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# ***Bryophyllum pinnatum* markers: CPC isolation, simultaneous quantification by a validated UPLC-DAD method and biological evaluations.**

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## **Abbreviations:**

Abs: Absorbance; Bp: *Bryophyllum pinnatum*; BpHE: *B. pinnatum* hydroethanolic extract; CPC: Centrifugal Partition Chromatography; CyHex: cyclohexane; DMSO: dimethyl sulfoxide; EMA: European Medicines Agency; EtOAc: ethyl acetate; EtOH: ethanol; FDA: Food and Drug Administration; HPLC: High-performance Liquid Chromatography; K<sub>D</sub>: Distribution coefficient; MS: Mass Spectrometry; NMR: Nuclear Magnetic Resonance; RDC: *Resolução de Diretoria Colegiada* (Brazilian legislation); RSD: Relative Standard Deviation; SISBIO: Brazilian Authorization and Biodiversity Information System; SISGEN: National System for the Management of Genetic Heritage and Associated Traditional Knowledge; TFA: trifluoroacetic acid; TLC: Thin Layer Chromatography; UPLC-DAD: Ultra-performance Liquid Chromatography coupled with Diode Array Detection; USP: United States Pharmacopeia; UV: Ultraviolet; WHO: World Health Organization; XO: Xanthine Oxidase.

## **1. Introduction**

Approximately a quarter of all approved drugs by Food and Drug Administration (FDA) and/or European Medicines Agency (EMA) are derived from plants [1]. Besides being sources of new hit compounds, medicinal plants are integral part of traditional medical systems worldwide and are the basis for the development of herbal medicines. During the last two decades, the demand for herbal medicines has been steadily increasing [2]. For this reason, the World Health Organization (WHO) has recommended that member countries should develop regulations and guidelines that strengthen quality, safety, appropriate use and effectiveness of traditional medicine [3].

The Brazilian Health Regulatory Agency - *Agência Nacional de Vigilância Sanitária* (Anvisa) - has established strict standards to ensure the quality and the safety of markers

present in the herbal extracts. The RDC 26/2014 (*Resolução de Diretoria Colegiada*), is the legislation that regulates the herbal medicines in Brazil. RDC determines the characterization of the marker and its subsequent quantification, ensuring its presence from the raw material (active pharmaceutical ingredient) to the finished product [4]. Furthermore, regarding the complex phytochemical composition of herbal substances and herbal preparations, their standardization is not an easy task [2] and requires the characterization of markers by previously validated analytical methods. Markers are chosen according to their pharmacological importance (constituents which are generally accepted to contribute to the therapeutic activity) or chosen because they are representative for analytical purposes (analytical markers) [5].

*Bryophyllum pinnatum* (Lam.) Oken is a succulent plant species native to Madagascar with several synonyms among which the most commonly used is *Kalanchoe pinnata* (Lam.) Pers. [6], and vernacular names like “miracle leaf” or “cathedral bells” in English, “feuille vivante” in French (New Caledonia), “saião” or “coirama” in Portuguese (Brazil). It is widely used in traditional medicine all over tropical areas for relief of inflammation-associated symptoms [7], most often as leaf juice, aqueous or hydro-alcoholic extracts, i.e. as solutions containing polar metabolites. In these preparations, major compounds are flavonoid glycosides, mostly quercetin and kaempferol derivatives. Among them, quercetin 3-*O*- $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- $\alpha$ -L-rhamnopyranoside containing a rare sugar moiety, was previously detected in high concentration in *B. pinnatum* extracts and associated to several biological effects, such as anti-inflammatory and leishmanicidal activities [8,9]. Thus, it appears as a potential marker for the species.

Our research group has been working with *B. pinnatum*, especially in phytochemistry field, through the analysis of hydroethanolic and aqueous extracts by HPLC-MS/MS, and on the evaluation of anti-inflammatory, antivenom against *Bothrops jararaca* poison and antiulcer activities [10–12]. Therefore, the isolation and identification of major compounds in *B. pinnatum* extracts is necessary for better understanding of its biological activities, as well as to be used as markers in the quality assessment of the raw material and finished product. Once this species is described in Renisus (*Relação Nacional de Plantas Medicinais de Interesse do Sistema Único de Saúde*) [13], a national list of 71 medicinal plants of interest to the Unified Health System in Brazil, it is necessary to ensure their efficacy, safety and quality.

Centrifugal partition chromatography (CPC) is a preparative support-free liquid-liquid separation technique, based on the difference in partitioning behavior of analytes between two immiscible phases. Due to the absence of any solid support, it provides many advantages over conventional liquid chromatographic techniques such as high selectivity through the infinite combination of biphasic solvent systems, high loading capacity, total recovery of the loaded sample without any risk of degradation, low solvent consumption and easy scale-up. Furthermore, the growing concern for lowering the environmental impact of purification processes makes CPC an attractive alternative, especially for high-value added compounds [14].

Xanthine oxidase (XO) catalyzes the oxidation of hypoxanthin to xanthin and uric acid in a process where reactive oxygen species (ROS) are released. This enzyme is involved in many pathological processes including inflammation, atherosclerosis and gout. The XO inhibitor allopurinol, sometimes prescribed in association with colchicine, indomethacine and/or other non-steroidal anti-inflammatory drugs in case of hyperuricemia and/or gout, may cause strong adverse effects such as nephropathy, hepatitis, skin rash. Thus, other effective treatments aiming to substitute allopurinol are urgently required. Flavonoid aglycones and glycosides have already shown promising *in vitro* XO inhibition [15] and are strong radical scavengers, able to prevent ROS damages.

The present study aims to isolate the major flavonoids of *B. pinnatum* leaves extract by CPC, to develop and to validate an UPLC-DAD method in order to simultaneously quantify these compounds in the context of the required quality assessment of starting plant materials, herbal preparations and herbal medicines. Additionally, *in vitro* XO inhibitory and antioxidant activities will also be evaluated in order to contribute to the understanding of the mechanism of action of *B. pinnatum* in relation to its use in folk medicine.

## 2. Materials and Methods

### 2.1. Plant Material

*Bryophyllum pinnatum* leaves were collected in Macaíba city, in the State of Rio Grande do Norte, Brazil, at coordinates 06° 05' 40" S 38° 22' 29" W, in January 2017. The botanical identification was done by Dr. Rúbia Santos Fonseca and a voucher specimen (n° 57335) was deposited at the Herbarium Prisco Bezerra of the Universidade Federal do Ceará, Brazil.

Permission to collect was issued from the Brazilian Authorization and Biodiversity Information System (SISBIO) (process number 35017) and National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) (process number A7EA798).

### 2.2. Preparation of *B. pinnatum* crude extract

*B. pinnatum* (6.5 Kg) fresh leaves were extensively washed with water and extracted with ethanol-water (1:1, v/v), plant/solvent ratio 1:1 (w/v) by turbo extraction for 5 minutes in an industrial blender. The extract was filtered and concentrated under reduced pressure using a rotaevaporator (model V-700, Buchi, Flawil, Switzerland) (temperature below 40 °C) in order to remove the ethanol and then freeze-dried yielding 130.0 g of a yellow dry extract.

### 2.3. Conditions for TLC and UPLC-DAD analysis

The extract was analyzed by thin layer chromatography (TLC) and UPLC-DAD. TLC were carried out on silica gel F<sub>254</sub> (Merck, Darmstadt, Germany) using two different mobile phases: (1) ethyl acetate - formic acid - methanol - water (10:0.5:0.6:0.2, v/v/v/v) and (2) ethyl acetate - formic acid - methanol - water (10:1.5:1.6:0.5, v/v/v/v). After development, the plates were dried, observed under UV light (254 and 365 nm), then sprayed with sulfuric vanillin and heated. UPLC-DAD system Shimadzu (Kyoto, Japan) Model LC-20AD, with DAD detector SPD-M20A and software LabSolutions (Shimadzu, Kyoto, Japan) was used, equipped with a Phenomenex (Torrance, California, USA) Kinetex RP-18 column (150 x 4.6 mm, 2.6 µm particle size) and a Phenomenex security guard column (4.0 x 2.0 mm i.d.). Elution was performed at step gradients of: (A) trifluoroacetic acid (TFA) 0.3% and (B) acetonitrile (7–15% v/v B, 0–3 min; 15–20% B, 3–12 min; 20–22% B, 12–30 min) at a flow rate of 0.7 mL/min, column temperature was 30 °C, 30 minutes total analysis time. The UV-DAD detector was set to the wavelength range of 200–500 nm and the chromatograms were plotted at 254 and 340 nm. Samples were resuspended in methanol-water, 1:1 (v/v) to the final concentration of 2 mg/mL, 12 µL of each sample were injected for analysis. HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were purchased from J. T. Baker (Brazil). Water was purified with a Milli-Q system (Millipore, Burlington, Massachusetts, USA). The samples and solvents were filtrated through a membrane (pore-size 0.45 µm) and degassed. Each sample was analyzed in triplicate.

### 2.4. CPC purification procedure

#### 2.4.1. Distribution coefficient ( $K_D$ ) estimation and solvents system selection

The solvents system was selected according to an estimation of the distribution coefficient  $K_D$ , defined as the ratio of concentrations of the targeted compounds between the two non-miscible phases.  $K_D$  was evaluated by shake-flask method and TLC observation as follows: a small amount (about 2 mg) of extract was added to the previously equilibrated biphasic solvents systems in test vials (Table A.1). The vials were vigorously shaken. After decantation, TLC examination under UV light (at 254 nm and 366 nm) and after spraying with a solution of sulfuric vanillin has led to choose system VIII (CyHex-EtOAc-EtOH-H<sub>2</sub>O, 0.5:9:3:5.5 (v/v/v/v)).

#### 2.4.2. Isolation of flavonoids

The CPC experiments were performed on a double rotor CPC-250 + 1000-B apparatus (Gilson Purification SAS, Saint-Avé, France, formerly Armen Instrument Sas) with an inbuilt quaternary high-pressure gradient pump and 1953 or 2016 ovoid twin-cell technology cells for the 250 mL column or for the 1000 mL column, respectively. The system (CyHex-EtOAc-EtOH-H<sub>2</sub>O, 0.5:9:3:5.5 v/v/v/v) was prepared in a separatory funnel, vigorously shaken and left to stand at room temperature until separation of the phases. The lower phase was chosen as the stationary phase and was injected in descending mode (flow rate 30 mL/min) into the 250 mL column previously filled with water, with a rotation of 500 rpm. After that, the speed was increased up to 1600 rpm in order to reach the desired centrifugal field. The superior mobile phase was then injected in the appropriate ascending direction (flow rate 15 mL/min). The displaced stationary phase volume was collected at the column exit until equilibrium was reached and the stationary phase retention (Sf) was measured at 85%.

A sample of extract (2.0 g) was dissolved in 10 mL of biphasic solvents system (7 mL of upper phase and 3 mL of lower phase) and injected through the 10 mL loop (final concentration of 200 mg/mL). A hundred and sixty fractions of 10 mL each, were collected during the elution. After that, for extrusion, lower phase was pumped with a flow rate of 30 mL/min, keeping the rotation at 1600 rpm, providing 30 fractions of 10 mL each. All of the fractions from elution-extrusion were evaluated by TLC using the two systems described above and were pooled into several groups.

#### 2.5. MS and NMR analysis

The MS spectra of the isolated compounds were performed with LCT Premier mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with a time-of-flight analyzer. The analysis was performed in positive and negative-ion mode with a mass range of  $m/z$  100–1100. The optimized ESI parameters were set as follows: source temperature of 80°C; desolvation temperature of 250 °C; source cone voltage of 30 V; capillary voltage of 2.5 kV. The instrument was controlled and the data acquisition was carried out by the software MassLynx version 4.0 (Waters).

The NMR spectra were recorded at 400 MHz for <sup>1</sup>H and 2D experiments on a Bruker Avance III 400 spectrometer, using Bruker pulse programs. NMR Fourier transform, integration and peak picking were done with Bruker TopSpin software version 3.2. Chemical shifts are reported in ppm, coupling constants ( $J$ ) in Hertz. The isolated compounds were dissolved in suitable deuterated solvents from Euriso-Top (Saint Aubin, France).

#### 2.6. Validation of the UPLC-DAD method for quantification of flavonoids.

The UPLC-DAD method for quantification was validated in accordance with Anvisa [16] as external validation where the standards used are present in the extract (compounds **1**, **2** and **3** isolated from *B. pinnatum*). The stock standard solution for each compound was prepared in methanol-water, 1:1 (v/v) to the final concentration 200 µg/mL. Selectivity,

linearity, limit of detection (LoD) and limit of quantification (LoQ), precision intra-day (repeatability) and inter-day (intermediary precision), accuracy and robustness were evaluated. The system suitability was performed according United States Pharmacopoeia (USP) to ensure adequate resolution ( $r > 1.5$ ), theoretical plates number ( $P > 2000$ ), tailing factor ( $T \leq 2.0$ ) and capacity factor ( $k' \geq 2.0$ ) for each peak under validation. The analysis of *B. pinnatum* hydroethanolic extract was performed with three replicates injections. Average and relative standard deviations (SRD) of area were calculated for each peak [17].

#### 2.6.1. Selectivity/Specificity

Selectivity was determined by analyzing and comparing the chromatographic data (retention time and peak purity) of the *B. pinnatum* hydroethanolic extract with corresponding data from the chromatograms of each standard solution (isolated compounds **1**, **2** and **3**) in triplicate.

#### 2.6.2. Linearity

Calibration curves with seven levels of concentration (175% to 25%) were plotted for each isolated compound (**1**, **2** and **3**), as well as area *versus* concentration for linearity evaluation. The linearity of the regression equation determined for each compound was investigated by means of the parameters correlation coefficient ( $r$ ). Stock solutions (70  $\mu\text{g/mL}$  for **1** and 10  $\mu\text{g/mL}$  for **2** and **3**) were diluted separately in MeOH-H<sub>2</sub>O (1:1, v/v) to provide the following concentrations: 8.75, 17.5, 26.25, 35.0, 43.75, 52.5 and 61.25  $\mu\text{g/mL}$  for flavonoid **1**; 1.25, 2.5, 3.75, 5.0, 6.25, 7.5 and 8.75  $\mu\text{g/mL}$  for flavonoid **2** and 0.75, 1.5, 2.25, 3.0, 3.75, 4.5 and 5.25  $\mu\text{g/mL}$  for flavonoid **3**. Each solution was analyzed in triplicate. Additionally, the linearity was also investigated for *B. pinnatum* hydroethanolic extract. A calibration curve based on five levels of concentration of hydroethanolic extract (1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL) was obtained. Each solution was prepared individually by dilution (w/v) of extract in MeOH-H<sub>2</sub>O (1:1, v/v). Peaks areas at 340 nm were plotted against the known concentrations of extract solutions for each peak concerning isolated compounds **1**, **2** and **3**, in order to establish the linearity of the regression equation for each peak compound of extract and to investigate the correlation coefficient ( $r$ ) for each one.

#### 2.6.3. LoD and LoQ

LoD and LoQ were calculated from the three analytical curves, using the values of standard deviation of the intercept ( $\delta$ ) and of the slope ( $S$ ):  $\text{LoD} = 3.3\delta/S$ ;  $\text{LoQ} = 10\delta/S$ .

#### 2.6.4. Precision

Precision was expressed as relative standard deviation (RSD) of the repeatability and of the intermediate precision analysis. Repeatability was determined by analyzing each isolated compound (**1**, **2** and **3**) in nine replicates ( $n=9$ ). Therefore, analyzes were performed at three concentrations levels (low, medium and high) for each compound with three replicates at each level covering the linear interval of the analytical method within one day. Intermediate precision was determined analyzing each isolated compound (**1**, **2** and **3**) in 27 replicates. Therefore, analyzes were performed at three concentrations levels (low, medium and high) for each compound with three replicates at each level covering the linear interval of the analytical method on three consecutive days, by three technicians.

#### 2.6.5. Accuracy

Accuracy of the method was evaluated according to a recovery test after spiking known amounts of the isolated compounds **1**, **2** and **3** on extract solution of known

concentration (2 mg/mL). The concentrations of isolated compounds used were low, medium and high with three replicates injection for each level contemplating the linear interval of the analytical method. Accuracy was expressed as the percentage and calculated by equation:  $\text{Accuracy (\%)} = [(\text{Peak area}_{\text{Extract+compound}} - \text{Peak area}_{\text{Extract}}) / \text{Peak area}_{\text{compound}}] \times 100$ .

#### 2.6.6. Ruggedness

Ruggedness was evaluated with variations of flow rate of mobile phase and column temperature.

#### 2.7. DPPH scavenging assay

Radical scavenging capacity was measured using DPPH (Sigma-Aldrich) according to previous report [18]. DPPH reagent solution (12.4 mg in 100 mL of EtOH) was freshly prepared just before the assay. *B. pinnatum* hydroethanolic extract was evaluated at the final concentration of 200 µg/mL. For IC<sub>50</sub> determination of isolated compounds, a range of final concentrations of 400-10 µg/mL was used. In each well were placed 10 µL of sample solution with 190 µL of DPPH solution. Plates were incubated at room temperature for 30 min protected from light. The absorbance was measured at 517 nm. The measurements were performed in triplicate. Gallic acid was used as positive control. Results are expressed as percentage of efficacy at 200 µg/mL of extract or as IC<sub>50</sub> for isolated compounds.

#### 2.8. ABTS Scavenging

Radical scavenging capacity was measured using ABTS reagent (Sigma Aldrich) according to previous report [19]. ABTS reagent was prepared as follows: 10 mL of 7 mM of ABTS solution were mixed with 164 µL of 140 mM of K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and stored overnight (16 h). The final solution was diluted with distilled H<sub>2</sub>O (1:20) to reach an absorbance of  $0.70 \pm 0.02$  before the assay. The extract was evaluated at the final concentration of 200 µg/mL and IC<sub>50</sub> was determined using a range of final concentrations of 400-10 µg/mL. In each well were placed 100 µL ABTS final solution and 50 µL of sample. Plates were incubated at room temperature for 10 min, away from light. The absorbance was measured at 734 nm. Measures were performed in triplicate. Quercetin was used as positive control. Results are expressed as percentage of efficacy at 200 µg/mL or as IC<sub>50</sub>.

#### 2.9. Xanthine Oxidase Inhibition Assay

Xanthine oxidase (XO) inhibition assay was performed using the modified protocol from [20]. Briefly, in a quartz 96-well plate, 62.5 µL of extract solution at the concentration of 800 µg/mL was added of 105 µL phosphate buffer (0.12 mM, pH 7.5) and 20 µL of XO (Sigma-Aldrich<sup>®</sup>) solution (0.125 units/mL in phosphate buffer) and then incubated at room temperature in the dark for 5 min. For IC<sub>50</sub> determinations, a range of concentrations of 400-10 µg/mL of each compound were used, 62.5 µL of xanthine (Sigma-Aldrich<sup>®</sup>) solution (200 µM in phosphate buffer) were added in each well and the absorbance was measured at 295 nm during 30 min (final DMSO concentration of 0.4 %). Negative controls were carried out, replacing XO solution by 62.5 µL of phosphate buffer. Initial rate was calculated and the percentage of inhibition was determined as follows:  $\text{Percentage of inhibition} = (1 - ((\Delta\text{Abs sample} - \Delta\text{Abs blank sample}) / (\Delta\text{Abs DMSO} - \Delta\text{Abs Blank DMSO}))) \times 100$ . Allopurinol was used as positive control.

### 3. Results

### 3.1. TLC profile of *B. pinnatum* hydroethanolic extract

Several spots corresponding to phenolic compounds were visualized on TLC under visible and UV light, some of them yellow colored after spraying with sulfuric vanillin followed by heating (Fig. A.1). The major spot has the same *R<sub>f</sub>* and color of a standard of flavonoid **1** available in the laboratory.

### 3.2. UPLC-DAD profile of *B. pinnatum* hydroethanolic extract

One major and five other peaks were observed at 340 nm on UPLC-DAD fingerprints of *B. pinnatum* hydroethanolic extract (Fig. 1). Their UV spectra are similar to those of quercetin and kaempferol (256 and 265 nm II band, and 355 nm I band, see Table A.2 and Fig. A.2). We hypothesized that the major peak corresponded to quercetin 3-*O*- $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- $\alpha$ -L-rhamnopyranoside **1**, reported in the literature as the major flavonoid glycoside of *B. pinnatum* [8,21]. This compound was chosen as a target for high-yield isolation, biological evaluation and as a potential marker for this species.

### 3.3. Flavonoids isolation and identification

A TLC of the extract side by side with a standard of flavonoid **1** was developed in order to determine its *R<sub>f</sub>*. Then, the spot putatively associated with this compound was selected for CPC experiment. This spot was chosen for *K<sub>d</sub>* estimation and selection of solvents system by shake-flask method. In a single run, four compounds were efficiently purified directly from the crude extract, all of them as amorphous yellow powder, with strong UV absorption (Fig.2, TLC and UPLC-DAD chromatograms available on Fig. A.3 and Fig. A.4). The process took two hours.

The compound corresponding to the major spot (peak 1, Fig.1) has the molecular formula C<sub>26</sub>H<sub>28</sub>O<sub>15</sub>, deduced from the pseudomolecular ion at *m/z* 603.1 [M + Na]<sup>+</sup> and *m/z* 579.1 [M-H]<sup>-</sup> on MS, in positive and negative mode, respectively. As expected, NMR data (Table A.3) are consistent with those of quercetin 3-*O*- $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- $\alpha$ -L-rhamnopyranoside [8,22].

One of the secondary compounds (peak 2, Fig.1) has the molecular formula C<sub>26</sub>H<sub>28</sub>O<sub>14</sub> deduced from the pseudomolecular ion at *m/z* 587.1 [M + Na]<sup>+</sup> (positive mode) and *m/z* 563.1 [M-H]<sup>-</sup> (negative mode) on MS. NMR data (Table A.3) are in agreement with those of kaempferol 3-*O*- $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- $\alpha$ -L-rhamnopyranoside, also known as kapinnatocide [8].

Another compound (peak 3, Fig.1) has the molecular formula C<sub>21</sub>H<sub>20</sub>O<sub>11</sub> deduced from the pseudomolecular ion at *m/z* 471.1 [M + Na]<sup>+</sup> (positive mode) and *m/z* 447.1 [M-H]<sup>-</sup> (negative mode) on MS. NMR data (Table A.3) are in agreement with those of quercetin 3-*O*- $\alpha$ -L-rhamnopyranoside (quercitrin) [8].

The metabolite associated with peak 4 (Fig.1) has the molecular formula C<sub>21</sub>H<sub>20</sub>O<sub>10</sub> deduced from the pseudomolecular ion at *m/z* 455.1 [M + Na]<sup>+</sup> (positive mode) and *m/z* 431.1 [M-H]<sup>-</sup> (negative mode) on MS. NMR data (Table A.3) are in agreement with those of kaempferol 3-*O*- $\alpha$ -L-rhamnopyranoside (afzelin) [23].

The injection of 2.0 g of extract provided 27.5 mg of **1**, 5.8 mg of **2**, 4 mg of **3** and 1 mg of **4**. The injection was repeated in the same conditions in order to isolate higher amounts of flavonoids. Same amounts of each compound were obtained.

### 3.4. Development and validation of method by UPLC-DAD for flavonoids quantification

Conditions already developed for HPLC-DAD for *B. pinnatum* [12] were optimized for UPLC. The method was performed using the Supelco Analytical HPLC Method Transfer Calculator (Sigma-Aldrich) software. Adaptations were done to establish a method capable of separating and evaluating the components of *B. pinnatum* hydroethanolic extract. The



obtained chromatogram using the most appropriate method (Fig. 1) showed symmetrical and resolved peaks, with a good baseline level and minimal tailing, when compared to the other systems. The method was validated for the quantification of the content of **1** (peak purity 98.3 %), **2** (peak purity 98.9 %) and **3** (peak purity 100 %). First, the method system suitability was assessed according to the parameters established in the USP [17] (Table 1). The system conformity was checked, according to the literature. Following RDC 166/2017 [16] the developed UPLC method was validated for selectivity, linearity, LoD and LoQ, repeatability and intermediary precision, accuracy and robustness.

#### 3.4.1. Selectivity

Selectivity was determined by analysis and comparison of chromatographic data (retention time and peak purity) and by no visible coelutions (shoulders) and very consistent DAD-spectra of *B. pinnatum* hydroethanolic extract with isolated compounds **1**, **2** and **3** in triplicate (Table 2). It should also be noticed that the UV spectra were also examined, showing no changes for the three evaluated peaks. Thus, the method was able to distinguish the analytes from the matrix.

#### 3.4.2. Linearity

The linearity of calibration curves was calculated by linear regression analysis of area versus solutions with seven points concentration levels of each flavonoid (**1**, **2** and **3**). The compounds were injected individually in triplicate and each point of calibration curves was the average of all three peak areas. The analysis generated a calibration curve for each isolated compound, whose equations were originated and the regression coefficients (*r*) were obtained, as summarized in Table 3. The *r* coefficient for each single compound was greater than 0.999 as described in RDC 166/2017, with RSD less than 5% for all points.

Linear regression was also applied to *B. pinnatum* hydroethanolic extract solutions at five different concentrations. The analysis generated a calibration curve for each retention time corresponding to each isolated compound (Table 4). The *r* coefficient for each peak evaluated was greater than 0.999 as described in RDC 166/2017, with RSD less than 5% for all points.

The values obtained with analysis of variance (ANOVA) showed that there are no significant differences between the different concentrations and the respective areas (*P*-value <0.05 and *F* > *F*<sub>critic</sub>), with a confidence interval of 95% ( $\alpha = 0.05$ ).

#### 3.4.3. LoD and LoQ

LoD and LoQ were estimated from mathematic models of analytical curves for each investigated flavonoid (Table 4).

#### 3.4.4. Precision

The precision of the method was assessed by RSD using peak areas of isolated compounds and showed a suitable range for each compound **1-3** (Table 3). Additionally, the repeatability was also performed for the *B. pinnatum* hydroethanolic extract, which presented RSD less than 5% for the three different extract concentrations, 1, 3 and 5 mg/mL, indicating the precision of the analytes (Table 4).

#### 3.4.5. Accuracy

To assess the accuracy of the method for complex matrices, known amount of isolated compounds were added to the extract solution. In this case, three different concentration levels (low, medium and high) were used. The percentage recoveries were calculated which were always higher than 80.757% (Table 3).

### 3.4.6. Robustness

The robustness of the analytical method was evaluated by triplicate injections of the extract with variations of mobile phase flow and column oven temperature (Fig. A.4). Although there was a slight variation in the retention time of the compounds, there was no change in system suitability parameters. Moreover, it is important to highlight that the decrease in flow practically did not change the retention time of the major peak but caused a delay in the elution of the other peaks. This was expected and due to the modification of the mobile phase viscosity and of the compound solubility.

### 3.5. Flavonoids quantification

The flavonoids **1-3** were quantified from the individual calibration curves determined for each compound (Table A.3). *B. pinnatum* hydroethanolic extract was evaluated in triplicate at five different concentrations (1, 2, 3, 4 and 5 mg/mL) and the averages of each peak areas were replaced by the y value of the linear regression equation to find the concentration (expressed in µg/mL) of the compound in the extract. After that, the compound amount in mg/g of extract was calculated (Table A.4). It is noteworthy that the RSD for the various concentrations of the analyzed extracts was below 5% indicating a minimum coefficient of variance in relation to the obtained mean. Regarding the linearity aspect of the developed analytical methodology, it is clear from the results that the method is in accordance with the specifications of the ANVISA regulations [16], since it was possible to observe the linear relation between the concentrations of the isolated compounds with the hydroethanolic extract. Proportions of **1**, **2** and **3** were respectively 2.43, 0.25 and 0.33 % (24.3 mg/g, 0.25 mg/g and 0.33 mg/g of extract) (Fig. 3).

### 3.6. DPPH and ABTS scavenging assay

DPPH and ABTS inhibition of *B. pinnatum* hydroethanolic extract at 200 µg/mL were 43.9% and 93.7%, respectively. IC<sub>50</sub> was then determined for extract and flavonoids **1-3** for DPPH scavenging activity as 247 µg/mL, 143 µM, 456 µM and 190 µM, respectively (Table 5).

### 3.7. Xanthine Oxidase Inhibition Assay

Percentages of inhibition of XO by *B. pinnatum* hydroethanolic extract at 50 and 100 µg/mL were 4.1 and 12.9, respectively (Table 5). Flavonoids **1-4** were evaluated at 200 µM; IC<sub>50</sub> were then determined for the most active derivatives **2** and **3** as 168 µM and 124 µM, respectively (Table 5).

## 4. Discussion

Alongside numerous indications in traditional medicines worldwide, *B. pinnatum* is registered as medicinal plant in several countries such as Germany, Switzerland and France [24]; in Brazil, it has been included in a Renisus [13]. In this context, studies dealing with safety, efficacy and quality of potential herbal medicine are strongly needed. In order to improve quality control, authentication and standardization, marker(s) has to be chosen and an analytical method for quantification must be validated.

In polar extracts of *B. pinnatum*, flavonoid glycosides have been identified as major metabolites. Among them, compounds **1** and **2** possess very common aglycons bearing the rare osidic moiety 3-*O*-α-L-arabinopyranosyl-(1→2)-*O*-α-L-rhamnopyranoside [25]. Except from *B. pinnatum*, **1** has only been reported from few species including *Alphitonia philippinensis* (Rhamnaceae) [22], while **2** has never been isolated and characterized from any other sources, except HPLC-MS detection from flowers of *Kalanchoe blossfeldiana* [26].

Moreover, both compounds have already demonstrated biological activities, such as anti-inflammatory [9,27] and leishmanicidal [8,24]. Flavonoids **1** and **2** may therefore be considered as specific analytical and/or active markers for *B. pinnatum* hydroethanolic extract. Quercitrin **3** and afzelin **4** are much more ubiquitous, mainly due to the large occurrence of 3-*O*-rhamnosyl transferase in comparison with 3-*O*-arabinosyl transferase [25].

Previous isolations of flavonoids from crude *B. pinnatum* extracts have been achieved by column chromatography or high-performance liquid chromatography [7-9,27]. These solid-liquid techniques are efficient but tedious, time- and solvent-consuming. In order to overcome these obstacles, we have decided to use CPC. This technique was already implemented for the quick fractionation of a methanolic extract of the same plant species, aiming to identify antimicrobial and cytotoxic properties [28]. Three complex fractions were obtained. The composition of one of them was tentatively identified by LC-UV and LC-MS/MS. Structures of flavonoids were proposed, but not confirmed by NMR data because no individual compounds were isolated. Determination of an appropriate solvents system is the key-step of CPC procedure. In reference [28], the system ethyl acetate-ethanol-water 3:1:3 v/v/v was selected and the experiment was run in descending ("reversed phase") mode. Indeed, considering the polar nature of the extract to partition, a mixture composed of water, a short-chain alcohol and a solvent of medium polarity immiscible with water (*n*-butanol or ethyl acetate) is generally efficient [29]. Nevertheless, our goal was not to fractionate the extract, but to isolate pure glycosides. To do so, it was necessary to run our experiment in ascending "normal phase" mode because in descending mode, due to their high affinity for the lower phase, the targeted flavonoids would not have been properly separated. Moreover, in order to make the *K<sub>d</sub>* of these compounds higher, the polarity of the mobile phase was reduced by increasing the proportion of ethyl acetate and by adding a low proportion of cyclohexane to the solvents mixture. All this also generated a quicker and more stable separation of the phases. Several systems have been tried and a satisfactory result was found with the combination cyclohexane-ethyl acetate-ethanol-water 0.5:9:3:5.5 v/v/v/v. This is the first report of CPC isolation of metabolites from this plant species.

Considering the potential health applications of some of the flavonoids and the fact that the industrial scale-up of the technique compatible with some principles of green chemistry (reduced solvents consumption, no polluting solid support such as silica gel) is available, this work highlights CPC as a choice technology and provides useful data for efficient recovery with low environmental impact of bioactive natural products, which are also highly relevant for quality assessment.

To the best of our knowledge, there were no studies dedicated to validation of analytical method for flavonoids quantification of *B. pinnatum* hydroethanolic extract. Some papers have reported the quantification of major flavonoids in the aqueous extract of the species, especially of compounds **1** and **3** by HPLC-DAD; however, the results were expressed in terms of rutin using a correction factor of molecular weight (MM) (correction factor=MM<sub>flavonoid</sub>/MM<sub>rutin</sub>) or quercitrin was used to build the analytical curves [21,30]. In this context, this work was the first to validate an UPLC-DAD analytical method for the simultaneous quantification of the three major flavonoids **1-3** of the hydroethanolic extract. The validation parameters are fundamental for evaluation of herbal medicine quality.

The quantification values can be compared with previous studies [21,30]. In one of them [30], plant material collected at different seasons under the sun or in the shadow was extracted with water at 50°C. The best yield was obtained for specimens cultivated under the sun, during the summer: **1** was quantified at 2.02%, **2** at 0.068% and **3** at 0.241%, quite similar to our results, that are 2.433%, 0.249% and 0.333%, respectively. Several factors may interfere with metabolite levels in a plant species and explain the tiny differences. In this case, the period and place of collection can be mentioned, as well as the extraction method and

solvent (hot water at a plant-solvent ratio of 1:5 w/v for [30], 50% hydroethanolic extract at 1:1 w/v in our case).

In another report, plant material was freeze-dried prior to the extraction and extracted with water; the methodology was optimized to obtain the highest yield of **1** by changing the temperature and extraction time [21]. In our study, a simple turbolysis with 50% EtOH for 5 min directly on fresh leaves at room temperature has provided 24.3 mg of **1** per g of extract, suggesting that our protocol is also suitable for obtainment of flavonoid glycosides.

As **1** is an unusual and strongly bioactive flavonoid found almost only in *B. pinnatum*, our results confirm that this compound is a specific marker for this species, as previously mentioned [21,30].

Inflammation is a complex physiological process where numerous pathways are involved. As many common uses of *B. pinnatum* deal with the field of inflammation, some evaluations aiming to validate these traditional indications have already been reported for leaves and flowers polar extracts [7,9,10-12]. For example, aqueous extract of flowers and flavonoid **1** have reduced TNF $\alpha$  concentration and have inhibited COX-1 and COX-2 [9]. Recently, the same compound has demonstrated to be a selective PDE4B inhibitor [27]. In order to further investigate the anti-inflammatory mechanism of action of this species, the XO inhibitory activity of *B. pinnatum* hydroethanolic extract and of the isolated flavonoids was evaluated. The activity of the extract and of compound **1** were low, while the IC<sub>50</sub> of compounds **2** and **3** were calculated as 168  $\mu$ M and 124  $\mu$ M, moderate when compared with standard drug allupurinol (Table 5). Furthermore, *B. pinnatum* hydroethanolic extract mainly through quercetin glycosides, has also shown DPPH and ABTS scavenging activity (Table 5).

## 5. Conclusions

Four flavonoids glycosides were efficiently isolated from *B. pinnatum* hydroethanolic extract using CPC. Considering that this technique is available for industrial production, the plant species, which is easy to cultivate, should be regarded as a source of commercially exploitable natural products with antioxidant properties. This paper reports also the development, validation and application of an UPLC-DAD method for direct and simultaneous detection and quantification of three flavonoids in this extract. The efficiency of the chromatographic system was proven and the peaks were well separated in the chromatogram. Therefore, based on the obtained results, the developed UPLC-DAD method can be an important tool for the quality assessment of raw material and herbal preparations, given that *B. pinnatum* is widely used as medicinal plant in several countries.

## Conflict of interest

The authors declare no conflict of interest.

## Author Contributions

**Júlia Fernandes:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data-Curation, Writing-Original Draft, Visualization. **Sergio Ortiz:** Biological Methodology, Biological Data-Curation. **Raquel Tavares:** Methodology. **Edilane Araújo:** Methodology. **Tsvetelina Mandova:** Methodology. **Anderson Andrade:** Methodology. **Sylvie Michel:** Resources. **Raphaël Grougnet:** Conceptualization, Methodology, Validation, Resources, Supervision, Writing-Review & Editing. **Silvana Zucolotto:** Conceptualization, Methodology, Investigation, Resources, Writing-Review & Editing, Supervision, Visualization, Project administration, Funding acquisition.

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## 21 Table Legends

23 **Table 1.** System suitability parameters for each flavonoid in the *B. pinnatum* hydroethanolic  
24 extract. Mean  $\pm$  SD for accuracy; n = 5. R: resolution; t: tailing factor; N: theoretical plates ; k':  
25 capacity factor.

27 **Table 2.** Selectivity parameters evaluated for each flavonoid in the *B. pinnatum*  
28 hydroethanolic extract. Mean  $\pm$  SD for accuracy; n = 5.

30 **Table 3.** UPLC-DAD method analytical parameters for each flavonoid in *B. pinnatum*  
31 hydroethanolic extract. Mean  $\pm$  SD for accuracy; n= 7 for linear range, limit of detection and  
32 intermediate precision, n=3 for accuracy and limit of quantification. LoD: limit of detection;  
33 LoQ: limit of quantification.

35 **Table 4.** UPLC-DAD method analytical parameters for each flavonoid in *B. pinnatum*  
36 hydroethanolic extract. Mean  $\pm$  SD for accuracy; n = 5 for linear range and repeatability.

38 **Table 5.** IC<sub>50</sub> of *in vitro* activities of *B. pinnatum* hydroethanolic extract and isolated  
39 flavonoids.

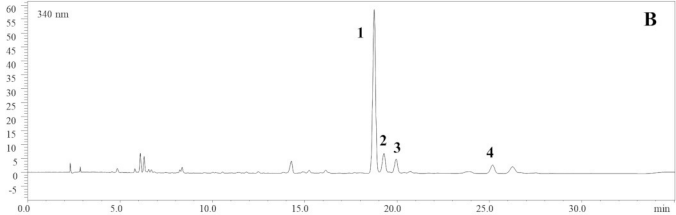
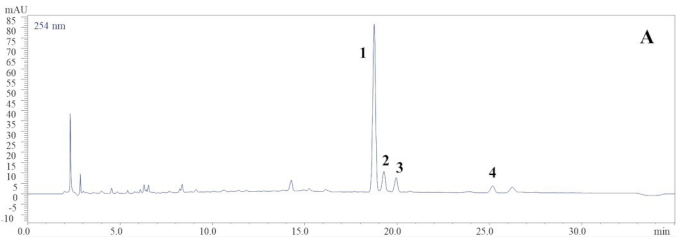
## 41 Figures Legends

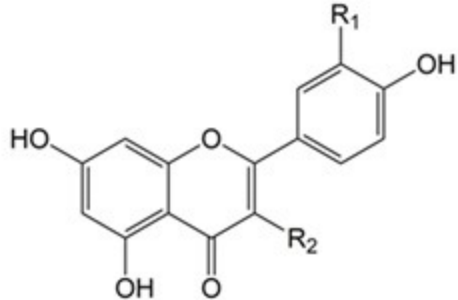
43 **Fig. 1.** UPLC-DAD chromatographic profile of *B. pinnatum* hydroethanolic extract.  
44 *B. pinnatum* hydroethanolic extract was chromatographed on a Phenomenex Kinetex RP-18  
45 column (150 x 4.6 mm, 2.6  $\mu$ m) and the column was eluted with a gradient of acetonitrile  
46 (ACN) and 0,3% TFA at a flow rate of 0.7 mL.min<sup>-1</sup>. The elution profile was monitored at  
47 (A) 254 nm and (B) 340 nm.

49 **Fig. 2.** Flavonoids isolated by CPC from *B. pinnatum* hydroethanolic extract.

1    **Fig. 3.** Concentration of isolated flavonoids in *B. pinnatum* hydroethanolic extract.







Flavonoid	R <sub>1</sub>	R <sub>2</sub>
1	H	O- $\alpha$ -L-rhamnopyranoside
2	OH	O- $\alpha$ -L-rhamnopyranoside
3	H	O- $\alpha$ -L-arabinopyranosyl (1 $\rightarrow$ 2) $\alpha$ -L-rhamnopyranoside
4	OH	O- $\alpha$ -L-arabinopyranosyl (1 $\rightarrow$ 2) $\alpha$ -L-rhamnopyranoside

Concentration (%)

2.433



0.249



0.330



1

2

3

1 **Table 1.** System suitability parameters of *B. pinnatum* hydroethanolic extract.

Flavonoid	R	t	N	k'
1	1.767 ± 0.037	1.426 ± 0.602	51218.33 ± 429.712	7.520 ± 0.278
2	2.114 ± 0.032	1.213 ± 0.035	39904.33 ± 304.733	7.869 ± 0.288
3	2.820 ± 0.041	1.057 ± 0.005	48178 ± 356.215	10.545 ± 0.378

2 Mean ± SD for accuracy; n = 5. R: resolution; t: tailing factor; N: theoretical plates ; k': capacity factor.

3

4

5 **Table 2.** Selectivity parameters evaluated for *B. pinnatum* hydroethanolic extract.

Flavonoid	Peak purity	Retention time (Rt)
1	0.98 ± 0.01	19.20 ± 0.26
2	0.95 ± 0.005	19.87 ± 0.28
3	Not calculated	25.58 ± 0.20

6 Mean ± SD for accuracy; n = 5.

7

1 **Table 3.** UPLC-DAD method analytical parameters for each flavonoid in *B. pinnatum* hydroethanolic extract.

Flavonoid	RT (min)	Curve calibration	Linear range (µg.mL <sup>-1</sup> )	r	LoD (ng.g <sup>-1</sup> )	LoQ (ng.g <sup>-1</sup> )		
<b>1</b>	19.20	y = 463320x - 11163	8.75-6.25	0,9997	0,00871	0,0263		
<b>2</b>	19.87	y = 91121x – 789,67	1.25-8.75	0,9996	0,7442	2,2553		
<b>3</b>	25.58	y = 21351x + 199,57	0.75-5.25	0,9997	1,984	6,012		
Flavonoid	Repeatability (%RSD)			Intermediate precision (%RSD)			Accuracy (%)	
	Low level	Medium level	High level	Low level	Medium level	High level	Low level	High level
<b>1</b>	0.985	0.199	0.477	3.581	3.342	0.558	102.387	83.131
<b>2</b>	1.109	2.253	0.382	1.742	1.090	1.024	109.186	80.757
<b>3</b>	2.224	2.628	1.707	3.465	3.314	1.445	99.344	134.985

2 Mean  $\pm$  SD for accuracy; n= 7 for linear range, limit of detection and intermediate precision, n = 3 for accuracy and limit of quantification.

3 LoD: limit of detection; LoQ: limit of quantification.

4

5

6 **Table 4.** UPLC-DAD method analytical parameters for each flavonoid in *B. pinnatum* hydroethanolic extract.

Flavonoid	RT (min)	Curve calibration	r	Repeatability (%RSD)		
				Low level	Medium level	High level
<b>1</b>	19.20	$y = 308793 - 19685$	0.9994	0.159	0.160	0.138
<b>2</b>	19.87	$y = 45227x - 382.27$	0.9997	1.192	1.610	0.038
<b>3</b>	25.58	$y = 23122x + 1113.2$	0.9996	1.097	0.131	0.384

7 Data obtained through the analysis of peak areas of compounds 1-3. Mean  $\pm$  SD for accuracy; n = 5 for linear range and repeatability.

8

1 **Table 5.** IC<sub>50</sub> of *in vitro* activities of *B. pinnatum* hydroethanolic extract and isolated flavonoids.  
2

Sample	Xanthine oxidase	DPPH
Extract	> 200 µg/mL	247 µg/mL
Flavonoid 1	> 200 µg/mL	143 µM
Flavonoid 2	168 µM	456 µM
Flavonoid 3	124 µM	190 µM
Control	3.5 µM (Alluporinol)	29.4 µM (Gallic acid)

3 Controls – Xanthine oxidase: allopurinol; DPPH: gallic acid.



*Bryophyllum pinnatum*



HE extract

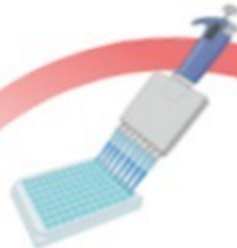


CPC isolation



Method validation of flavonoids  
quantification by UPLC-DAD

In vitro  
activities



Xanthine oxidase  
inhibitory activity

Antioxidant capacity  
DPPH  
ABTS

