Camu *Myrciaria dubia* Report

June 22, 2023

Project Objective

Identity verification of *Myrciaria dubia* samples using two methods, including

1) DNA test-Polymerase Chain Reaction (PCR)
2) Nuclear Magnetic Resonance (NMR)

Project Outline

I. DNA ID
- DNA extraction
- DNA archival in -80° Celsius freezer
- DNA Sybr Green PCR validated test method using specific markers

II. NMR fingerprinting
- Sample preparation
- Methanol extraction for NMR
- Spectra acquisition for NMR
- Data processing and Analysis for NMR
- Elucidation of bioactive compounds from NMR spectra using reference spectra

III. Report on the identity of the source material using DNA and NMR-based methods. This will include methods, analysis, and the interpretation of the results.

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Project Report

Sample information

Sample Number	BRM Number	Target species
PIQ05092023-1401	BRM1507	*Myrciaria dubia*

Methods

DNA Purification and Quantification

Genomic DNA from the samples were extracted using the Nucleospin Plant II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) to get high-quality DNA. Extractions were performed using 100 mg of each sample according to the manufacturer's instructions. DNA quantification for both targets and non-targets was performed using the Qubit™ 3.0 Fluorometer (Invitrogen, Carlsbad, CA).

End Point PCR Amplification

The PCR was performed under standard conditions for the primer pairs: species-specific mini marker, *trnH-psbA*, and *ITS2* as described in Newmaster *et al.*, 2013.

DNA Sequencing

Three genomic markers were sequenced, including chloroplast and nuclear regions of the plant genome: trnh-psbA, ITS and a species-specific mini nuclear marker. Chromatographic traces and contiguous alignments of the sequences obtained after sequencing were edited using the DNASTAR offline software (http://www.dnastar.com/). The sequences were then aligned using Clustal W (Thompson, Higgins, & Gibson, 1994). The genetic distances were calculated using the Kimura2Parameter (K2P) model in Mega5 (Tamura *et al.*, 2011).

NMR Metabolite Fingerprinting Reference Library

The Biological Reference Material (BRM) for *Myrciaria dubia* included eight validated samples with NMR metabolite spectra in the NHPRA reference library. All BRM reference materials are archived at the NHPRA, University of Guelph, Canada.

Sample preparation

The standard sample preparation for NMR processing requires 300 mg of homogenized tissues and dissolved in 2 ml of deuterated methanol (CD_3OD). The solvent was chosen for its greater solubility towards diverse chemical compounds. Samples were incubated in the sonicating bath for 30 min at room temperature. Sonicated samples were centrifuged for 15 min at 6000 rpm, and then 650 µL of clear supernatant was collected in an NMR tube.

Spectra Acquisition for NMR:

To analyze the chemical fingerprints of the samples, ^{1}H-NMR spectra were acquired using 400 MHz Bruker Avance III NMR equipped with a 5 mm "BBI" room temperature probe. To acquire data, we used Bruker pulse program "noesygpps1d" and the acquisition mode "DQD" including the following parameters as follows: number of complex points, 32768; dummy scans, 4; number of scans, 64; acquisition time, 3.98 sec; delay time, 15.0 sec; spectral width, 8223 Hz; fid resolution, 0.25 Hz.

Data Processing and Analysis for NMR:
^{1}H-NMR spectra were processed using TopSpin 4.0.7. Phase and baseline were carefully checked and corrected. Spectra were calibrated to internal standard TMS at 0.0 ppm. Processed spectra were bucketed with simple rectangular buckets of positive intensities without scaling (AMIX 4.0.1). The chemical range utilized for bucketing was -1 to 12 ppm, with a width of 0.01 ppm. While bucketing, the residual solvent signals of water, methanol, and TMS were removed at the regions 4.75-5.06, 3.16-3.45 and -0.05 to 0.05 ppm, respectively. After bucketing, each spectrum was normalized by setting the below means as 0, and the above means were binned from 1 to 100 (Martinez-Farina *et al.*, 2019).

Elucidation of bioactive compounds from NMR spectra using reference spectra:
The structural elucidation of bioactive compounds in the NHPs is done using 1D NOESY and 2D COSY/TOCSY experiments. The library of reference bioactive compounds at NHPRA are assigned individually and spiked with the NHPs. The chemical shift problems are rectified using spiking studies.

Results and discussion
DNA analysis: DNA from the test samples was amplified with mini-DNA barcodes. All samples were verified with 100% matched to the reference library using positive and negative controls.

Sample ID: PIQ05092023-1401
Lot number: CC319P
Species name: *Myrciaria dubia*

DNA sequences:
>*Myrciaria-dubia*_564p_specific-marker
AGGAGCAATAACCAACACTCTTGATAGAATAAGAAGTTGGTTATTGTTCCTTTATTTAGTGCTCTTTTCT
TTACATAAGTGTTTCTTTTCTTCAACATAAGAAAAGGTATTCGAGTATTTAGGGATTGTTTTATGATTGC
GTATCATACTTTAGATATGAATTTCGAATTTATATACATTCTTTTCAACCCTTTGTAAGTCTTTGTGAGA

>*Myrciaria-dubia*_ITS2
TGCAGAATCCCGTGAACCATTGAGTCTTTGAACGCAAGTTGCGCCCGAGTCCATTTGGACGAGGGCACG
TTTGCCTGGGTGTCACACACGGCGTTGCCCCTAATCCCACGCCTTGAATCGGGCGCGCGAGACTCGGGT
GCGTATGTTGGCCTCCCGTGACGACTTTCGTCCCGGTTGGCCCAAAATCGAGCGCTGGAGCGATCAGCA
CCACGACATTCGGTGGTTGATGAGACCCCAATGATCAATGTCATGCGCGTCGCTCGTGCAAGTGCTCCA
TGAATCTACTCTTTACCAACGCGACCCCAGGTCAAGCGGGGCTACCCGCTGAGTTTAA

>*Myrciaria-dubia*_trnH-psbA
ATAACTTCCCTCTAGATCTAGCTGCTGTCGAAGCTCCATCTACAAATGGATAAGACTTTGGTCTTAGTAT
AAGTGTAGTTGAGTTTTTGATTGCAAAATAAAGGAGCAATAACCAACACTCTTGATAGAATAAGAAGTT
GGTTATTGTTCCTTTATTTAGTGCTCTTTTCTTTACATAAGTGTTTCTTTTCTTCAACATAAGAAAAGGTA
TTCGAGTATTTAGGGATTGTTTTATGATTGCGTATCATACTTTAGATATGAATTTCGAATTTATATACATT
CTTTTCAACCCTTTGTAAGTCTTTGTGAGATTATTATCTTCTAGTTTTTTTTTTCGAACAAAATACAAAGG
TTAGCATTTTCCGCTTCTTCTATCTCATAAGTAAGGTAAAAATGTTAAAAATTAACAATCGAAATGAAAT
ATTTTCCATTCTTAATTTATTTAATTCAAAATGAATTTAATTGAAAATTGAATATTTTTTGAAAATAAAA
ATAAATAGAAATACTAGTAATAGTAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGT

Chemical Fingerprinting: In this study, we have included the test samples and corresponding retained BRM samples to determine their consistency with the retained reference samples using chemometric modelling of ^{1}H-NMR. All the given test samples are chemically consistent with the retained samples; this can be inferred from the below given NMR spectral comparison.

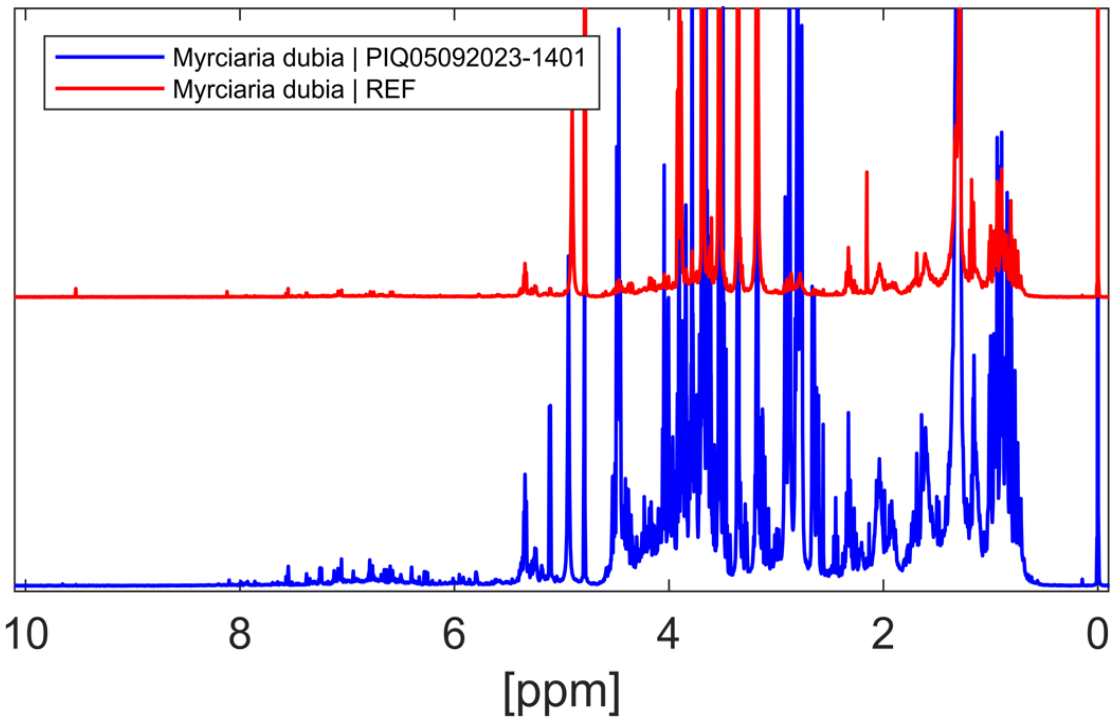

Figure 1: ^{1}H-NMR spectral comparison of the BRM reference and test samples. The chemical profile of the test and BRM samples are well aligned.

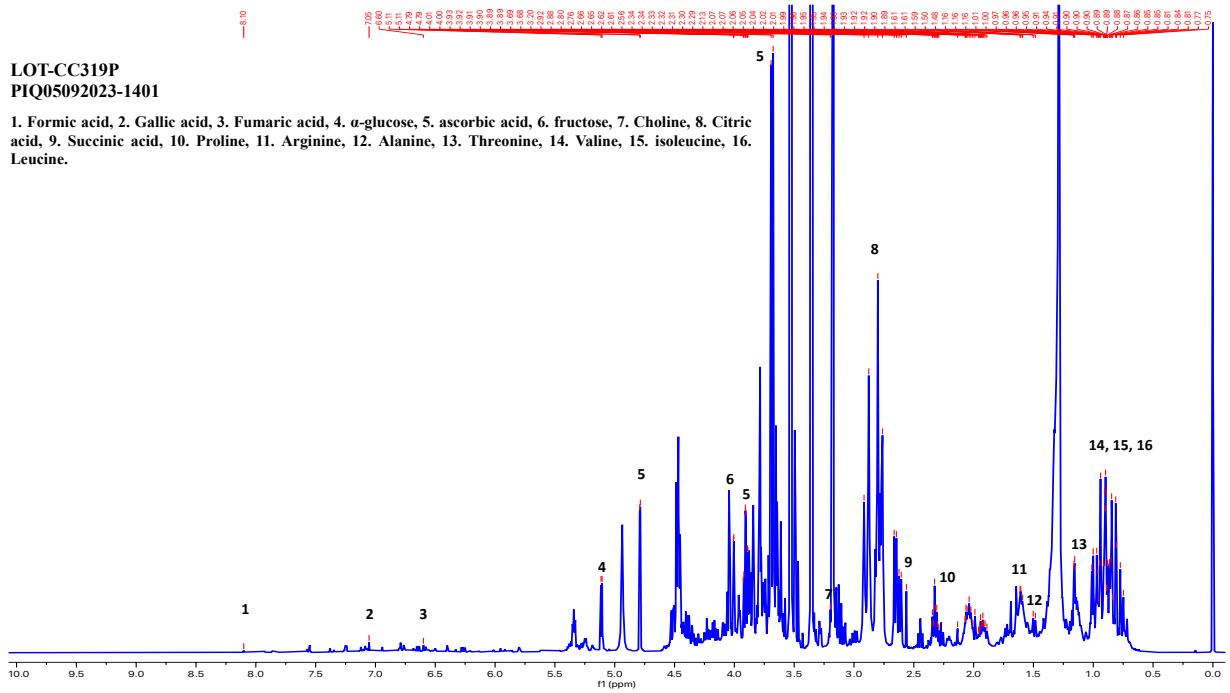

Figure 2. The ^{1}H-NMR spectrum of the test sample (Camu Camu) at δ 0.0–10.0 ppm shows characteristic signals for primary and secondary metabolites.

Molecule	Chemical shifts – ppm (multiplicity)
Ascorbic acid	4.79 (d)
Formic acid	8.10 (s)
Gallic acid	7.05 (s)
Fumaric acid	6.60 (s)
α-glucose	5.11 (s)
Fructose	4.01 (s)
Choline	3.20 (s)
Citric acid	2.62 and 2.80 (dd)
Succinic acid	2.56 (s)
Proline	2.03 and 2.35 (m)
Arginine	1.59 and 1.61 (m)
Alanine	1.49 (d)
Threonine	1.17 (d)
Valine	0.99 (d)
Isoleucine	0.93 (m)
Leucine	0.85 (m)

Table 1: List of molecules observed from ^{1}H-NMR.

The metabolomic profiling of the test samples using proton NMR confirms the species identity as *Myrciaria dubia.* The complete chemical profile (untargeted method) aligns well with the Botanical Reference Material (BRM), shown in Figure 1. In addition to the untargeted method, we elucidated the list of 16 molecules (Table 1 & Figure 2), which includes the characteristic bioactive molecule vitamin-C (Fortes, Naves, Ferri, & Santos, 2012) from the test sample. The proposed mechanism for the nitrosation and oxidation of ascorbic acid (AA, 1) by nitric oxide (NO) is shown in Figure 3. The initial nitrosation occurs on the C3 hydroxyl of ascorbic acid to form 3-nitrosoascorbic acid (2). The further nitrosation of the nitroso-ascorbic acid intermediate (2) to form the 2,3-dinitrosoascorbic acid intermediate (3). Finally, the 2,3-dinitrosoascorbic acid (3) eliminates NO to form oxidized molecules of dehydroascorbic acid (DHA, 4). Intermediates proposed in Figure 3 are not observed in the test sample (Figure 4). But the presence of ascorbic acid in the test sample is prominent in the ^{1}H NOESY NMR (Figure 4). The peak assignment details of the ascorbic acid are as follows (Figure 4), H1 proton was observed at 4.79 ppm (d); H2 protons at 3.89 to 3.93 ppm (m); and those around 3.68 and 3.69 ppm (d) are of H3 protons, and two hydroxy protons (OH) are interchangeable.

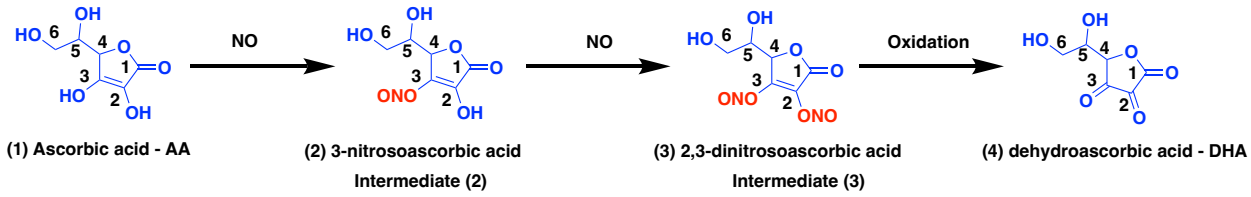

Figure 3: A scheme of a proposed mechanism of ascorbic acid nitrosation

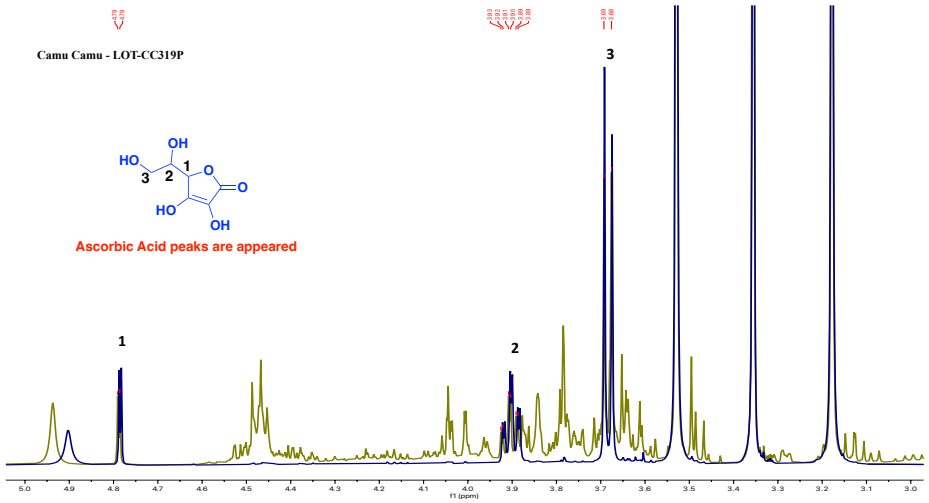

Figure 4: Overlay of ^{1}H-NMR spectra of Camu test sample (yellow green) and ascorbic acid reference (Navy blue). The presence of ascorbic acid (Vitamin-C) is confirmed.

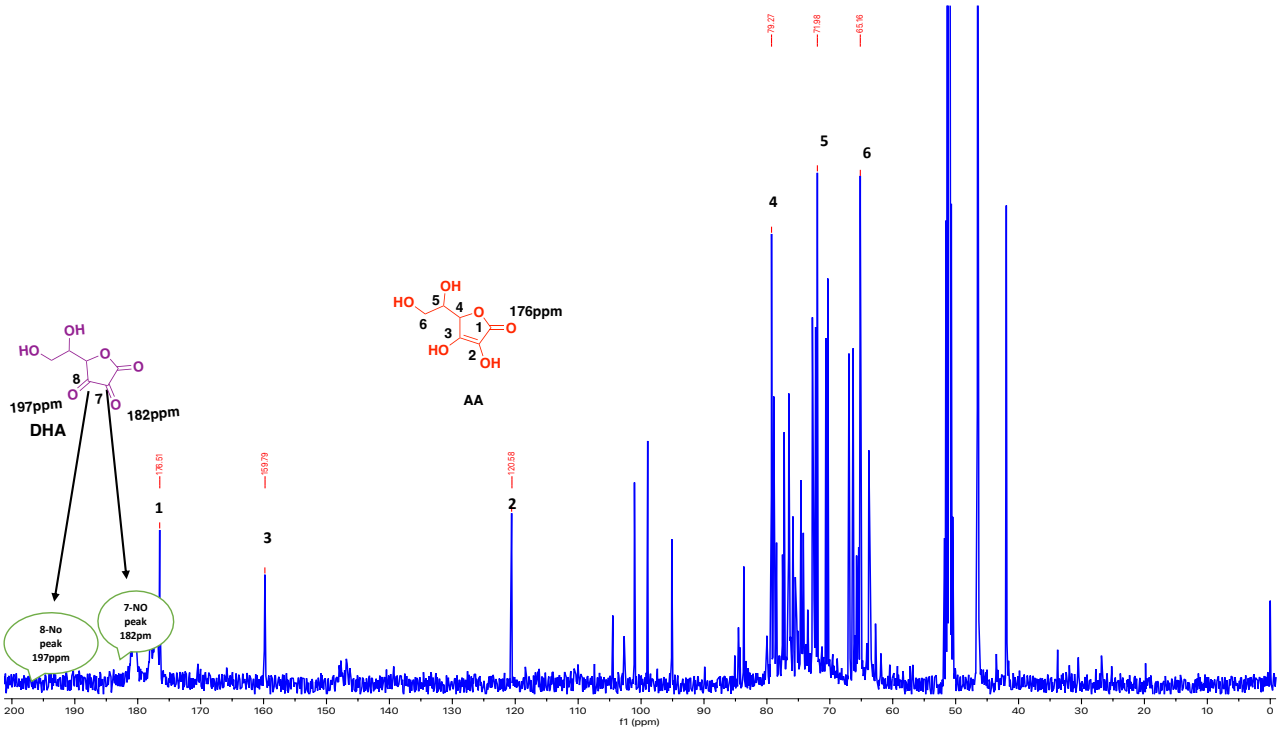

Figure 5: ^{13}C-NMR spectrum of Camu Camu at δ 0.0–200.0 ppm showing characteristic signals for primary and specialized metabolites or compounds (400 MHz, 300 K, 9:1 Methanol).

Further analysis using ^{13}C-NMR spectrum confirmed the peaks of ascorbic acid (AA) molecules five carbon and one carbonyl carbon signal was observed and showed in Figure 5. The ^{13}C peaks of ascorbic acid signals appear at 65.18, 71.96, 79.27, 120.58 and 159.78 ppm, and carbonyl carbon signal appeared at 176.51 ppm. In contrast, nitric oxide (NO) involving an ascorbic acid (AA) to form oxidized molecule of dehydroascorbic acid (DHA) did not have three-carbonyl carbon signals such as 176.51, 182.0 and 197.0 ppm. There was no evidence of carbonyl carbon signals at 182.0 and 197.0 ppm in the ^{13}C NMR spectrum, thereby we can conclude there are NO intermediates found in the sample. This is expected because NO is not produced in plants, but rather it is produced in animal systems using ascorbic acid. There are three ways the ascorbic acid can increase nitric oxide (NO) levels in humans: Mode of Action (MOA)-01 – endothelial nitric oxide synthase (eNOS) expression, enzyme production/translation; MOA-02 – ascorbic acid helps in posttranslational modifications (phosphorylation and S-Nitrosylation) of NOS to facilitate the NO production; MOA-03 – reduction of a cofactor (BH4) involved in the NO pathway, thereby increasing the levels of NO. These systems are defined below.

Mode of action 01 (MOA-01)

An oral administration of camu camu fruit juice study by Tanaka & Kashimura, 2014 on spontaneously hypertensive rats (SHR) showed a significant expression level of aortal endothelial nitric oxide synthase (eNOS – an enzyme) compared to control rats. The increased levels of eNOS enzyme on SHR are directly associated with lowering blood pressure by producing nitric oxide (NO).

Mode of action 02 (MOA-02)

Posttranslational modification is a necessary step for the functioning of an enzyme. The eNOS must undergo phosphorylation and S-Nitrosylation for the NO production in the cell. The structural motif characterization of eNOS by Ladurner *et al.*, 2012; Rafikov *et al.*, 2011 and Erwin *et al.*, 2005 have determined that the saturable level of ascorbate in the cell has an important role in the structural modification of eNOS and increases the levels of NO in the cell. The phosphorylation enzymes PPA2 and AMPK do modifications at amino acids Ser^{1177} and Thr^{495} (Ladurner *et al.*, 2012; Rafikov *et al*., 2011). The S-Nitrosylation on eNOS is important for maintaining the active conformations (dimerization) by modifying amino acids Cys^{96} and Cys^{101}. It stabilizes the dimeric association of eNOS and reduces the inhibition of eNOS (eNOS is regulated antagonistically) activity (Erwin *et al.*, 2005).

Mode of action 03 (MOA-03)

The nitric oxide pathway in endothelial cells generates NO by converting L-arginine to L-citrulline with the help of many cofactors (FAD-Flavin adenine dinucleotide, FMN-Flavin mononucleotide, and BH4-tetrahydrobiopterin). A chemical stabilization study by Huang *et al.*, 2000 and Heller *et al.*, 2001 showed that the saturated levels of ascorbic acids in the endothelial cells are important to prevent oxidation of BH4 and increase NO levels in the cell. The ascorbic level in the cell plays a vital role in each step of NO production, from the beginning (eNOS expression) to the end (reducing cofactors involved in the pathway).

Conclusion

Your sample: Sample_id-PIQ05092023-1401; Lot number: CC319P
Species name: *Myrciaria dubia*

- DNA tested positive for *Myrciaria dubia*.
- NMR fingerprinting tested positive *Myrciaria dubia*.
- Ascorbic acid was detected, but NO intermediates were not found in the sample.

References

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