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Evaluation of Solvent Effects on NMR Metabolic Profiling of *Passiflora cincinnata* Mast. Cv. BRS Sertão Forte

Avaliação dos Efeitos do Solvente Sobre o Perfil Metabólico de RMN de Passiflora cincinnata Mast. Cv. BRS Sertão Forte

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Passiflora cincinnata Mast. is a native species from the Caatinga biome (Brazil), commonly known as "maracujá-do-mato" and "maracujá-da-Caatinga". *P. cincinnata* leaves were subjected to microextraction with 70% ethanol-water mixture as follows: 1.5 mL of the solvent mixture was added in 50 mg of powdered leaves and sonicated for 15 min. After solvent removal, the samples were analyzed by nuclear magnetic resonance (NMR). Besides signals from primary metabolites, the chemical profile revealed the presence of isovitexin, vitexin-2"-*O*-xyloside, and trigonelline. Tracking these compounds might be of interest to metabolomic studies.

Keywords: *Passiflora cincinnata*; chemical profiling; flavonoids; Caatinga.

1. Introduction

The Passifloraceae family contain two tribes, 17 genera, and approximately 700-750 species.[1] The genus *Passiflora* is the largest and most important of this family, comprising about 630 species. In Brazil, about 87 out of approximately 150 existing *Passiflora* species are considered endemic.[2] Aerial parts of some *Passiflora* species have been traditionally used to treat anxiety, insomnia, and nervousness,[3] while fruits can be consumed or used in preparation of juices and ice creams. The phytochemistry of the genus *Passiflora* is characterized by the presence of flavonoid glycosides (*C*- and *O*-glycosides), saponins, cyanogenic glycosides, and β-carboline or indole alkaloids.[4-5]

Passiflora cincinnata is a species native to the Caatinga biome, popularly known as "maracujá-do-mato" and "maracujá-da-Caatinga". It is a species resistant to water deficit and adapted to the semi-arid climate. Also, *P. cincinnata* has edible fruits, ornamental appeal, and medicinal purpose, presenting biological activities such as antibacterial,[6-7] antioxidant,[8] antinociceptive, and anti-inflammatory.[9]

Launched by the Brazilian Agricultural Research Corporation (EMBRAPA), *P. cincinnata* BRS Sertão Forte is the first variety of the species *P. cincinnata* available for commercial cultivation. This cultivar consists of a genetically improved version obtained from the selection of several wild passion fruit accessions of *P. cincinnata*, collected in different areas of the Caatinga biome in Brazil.[10]

P. cincinnata BRS Sertão Forte (BRS SF) has higher field yield and pulp productivity when compared to native plants. This fruit can be considered as an alternative for the exotic fruit market intended for "in nature" consumption for its processing in agro-industrial products such as juices, fruit pulp, among others. The previous study carried out with this variety analyzed the physicochemical composition and *in vitro* antioxidant activity, as well as the chemical composition by high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD).[10]

The improvement of analytical tools, such as nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) has allowed the analysis of complex mixtures, without a necessity of isolation procedures.[11] Such approach has gained strength in the scientific community as it allows the monitoring of multiple compounds. From this scenario emerged two powerful approaches: metabolomics and dereplication.

^{1}H NMR has been the predominant profiling method because it is fast and simple, and the technique has been used as a major analytical tool for many applications in plant metabolomics,

for example, for quality control, chemotaxonomy (classification and characterization), analysis of the equivalence of genetically modified plants, and interaction with other organisms and the environment.[12]

In the present study we aimed to investigate NMR metabolic profiles of *P. cincinnata* BRS Sertão Forte using different solvents. We wanted to extract, as much as possible, primary and secondary metabolites by using simple, fast, and cheap microextraction procedures. Our objective is to contribute to the chemical study of this variety by exploring NMR chemical profiles, which can be used in further metabolomic studies to respond to more specific questions on the agronomical-chemical or chemical-bioactive aspects.

2. Materials and Methods

2.1. Plant material

Leaves of *P. cincinnata* var. BRS Sertão Forte were collected at EMBRAPA Semiárido (geographical coordinates: 09°13'S; 40°29'W), in Petrolina, State of Pernambuco, Brazil, in August 2018. The samples were identified by a botanist and compared with a voucher specimen (#22870) deposited at the Herbarium Vale do São Francisco (HVASF) of the Federal University of Vale do São Francisco (UNIVASF). All procedures for access to genetic patrimony and associated traditional knowledge were carried out and the project was registered in SisGen (Register #A0B9F84).

2.2. Preparation of plant microextracts

Plant material was dried in an oven with air circulation at temperature of 45 °C for 72 h, and then powdered in a mechanical mill and submitted to microextractions. Initially, tests with different solvents (chloroform, ethyl acetate, acetone, ethanol, 70% ethanol-water mixture, and water) were performed. In these experiments, microextractions were carried out with 25 mg of powdered leaves in 1 mL of solvent. The resulting mixture was sonicated at 30 °C for 15 minutes in a SB-DTN Ultrasonic Cleaner (Logen Scientific). The solutions obtained in the microextractions were separated from the plant powder and evaporated in rotavapor, and then they were resuspended in 500 µL of deuterated solvent for analysis using nuclear magnetic resonance (NMR).

Once figured out the best solvent for our proposal a few modification was adopted, and then the microextraction was carried out as follows: 50 mg of powder from the leaves of the species, previously sieved in 425 µm mesh sieves, was used for extraction with 1.5 mL of ethanol:water (70:30) for 15 minutes in sonicator. Subsequently, the material was centrifuged at 2000 rpm for 15 minutes, separating the vegetable powder from the extractive supernatant solution, which was transferred to a tube that was taken to dry the solvent in an oven with circulating air. Finally, the samples were solubilized in 500 **µL** of deuterated solvent and analyzed by NMR.

2.3. Nuclear Magnetic Resonance analysis

For spectra acquisition, the samples were solubilized in 500 µL of deuterated solvents and transferred to 5 mm NMR tubes. The chemical characterization of extracts was performed by Nuclear Magnetic Resonance (NMR) analysis. 1D and 2D NMR data were acquired at 298 K in DMSO-d_6 on a Bruker AVANCE III 400 NMR spectrometer operating at 9.4 T, observing ^{1}H and ^{13}C at 400 and 100 MHz, respectively. The NMR spectrometer was equipped with a 5-mm direct detection probe (BBO) with z-gradient. The hydrogen spectra were acquired using the zg30° pulse sequence with the following parameters: 68 transients, 3.0 s between cycles - relaxation delay (d1), acquisition time (AQ) equal to 5.0 seconds, 64k of number of points, and a spectral window of approximately 16 ppm. The spectra were processed in the TOPSPIN software with 64k points by applying an exponential multiplication of the FIDs by a factor of 0.3 Hz. Homonuclear (^{1}H-^{1}H COSY), one-bond (^{1}H-^{13}C HSQC) and long-range (^{1}H-^{13}C HMBC) NMR heteronuclear correlation experiments were optimized for average coupling constant ^{1}J(C,H) and ^{LR}J(C,H) of 140 and 8 Hz, respectively. All ^{1}H and ^{13}C NMR chemical shifts (δ) are given in ppm related to the TMS signal at 0.00 ppm as an internal reference, and the coupling constants (*J*) in Hz.

3. Results and Discussion

NMR-based chemical profiles obtained by the different microextractions of *P. cincinnata* BRS Sertão Forte offered a general view of solvent-dependent compound classes. These profiles are depicted in Figure 1. Visual inspection of ^{1}H NMR spectra allow classifying the chemical profiles into three groups:

- **Group 1:** extractions performed with chloroform, ethyl acetate, and acetone. With small differences between them, the chemical profiles obtained with these solvents were characterized mainly by the presence of saturated and unsaturated fatty acids.
- **Group 2:** extractions performed with ethanol and 70% ethanol:water. The spectral profiles obtained with these solvents showed several signals referring to different chemical classes: fatty acids, amino acids, organic acids, carbohydrates, and aromatic compounds. However, based on the signal intensities, extraction with ethanol 70% was more effective since it offers a higher signal/noise ratio.
- **Group 3:** extraction with water. The spectral profile with low signal intensity revealed the low power of water to extract compounds. A more careful analysis showed that a good part of the signs presents in the profile of

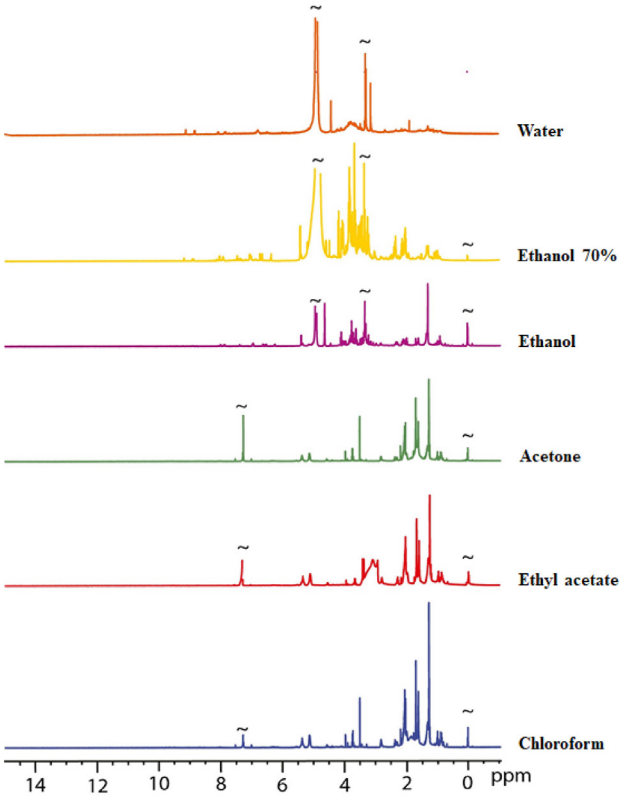

Figure 1. ^{1}H-NMR spectra of different extracts obtained from the leaves of *P. cincinnata* var. BRS Sertão Forte (Water extract was solubilized in D_2O; Ethanol and ethanol 70% were solubilized in DMSO-d_6; Acetone, ethyl acetate and chloroform extracts were solubilized in $CDCl_3$, 400 MHz)

group 2 are potentially in the profile obtained with water. However, with considerably reduced intensity. This problem could be overcome by increasing the sample amount in the extraction procedure.

Based on these experiments, extraction with ethanol 70% seemed to be the most appropriate when it is desired to extract a broad range of compounds with a good signal/noise ratio. Thus, the solvent system ethanol:water (70:30% v/v) was established for metabolite characterization. The influence of previous degreasing was also evaluated for 70% ethanol microextraction to verify if the removal of fats present in the leaves would improve the extraction of metabolites. The experiments showed a reduction in signals related to lipids but did not improve the intensity of the other compounds (data not shown). Thus, the insertion of this stage in the extraction methodology was considered unnecessary.

The general identification of the extracted metabolites revealed signals from primary and secondary metabolites, with the former being the most abundant. For a better visualization of the signals, a ^{1}H-NMR spectrum with saturation of the residual water signal was acquired (Figure 2). Saturated and unsaturated fatty acids, amino acids, carbohydrates, organic acids, and aromatic compounds are examples of chemical classes shown in Figure 2.

The information obtained suggested the presence of typical signals from rings A and B of conjugated flavones. In addition, the spectral profile indicated the presence of glucose units linked to these compounds, confirmed by doublets at 4.79 and 4.58 ppm with a coupling constant (*J*) of 10 and 9.7 Hz, respectively. The results presented in this work corroborated previous studies carried out with *Passiflora* species that resulted in the identification of the flavonoids vitexin and isovitexin in mixture, supporting the viability of this proposal .[11] The identification of the chemical constituents present in the ethanol 70% extract from the leaves of *P. cincinnata* BRS Sertão Forte was confirmed by two-dimensional NMR (2D NMR) experiments, as well as

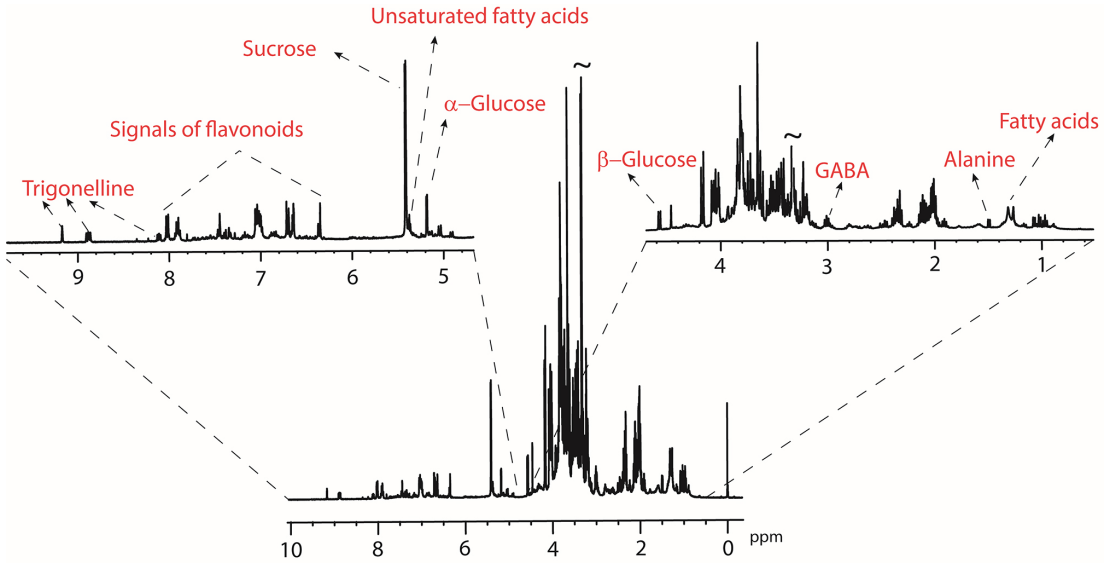

Figure 2. ^{1}H-NMR spectrum of ethanol 70% extract from the leaves of *P. cincinnata* BRS Sertão Forte showing characteristic signs for primary and secondary metabolites. The regions were expanded: δ 0.5-4.6 ppm and δ 4.8-9.6 ppm (DMSO-d_6, 400 MHz)

by comparison with data from the literature. The primary metabolites alanine (**1**), γ-aminobutyric acid (**2**), sucrose (**3**), α-glucose (**4**), β-glucose (**5**); and secondary metabolites such as the alkaloid trigonelline (**6**) and the flavonoids isovitexin (**7**) and vitexin-2''-*O*-xyloside (**8**) were identified (Figure 3). Compounds **7** and **8** were identified based on literature values of chemical shift.[13-14] The spectral data of the compounds are shown in Table 1. A more exhaustive compound identification can be performed depending on the goal of the study.

The chemistry of different parts of *P. cincinnata* BRS Sertão Forte has been investigated. Eleven phenolic compounds were identified and quantified by HPLC in "maracujá-do-mato" pulp at intermediate and ripe stages.[10] The authors reported five phenolic acids (caffeic acid, caftaric acid, *p*-coumaric acid, ferulic acid, and gallic acid) and six flavonoids (kaempferol, isoquercetin, isorhamnetin, miricetin, piceatannol, and rutin), identified based on retention time and UV spectra of standard compounds. Also, *trans*-ferulic acid, chlorogenic acid, *p*-coumaric acid, quercetin, rutin, naringenin, and gallic acid were found in the fruit pulp.[8] Chemical composition of extracts obtained from fruit peels, flowers, leaves, seeds, and stems was assessed by chromatography coupled to a diode array detector and mass spectrometry (HPLC-DAD-MS/MS). Fourteen substances were identified, highlighting the presence of *O*- and *C*-glycosylated flavonoids derived from apigenin, orientin, isoorientin, vitexin, and isovitexin, which are considered chemotaxonomic markers for *Passiflora* species.[15]

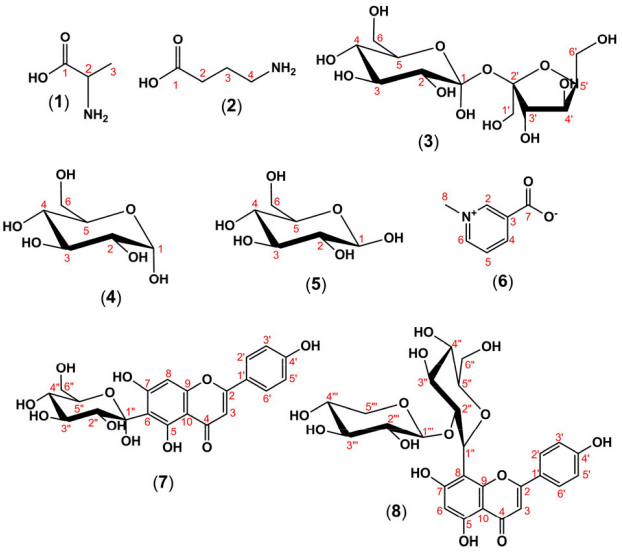

Figure 3. Chemical structures of main metabolites identified in the leaves of *P. cincinnata* BRS Sertão Forte

Beyond the chemical information of primary metabolites, that can be very informative to studies focused on the physiological and biochemical aspects of the plant, a few secondary metabolites the flavonoids isovitexin (**7**) and vitexin-2''-*O*-xyloside (**8**), and the alkaloid trigonelline (**6**)

Table 1. NMR data of ^{1}H-NMR (400 MHz) and ^{13}C NMR (100 MHz) of key metabolites of *Passiflora cincinnata* BRS Sertão Forte extract in DMSO-d_6

Metabolites	δ Chemical shift (mult., J in Hz, position)
Primary metabolites	
Alanine (**1**)	**δ ^{1}H**: 1.43 (d, 7.4, H-1)
γ-Aminobutyric acid (**2**)	**δ ^{1}H**: 2.17 (t, 7.4, H-2), 2.17 (m, H-3), 3.01 (m, H-3)
	δ ^{13}C: 174.5 (C-1), 33.6 (C-2), 24.5 (C-3), 45.1 (C-3)
Sucrose (**3**)	**δ ^{1}H**: 5.38 (d, 3.9, H-1)
α-Glucose (**4**)	**δ ^{1}H**: 5.18 (d, 3.7, H-1), 3.18 (dd, 9.4 and 3.7, H-2)
	δ ^{13}C: 91.8 (C-1), 71.7 (C-2)
β-Glucose (**5**)	**δ ^{1}H**: 4.57 (m, H-1)
Secondary metabolites	
Trigonelline (**6**)	**δ ^{1}H**: 9.21 (s, H-2).8.76 (br d, 8.0, H-4), 8.03 (d, 8.0 and 6.4, H-5), 8.88 (br d, 6.4, H-6), 4.36 (s, H-8)
	δ ^{13}C: 146.1 (C-2), 144.4 (C-4), 144.9 (C-6), 47.4 (C-8)
Isovitexin (**7**)	**δ ^{1}H**: 6.66 (s, H-3), 13.55 (s, OH-5) 6.57 (s, H-8), 7.93 (d, 8.7, H-2'/H-6'), 6.95 (d, 8.7, H-3'/H-5'), 4.58 (d, 9.7, H-1''), 3.43 (m, H-6''a), 3.57 (m, H-6''b)
	δ ^{13}C: 163.2 (C-2), 102.7 (C-3), 93.6 (C-8), 156.6 (C-9); 120.9 (C-1'); 128.6 (C-2'/C-6'), 116.1 (C-3'/C-5'), 161.4 (C-4'), 72.9 (C-1''), 61.0 (C-6'')
Vitexin-2''-*O*-xyloside (**8**)	**δ ^{1}H**: 6.77 (s, H-3), 13.14 (s, OH-5) 6.29 (s, H-6), 8.01 (d, 8.8, H-2'/H-6'), 6.92 (d, 8.8, H-3'/H-5'), 4.79 (d, 10.0, H-1''), 3.41 (m, H-6''a/H-5'''b), 3.58 (m, H-6''b/H-5'''b), 3.88 (d, 8.0, H-1''')
	δ ^{13}C: 163.6 (C-2), 102.4 (C-3), 160.2 (C-5), 98.3 (C-6), 162.9 (C-7), 103.5 (C-10), 121.8 (C-1'); 129.0 (C-2'/C-6'), 116.0 (C-3'/C-5'), 161.1 (C-4'), 71.5 (C-1''), 60.8 (C-6''), 105.8 (C-1'''), 62.0 (C-5''')

were identified and can be qualitatively and quantitatively monitored by using the microextraction with 70% ethanol-water mixture. These compounds can be related to the quality control of leaves or even be correlated with biological properties. Questions on these matters have been under investigation in our research group via NMR-based metabolomic approach.

4. Conclusion

In this work we described, for the first time, the NMR-chemical profiling of *P. cincinnata* BRS Sertão Forte leaves. Microextractions performed with 70% ethanol-water mixture presented chemical profiles composed of primary and secondary metabolites, including isovitexin, vitexin-2"-*O*-xyloside, and trigonelline that might be considered chemical markers for quality control of the species. Besides contributing to best knowledge of the chemical composition of *P. cincinnata* BRS Sertão Forte, these results can be very useful to the development of metabolomic methods of *P. cincinnata*, to elucidate the resistance to water deficit or even the relation of chemical composition and bioactivity of this species.

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Conflict of Interest

The authors declare that there are no conflicts of interests and they affirm that this paper consists of original and unpublished work.

Author Contributions

RFS and ADCS designed the experiments; RFS and ADCS conducted the experiments; LMD, APO and JRGSA analyzed the results; RFS, ADCS, APO, LMD, PHVT, NFM and JRGSA wrote the manuscript.

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