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In Vitro Evaluation of the Potential Antioxidant of *Bidens segetum* Mart. ex Colla (Asteraceae) in Melanocyte and Melanoma Cells

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Abstract

Bidens segetum Martius ex Colla known as the "picão do mato", is an herbaceous plant that occurs in the Cerrado biome of some Brazilian states. Among the species of *Bidens*, we highlight *B. pilosa* known as "picão preto", of which several activities are reported as antioxidant and antibacterial. Ethanolic extract from *Bidens segetum* (EEBs) showed antioxidant potential when analyzed by free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and antifungal activity against *Cladosporium cladosporiodes* and *C. sphareospermum* fungi. PFFR3.3 subfraction from EEBs has 81.5% of 5-O caffeoylquinic acid (5-CQA) and potential antioxidant (DPPH). However, PFFR3.3 did not decrease superoxide anion in metastatic melanoma cells by dihydroethidium assay (DHE). PP4 subfraction is a mixture of polyacetylenes that has antifungal (*Cladosporium*) and antioxidant activity, since reduced superoxide anion amount in melanoma cells after 5 min of treatment. However, no dose-response and time-response curve were observed, not even with the authentic standard (5-CQA). Complementary chemical studies will be performed to confirm the polyacetylenes and 5-CQA structures present in the EEBs from *B. segetum* and new methodologies should be performed to confirm the antioxidant activity of these compounds and the effects on melanocytes and melanomas.

Keywords

Asteraceae, Antioxidants, Melanoma, Melanocytes, Polyacetylenes, Chlorogenic Acid

1. Introduction

Bidens genus pertaining to Heliantheae tribe (Asteraceae) has approximately 240 species distributed throughout the world, mainly in North and South America. In Brazil, around 13 species are found in some biomes [1] [2] [3] [4]. The main ethnopharmacological applications of the species include the treatment of diabetes, malaria, antioxidant and antibacterial activities and the most used species are *Bidens pilosa* and *Bidens alba* [5]-[10]. Among the most common chemical constituents are reported polyacetylenes (34%), chalcones (12%), flavonoids (9%) and phenylpropanoids (9%) [11]. Polyacetylenes present in *Bidens* are relatively unstable long chain hydrocarbons and highly sensitive to light exposure [11]. On the other hand, polyacetylenes isolated from *Bidens pilosa* could modulate the differentiation of human helper T cells and prevent autoimmune diabetes in mice and demonstrating antiproliferative effect on normal and rapidly growing human transformed cell lines [12] [13]. *Bidens segetum* is still little studied, but tests with ethanolic extract of flowers, stems and leaves showed cytotoxic effect against cancer cells and antioxidant activity from the ethyl acetate fraction [14].

Reactive oxygen species (ROS) are produced during the body's metabolic processes [14] [15] and superoxide anion (O_2^-) is formed by the reduction of molecular oxygen (O_2) [16]. The uncontrolled production of ROS overloads the antioxidant defense mechanisms, generating oxidative stress, increasing cell proliferation, apoptosis, cytotoxicity, and aging [17] [18]. In carcinogenesis, ROS are involved in all three stages (initiation-promotion-progression) and malignant transformation of melanocytes has been recognized as associated with increased ROS production [19] [20] [21].

Melanocytes are responsible for the production of melanin pigment that colors the skin and protects it from UV exposure [22] [23]. Besides increased UV exposure leads to genetic and epigenetic alterations, it also induces a chronic inflammation and ROS accumulation, which in turn, is associated with melanocytes malignant transformation and melanoma development [24]. Melanoma is the least frequent type of skin cancer (3% to 4%) of cases, but its incidence is lethal in about 75% to 80%. The prognosis of death for this type of tumor is 6 to 9 months and the chances of cure are greater the sooner the disease is diagnosed and treatment started [25] [26] [27] [28]. Therefore, antioxidant administration could be a chemoprevention strategy. This work aims to evaluate the antioxidant potential of *Bidens segetum* species in decreasing superoxide anion (O_2^-) levels in melanoma cells and melanocytes based on dihydroethidium oxidation (DHE).

2. Material and Methods

2.1. Plant Material

Leaves of *Bidens segetum* (Asteraceae) were collected in March 2017 (**Figure 1**). The specimens were obtained from plants grown in beds at the Instituto de

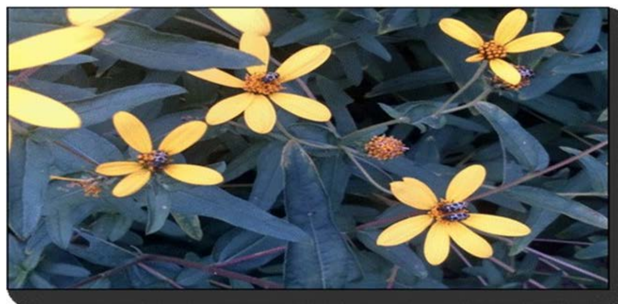

Figure 1. *Bidens segetum* Mart. ex Colla (pollinator visit.) from plant collections of the Research Center in Physiology and Biochemistry, Botany Institute, São Paulo, SP, Brazil.

Botânica-SP (Atlantic Forest domain) (Figure 2) from seeds from a wild population of *B. segetum*, brought from the Biological Reserve and Experimental Station of Mogi-Guaçu (Cerrado domain) São Paulo State, Brazil. The botanical identity of the plant material was confirmed by Dr. Inês Cordeiro (Center for Research in Vascular Plants, Instituto de Botânica, São Paulo, Brazil). A voucher specimen (Nascimento 452794-SP) was deposited at the Herbarium Maria Eneyda P. Kaufmann Fidalgo (SP), São Paulo, Brazil.

2.2. Extraction and Bio-Guided Fractionation

Extraction

Crude ethanolic extract (EEBs. 123.284 g, 23% yield) was obtained from fresh leaves (1.345 kg), frozen and lyophilized (485 g, 27%) by exhaustive extraction (6 days), ethanol 93˚ (485 g·6 L^{-1}) at room temperature, filtration, concentration under reduced (Büchi® rotary evaporator) pressure and drying in a water bath 40˚C.

Bio-guided fractionation and Analyses

EEBs (45.0 g) was performed by flash chromatography in vacuum system on column (silica gel 60 g, Merck 200 - 500 mμ) and gradient system with the solvents: hexane (100%); hexane + ethyl acetate 5:5 (v:v); ethyl acetate (100%); methanol + ethyl acetate 5:5 (v:v); methanol (100%) methanol + H_2O 2:8 (v:v). The ethyl acetate + methanol (FAcEOt + MeOH; 35% yield) active fraction on the DPPH assay was solubilized in MeOH (10 mL) and fractionated by exclusion liquid chromatography on a 25 cm open top chromatographic column in length and 5 cm in diameter prepared with Sephadex LH 20 (35 g, Pharmacia) and 150 mL MeOH (Merck PA) and eluted with 1400 mL MeOH (Merck PA) yielding 47 subfractions (AM1-AM47). The AM17-AM23 active subfractions were pooled and fractionated by reverse phase chromatography (SPE Discovery DSC-18 Tube, 10 g, 60 mL, SUPELCO®) with gradient solvent: 100 mL Milli-Q® H_2O, 100 mL Milli-Q® H_2O + MeOH (8:2, v:v), 100 mL Milli-Q® H_2O + MeOH (5:5, v:v) and 100 mL 100% MeOH and were obtained seven new subfractions. The FFR3 (23.6 mg) was fractionated by preparative thin layer chromatography (PTLC, 60 F_{254}, silica gel, 1 mm, Merck, Darmstadt, Germany) furnishing three active fractions (DPPH): PFFR3.1 (Rf = 0.36); PFFR3.2 (Rf = 0.63 and 0.48) and PFFR3.3 Rf =

Figure 2. *Bidens segetum* Mart. ex Colla leaves from plants collections of the Research Center in Physiology and Biochemistry, Botany Institute, São Paulo, SP, Brazil. Date 29.10.2019.

0.36) with solvent system (BAW):n-Butanol:acetic acid: water (4:1:5, upper phase). All fraction was analyzed by HPLC-DAD.

Another part of the EEBs (75.0 g) was suspended in Milli-Q® water and fractionated by liquid-liquid partition with solvents: hexane (Hex) and ethyl acetate, (FAcEOt) and was fractionated by preparative thin layer chromatography PTLC on 60 F_{254} silica gel (1 mm; Merck, Darmstadt, Germany), eluted with 8:2 (v:v) $CHCl_3$: MeOH furnished the active PP4 subfraction that inhibits the growth of the spores of the *Cladosporium sphaerospermum* and *Cladosporium cladosporioides* fungi by bioautographic assay.

Analytical HPLC-DAD and semi-preparative HPLC-DAD

Analytical scale HPLC-DAD was performed on an Agilent model 1260 chromatograph consisting of a G1316A thermostatic furnace, a G1329B automatic injector, a G1330B thermostatic sample compartment, a G1311B pump equipped with a spectrum scan detector. Ultraviolet by arrangement of 60 mm flow cell photodiodes. The stationary phase for analytical scale analyzes was carried out using a reverse phase C_{18} Zorbax Eclipse plus column (4.6 × 150 mm) with 3.5 μm particle diameter and a flow rate of 1.0 $mL \cdot min^{-1}$. The elution system employed was the gradient mode initiated and consisted of a mixture of 75% acidified H_2O with 0.1% acetic acid and 25% acetonitrile (ACN) for 2 min, from 2 to 7 min reaching 50% of ACN, from 7 to 25 min reaching 90% ACN, from 25 to 26 min 100% ACN, which was held for one more minute.

Semi-preparative scale HPLC-DAD was performed on Agilent model 1200 chromatograph and a C_{18} reverse phase Zorbax eclipse plus LC-18 column (25 cm × 10 mm) with 5 μm diameter particles and a flow rate of 4.0 $mL \cdot min^{-1}$. The elution system employed was the same as that used in the analytical scale HPLC. All solvents used were HPLC grade (T. J. Baker).

LC-DAD-MS/MS/ESI⁺ and NMR

Analytical LC-DAD-MS/MS/ESI⁺ spectra the PP4 was performed on Shimadzu model CBM-20A chromatograph, pumps: LC-20AD, with detector:

SPD-20A-Oven: CTO-20A and autoinjector: SIL 20AC (Shimadzu) and Esquire 3000 plus—Bruker Daltonics mass spectrometer, with 4000 V capillary, 27 Psi nebulizer, 300˚C and 7 $L \cdot min^{-1}$ gas flow. Operating in MS and MS/MS data acquisition mode with electron spray source in positive mode (ESI^+). Subfraction PP4 (5mg) obtained from EEBs was solubilized in MeOH (Merck, PA). Chromatography conditions. Phenomenex Luna C18 column (250 × 4.6 mm – 5 um). The mobile phase was solvent A: H_2O acidifies with 0.1% formic acid and solvent B acetonitrile (ACN, HPLC grade). The solvent system was isocratic as follows: 0 min 95% A and 5% B, 50 min 0% A, 100% B. The flow rate was 1 $ml \cdot min^{-1}$. Temperature 40˚C and injection volume: 10 $\mu L \cdot min^{-1}$ (1 $mg \cdot mL^{-1}$). The chromatograms were monitored at 315 nm and 250 nm and retention times (Rt) detected. The TIC (Total ions chromatographic) were analyzed based on ion of mass/charge (*m/z*) of the $[M + H]^+$ Dalton unit (Da) detected in the MS^1 spectrum. The fragment ion 100% it's chromatography peak from (MS^2) spectrum of major relative abundance.

NMR spectra of PP4 were performed on Bruker III 500 MHz spectrometer. PP4 in deuterated chloroform ($CDCl_3$) was analyzed by ^1H NMR and bidimensional HSQC and HMBC (http://ca.iq.usp.br/novo/).

Bioautographic Assay

Strains used in the antifungal assays belonged to *C. sphaerospermum* Penzig and *C. cladosporioides* (Fresen) de Vries, from the mycology collection of Instituto de Botânica, São Paulo, Brazil (CCIBt 491; CCIBt140). To obtain spore solutions for the bioautographic assay, *C. sphaerospermum* and *C. cladosporioides* were maintained on plates with potato-dextrose-agar (PDA) medium, incubated at 27˚C in the dark for 14 days. Conidial suspensions of fungi were obtained in salt and glucose solution prepared as follows: a stock solution contains 8.4 g KH_2PO_4; 2.4 g $Na_2HPO_4 + 2H_2O$; 4.8 g KNO_3; 1.2 g $MgSO_4$, $7H_2O$; 1.2 g NaCl per 1200 mL of distilled water. The solution is homogenized at 120˚C for 20 min. Just before making the conidial suspension 10 mL of a 30% aqueous solution of glucose is added per 60 ml of this solution. The conidial suspension is stored in a freezer.

The antifungal activity of the extracts and subfractions was evaluated by qualitative bioautographic method on TLC [29]. *Cladosporium sphaerospermum* and *C. cladosporioides* were used in bioassays. For this assessment, samples were solubilized separately in methanol, and aliquots of 200 μg of samples were applied to silica gel 60 F_{254} (0.2 mm; Merck, Darmstadt Germany) TLC and eluted in the solvent system $CHCl_3$: MeOH (9:1 v:v), for about 1 h, using nystatin standard and cinnamic acid (Sigma-Aldrich) as positive control (1 μg). The TLC plates were sprayed separately with the conidia fungi suspension of each species ($>5 \times 10^7$ conidia mL^{-1}) and incubated in a moist chamber at 27˚C for 48 h in darkness. A clear inhibition zone appeared against a dark background, indicating the presence of fungitoxic compounds since the dark background is due to fungal growth, and the lack of growth indicates fungicidal activities. Retention factor values (Rf) of the spot are determined for each inhibition zone observed.

Each bioautography was repeated three times for each of the fungi tested and photographed in the Camag System—Repostar 3.

DPPH free radical scavenging activity

This assay was performed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable free radical photocolorimetry method [30] [31]. Aliquots of 100 μg the PFRR3.3 subfraction and the 5-CQA standard (1 mg·mL^{-1}, Sigma-Aldrich) were analyzed by TLC eluting with the upper phase of BAW (n-butanol: glacial acetic acid: H_2O 4:1:5, v:v). The plates were sprayed with a methanolic solution of the 2,2-diphenyl-1-picrylhydrazyl radical (0.2%—DPPH). After approximately 30 min white spots appeared under a purple background indicating that the DPPH radical was active in free radical DPPH scavenging. Chromatography profile was visualized under white light (Camag System—Repostar 3) and the retention factor (Rf) calculated. The active PFFR3.3 subfraction and 5-CQA standard (Sigma-Aldrich) by DPPH assay were quantified in 96-well microplate in triplicate (KC4-Bio-Tek Instrument INC., USA). For this purpose, 70 μL of methanol solution of DPPH (0.3 mM) and 178.5 μL of the sample (concentrations of 25, 50, 100, 150, 200, 250, 300 μg·mL^{-1}) and authentic standard 5-CQA to determine the percentage of purple-colored DPPH free radical scavenger absorbing at 518 nm. IC_{50} determination was calculated using the GraphPad Prims 5 program.

Cell Culture

The human melanocyte cell line (NGM) was acquired from the Rio de Janeiro Cell Bank (code BCRJ 0190) and was grown in DMEMf12 medium (Thermofisher, Carlsbad, CA) supplemented with20@ fetal bovine serum (Invitrogen, Scotland, UK), 2 mM glutamine, 1.4 μM hydrocortisone, 1 nM T3 hormone, 10 μg·mL^{-1} insulin (Sigma), 10 μg·mL^{-1} transferrin and 10 ng·mL^{-1} epidermal growth factor. Human melanoma lineages corresponding to different stages of tumor progression: radial growth melanoma (Wm1552), vertical growth melanoma (Wm1366) and metastatic (Lu1205) cell lines were kindly provided by Dr. Meenhard Merley from Institute Wistar (https://wistar.org/our-scientists/meenhard-herlyn) were cultured in 20% Leibovitz medium (GIBCO, Carlsbad, CA) at pH 7.2 plus 80% MCD153 medium (Sigma) supplemented with 2% fetal bovine serum and 1.68 mM $CaCl_2$. The cultures were kept in incubator at 37˚C in a humidified atmosphere containing 5% CO_2.

Dihydroethidium (DHE) antioxidant activity in melanoma and melanocyte cells

Intracellular superoxide anion (O^-) amount was measured using dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA), a non-fluorescent cell permeable indicator for superoxide anion and analyzed by fluorometric assay. Melanocytes and human melanoma cell lines Wm1552, Wm1366 and Lu1205 were treated or not (control) with 0.5, 1.0, 10 and 100 μg·mL^{-1} of PFFR3.3 and PP4 subfractions for 5, 15 and 30 min. Then, medium containing the treatments was removed, cells were washed with phosphate-buffered saline (PBS) and incubated

with 25 μm DHE for 40 min at 37˚C in the dark. After washing, fluorescence was evaluated in Spectromaxi3 (Molecular Devices) (excitation wavelength 480 nm; emission wavelength 567 nm).

To evaluate the results, we relied on Student's t-test for two-group experiments and analysis of variance (factorial ANOVA) with Dunnett's post-test for experiments with three or more groups using the GraphPad Prism 7.0® statistical software (GraphPad, San Diego, CA). The significance level was established at $p < 0.05$.

3. Results and Discussion

Previously data analysis from *B. segetum* ethanolic extract (EEBs, 24.46 g; 13.22%) with free radical scavenging assay (DPPH) and bioautographic assay (*Cladosporium* genus) showed that EEBs is active in these assays. EEBs by bio-guided fractionation furnished the PFFR 3.3, a subfraction active in DPPH assay, therefore with antioxidant potential and the PP4, subfraction with antifungal activity on bioautography assay (**Figure 3**).

The subfraction PFF3.3 obtained of EEBs corresponds to 81.5% of the 5-CQA by HPLC-DAD (**Figure 4**) based on the data network. The IC_{50} of 146.8 μg/mL

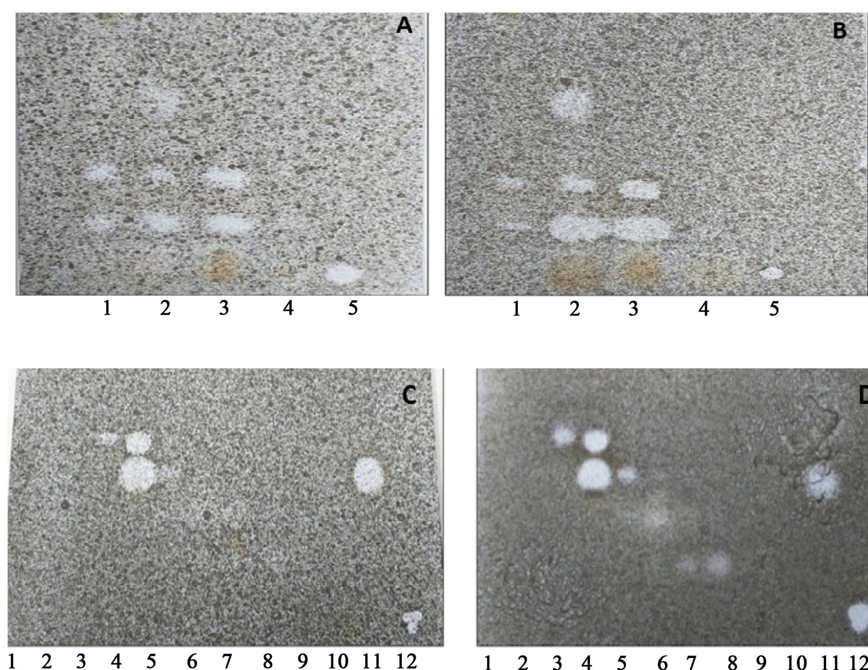

Figure 3. Bioautography (TLC on F_{254} silica gel eluted in 9:1 $CHCl_3$: MeOH visualized under white light (Camag System): Fractions of ethanolic extract (EEBs) from *Bidens segetum*: 1-FH, 2-FAcEOt, 3-FAcEOt, 4-MeOH, 5-Nystatin. A-Developed with *Cladosporium cladosporioides* spores (2 and 3 from FAcOEt) showed different colors and were analyzed separately and then pooled based on Rf and B-Developed with *Cladosporium sphaerospermum* spores. FAcEOt subfractions: 1-PP1, 2-PP2, 3-PP3, 4-PP4, 5-PP5, 6-PP6, 7-PP7, 8-PP8, 9-PP9, 10-PP10, 11-Cinnamic acid authentic standard (Sigma), 12-Nystatin. C-Developed with spores of *C. cladosporioides* and D-Developed with spores of *C. sphaerospermum*.

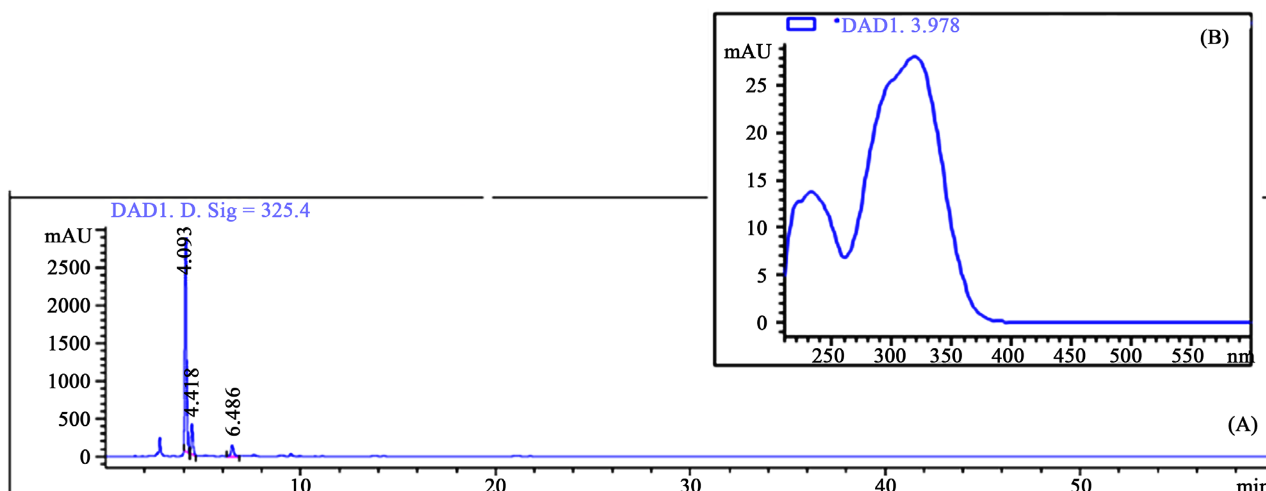

Figure 4. Chromatographic profile (Analytical HPLC-DAD) of PFFR3.3 subfraction obtained of ethanolic extract (EEBs) from *Bidens segetum* A-λ = 325 nm and B-UV spectrum at R_t = 4.09 min.

(DPPH assay) of PFFR3.3 obtained based on data from the 5-CQA standard curve. The caffeoylquinic acids (CQAs) show a variety of biological activities like antioxidant, anti-inflammatory, inhibit mutagenesis and carcinogenesis, anti-HIV, anti-HBV, radical scavenging, and are considered to be beneficial to human health [32] [33] [34] [35] [36].

A study radical scavenging DPPH with 5-CQA had an IC_{50} value of 7.4 µM and a percentage of DPPH inhibition of 10.3% [34]. The 3,4- and 4,5-dicaffeoylquinic acids isolated from various parts of *Bidens pilosa* showed also antioxidant potential using the DPPH assay [37]. *In vitro* evaluation of the potential antioxidant activity of PFFR3.3 and PP4 subfractions in melanocytes and melanoma cells, Wm1552 (1.0, 10.0, 100 $\mu g \cdot mL^{-1}$), Wm 1366 (0.5. 10.0, 100, 200, 400 and 500 $\mu g \cdot mL^{-1}$), and Lu 1205 (0.5, 5.0, 50, 100, 200 $\mu g \cdot mL^{-1}$) (DHE assay) showed that 30 min of treatment not reduced superoxide anion (data not shown). However, 5 min of treatment of metastatic melanoma cell Lu1205 with PFFR3.3 at concentrations of 1.0, 10 and 100 $\mu g \cdot mL^{-1}$ showed a downward trend in superoxide anion levels *in vitro* which was not observed at the same concentrations after 15 min (**Figure 5(A)**). Instead, we observed an increase in superoxide anion levels at 10 µg/mL (**Figure 5(B)**). These results suggest that the oxidation-reduction reaction kinetics is dependent on the concentration and time of action of PFFR3. By other hand, treatment of melanocytes for 30 min with 0.5 $\mu g \cdot mL^{-1}$ PFFR3.3 reduced superoxide anion levels compared to the control (NT) **Figure 6**.

The PP4 chromatographic profiles by LC-MS/MS/ESI^+ data showed chromatography peak with Rt = 17.2 min and $[M + H]^+$ with *m/z* = 419 Da and M = 418 Da to $C_{22}H_{27}O_8$. MS^2 spectrum showed a fragment ion with a 100% relative abundance of *m/z* = 257 Da. $[256 + H]^+$ and molecular formula (MF) $C_{16}H_{17}O_3$, compatible for loss of one hexose unit $[M - 162]^+$ and attributed to glycoside polyacetylene (Tr = 17.2 min), Chromatography peak detected at Rt = 22.6 min

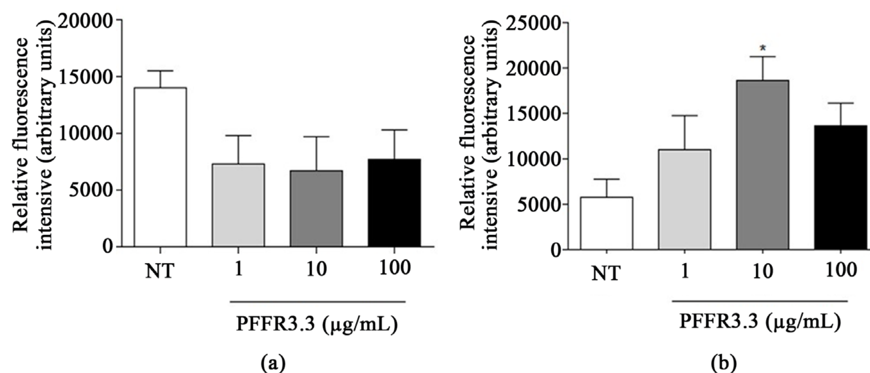

Figure 5. Superoxide anion levels in Lu1205 metastatic melanoma cells were analysed using DHE by fluorimetric assay. Metastatic melanoma cell Lu 1205 treated with different concentrations of PFFR3.3 subfraction for 5 min (A) and 15 min (B) using untreated cell line as control (NT). Values are reported in the bar graphs and expressed as the means ± SD. The experiments were performed in triplicate and the bar graphs and expressed as the means ± S.D. The experiments were performed in triplicate and $p < 0.05$.

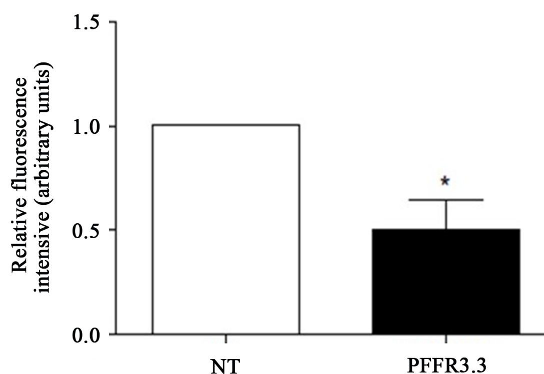

Figure 6. Superoxide anion levels in melanoctyes were analyzed using DHE by fluorimetric assay. Melanocytes treated with 0.5 mg/mL PFFR3.3 subfraction for 30 min using untreated cell line as control (NT). Values are reported in the bar graphs and expressed as the means ± SD. The experiments were performed in triplicate and the bar graphs and expressed as the means ± S.D. The experiments were performed in triplicate and $p < 0.05$.

($C_{31}H_{34}O_{10}$) *m/z* = 567 Da $[M + H]^+$ and MS^2 spectrum showed a fragment ion with a 100% relative abundance of *m/z* = 257 Da. $[256 + H]^+$ similar to polyacetylene (Rt = 17.2 min) and fragment ion of *m/z* = 309 Da $[M + H]^+$ and MF $C_{15}H_{16}O_7$ was attributed to radical -O-caffeoyl-2-methyl-D-erythonic acid (Rt = 22.6 min [38]. The structures of the derivate polyacetylenes were proposed based on data from ^1H NMR and HSQC and HMBC (Data not showed).

PP4 subfraction treatment of metastatic melanoma cell Lu1205 at 1 μg/mL for 5 min decreased the amount of superoxide anion (**Figure 7(A)**). However, when incubation time was increased to 15 minutes, superoxide anion levels augmented (**Figure 7(B)**). The polyacetylene rich extracts of *Bidens* species (34%) and other species of the Asteraceae family present cytotoxic activity against fungi, bacteria and insect larvae, evidencing the relationship between cytotoxic activity and these compounds [9].

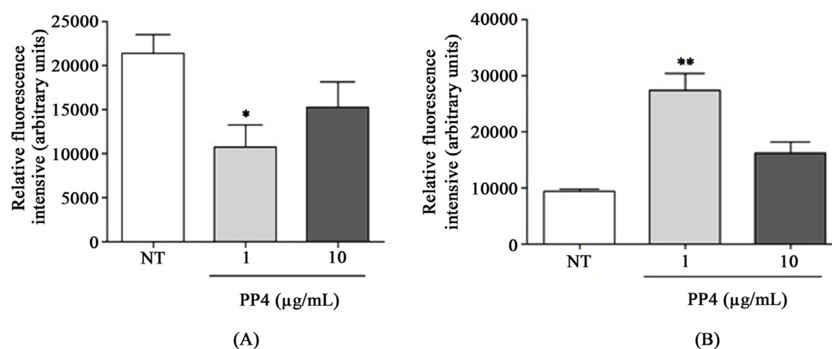

Figure 7. Superoxide anion levels in Lu1205 metastatic melanoma cells were analyzed using DHE by fluorimetry assay. Metastatic melanoma cell Lu 1205 treated with different concentrations of PFFR3.3 subfraction for 5 min (A) and 15 min (B) using untreated cell line as control (NT). Values are reported in the bar graphs and expressed as the means ± SD. The experiments were performed in triplicate and the bar graphs and expressed as the means ± S.D. The experiments were performed in triplicate and $p < 0.05$.

The subfraction PP4 was fractionated by semi-preparative HPLC, obtaining five subfractions (PP4.1 - PP4.5). PP4.1 presented the highest relative abundance peaks (66.22%) of PP4 (λ = 370, 330, 230 nm and R_t = 49.934 min), but it is not the antifungal active subfraction of PP4 and not was submitted to DHE assay. Polyacetylene UV data were observed for the compound at R_t = 28.669 min [39] (**Table 1**).

4. Conclusion

Ethanolic extract (EEBs) from *Bidens segetum* leaves present in their composition glycoside polyacetylenes and phenolic compounds such as 5-O-caffeoylquinic acid (5-CQA), like other *Bidens* species. The 5-CQA was identified in the subfraction PFFR3.3 (81.5% purity) and showed potential antioxidant when evaluated in the DPPH assay. Treatment of melanocytes for 30 min with PFFR3.3 decreased superoxide anion amount; however, in metastatic melanoma cells superoxide anion levels increased after 15 min. PP4 subfraction is a mixture, among polyacetylenes, has antifungal (*C. cladosporioides* and *C. shaerospermum*) and antioxidant activity. PP4 reduced superoxide anion amount in metastatic melanoma cells Lu1205 after 5 min at concentrations of 1.0 μg·mL^{-1}. However, superoxide levels increased after 15 min. Therefore, we cannot say that PFFR3.3 subfractions have activity in inhibiting superoxide anion amount because the results of inhibition of O_2^- were not conclusive; in most of the analyzed concentrations, there is no concentration-response relationship and time of action. *In vitro* assays to determine inhibition of superoxide anion production in melanoma and melanocytes using caffeoylquinic acids and polyacetylenes have not been reported in the literature. Complementary chemical studies will be performed to confirm the polyacetylenes and 5-CQA structures present in *B.segetum* leaves and new methodologies should be performed to confirm the antioxidant activity of these compounds and the effects on melanocytes and melanomas.

Table 1. Data of PP4 subfraction from *Bidens segetum* (EEBs) by HPLC-DAD and ultraviolet (UV) spectra.

PP4						
PP4	352 nm	280 nm	325 nm	UV		
R*t* = min	%	%	%	min	Ref.	λ = nm
27.879	5.63	nd	nd	27.845	27.530	370, (290), 230
28.669	7.12	9.63	2.94	28.666	28.251	380, 350, 320, 300, 280, 260, 240
49.671	14.04	65.10	56.73	49.608	49.402	320, (290), 220
49.934	66.22	16.66	30.81	49.938	49.402	370, 330, 230
50.307	3.41	nd	1.05			
51.033	3.56	3.24	3.49	51.030	49.402	(360), 330, 220

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Conflicts of Interest

Authors have no conflict of interests of any nature to state in this section.

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