NHP RESEARCH ALLIANCE

***Myrciaria dubia* samples test report** **October 6, 2022**

Project Objective:

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

1) DNA test-Polymerase Chain Reaction.
2) NMR metabolites variability and elucidation of bioactive compounds from NMR spectra using reference spectra

***Myrciaria dubia* test samples from July 2022 DNA and NMR screen Test Steps:**

1. DNA ID
    a. DNA extraction
    b. DNA archival in -80 Celsius freezer
    c. DNA Sybr Green PCR validated test method using specific markers

2. NMR fingerprinting
    a. Sample preparation
    b. Methanol extraction for NMR
    c. Spectra acquisition for NMR
    d. Data processing and Analysis for NMR
    e. Elucidation of bioactive compounds from NMR spectra using reference spectra

3) Final 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.

Dr Steven Newmaster,
Director, NHP Research Alliance

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Report

Sample information:

Sample	BRM	Target species
BT5005LF5606	BRM1507	*Myrciaria dubia*
BT5005LF5608	BRM1508	*Myrciaria dubia*
BT5005LF5611	BRM1509	*Myrciaria dubia*
BT5005LF5609	BRM1510	*Myrciaria dubia*

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, *psbA-trnH*, and *ITS2* as described in Newmaster et al 2013.

DNA sequencing

Four 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 et al. 1994). The genetic distances were calculated using the Kimura2Parameter (K2P) model in Mega5 (Tamura et al. 2011).

NMR fingerprinting

The Biological Reference Material (BRM) *Myrciaria dubia* of all test samples was used for preparing a NMR fingerprint library. This NMR library was assembled from six retained samples that were available within the NHPRA reference archive of known samples and respective test species reference vouchers from our NHPRA collection. All BRM refence 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 600uL of clear supernatant was collected in an NMR tube.

Spectra Acquisition for NMR:
To analyze chemical fingerprints of the samples, 1H-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:
1H-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 and 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 below means as 0 and above means were binned from 1 to 100.

Elucidation of bioactive compounds from NMR spectra using reference spectra:
The structural elucidation of bioactive compounds in the NHPs are 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

DNA analysis: DNA from the Piping Rock test samples were amplified with mini-DNA barcodes. All samples were successfully matched with 100% identification matches using suitable positive and negative controls of respective test samples vs with our BRM Reference.

sample_id	BRM_id	Target sp.
BT5005LF5606	BRM1507	*Myrciaria dubia*
BT5005LF5608	BRM1508	*Myrciaria dubia*
BT5005LF5611	BRM1509	*Myrciaria dubia*
BT5005LF5609	BRM1510	*Myrciaria dubia*

Chemical Fingerprinting: In this study, we have included all the test samples and its corresponding retained/BRMs samples to determine its consistency with the retained reference samples using chemometric modelling of 1H NMR. All the given test samples are chemically consistent with he retained samples; this can be inferred from the below given NMR spectral comparison.

Individual Sample Results

DNA/NMR Sequence Analysis
Sample_id: BT5005LF5606
Species name: *Myrciaria dubia*

DNA:>

>Myrciaria-dubia_564p_Specific marker
AGGAGCAATAACCAACACTCTTGATAGAATAAGAAGTTGGTTATTGTTCC
TTTATTTAGTGCTCTTTTCTTTACATAAGTGTTTCTTTTCTTCAACATAA
GAAAAGGTATTCGAGTATTTAGGGATTGTTTTATGATTGCGTATCATACT
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CTTTGTGAGA

>Myrciaria-dubia_ITS2
TGCAGAATCCCGTGAACCATTGAGTCTTTGAACGCAAGTTGCGCCCGAGT
CCATTTGGACGAGGGCACGTTTGCCTGGGTGTCACACACGGCGTTGCCCC
TAATCCCACGCCTTGAATCGGGCGCGCGAGACTCGGGTGCGTATGTTGGC
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>Myrciaria-dubia_psbA
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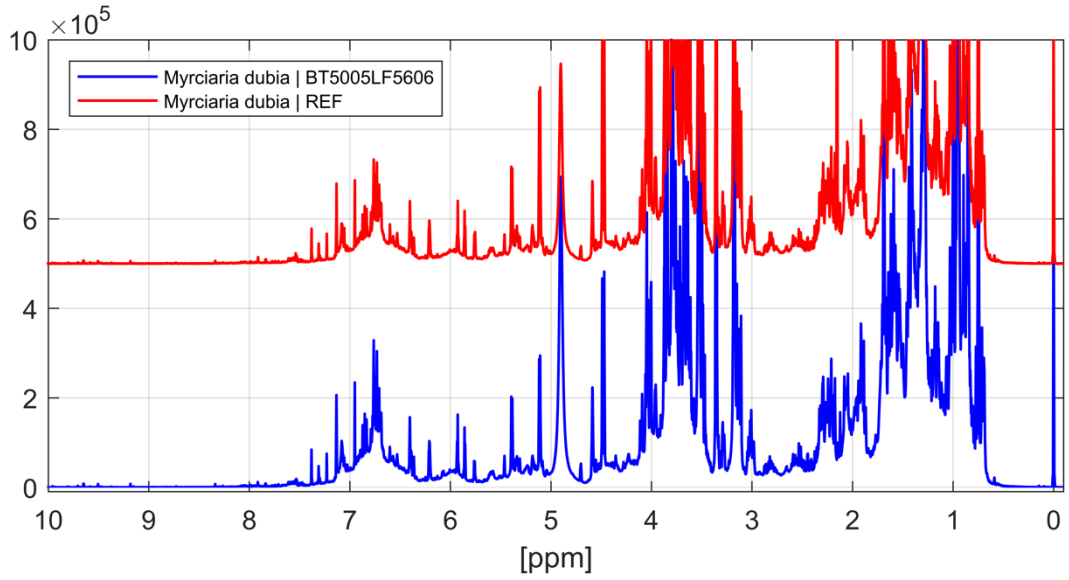
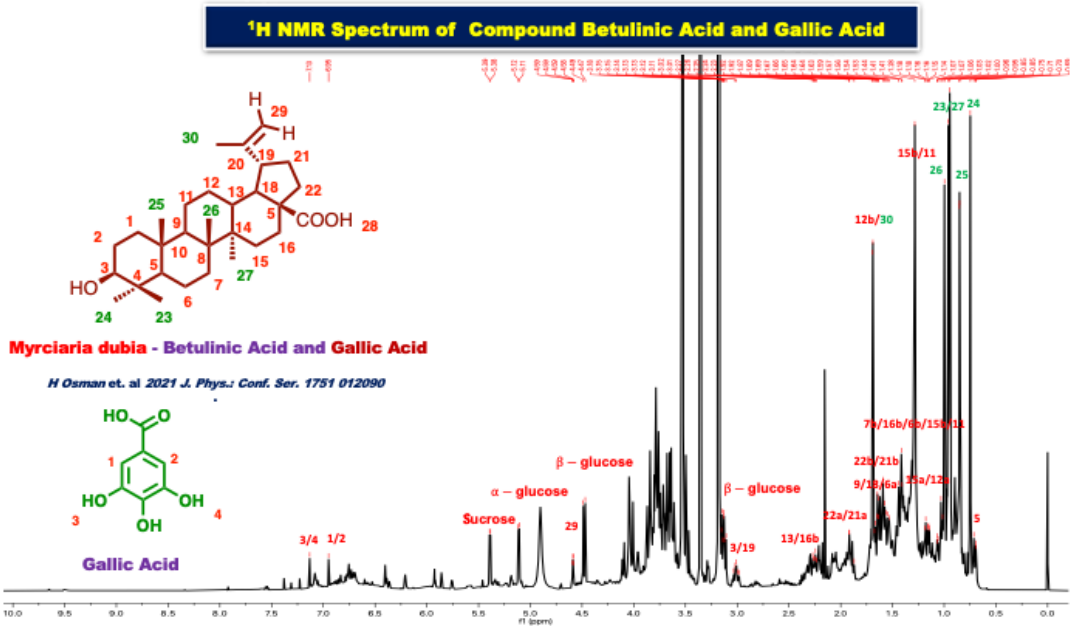
NMR Spectrum



Bioactive molecule elucidation:



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Sample_id: BT5005LF5608
Species name: *Myrciaria dubia*

DNA:>
>Myrciaria-dubia_564p_Specific marker
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CTTTGTGAGA

>Myrciaria-dubia_ITS2
TGCAGAATCCCGTGAACCATTGAGTCTTTGAACGCAAGTTGCGCCCGAGT
CCATTTGGACGAGGGCACGTTTGCCTGGGTGTCACACACGGCGTTGCCCC
TAATCCCACGCCTTGAATCGGGCGCGCGAGACTCGGGTGCGTATGTTGGC
CTCCCGTGACGACTTTCGTCCCGGTTGGCCCAAAATCGAGCGCTGGAGCG
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>Myrciaria-dubia_psbA
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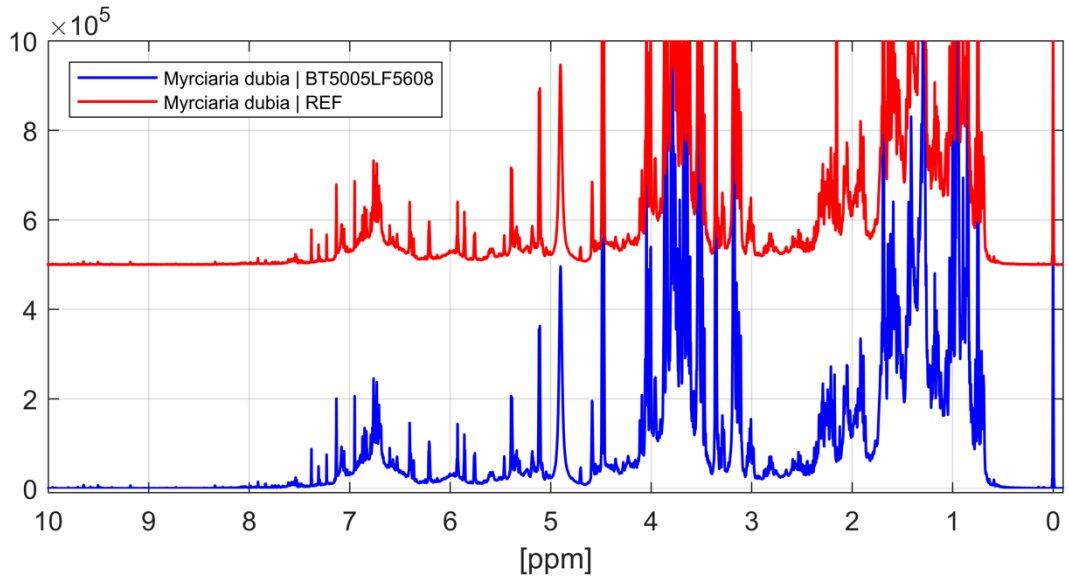
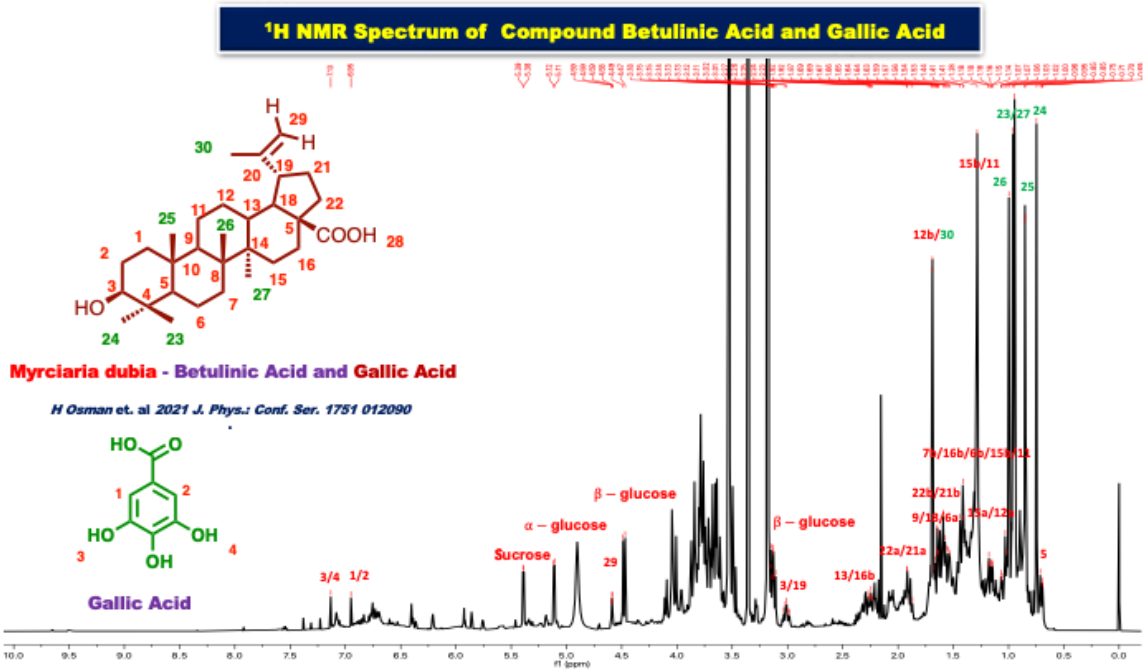
NMR Spectrum:



Bioactive molecule elucidation:



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Sample_id: BT5005LF5609
Species name: *Myrciaria dubia*

DNA:>
>Myrciaria-dubia_564p_Specific marker
AGGAGCAATAACCAACACTCTTGATAGAATAAGAAGTTGGTTATTGTTCC
TTTATTTAGTGCTCTTTTCTTTACATAAGTGTTTCTTTTCTTCAACATAA
GAAAAGGTATTCGAGTATTTAGGGATTGTTTTATGATTGCGTATCATACT
TTAGATATGAATTTCGAATTTATATACATTCTTTTCAACCCTTTGTAAGT
CTTTGTGAGA

>Myrciaria-dubia_ITS2
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CCATTTGGACGAGGGCACGTTTGCCTGGGTGTCACACACGGCGTTGCCCC
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>Myrciaria-dubia_psbA
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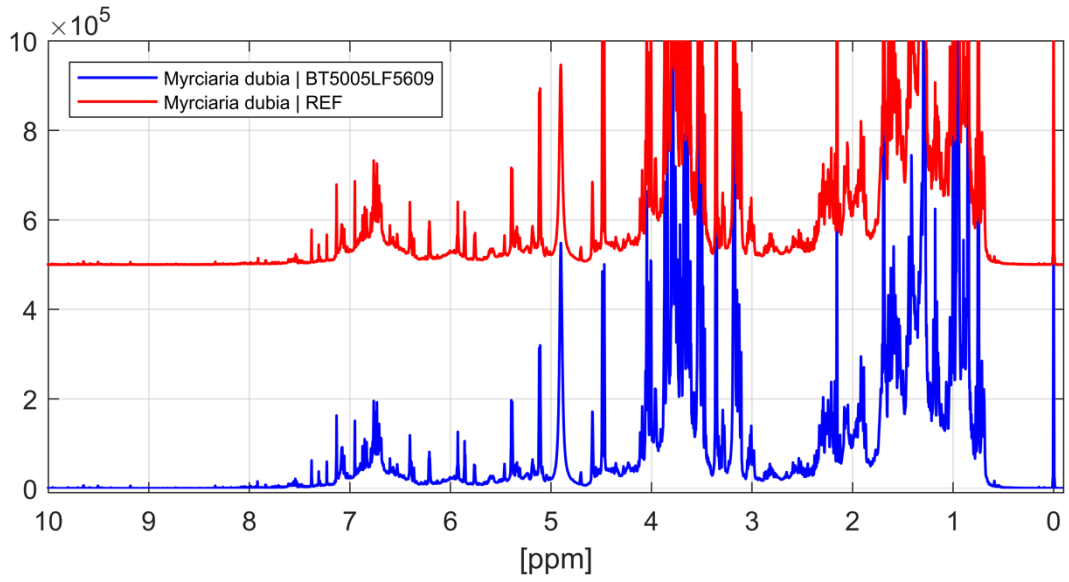
NMR Spectrum:



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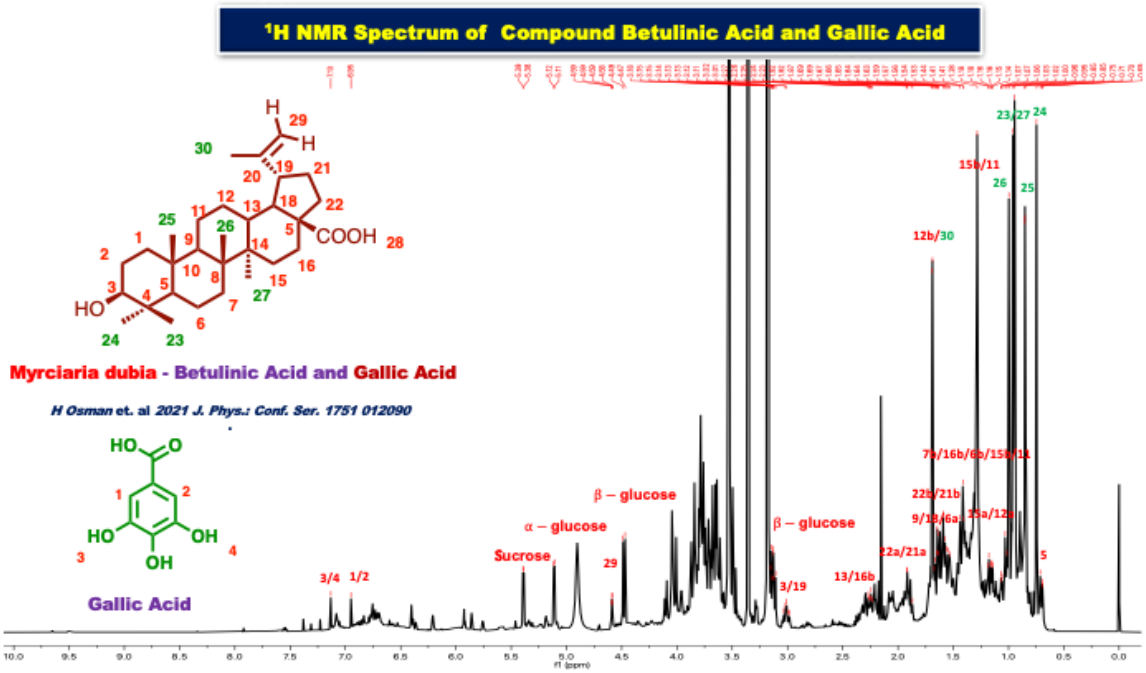

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Sample_id: BT5005LF5611
Species name: *Myrciaria dubia*

DNA:>

>Myrciaria-dubia_564p_Specific marker
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CTTTGTGAGA

>Myrciaria-dubia_ITS2
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>Myrciaria-dubia_psbA
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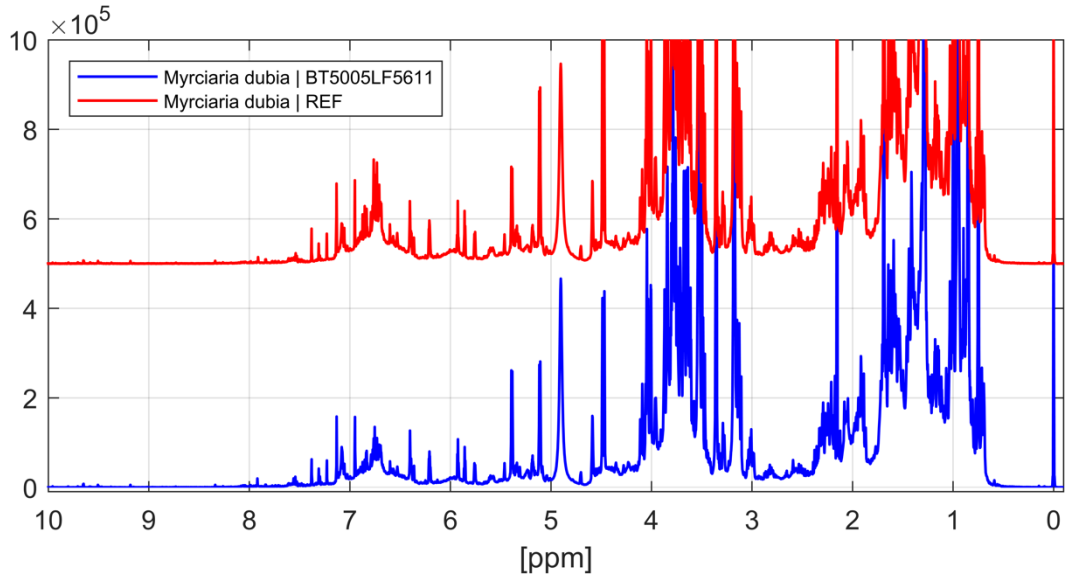
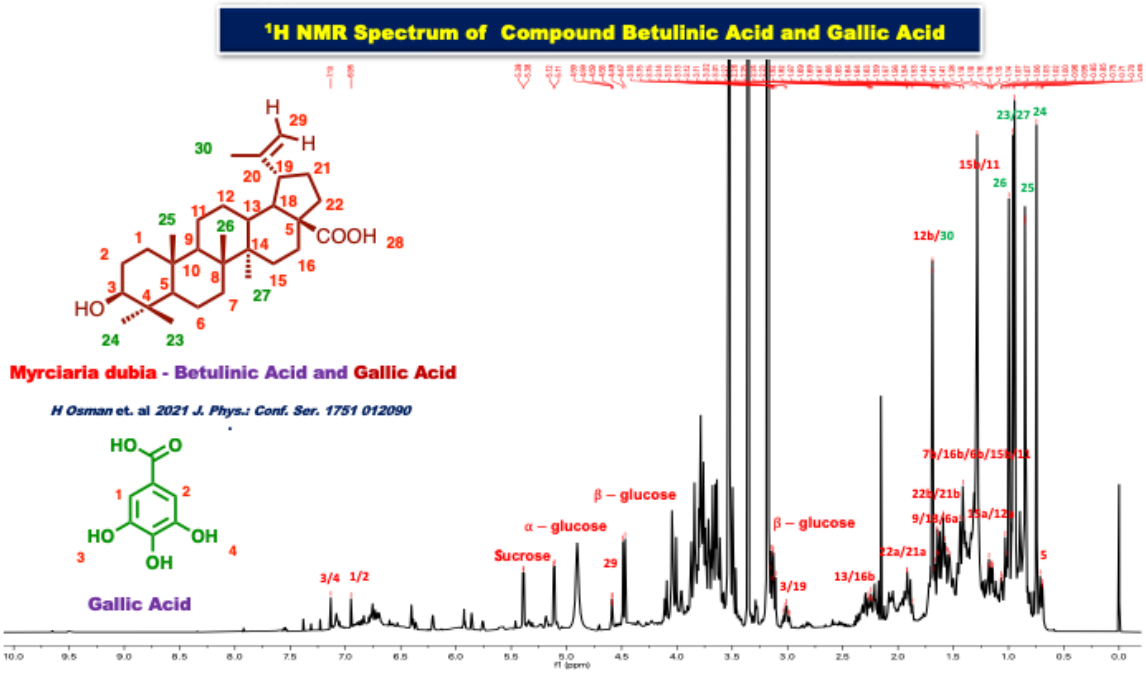
NMR Spectrum:



Bioactive molecule elucidation:



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Conclusion

All samples were identified using DNA/genomics and NMR metabolomics. This study demonstrates the fine-scale identification using NMR metabolites diversity derived from 1H NMR is an efficient tool for fingerprinting and determining differences in reference samples with identity of potential adulterants. This provides a quality assurance tool that can be used to verify authenticity of suppliers and the quality and consistency of the product ingredient. All plant material harvested and donated by **Jeff Moats** of Nitro-C, LLC Naples, FL. USA

Literature cited

Broeders S. Huber I. Grohmann L. Berben G. Taverniers I. Mazzara M. Roosens NH. Morisset D. Guidelines for validation of qualitative Real-Time PCR methods. Trends Food SciTech. 2014;37:115–126. doi: 10.1016/j.tifs.2014.03.008.

ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology NOTE FOR GUIDANCE ON VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY (CPMP/ICH/381/95) APPROVAL BY CPMP November 1994

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