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

3  
4 Júlia Morais Fernandes<sup>a</sup>, Sergio Ortiz<sup>b</sup>, Raquel Padilha M. Tavares<sup>a</sup>, Tsvetelina Mandova<sup>b,c</sup>,  
5 Edilane Rodrigues D. Araújo<sup>a</sup>, Anderson Wilbur L. Andrade<sup>a</sup>, Sylvie Michel<sup>b</sup>, Raphaël  
6 Grougnet<sup>b,\*</sup>, Silvana Maria Zucolotto<sup>a,\*</sup>

7  
8 <sup>a</sup> Grupo de Pesquisa em Produtos Naturais Bioativos, Departamento de Farmácia,  
9 Universidade Federal do Rio Grande do Norte, 59.012-570 Natal, Brazil.

10 <sup>b</sup> Equipe Produits naturels, analyse, synthèse, UMR CNRS 8038, Faculté de Pharmacie,  
11 Université Paris Descartes/Université de Paris, 75006 Paris, France

12 <sup>c</sup> Gilson Purification SAS, Saint-Avé, France

13  
14 \*Corresponding authors

15 Raphaël Grougnet, UMR CNRS 8038, Faculty of Pharmacy, Paris Descartes University,  
16 4 avenue de l'Observatoire, 75006 Paris – France

17 Tel: +3353739806

18 Email: raphael.grougnet@parisdescartes.fr

19  
20 Silvana Maria Zucolotto, Grupo de Pesquisa em Produtos Naturais Bioativos, Departamento  
21 de Farmácia, Universidade Federal do Rio Grande do Norte, 59.012-570 Natal, Brazil.

22 Tel: +558432429818

23 Email: szucolotto@hotmail.com

24  
25  
26 **Abbreviations:**

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

39  
40 **1. Introduction**

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

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

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

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

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

32 Centrifugal partition chromatography (CPC) is a preparative support-free liquid-liquid  
33 separation technique, based on the difference in partitioning behavior of analytes between two  
34 immiscible phases. Due to the absence of any solid support, it provides many advantages over  
35 conventional liquid chromatographic techniques such as high selectivity through the infinite  
36 combination of biphasic solvent systems, high loading capacity, total recovery of the loaded  
37 sample without any risk of degradation, low solvent consumption and easy scale-up.  
38 Furthermore, the growing concern for lowering the environmental impact of purification  
39 processes makes CPC an attractive alternative, especially for high-value added compounds  
40 [14].

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

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

## 7 8 **2. Materials and Methods**

### 9 10 *2.1. Plant Material*

11 *Bryophyllum pinnatum* leaves were collected in Macaíba city, in the State of Rio  
12 Grande do Norte, Brazil, at coordinates 06° 05' 40" S 38° 22' 29" W, in January 2017. The  
13 botanical identification was done by Dr. Rúbia Santos Fonseca and a voucher specimen (n°  
14 57335) was deposited at the Herbarium Prisco Bezerra of the Universidade Federal do Ceará,  
15 Brazil.

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

### 20 21 *2.2. Preparation of B. pinnatum crude extract*

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

### 27 28 *2.3. Conditions for TLC and UPLC-DAD analysis*

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

### 48 49 *2.4. CPC purification procedure*

50

#### 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,

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

#### 8 9 2.6.1. Selectivity/Specificity

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

#### 14 15 2.6.2. Linearity

16 Calibration curves with seven levels of concentration (175% to 25%) were plotted for  
17 each isolated compound (**1**, **2** and **3**), as well as area *versus* concentration for linearity  
18 evaluation. The linearity of the regression equation determined for each compound was  
19 investigated by means of the parameters correlation coefficient ( $r$ ). Stock solutions (70  $\mu\text{g/mL}$   
20 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  
21 the following concentrations: 8.75, 17.5, 26.25, 35.0, 43.75, 52.5 and 61.25  $\mu\text{g/mL}$  for  
22 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,  
23 3.0, 3.75, 4.5 and 5.25  $\mu\text{g/mL}$  for flavonoid **3**. Each solution was analyzed in triplicate.  
24 Additionally, the linearity was also investigated for *B. pinnatum* hydroethanolic extract. A  
25 calibration curve based on five levels of concentration of hydroethanolic extract (1.0, 2.0, 3.0,  
26 4.0 and 5.0 mg/mL) was obtained. Each solution was prepared individually by dilution (w/v)  
27 of extract in MeOH-H<sub>2</sub>O (1:1, v/v). Peaks areas at 340 nm were plotted against the known  
28 concentrations of extract solutions for each peak concerning isolated compounds **1**, **2** and **3**, in  
29 order to establish the linearity of the regression equation for each peak compound of extract  
30 and to investigate the correlation coefficient ( $r$ ) for each one.

#### 31 32 2.6.3. LoD and LoQ

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

#### 35 36 2.6.4. Precision

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

#### 46 47 48 2.6.5. Accuracy

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

1 concentration (2 mg/mL). The concentrations of isolated compounds used were low, medium  
2 and high with three replicates injection for each level contemplating the linear interval of the  
3 analytical method. Accuracy was expressed as the percentage and calculated by equation:  
4 Accuracy (%) = [(Peak area<sub>Extract+compound</sub> – Peak area<sub>Extract</sub>)/Peak area<sub>compound</sub>] × 100.

#### 6 2.6.6. Ruggedness

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

#### 10 2.7. DPPH scavenging assay

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

#### 21 2.8. ABTS Scavenging

22 Radical scavenging capacity was measured using ABTS reagent (Sigma Aldrich)  
23 according to previous report [19]. ABTS reagent was prepared as follows: 10 mL of 7 mM of  
24 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).  
25 The final solution was diluted with distilled H<sub>2</sub>O (1:20) to reach an absorbance of 0.70 ± 0.02  
26 before the assay. The extract was evaluated at the final concentration of 200 µg/mL and IC<sub>50</sub>  
27 was determined using a range of final concentrations of 400-10 µg/mL. In each well were  
28 placed 100 µL ABTS final solution and 50 µL of sample. Plates were incubated at room  
29 temperature for 10 min, away from light. The absorbance was measured at 734 nm. Measures  
30 were performed in triplicate. Quercetin was used as positive control. Results are expressed as  
31 percentage of efficacy at 200 µg/mL or as IC<sub>50</sub>.

#### 33 2.9. Xanthine Oxidase Inhibition Assay

34 Xanthine oxidase (XO) inhibition assay was performed using the modified protocol  
35 from [20]. Briefly, in a quartz 96-well plate, 62.5 µL of extract solution at the concentration  
36 of 800 µg/mL was added of 105 µL phosphate buffer (0.12 mM, pH 7.5) and 20 µL of XO  
37 (Sigma-Aldrich<sup>®</sup>) solution (0.125 units/mL in phosphate buffer) and then incubated at room  
38 temperature in the dark for 5 min. For IC<sub>50</sub> determinations, a range of concentrations of 400-  
39 10 µg/mL of each compound were used, 62.5 µL of xanthine (Sigma-Aldrich<sup>®</sup>) solution (200  
40 µM in phosphate buffer) were added in each well and the absorbance was measured at 295 nm  
41 during 30 min (final DMSO concentration of 0.4 %). Negative controls were carried out,  
42 replacing XO solution by 62.5 µL of phosphate buffer. Initial rate was calculated and the  
43 percentage of inhibition was determined as follows: Percentage of inhibition = (1-((ΔAbs  
44 sample – ΔAbs blank sample)/(ΔAbs DMSO – ΔAbs Blank DMSO))) x 100. Allopurinol was  
45 used as positive control.

### 47 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



1 obtained chromatogram using the most appropriate method (Fig. 1) showed symmetrical and  
2 resolved peaks, with a good baseline level and minimal tailing, when compared to the other  
3 systems. The method was validated for the quantification of the content of **1** (peak purity 98.3  
4 %), **2** (peak purity 98.9 %) and **3** (peak purity 100 %). First, the method system suitability  
5 was assessed according to the parameters established in the USP [17] (Table 1). The system  
6 conformity was checked, according to the literature. Following RDC 166/2017 [16] the  
7 developed UPLC method was validated for selectivity, linearity, LoD and LoQ, repeatability  
8 and intermediary precision, accuracy and robustness.

#### 9 10 3.4.1. Selectivity

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

#### 17 18 3.4.2. Linearity

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

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

31 The values obtained with analysis of variance (ANOVA) showed that there are no  
32 significant differences between the different concentrations and the respective areas ( $P$ -value  
33  $<0.05$  and  $F > F_{critic}$ ), with a confidence interval of 95% ( $\alpha = 0.05$ ).

#### 34 35 3.4.3. LoD and LoQ

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

#### 38 39 3.4.4. Precision

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

#### 45 46 3.4.5. Accuracy

47 To assess the accuracy of the method for complex matrices, known amount of isolated  
48 compounds were added to the extract solution. In this case, three different concentration  
49 levels (low, medium and high) were used. The percentage recoveries were calculated which  
50 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  $\mu\text{g/mL}$ ) 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  $\mu\text{g/mL}$  were 43.9% and 93.7%, respectively.  $\text{IC}_{50}$  was then determined for extract and flavonoids **1-3** for DPPH scavenging activity as 247  $\mu\text{g/mL}$ , 143  $\mu\text{M}$ , 456  $\mu\text{M}$  and 190  $\mu\text{M}$ , respectively (Table 5).

### 3.7. Xanthine Oxidase Inhibition Assay

Percentages of inhibition of XO by *B. pinnatum* hydroethanolic extract at 50 and 100  $\mu\text{g/mL}$  were 4.1 and 12.9, respectively (Table 5). Flavonoids **1-4** were evaluated at 200  $\mu\text{M}$ ;  $\text{IC}_{50}$  were then determined for the most active derivatives **2** and **3** as 168  $\mu\text{M}$  and 124  $\mu\text{M}$ , 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 Rénisus [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*- $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- $\alpha$ -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].

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

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

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

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

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

1 solvent (hot water at a plant-solvent ratio of 1:5 w/v for [30], 50% hydroethanolic extract at  
2 1:1 w/v in our case).

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

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

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

## 23 24 **5. Conclusions**

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

## 35 36 **Conflict of interest**

37 The authors declare no conflict of interest.

## 38 39 **Author Contributions**

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

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- 19  
20

## 21 Table Legends

22  
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.

26  
27 **Table 2.** Selectivity parameters evaluated for each flavonoid in the *B. pinnatum*  
28 hydroethanolic extract. Mean  $\pm$  SD for accuracy; n = 5.

29  
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.

34  
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.

37  
38 **Table 5.** IC<sub>50</sub> of *in vitro* activities of *B. pinnatum* hydroethanolic extract and isolated  
39 flavonoids.

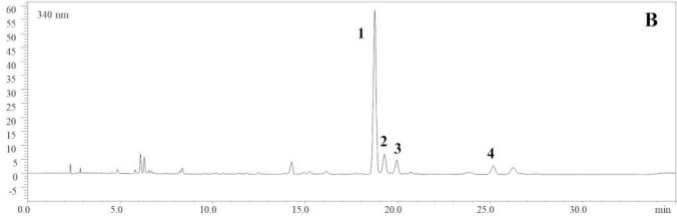
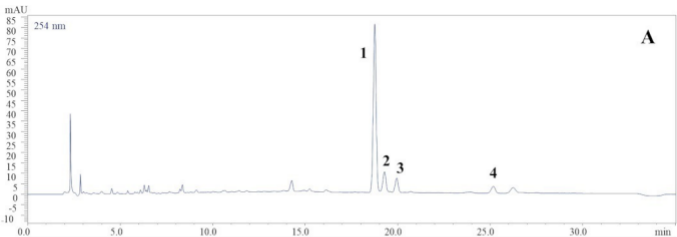
## 40 Figures Legends

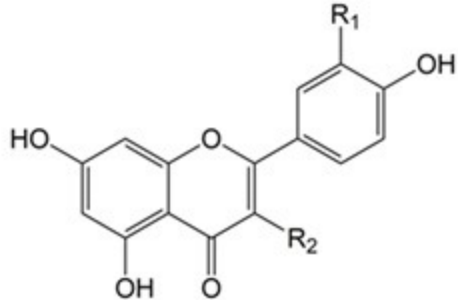
41  
42  
43 **Fig. 1.** UPLC-DAD chromatographic profile of *B. pinnatum* hydroethanolic extract.  
44 *B. pinnatum* hydroethanolic extract was chromatographed on a Phenomenex Kinetex RP-18  
45 column (150 x 4.6 mm, 2.6  $\mu$ m) and the column was eluted with a gradient of acetonitrile  
46 (ACN) 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.

48  
49 **Fig. 2.** Flavonoids isolated by CPC from *B. pinnatum* hydroethanolic extract.

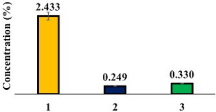
1 **Fig. 3.** Concentration of isolated flavonoids in *B. pinnatum* hydroethanolic extract.







| 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 |



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

| <b>Flavonoid</b> | <b>R</b>      | <b>t</b>      | <b>N</b>           | <b>k'</b>      |
|------------------|---------------|---------------|--------------------|----------------|
| <b>1</b>         | 1.767 ± 0.037 | 1.426 ± 0.602 | 51218.33 ± 429.712 | 7.520 ± 0.278  |
| <b>2</b>         | 2.114 ± 0.032 | 1.213 ± 0.035 | 39904.33 ± 304.733 | 7.869 ± 0.288  |
| <b>3</b>         | 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.

| <b>Flavonoid</b> | <b>Peak purity</b> | <b>Retention time (Rt)</b> |
|------------------|--------------------|----------------------------|
| <b>1</b>         | 0.98 ± 0.01        | 19.20 ± 0.26               |
| <b>2</b>         | 0.95 ± 0.005       | 19.87 ± 0.28               |
| <b>3</b>         | 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 ( $\mu\text{g}\cdot\text{mL}^{-1}$ ) | r                             | LoD ( $\text{ng}\cdot\text{g}^{-1}$ ) | LoQ ( $\text{ng}\cdot\text{g}^{-1}$ ) |              |              |            |
|-----------|----------------------|-----------------------|---|-------------------------------|---------------------------------------|---------------------------------------|--------------|--------------|------------|
| <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    | Medium level | High level |
| <b>1</b>  | 0.985                | 0.199                 | 0.477   | 3.581                         | 3.342                                 | 0.558                                 | 102.387      | 98.443       | 83.131     |
| <b>2</b>  | 1.109                | 2.253                 | 0.382   | 1.742                         | 1.090                                 | 1.024                                 | 109.186      | 110.595      | 80.757     |
| <b>3</b>  | 2.224                | 2.628                 | 1.707   | 3.465                         | 3.314                                 | 1.445                                 | 99.344       | 110.847      | 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.** IC50 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<br>(Alluporinol) | 29.4 µM<br>(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

