

Variation of caffeine and related alkaloids in *Ilex vomitoria* Ait. (Yaupon holly): A model of intraspecific alkaloid variation

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Abstract: *Ilex vomitoria* Ait. (Aquifoliaceae), yaupon holly, is a North American evergreen shrub from which indigenous groups of the southeastern United States produced a stimulant and emetic leaf tea known as *cassina* or black drink to Europeans. Being the only North American plant species to contain caffeine, *I. vomitoria* was a valuable resource competitive with tea and coffee during the colonial period and now may be regaining popularity. Since considerable intraspecific variation in plant alkaloid production is known to occur, as in sanguinarine in *Sanguinaria canadensis* L. and piperidine alkaloids in *Pinus ponderosa* Dougl. ex Loud, I have explored phytochemical variation within individuals of *I. vomitoria* as it relates to tissue type and tissue age. Further analysis will determine the relative influence of biotic and abiotic environmental factors on alkaloid variation. To this end, *I. vomitoria* will be used as a model for investigating the relationship between alkaloid levels and ecogeographic variables such as light conditions, soil nutrients, and geographic location.

I. Introduction

Alkaloids are a class of phytochemicals with undeniable value to humans as medicines, hallucinogens, stimulants, and precursors for synthesis reactions. Since useful plants and their chemical constituents are part of a broad framework of ecological and evolutionary processes, a host of internal and ecogeographical variables affect the amount and type of phytochemicals that plants produce (1, 2, 3). Though researchers have examined in detail the biosynthesis of alkaloids, their role in mediating plant-insect interactions, and their utility to humans (4), alkaloid variation remains less well understood.

II. Research questions and hypotheses

- How do alkaloids vary within a species?
- How do alkaloids of one species vary within individuals of that species?
- Hypothesis: Alkaloids are concentrated in some tissue types, while absent or present in small amounts in others.
- Hypothesis: New leaves have a higher alkaloid concentration than mature leaves.

III. Model species: *Ilex vomitoria* Ait. (Aquifoliaceae)

- North American evergreen shrub with elliptical, crenate leaves
- Grows in moist, well-drained soils of coastal regions and hammocks of southern mixed hardwood forests (5)
- *Ilex vomitoria* (yaupon holly) is a suitable model for this investigation for three reasons:
 1. *I. vomitoria* synthesizes the xanthine alkaloids caffeine and theobromine, making it one of over sixty higher plants that produce purine alkaloids (6) and the only caffeine producing plant native to North America (5).
 2. The range of *I. vomitoria* extends south from Virginia to central Florida and west to Texas, making it a convenient model for testing the above and other related hypotheses due to its proximity to Miami, FL.
 3. Like its congeners *I. paraguariensis* St. Hil. (yerba maté) and *I. guayusa* Loes. (guayusa) (7) of South America, *I. vomitoria* is an example of a plant involved in the broad framework of human interactions with plant chemicals. Indigenous groups of the southeastern United States utilized yaupon in the production of *cassina* or black drink, a caffeinated leaf tea (8). Prepared from the young leaves and shoots of *I. vomitoria* (8, 9), *cassina* was taken during ceremonial events as an emetic and a stimulant, the latter property owed to caffeine's direct stimulation of the central nervous system (10).



Figure 1: Distribution of *Ilex vomitoria* – reprinted from (11)



Figure 2: *Cassina* ceremony – reprinted from (8). Indigenous groups of the southeastern U.S. made a stimulant and emetic leaf tea known as *cassina* from the young leaves and shoots of *I. vomitoria*. The tea was consumed in ritual, ceremonial, and festival contexts.

IV. Materials and methodology

Plant material: *Ilex vomitoria* cultivars were obtained locally in Miami, FL and grown in the greenhouse at FIU.

Standards: Caffeine, theobromine, and theophylline standards were obtained from Sigma Chemical Company (St. Louis, MO, USA). 50mg of each standard were dissolved in 50ml of a water:methanol (7:3) solvent in a 50ml Erlenmeyer flask placed in a boiling water bath for 5 minutes.

Extraction: 2.5g of fresh plant material (leaves, stems, or roots) were soaked for 24 hours in 25ml of a water:methanol solvent (1:1). After 24 hours, 500µl aliquots were taken and washed twice with 500µl of chloroform. After each wash, the organic chloroform layer was removed from the sample vial and placed in a 10ml test tube. The two chloroform washes for each sample were combined and dried under nitrogen. The dried sample was then suspended in 500µl of the water:methanol solvent (1:1). 10µl aliquots of each sample were then utilized in chromatographic analyses.

Liquid chromatography: A reversed phase system was used with a Hewlett-Packard 1090 Liquid Chromatograph system (ternary pump, diode array detector, autosampler), with instrument control via an HP Kayak XA computer running HP Chemstation software Rev. A.06.03 (Waldborn, Germany). The separation method utilized a Hewlett-Packard ODS Hypersil column (100x2.1mm). Optimal separation was achieved with methanol and water (pH 3.5) using a phosphoric acid buffer. The gradient consisted of 0-40% methanol over 4 min and 40-10% over the next 4 min, completing the run with 100% water for 2 min at a flow rate of 1ml/min and a column temperature of 40°C. Total run time was 10 min per sample. Detection was set at 271 nm. Standards were run both separately and mixed to determine retention times and generate calibration curves. Peak identity was verified by retention time, peak purity analysis, and LC-MS.

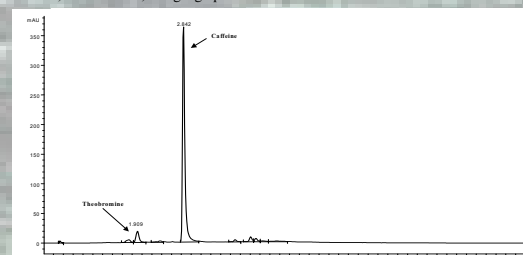


Figure 3: HPLC chromatogram from an extract of leaves of *I. vomitoria*. The caffeine and theobromine peaks are labeled.

Table 1: Standard retention times

Alkaloid	Retention time
Caffeine	2.896
Theobromine	1.910
Theophylline	2.282

V. Results

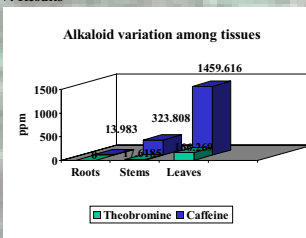


Fig. 6. Root, stem, and leaf tissue from two individuals of *I. vomitoria* were analyzed as described in methods.

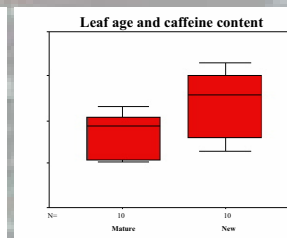


Fig. 7. Box plots represent sample....

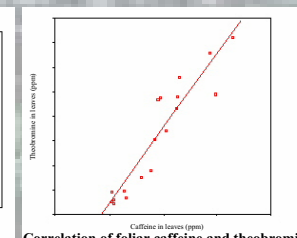


Fig. 8. Graph of correlation between caffeine (ppm) and theobromine (ppm) in foliage of individuals of *I. vomitoria* (R = ...)

- Caffeine concentration in leaves of *I. vomitoria* ranged from 0.0038 to 0.2288 % fresh weight
- Theobromine concentration in leaves of *I. vomitoria* ranged from 0.0009 to 0.0300 % fresh weight

VI. Conclusions

- Notable variation in alkaloid concentration exists within individuals of *I. vomitoria*. Within the the foliage, caffeine concentration is different between new and mature leaves (two-sample test of equality, $t = \text{????}$, $df = \text{????}$, $P = 0.036$), with new leaves having a higher caffeine concentration (Fig. 7). The limited supply of defensive compounds in a plant dictates that they be concentrated in regions of a plant where their presence would most increase the plant's fitness (12). For vegetative tissue such as leaves, the need for protection is greater in young tissue than in old since young tissues are both "valuable" due to their high nutrient concentration and photosynthetic potential and "vulnerable" due to a general lack of protective mechanisms. The higher caffeine concentration in new leaves also provides a phytochemical basis for explaining the use of young leaf and shoot material by the indigenous groups in *cassina* preparation. Younger leaves mean more caffeine, which makes a stronger stimulant.
- Another source of variation in alkaloid concentration within individuals is the difference among tissue types (Fig. 6). Leaves have the highest concentration of both caffeine and theobromine in *I. vomitoria*, while stems contain more of these alkaloids than roots. Only theobromine was detected in root tissue.
- Theobromine and caffeine concentration are correlated in individuals of *I. vomitoria* (bivariate correlation, $R = \text{????}$, $df = \text{????}$, $P < .001$, Fig. 8). Theobromine is a precursor of caffeine on the biosynthetic pathway of these alkaloids, which could be part of the explanation of the observed correlation.

VII. Future directions

The research I have presented is the first part of a three-tiered approach to studying alkaloid variation in a model species. The second part of my proposed research involves quantifying variation in purine alkaloids in *I. vomitoria* at the population level. The third and final part of my proposed research will involve quantifying alkaloid variation in *I. vomitoria* across its range. This third portion of my research will involve both identifying ecogeographical variables such as sunlight, soil characteristics, and geographical location that correlate with alkaloid expression in *I. vomitoria* and determining the roles each play in such variation.

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Acknowledgments: I gratefully acknowledge input and reviews from Dr. Bradley C. Bennett, Dr. Kelsey R. Downum, Dr. Martin Quirke, Dr. Suzanne Koptur, Dr. Martin Quirke, and Dr. James Graham. This research was funded by the Center for Ethnobiology and Natural Products (CENaP) at FIU.