howler monkeys (Righini et al., 2017). Our findings indicate that based on DM, the MF of *S. mombin* and *M. tinctoria* (Table 1) were consumed most by the howlers. These fruits provide easily digestible soluble carbohydrates and lipids and are low in undigestible fiber (Righini et al., 2017). The abundance and distribution of these fruits likely influenced their consumption during the study period (October, rainy season), and they represent a readily available high-energy resource for black howlers (Dunn et al., 2010; Palma et al., 2011).

All MF consumed in our study contained TP and CT (%DM measured as gallic acid and catechin equivalents, respectively). Furthermore, tannins have been reported to play a role in mitigating the risk of acidosis by slowing down the fermentation process (Davies et al., 1988; Goltenboth, 1976). Fruits have a rapid rate of fermentation which may cause hyperacidity of the forestomach fluid (Goltenboth, 1976). A study by Kool (1992) found that fruits consumed by silver leaf monkeys (*Trachypithecus auratus sondaicus*)

had higher concentrations of CT and TP (and their PP capacity) compared to fruits that were not eaten. A similar case reported by Norconk and Conklin-Brittain (2004) suggested that white-faced sakis (*Pithecia pithecia*) might be willing to ingest fibrous or astringent food items if they are also rich in lipids. Bai Hokou gorillas (*Gorilla gorilla gorilla*) consumed fruits with high CT levels when the iron content exceeded 100 mg/kg (Remis et al., 2001). Tannins may assist in regulating iron metabolisms by binding to excess dietary iron (Roy & Mukherjee, 1979).

It is brought into notice that the MF of *Ficus* sp., YL of *I. edulis*, YL and ML of *G. ulmifolia*, ML of *Tabebuia rosea*, MF of *Psidium guajava*, MF of *Maclura tinctoria*, YL of *Enterolobium cyclocarpum*, and YL of *Phitecellobium lanceolatum* had been reported as food plants of black howlers, which aligns with the findings of this study (Aristizabal et al., 2017; Righini et al., 2017; Silver et al., 1998).

In conclusion, tannins in the black howler monkey diet play a complex role beyond mere dietary choice, involving adaptive mechanisms, ecological context, and potential physiological benefits. To comprehend the ecological role of tannins, a deeper understanding is necessary. This involves examining the nutritional and anti-nutritional effects of PSMs consumed by a wide range of animal species. Additionally, gaining insights into the daily variation of phenolic PSM consumption by wild black howlers contributes to a more detailed understanding of how these compounds influence their dietary patterns over time.

However, the analysis of a heterogeneous group of molecules like tannins poses unique challenges. Careful considerations are crucial when selecting assays to accurately address research questions, as each method exhibits specific selectivity and sensitivity to different types of phenolics or alkaloids (Scalbert, 1992). This comprehensive approach not only enhances our understanding of tannins in primate diets but also underscores the necessity for meticulous methodology in future studies within this field.

## AUTHOR CONTRIBUTIONS

**María Fernanda Alvarez-Velazquez:** Data curation (equal); formal analysis (equal); investigation (equal); validation (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **Fabiola Carolina Espinosa-Gómez:** Conceptualization (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); writing—review and editing (equal). **John F. Aristizabal:** Data curation (equal); methodology (equal); writing—review and editing (equal). **Paul A. Garber:** Supervision (equal); writing—original draft (equal). **Juan Carlos Serio-Silva:** Investigation (equal); methodology (equal); supervision (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal).

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#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT


All data used in this study are either presented in the supplemental materials or are available upon reasonable request.

#### ETHICS STATEMENT

This research adhered to the American Society of Primatologists Principles for the Ethical Treatment of Primates and to the laws governing primate research in Mexico.

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