**Passiflora mucronata** leaves extracts obtained from different methodologies: a phytochemical study based on cytotoxic and apoptosis activities of triterpenes and phytosterols constituents

Isabel Cristina Vieira da Silva¹,³, Pollyana Felix de Oliveira¹,², Gleyce Moreno Barbosa³, Ludger A. Wessjohann⁴, Lucio Cardozo-Filho⁵, Carla Holandino³, Michelle Frazão Muzitano²,³, Ivana Correa Ramos Leal¹,³*

¹Pharmacy Faculty, Natural Products and Food Department, Laboratory of Natural Products and Biological Assays (LaProNEB), Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil, ²Pharmacy Faculty, Bioactive Products Laboratory, Federal University of Rio de Janeiro Campus Macaé, RJ, Brazil, ³Pharmacy Faculty, Pharmaceutical Sciences Post-graduate Programme, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil, ⁴Bioorganic Chemistry Department, Leibniz Institute of Plant Biochemistry, IPB, Weinberg 3, Halle an der Saale, Germany, ⁵Centro Universitário Fundação de Ensino Octávio Bastos, São João da Boa Vista-SP

Cancer is one of the most prevalent diseases worldwide and the natural products could be a source of bioactive compounds. *Passiflora mucronata* (PM) belongs to a very known vegetal genus, although, there are no studies about cytotoxic activity or isolated compounds. Different extracts from PM were obtained by liquid-liquid partition (P), Soxhlet (Sox) and supercritical fluid (SFE1-5) extraction techniques, being compared concerning their yields, chemical profile and cytotoxicity. The Sox extracts showed the highest yields (6.03%: hexane; 2.51%: dichloromethane) followed by SFE (from 4.34 to 1.63%) and partitions (1.06 and 2.26%). The hexane partition (HP) showed the best cytotoxic activity against K562 cell line (IC₅₀ = 18.72 µg.mL⁻¹). From HP, the following compounds were identified and analysed its cytotoxic activities: β-amyrin (IC₅₀ = 3.92 µg.mL⁻¹), β-sitosterol (IC₅₀ = 3.37 µg.mL⁻¹), stigmasterol (IC₅₀ = 3.31 µg.mL⁻¹) and oleanolic acid. Stigmasterol induced about 75% of K562 total apoptosis. The compounds were tested against MA-104 cell line and the selective index (SI) attributed (SI >10 for all compounds). This indicates good selectivity to K562 cell line at the expense of MA-104. This is the first time, identifying those compounds to PM.

Keywords: Apoptosis. Cytotoxic. *Passiflora mucronata*. Stigmasterol. Triterpenes.

**INTRODUCTION**

Natural products such as polyphenols, alkaloids and terpenes are important sources of compounds able to treat several diseases due to their diverse pharmacological properties, including cytotoxic and cancer chemo prevents disturbances effects (De Almeida et al., 2013). The vast diversity of the Brazilian flora stimulates the exploration of plant extracts with medical and biological activities (Rennó et al., 2008) including with anticancer properties. Cancer is one of the most prevalent diseases worldwide in which, in accordance to WHO, 8.2 millions of people die annually. In addition, there are expected 70% of new cases of cancer worldwide in the next two decades (World Health Organization, 2017). In Brazil, in 2015, the estimative showed that it has occurred about 576,000 of new cases, including 5,050 new registers for leukemia in men and, 4,320 registers in women (Instituto Nacional do Cancer, 2016).

The vegetal species *Passiflora mucronata* belongs to the Passifloraceae family and it is known in Brazil as Maracujá Mirim. Its chemical compounds have never been studied before, besides the genus has hardly never been investigated in the scientific literature. Researches

---

*Correspondence: I. C. R. Leal. Laboratório de Produtos Naturais e Ensaios Biológicos (LaProNEB), Departamento de Produtos Naturais e Alimentos, Faculdade de Farmácia – Programa de Pós-graduação em Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, 21941-902, Rio de Janeiro, RJ, Brazil. Phone: + 55 21 39386422. E-mail: ivanafarma@yahoo.com.br
focusing on species from the genus *Passiflora* described the presence of flavonoids, alkaloids, essential oils and glycosylated triterpenes (Dhawan, Dhawan, Sharma, 2004; Ingale, Hivrale, 2010; Otsuka et al., 2010; Wang et al., 2013) that are important to the anxiolytic, sedative and diuretic properties commonly observed in *Passiflora* species. A highly important chemical class is the triterpenes, mainly because of their medicinal properties. Different kinds of triterpenes are correlated to anti-cancer activities, such as the pentacyclic skeletons (de Almeida et al., 2013; Hao et al., 2013). Moreover, the research for new anti-cancer drugs shows that natural pentacyclic triterpenes rarely exhibit severe side effects (Lemos et al., 2012). However, for extracting and isolating different secondary metabolites, including triterpenes, it is hardly necessary to use large amounts of solvents, especially by the conventional extraction methodologies, such as Soxhlet and maceration. These techniques are considerably time-consuming as well as are not environmentally friendly, so, by this reason, it is important to search for new alternatives, which require small amounts of solvents or do not use it in any step. The Supercritical Fluid Extraction (SFE) is an interesting method that can be conducted free of organic solvents or, with minor amounts (co-solvents). This extraction technique is influenced mainly by the following parameters: temperature, pressure, solvent flow rate and solubility of the compound of interest (Reverchon, De Marco, 2006; Meireles, Angela, 2009). By this technique, carbon dioxide is one of the most used solvent since it is environmentally safe, non-explosive, and, it can be easily removed from the final desired products (De Melo, Silvestre, Silva, 2014).

The aim of the present study was to compare *Passiflora mucronata* leaves extracts, obtained by different extractive methodologies, including maceration, Soxhlet and Supercritical Fluid, that had never been studied before and are rich in triterpenes and phytosteroids. Extracts were compared regarding their yields and analyzed by Gas Chromatography coupled to Mass Spectrometry (GC-MS) in order to investigate their chemical profiles concerning the secondary metabolites. The viability and the metabolism of myeloid leukemia cell line (K562) in culture, as well as the selective index (on MA-10⁴ cell line) were also investigated for the fractions. The most active extract was submitted to a chromatographic purification step followed by the cytotoxic activity investigation aiming to isolate the bioactive compounds and, to evaluate the apoptosis behavior of the most active by flow cytometry.

**MATERIAL AND METHODS**

**Plant material**

The vegetal species *P. mucronata* was collected in July 2013 on the Jurubatiba’s Shoal, located in the Northeastern of the Rio de Janeiro City, in Quissamã, RJ, Brazil. A voucher specimen was deposited in the Herbarium of the Biological Science Institute, located at the Federal University of Rio de Janeiro under the number RFA38758. The leaves were left at room temperature, in darkness, until dryness (by seven days). The dried leaves were crushed and then sieved in a 20-30 mesh sieve. The powder was finally separated for Maceration, Soxhlet and Supercritical fluid extractions.

**Extractive methods**

**Maceration**

The dried and triturated leaves (50.4 g) were macerated by using ethanol:water (9:1), by three days, with renewal of the solvent. After one week, the hydroalcoholic solution was concentrated by vacuum in a rotatory evaporator (Fisatom®), and, the crude extract obtained (CE) (7.42 g) was frozen and lyophilized (Eppendorf®). A total of 6.96 g of the CE was solubilized in 160 mL of methanol: water (9:1) and, the solution was partitioned with the following solvents (Tedia®) to furnish the correspondent partitions: hexane (HP), dichloromethane (DP), ethyl acetate (EAP), butanol (BP) and the residual aqueous partition (RAP). The yields obtained, applying each solvent as extractor, were expressed as percentage in relation to the initial mass of the vegetal material.

**Soxhlet (Sox)**

The extraction was also performed by using the Soxhlet (Sox) apparatus with 5.87 g of the dried triturated leaves. The leaves were deposited inside the glass balloon with hexane or dichloromethane (Tedia®), giving the correspondent fractions: HSox and DSox, respectively. The yields obtained were expressed as percentage in relation to the initial dry weight of the leaves wrapped.

**Supercritical fluid (SF)**

Considering that the hexane (HP) and the dichloromethane (DP) partitions presented the highest cytotoxic activity against K562 cell (item 3.4.1), the SFE technique was selected as an alternative green extraction method. For this procedure, CO₂ was selected as gas carrier system (Zuculotto et al., 2012) considering the...
more nonpolar characteristic of the bioactive partitions (HP and DP). This experiment was performed on a bench scale unit, gathering a CO$_2$ cylinder (Air liquid 95% purity), two thermostatic baths, two syringe pumps (ISCO, Model 500D) and one extractor with internal volume of approximately 170 cm$^3$. There were applied pressures between 15.0 MPa and 20.0 MPa and, temperatures from 298.15K to 318.15K (Table I). The extractor was evenly fed with 10 g of the triturated and dried leaves, approximately, for each sample to be analyzed. The remaining extraction cell space was filled with glass beads (inert bed). After reaching the desired temperature, the pump and the extractor were simultaneously pressurized. After reaching the pressure, the system was left at equilibrium by 30 min and the extractions were performed by 120 min. These set parameters were based on previous works for isolation of terpenoid compounds (Lemos et al., 2012). This procedure originated five different samples that were obtained from different pressures and temperatures conditions, as we can see in Table I.

**TABLE I - Supercritical fluid extraction conditions for *P. mucronata* leaves**

<table>
<thead>
<tr>
<th>Supercritical Fluid Extract (SFE) codes</th>
<th>Temperature (K)/Pressure (MPa)</th>
<th>Density of CO$_2$ (kg/m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFE 1</td>
<td>298/15</td>
<td>877.31</td>
</tr>
<tr>
<td>SFE 2</td>
<td>318/15</td>
<td>742.55</td>
</tr>
<tr>
<td>SFE 3</td>
<td>308/18.5</td>
<td>853.28</td>
</tr>
<tr>
<td>SFE 4</td>
<td>298/22</td>
<td>927.39</td>
</tr>
<tr>
<td>SFE 5</td>
<td>318/22</td>
<td>833.21</td>
</tr>
</tbody>
</table>

**Purification of the hexane partition (HP)**

Considering that the hexane partition (HP) presented the best cytotoxic activity it was selected for further purification step by using classical chromatographic column. The HP (501.6 mg) was solubilized in ethanol (few volume) and then mixed to 100 mg of silica (Silicycle-ultrapure silica gel-Siliaflash® GCO:70-230 mesh) in order to obtain a pastille. The mixture was homogenized and conducted to a rotatory evaporator until complete drying and powder formation. The pastille was then transferred to a column full filled with a normal silica gel (L=80 cm; d=2 cm). The mobile phase adopted was constituted by two gradients of solvents (hexane and ethyl acetate) with a flow rate of 0.5 mL.min$^{-1}$.

**Chemical profile analysis: Gas chromatography coupled to mass spectrometry**

The chemical profile of the extracts was investigated by the GC-MS equipment. The GC-MS analyses was performed in a Shimadzu 2010 apparatus with interface GCMS-QP2010 and electronic impact 70 eV. The column used was RTx-5Ms (L=30 m; d=0.25 µm). The GC conditions adopted were: helium as carrier gas at 1.2 mL.min$^{-1}$ and Split injection rate of 1:40. The temperature ramp was programmed as follows: from 60 °C (1 min) to 290 °C in a speed rate of 10 °C.min$^{-1}$, remaining for added 16 min at the end. The total run time was 40 min. The injection temperature was 250 °C; the ionization source temperature was at 250 °C and interface at 300 °C. The injection volume was 1 µL (1 mg.mL$^{-1}$ in hexane).

**Calibration curve of β-amyrin by Gas Chromatography (GC)**

The β-amyrin was the major active triterpene detected in both partitions (HP and DP), as well as on the supercritical fluid (SF) and Soxhlet (HSox and DSox) extracts. For this reason, the β-amyrin, isolated by our research group on this work, was used as standard for being quantified (mg.mL$^{-1}$) by GC-MS on each of the referred extracts. So, there were prepared five different concentrations of the standard (5; 2.5; 1.25; 0.75 and 0.185 mg.mL$^{-1}$), as follows: 12.5 mg of β-amyrin were previously derived with 625 µL of MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide) (Sigma-aldrich®) and then diluted at 5 mg.mL$^{-1}$ with dichloromethane for the serial dilution. The extracts (2 mg) were also diluted in 1 mL dichloromethane. All analyses were performed in triplicate on the same experimental conditions previously described.

**Cell culture**

The leukemia cell line (K562) and fibroblasts obtained by embrionary *Rhesus* monkey kidneys (MA-104) were kindly provided by Dr. Vivian Rumjanek (Medical Biochemistry Department, Federal University of Rio de Janeiro, Brazil). Cells were grown at 37 °C and 5% CO$_2$ in 25 cm$^2$ culture flasks containing Dulbecco’s modified Eagle’s medium (DMEM- Gibco Cell Culture Media) supplemented with 10% of fetal bovine serum (FBS) (Gibco®), 1% PSA (10,000 U.L.mL$^{-1}$ penicillin G sodium (Gibco®), 10 000 g.mL$^{-1}$ streptomycin sulfate (Gibco®) and 25 μg. mL$^{-1}$ amphotericin B (Xellia Pharmaceutics).
Cytotoxicity assay

For the cytotoxicity assays, 10⁶ cell.mL⁻¹ leukemia cells were suspended in DMEM/FBS and plated into a 96-well culture plates (FALCON 3072). The extracts (CE, HSox, DSox and all SF) and partitions (HP, DP, EAP, BP, AQR) were investigated in different concentrations (10, 30 and 90 µg.mL⁻¹). The purified compounds tested (β-amyrin, β-sitosterol, stigmasterol) were treated with different concentrations (270, 150, 90, 30, 10, 3.3 and 1.1 µg.mL⁻¹). The samples were dissolved in DMSO and, then, diluted with medium. The cells were incubated at 37 °C and 5% CO₂ and, after 48 h, the viability was evaluated by permeability of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma). The number of viable cells in the different systems was determined as Yang et al. (2012). For the procedure, there were carried 10 µL of MTT in each well, and, after incubation for 2 h at 37 °C, with 5% CO₂, there were added 100 µL/well of SDS 10% solution with 0.01 N HCl to the culture. The extent of formazan crystal production was measured by absorbance at 570 nm (microplate reader μQuant, Bio-Tek Instruments, Inc.) after plate incubation for 48 h at 37 °C. Control cultures did not receive the tested samples. To calculate the percentage of cytotoxicity for all samples, there were used a Triton X-100 (CT), as positive control, and cells, without Triton (C), as negative control. Triton –X -100 is one of the most widely used nonionic surfactants for lysing cells organelles. This toxicity arises because of the disruptive action of its polar head group on the hydrogen-bonding present within the cell’s lipid bilayer, leading to the destruction of the compactness and integrity of the lipid membrane (Wang, Ren, Xi, 2012). To the cytotoxic tests, the antineoplastic drug cisplatin (FAUDCISPLATINA, onjet solution LIBBS) and the flavonoid quercetin (SIGMA) were used as standards drugs at 90, 30 and 10 µg.mL⁻¹. Cisplatin is an antitumoral drug used to the treatment of versatile solid tumors (Vázquez, Palazon, Navarro-Ocanã, 2012). The cells control was constituted by 10⁶ cell.mL⁻¹ and the DMEM medium.

Cell death analysis

The apoptosis analysis was assessed using a quantification measurement by flow cytometry. Previously, K562 cells (10⁶ cells.mL⁻¹) were incubated either in the absence or presence of stigmasterol (3.308 µM) and H₂O₂ (10 µM) for 4 h and 24 h at 37°C and 5% CO₂. Phosphatidylserine (PS) exposure on the outer layer of the plasmatic membrane of apoptotic cells was determined by Annexin-V labeling according to the manufacturer’s instructions (Annexin V FITC apoptosis kit, BD Pharmingen, CA, USA). Briefly, cells were washed twice with cold PBS, and suspended in 1 mL binding buffer. There were transferred 100 µL of the solution (10⁶ cells) to a 5 mL culture tube. Then, the Annexin V-FITC (5 µL), PI solution (5 µL), and the binding buffer (400 µL) were added. After 15 min at room temperature the cells were analyzed in the FACSCalibur cytometer (Beckton Dickson, USA). Data were analyzed in Cylflog software.

Statistical analyses

The 50% of Inhibitory Concentration (IC₅₀) values were calculated by non-linear regression analysis, using the GraphPad Prism 5.0 software. Statistical analysis were performed by One Way Test ANOVA followed by the test Tukey (p<0.05).

RESULTS

Different extractive methodologies analyses

Yields obtained by Partition x Supercritical Fluid x Soxhlet

The yields of the leaves extracts obtained by Partition (P), Soxhlet (Sox) and Supercritical fluid (SF) are represented in Table II. The Soxhlet extracts presented the highest yield (6.03% for the hexane (HSox) and 2.51% for diclorometane (DSox) extracts) compared to the fractions obtained by the liquid-liquid partition (2.26 and 1.06%, for the hexane (HP) and the diclorometane (DP), respectively). It was possible to obtain satisfactory yields by the SFE, which varied from 1.63 to 4.34%. This value (4.34%) is higher compared to the partitions yields and, close to that obtained by the Soxhlet method (6.03%).

Supercritical fluid extraction kinetics

For CO₂ extractions, it was set five conditions (SFE 1-5) combining distinctive pressures and temperatures, as follows: SFE 1 (298K/15.0 MPa); SFE 2 (318K/15.0 MPa); SFE 3 (308K/18.5 MPa); SFE 4 (298K/22.0 MPa) and SFE 5 (318K/22.0 MPa) (De Melo et al., 2014). The kinetic curves for each extractive condition, correlating the crude yield obtained, according to the time, for each 10 and 20 min are plotted in Figure 1.

By the kinetic curves, it was possible to observe in Figure 1 that the yields obtained throughout 120 min of extraction increased in all conditions adopted, suggesting that the extractive process was satisfactory and time-dependent. Comparing the extractions conducted at the same temperature (SFE 1 x SFE 4 - 298 K; SFE 2 x SFE 5 - 318 K), it was possible to notice that those under higher pressures (22 MPa) resulted in best yields (SFE 4 and SFE 5). Among those, the highest
yield (4.34%) was observed for the extraction conducted with the highest temperature (318 K) (SFE 5). In order to evaluate the chemical profile of the SFE samples and their chromatographic similarity with those obtained by the conventional techniques, the samples were analyzed by GC-MS and this point will be discussed later.

**TABLE II - Yields (%) of the leaves extracts obtained from *P. mucronata* by partition, Soxhlet and Supercritical Fluid in different conditions**

<table>
<thead>
<tr>
<th>EXTRACTIVE METHODOLOGIES</th>
<th>Yields (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Partition</strong></td>
<td></td>
</tr>
<tr>
<td>Partitions obtained by liquid-liquid partition of the crude extract</td>
<td></td>
</tr>
<tr>
<td>HP 1 atm</td>
<td>2.26 a</td>
</tr>
<tr>
<td>DP 1 atm</td>
<td>1.06 c</td>
</tr>
<tr>
<td><strong>Soxhlet</strong></td>
<td></td>
</tr>
<tr>
<td>HSox 1 atm</td>
<td>6.03 f</td>
</tr>
<tr>
<td>DSox 1 atm</td>
<td>2.51 a</td>
</tr>
<tr>
<td><strong>Supercritical Fluid</strong></td>
<td></td>
</tr>
<tr>
<td>Temperature (K)/Pressure (MPa)</td>
<td>Density of CO₂ (kg/m³)</td>
</tr>
<tr>
<td>SFE 1 298 / 15</td>
<td>877.31</td>
</tr>
<tr>
<td>SFE 2 318 / 15</td>
<td>742.55</td>
</tr>
<tr>
<td>SFE 3 308 / 18.5</td>
<td>853.28</td>
</tr>
<tr>
<td>SFE 4 298 / 22</td>
<td>927.39</td>
</tr>
<tr>
<td>SFE 5 318 / 22</td>
<td>833.21</td>
</tr>
</tbody>
</table>

Superscript letters represent significant statistical differences among the yields obtained on the different extraction conditions; *Yields percentage (%) was calculated in relation to the mass of dry plant leaf used for the extraction procedure.

**FIGURE 1 - Kinetic of extraction (crude yield x time) obtained by SFE in different temperature and pressure conditions.**

**Purification and chemical identification of the bioactive hexane partition (HP)**

Isolation of β-amyrin, oleanolic acid, β-sitosterol and stigmasterol from the hexane sample (HP) obtained by liquid-liquid partition of the crude macerated extract.

As HP showed the best cytotoxic profile as well as the best yield, HP was selected for further purification steps. The HP was chromatographed in a normal phase silica gel column chromatography and there were obtained 271 fractions. The fractions were analyzed by thin layer chromatography (normal phase silica-TLC F<sub>254</sub>) with hexane: ethyl acetate (7:3) as mobile phase and, sulfuric acid followed by vanillin, under heating, as chromogenic reagents. The fractions were assembled based on their similar profiles by TLC. There were obtained the following sample groups: 112-118 (108 mg) (21.53% yield): β-amyrin; 119-123 (18.1 mg) (5.32% yield): β-sitosterol and stigmasterol; 125-130 (4 mg) (1.27% yield): oleanolic acid (Figure 2). The fractions were analyzed by 1H and 13C NMR techniques and, by Atmosphere Pressure Chemical Ionization/Mass Spectrometry (APCI/MS) in order to structurally elucidate the compounds (Figures 3A and 3B).
Identification of the phytosterols and triterpenes

Four compounds were identified by mono and bidimensional NMR \(^1\)H and \(^13\)C techniques.

\(\beta\)-Amyrin (1) (Figure 2) (sub-fraction 112-118: 108 mg): H-3 (3.22 ppm); H-5 (0.7 ppm); H-12 (5.18 ppm); H-15 (1.88 ppm); H-16 (1.68 ppm); H-22 (1.88 ppm); H-23 (0.75 ppm); H-24 (0.94 ppm); H-25 (0.73 ppm); H-26 (0.97 ppm); H-27 (1.13 ppm); H-28 (1.07 ppm); H-29 (0.87 ppm); H-30 (0.79 ppm) C-1 (38.7 ppm); C-2 (27.13 ppm); C-3 (79.26 ppm); C-4 (38.65 ppm); C-5 (38.65 ppm); C-6 (27.13 ppm).
Passiflora mucronata leaves extracts obtained from different methodologies

Braz. J. Pharm. Sci. 2020;56:e17666

(55.08 ppm); C-6 (19.00 ppm); C-7-C-8 (39.9 ppm); C-9 (47.69 ppm); C-10 (37.13 ppm); C-11 C-12 (121.9 ppm); C-13 (145.2 ppm); C-14 (41.7 ppm); C-15 (26.2 ppm); C-16 (26.1 ppm); C-17 (32.6 ppm); C-18 (47.2 ppm); C-19 (46.8 ppm); C-20 (31.8 ppm); C-21 (34.7 ppm); C-22 (37.0 ppm); C-23 (28.0 ppm); C-24 (15.4 ppm); C-25 (15.4 ppm); C-26 (16.8 ppm); C-27 (25.9 ppm); C-28 (28.4 ppm); C-29 (33.8 ppm); C-30 (23.7 ppm) (Zhang, Men, Lei, 2014). The APCI analyses by the negative mode analysis (Figure 3B) showed the molecular ion m/z 409 [M-H]. Other peaks were also observed such as m/z 231, m/z 245, m/z 259, m/z 271 and m/z 313, correspondent to the mass fragmentation of β-amyrin (Uddin et al., 2014).

Stigmasterol (2) and β-sitosterol (3) (Figure 2) (sub-fraction 119-123 (18.1 mg): H-3 (3.56 ppm); H-6 (5.35 ppm); H-23 (5.16 ppm); H-22 (4.65 ppm); C-1 (38.0 ppm); C-2 (32.0 ppm); C-3(72.0 ppm); C-4 (42.0 ppm); C-5 (142.0 ppm); C-6 (122.0 ppm); C-7 (32.0 ppm); C-8 (32.0 ppm); C-9 (50.0 ppm); C-10 (36.36 ppm); C-11(20.04 ppm); C-12 (40.0 ppm); C-13 (42.0 ppm); C-14 (57.0 ppm); C-15 (24.5 ppm); C-16 (29.0 ppm); C-17 (56.0 ppm); C-18 (12.0 ppm); C-19 (20.0 ppm); C-20 (40.0 ppm); C-21(20.0 ppm); C-22 (138.0 ppm); C-23(130.0 ppm); C-24 (50.0 ppm); C-25 (32.0 ppm); C-26 (20.0 ppm); C-27 (19.0 ppm) C-28(25.0 ppm) C-29 (12.0 ppm) (Ovesná, Kozics,Slame, 2006).

Oleanolic acid (4) (Figure 2) (sub-fraction 125-130: 4 mg): H-3 (3.22 ppm); H-12 (5.49 ppm); H18 (2.52 ppm); H23 (1.0 ppm); H24 (0.9 ppm); H25 (0.9 ppm); H26 (0.8 ppm); H29 (0.9 ppm); H30 (0.9 ppm); C3 (79.2 ppm); C12 (122.0 ppm), C18 (41.27 ppm); C23 (28.0 ppm); C24 (16.0 ppm); C25 (15.0 ppm); C26 (17.0 ppm); C29 (32.0 ppm); C30 (23.0 ppm) (Luo et al., 2014). The APCI negative mode analysis (Figure 3B) showed the molecular ion peak at m/z 455 [M-H] correspondent to the oleanolic acid triterpene (Vechia, Gnoatto, Gosmann, 2009).

Gas chromatography analysis: Identification and quantification of β-amyrin on the partitions and extracts

By the CG-MS analyses it was possible to realize that the hexane (HP) and the dichloromethane (DP) partitions, as well as the hexane extract obtained by Soxhlet (HSox) presented the same chromatographic profile. For all it was possible to suggest, based on the GC-MS NIST library, substances with fragments compatible with β-amyrin, β-sitosterol, stigmasterol and oleanolic acid. By the chromatogram it was also possible to identify fatty acids at about 17.5 and 19.2 min, correspondent to the palmitic and stearic acids, respectively. The β-sitosterol and stigmasterol were detected between 30 and 31 min. The oleanolic acid was identified at 32.4 min. At 34.56 min it was detected the presence of other triterpene, not identified yet. The β-amyrin triterpene was identified as the major compound on the HP, as the peak eluting at 31.8 min, as well as on the DP and HSox extracts at 31.6 min and 31.3 min, respectively. Additionally, on the HSox it was also possible to detect the presence of the following substances: squalene at 24.55 min, α-tocopherol at 27.67 min and, α-amyrin acetate at 31.30 min. The DSox extract presented unknown peaks and, by this reason, it was not considered for this discussion. All the chromatographic profiles are presented in Figure 4.

In order to confirm the presence of β-amyrin as correspondent to the peak eluted at 31.8 min, the isolated compound I was also analyzed by GC-MS. According to the mass spectrometry fragmentation, to the molecular ion (m/z 426), and to the previous NMR elucidation technique, the β-amyrin compound was confirmed (Figure 5). These data are in accordance with the literature descriptions (Barros, de Assis, Mendes, 2014). Considering that β-amyrin was detected as the main constituent in HP, DP and HSox, this substance was used as standard for further quantification studies.

The β-amyrin contents, in each extract, were calculated by plotting a calibration curve performed by gas chromatography (R²= 0.976). The Table III shows that all extracts obtained by SFE (SFE 1-5) presented higher β-amyrin contents (from 1.61 ± 0.01 to 2.03 ± 0.14 mg.mL⁻¹) compared to the other extracts (Figure 6). Concerning the extracts obtained by the conventional methods (Soxhlet x liquid- liquid partition), the β-amyrin concentrations varied from 0.51 ± 0.01 to 1.06 ± 0.02 mg.mL⁻¹. In contrast to the SFE samples, these fractions presented fatty acids as main compounds, showing that the conventional methods exhibited lower selectivity for the triterpenes extraction.

Cytotoxic activity

Liquid-liquid partition, Soxhlet and supercritical fluid extracts

The samples obtained by the maceration, soxhlet and supercritical fluid (SF) techniques were tested against the K562 cancer cell line at 90, 30 and 10 µg.mL⁻¹. The cytotoxic results for K562 showed that the crude extract (CE), the hexane (HP) and dichloromethane (DP) partitions presented significant cytotoxic effect at 90 µg.mL⁻¹ (83.70 ±
1.17%; 89.80 ± 1.05% and 65.35 ± 1.08%, respectively), compared to the following controls: Control without Triton (C) (0%), Control with Triton (CT) (100%) and cisplatin (81.20 ± 1.28%). The ethyl acetate (EAP) and butanol (BP) partitions showed moderate but significative activity in relation to the (C) at the highest concentration (90 µg.mL⁻¹) (37.98 ± 1.33% and 49.7 ± 1.31%, respectively) (Figure 7). The hexane partition (HP) exhibited the best IC₅₀.

**FIGURE 4** - GC-MS chromatogram profiles of the fractions from the liquid-liquid partition: hexane (HP) (A), dichloromethane (DP) (B) and the extract from Soxhlet: hexane (H Sox) (C).

**FIGURE 5** - Chromatogram obtained by GC-MS of β- amyrin obtained from the hexane partition (HP) of *P. mucronata* crude extract.
Passiflora mucronata leaves extracts obtained from different methodologies

(18.72 ± 1.05 µg.mL⁻¹) compared to the crude extract (CE) (26.18 ± 1.17 µg.mL⁻¹), to the dichloromethane (DP) (27.49 ± 1.08 µg.mL⁻¹) and to the other partitions and Soxhlet extracts (> 90 µg.mL⁻¹). The IC₅₀ data are presented.

FIGURE 6 - Chromatograms obtained by GC-MS correspondent to the supercritical fluid extracts (SFE) obtained by different temperature and pressure conditions.
in Table IV. For the CO₂ supercritical extracts, the cytotoxic results showed that SFE 3 (15 MPa; 298.15K) and SFE 2 (15 MPa; 318.15K) were the most active against K562 at 90 µg.mL⁻¹ (78.3 ± 0.12% and 75.2 ± 0.14% cytotoxicity, respectively), followed by SFE 1 (60.8 ± 2.67%), SFE 4 (52.7 ± 1.44%) and SFE 5 (52.9 ± 2.79%), at this same concentration (Figure 8).

β-amyrin, stigmasterol and β-sitosterol isolated substances

Considering that the HP partition exhibited the best IC₅₀ (18.72 ± 1.05 µg.mL⁻¹), HP was selected for further phytochemical studies resulting in some purified compounds that were promptly tested. By the Tuckey’s multiple comparison tests, it was possible to verify that the activity of the substances did not present significant variations. The results concerning the isolated compounds showed that all compounds presented high and very similar activity concerning its IC₅₀ values (β-amyrin: IC₅₀ = 3.918 ± 1.16; β-sitosterol: IC₅₀ = 3.370 ± 1.32; stigmasterol: IC₅₀ = 3.308 ± 1.33 µg.mL⁻¹) (Figure 9 and Table IV). These results are of extreme relevance considering that, in general, cisplatin is the antitumoral drug of choice in the treatment of a variety of tumors (Vázquez, Palazon, Navarro-Ocaná, 2012). For all compounds tested, it was observed about 100% of the cytotoxic effect at 270 µg.mL⁻¹ and, it is possible to note that the inhibition profile is dose-dependent for all samples. The statistical analysis did not show significant difference among them. In accordance with the literature, oleanolic acid is not recognized as a cytotoxic compound against the K562 cell line (Martelanc, Vovk, Simonovska, 2009), so, by this reason, this compound was not selected for testing.

Cell death analysis

Among all compounds tested against K562 cell, stigmasterol showed the lowest IC₅₀, so, it was chosen for flow cytometry analysis in order to evaluate the induced cell death type. In the right inferior quadrant (Figure 10-I) it is possible to see the early apoptotic cells, with low fluorescence of PI and high fluorescence to annexin V. The right superior quadrant represents cells in late apoptosis, with high fluorescence to annexin V and PI.

The experiment showed that, at 4 h, in addition to the hydrogen peroxide (used as apoptosis positive control) the stigmasterol also began to induce apoptosis.

![Figure 7](image_url)
(26.88 ± 3.51% of early apoptosis and 12.35 ± 1.30% of late apoptosis) compared to the non-treated control. At 24 h of experiment it is possible to see that stigmasterol, compared to the control, induced 50.67 ± 2.84% of early apoptosis and 23.63 ± 1.09% of late apoptosis, performing about 75% of total apoptosis (Figure 10-I and II). It is interesting to mention that all treatments induced very low levels of necrosis.

**Cytotoxicity to normal mammal's cells**

The cell MA-104 was chosen as a non-cancer cell in order to verify the selectivity of all samples. The results obtained for the hexane (HP) and the dichloromethane (DP) partitions showed a IC$_{50}$ higher than 100 ± 2.6 µg.mL$^{-1}$ for DP and a IC$_{50}$ of 38.90 ± 1.77 µg.mL$^{-1}$ for HP. The isolated compounds were also analyzed on MA104 cell line and the selective index further calculated (Table V). The selective index for almost all samples tested were, as follows: β-amyrin: SI=10.9; β-sitosterol: SI=12.3 and stigmasterol: SI=12.5, indicating a good selectivity against the cancer cell line (K562) in comparison to the normal cell line (MA104).

**DISCUSSION**

This study describes three different extractive methodologies followed by phytochemical and biological investigations of *P. mucronata* extracts. Concerning the crude extract yields, mainly the hexane Soxhlet extract (HSox) and the supercritical fluid (SF) extracts, such as the SFE 1, 3, 4 and 5, showed the highest total yields (HSox=6.03; SFE1=2.61±0.23; SFE3=3.60±0.12; SFE4=3.34±0.15; SFE5=4.34±0.01). These results are similar to the other data already reported on literature (Lemos *et al.*, 2012; Vazquez, Palazon, Navarro-Ocaná, 2012). Lemos and colleagues (2012) attributed the best extract yield obtained by the Soxhlet apparatus...
FIGURE 9 - Comparative cytotoxic activities in percentage values for the isolated compounds from the hexane partition (HP), as follows: β-amyrin, β-sitosterol and stigmasterol against K562 cell line, in different concentrations (270, 150, 90, 30, 10, 3 and 1 μg.mL⁻¹). The results express the average of three independent experiments in triplicate. The cells (10⁶.mL⁻¹) was incubated only with culture medium (control C), or, with different concentrations of the samples by 48 h, at 37ºC, in humid atmosphere, with CO₂ (5%). Statistical was performed by one-way ANOVA followed by Tukey’s t-test (p < 0.05).

FIGURE 10 - Effects of *Passiflora mucronata* samples on K562 cell apoptosis. The annexin V+-FITC-PI labeling cytograms (I) of control (A), stigmasterol (B), H₂O₂ (C) cultures in 4h and 24 h. Cells (10⁶.mL⁻¹) were incubated with culture medium (control), Apoptosis was investigated by flow cytometry analysis of cell shrinkage and Annexin V+-FITC labeling. The propidium iodide and Annexin V-FITC fluorescences were detected by the FL-3 and FL-1 channels, respectively. The graphic (II) represents the results expressed as average ± SD of two independent experiments. [*] p<0.05 and [***] p<0.001.
Passiflora mucronata leaves extracts obtained from different methodologies

The β-amyrin bioactive triterpene is being described for the first time in the Passiflora genus and, the oleanolic acid, on the species P. mucronata. Interestingly, β-amyrin was extracted by supercritical fluid for the first time, and, it was in a larger quantity (2.03 ± 0.14 mg.mL⁻¹) compared to the other conventional techniques studied here. In 2001, authors detected in P. alata three different glycosylated forms of the oleanolic acid (oleanolic acid 3-O-β-D-glucopyranoside, oleanolic acid 3-O-β-D-glucopyranoside (1→3) β-D-glucopyranosyl and oleanolic acid 3-O-β-D-glucopyranoside (1→2) β-D-glucopiranosyl), never detected before on this genus (Mosmann, 1983). In P. edulis it has already been detected the maslinic and oleanolic acids derivatives (Wang et al., 2013). In addition to the oleanane type triterpenes, other classes have already been isolated from Passiflora species, such as the cicloartanes triterpenes (passiflorine and ciclopassifloside) (Arechabala et al., 1999). The maslinic acid is an important compound recognized to induce apoptosis and, for blocking the growth of the Ehrlich HeLa carcinoma (de Almeida et al., 2012; Rusiecka et al., 2016). Concerning the phytosterols stigmasterol and β-sitosterol, identified in P. mucronata on the present study, we highlight that, unexpectedly, this is the first report describing their presence in the Passiflora genus. The glycosylated form of stigmasterol named as stigmasterol 3-O-β-D-glucopyranosyl, never detected before on this genus (Mosmann, 1983), being the only description so far for the genus.

The phytosterols and triterpenes are the most important compounds found in the plant kingdom (Arora, Kalia, 2013). In addition, many studies focusing on pentacyclic triterpenes revealed their anti-cancer activities (Reginatto et al., 2001; Huang et al., 2007; Zhang, Men, Lei, 2014). For β-amyrin and β-sitosterol, for example, many studies have already described activities against different types of cells lines, such as A549 (lung cancer).

to the highest temperature, solvent recirculation and interaction with the solute applied. In addition, by this type of extraction, generally, all steps are time-consuming compared to the other techniques, such as the liquid-liquid partition, conducting to higher amounts of the compounds extracted. The extraction by Sohxlet with n-hexanes solubilize many nonpolar compounds, indiscriminately, contributing also to higher yields (Lin, Tsai, Wen, 1999). SFE studies show that, during the extraction, some important factors can interfere on the final extract yields, in a general form, such as how drying the leaves after harvest and, the size of the plant material submitted to the extraction (Lemos et al., 2012). It is known that, the smaller the size of the vegetable material is, probably, the better the obtained yield will be, since the solvent will have better access to the tissue and, hence, the mass diffusion will be better (Lemos et al., 2012). Besides that, other factors can also interfere on the final yields. In general, the SFE yield observed on the present study showed to be quite interesting, conducting to further kinetic studies, in which it was obtained a free-solvent extract rich on the bioactive compound β-amyrin. It is known that, increasing the pressure on supercritical fluid systems it may lead to a decrease on the chemical selectivity, since it can infer, but not necessarily, on the co-extraction of undesired compounds. Therefore, it can result in a better final yield (Meireles, Angela, 2009; Lemos et al., 2012). Among the partitions, the hexane (HP) presented the best cytotoxic activity. Despite presenting a similar chromatographic profile to the SFE, the HP presented a slightly higher activity, probably because of the increased presence of fatty acids not detected in SF extracts, considering their high extraction selectivity. Fatty acids are markedly recognized for their cytotoxic activities, and, probably, it is favoring the K562 cell inhibition growth observed by this partition. Even so, it is important to highlight that the biological activity of the SFE was preserved, corroborating that the supercritical fluid showed to be a satisfactory alternative green method for obtaining a cytotoxic extract from P. mucronata leaves, rich in triterpenes and phytosterols.

### TABLE V - The selectivity index (SI) of each sample was calculated based on the IC50 MA-104 / IC50 K562 data

<table>
<thead>
<tr>
<th>Samples</th>
<th>K562 cell line IC50 (µg.mL⁻¹)</th>
<th>Ma-104 cell line IC50 (µg.mL⁻¹)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPa</td>
<td>18.72</td>
<td>38.90</td>
<td>2.07</td>
</tr>
<tr>
<td>DPa</td>
<td>27.49</td>
<td>100</td>
<td>3.63</td>
</tr>
<tr>
<td>β-amyrin</td>
<td>9.18</td>
<td>100</td>
<td>10.9</td>
</tr>
<tr>
<td>β-sitosterol</td>
<td>8.13</td>
<td>100</td>
<td>12.3</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>8.01</td>
<td>100</td>
<td>12.5</td>
</tr>
</tbody>
</table>

*HP=Hexane Partition; DPa=Dichloromethane Partition.*
and HL-60 (human promyelocytic leukemia), with IC\textsubscript{50} values at 46.2 and 38.6 µM, respectively (Arora, Kalia, 2013). Concerning the mechanism of action suggested, studies with triterpenes have showed that these secondary metabolites can promote apoptosis in lung cancer (Reginatto et al., 2001). So, our findings are new for K562 cell line.

The cells death by apoptosis is linked to the tumor appearance, and this is an important factor in the cytotoxicity induced by antineoplastic drugs (Silva et al., 2015), because of that, we investigated on the present study the mechanism of action of stigmasterol. Hydrogen peroxide, a reactive oxygen species (ROS), was used as apoptosis positive control. The H\textsubscript{2}O\textsubscript{2} shows a deleterious effect and can cross the lipid layers and can react with O\textsubscript{2}− and become HO\textsuperscript{−}, that is very reactive. When the amount of ROS is higher than the antioxidant elements in our body, the oxidative stress began and can cause damage in cells. This damage can be correlated with cells apoptosis (Lemos et al., 2012). The present study showed that stigmasterol induced cells apoptosis after 4 h and 24 h of treatment. Unless stigmasterol is not the major compound on the hexane partition (HP), it can help to induce cell death and can contribute to the higher cytotoxic effect observed by the MTT experiment. It is known that, during the apoptosis, the events observed in the beginning is the loss of membrane asymmetry as well as the plasmatic membrane phosphatidyserine externalization (Urech et al., 2005). The annexin V strongly bind to the phosphatidyserine phospholipid in the presence of Calcium ions. In life cells the phosphatidyserine phospholipid is found in the inner face of membrane and, when the cells are in early apoptosis, it migrates to the external face of the membrane and is marked by Annexin V (Silva et al., 2015). So, the fluorescence will be proportional to the death cells. The PI penetrates in non-intact membranes and binds in nucleic acids (Silva et al., 2015). On this investigation, stigmasterol performed about 75% of total apoptosis and induced very low levels of necrosis. This data is quite important since stigmasterol is present in some cytotoxic vegetal species extracts and can satisfactorily contribute to this activity (Ayer, Patil, 2012).

This study found that the cytotoxic compound β-amyrin was extracted in a higher amount by the supercritical fluid technique, a green and efficient method of extraction, compared to Soxhlet and maceration. Our research pointed out that the supercritical fluid can be, therefore, a viable alternative technique for obtaining bioactive triterpenes in the absence of the use of co-solvents, in a high quantity and as a selective form. Among all extracts evaluated against the K562 cell line, the hexane partition stood out and, interestingly, the triterpenes (β-amyrin and oleanolic acid) and phytosterols (β-sitosterol and stigmasterol) isolated from it showed significant cytotoxic activity compared to the standard cisplatin and, also, a good selectivity index. In addition, stigmasterol presented apoptosis induction. This study provided relevant scientific evidences since it correlates, for the first time, the cytotoxic activity of \textit{P. mucronata} extracts, obtained by different extractive technologies, with isolated constituents not previously identified on this species.

**CONFLICT OF INTEREST**

None

**ACKNOWLEDGMENTS**

This study was supported by CAPES scholarship, by the funding from Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq): Produtividade em Pesquisa/ PQ2014 (process 312045/2014-0); from Fundação de Amparo à Pesquisa Do Estado do Rio de Janeiro (FAPERJ): Emergentes (process E-26/110.127/2014) and Jovem Cientista do Nosso Estado/ JCNE (process E-26/202.817/2015). We also like to thank Andrea Portzel for the NMR analyzes and Thatiana Ventura for the support on the flow cytometry analysis.

**REFERENCES**


Passiflora mucronata leaves extracts obtained from different methodologies


Received for publication on 14th October 2017
Accepted for publication on 06th December 2018