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Original Article

In vitro Antitumor Effect on Melanoma Cell Line and Chemical Composition of *Diplopterys pubipetala* (A. Juss) W.R. Anderson and C. Davis

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ABSTRACT
The *Banisteriopsis pubipetala* A. Juss. came to be considered synonymous with *Diplopterys pubipetala* (A. Juss.) W.R. Anderson and C. Davis. *D. pubipetala* is a liana, occurring in the cerrado biome, belonging to a genus already described in the literature for the antioxidant action of some species and for being effective in controlling some types of cancer, including cutaneous melanoma, which is the most common malignant tumor in the whole human species. However, *D. pubipetala* is still poorly studied with regard to bioactive potential and chemical composition. The main objective of the work is to evaluate antitumor activity by evaluating migratory activity, detecting cell death and determining cell viability of the partitions and fractions of *D. pubipetala* leaf and stem. The fractions demonstrated the ability to induce death, decrease cell viability and even suppress B16F10 migration. This study is a pioneer in the antitumor evaluation of *D. pubipetala* and opens new lines of research for exploration. This demonstrates the promising possibility of using the species to develop new strategies for the treatment and prevention of cutaneous melanoma.
Key words: Antitumor activity, Cell death, Cutaneous melanoma, Malpighiaceae, Metastasis.

INTRODUCTION

Pure chemical substances of botanical origin are often used in the form of licensed medicines and even when produced synthetically, drugs capable of promoting the treatment of diseases were originally discovered from plant drugs.[1] The discovery of the isolation of natural products was decisive for the rise of the pharmaceutical industry.[2] From the revelation of the importance of natural products and the possibility of assessing the bioactive effect of these isolated substances, a new research trend emerged at the end of the 19th century; the chemistry of natural products. Drugs of natural origin have been shown to be effective for the treatment of infectious, cardiovascular diseases, premature aging, chronic diseases and have also stood out for the treatment of cancer.[3]

At the beginning of the 20th century, cancer, considered until then as a rare and exceptional disease, started to be seen as an increasingly frequent cellular disorder.[3,4] Cancer, which can also be called malignant neoplasm or malignant tumor, is the name given to a set of more than one hundred diseases that have in common the multiplication and propagation of abnormal cells in the body.[5] The main causes of cancer are related to external factors such as cigarette consumption, some types of viruses, eating and sexual habits, alcoholism, the practice of using medications and also, the excess of sun and exposure to ultraviolet rays (UV).[6]

Among the variations of the disease, cutaneous melanoma is an extremely common malignant tumor, it causes about 90% of deaths in the occurrences of all cases of skin cancer and the incidence is constantly increasing worldwide.[7,8] On average, one million new occurrences appear in the world each year and in Brazil, melanoma represents just over 25% of the total number of all types of cancer that affect Brazilians.[9] Because they are quite variable, with regard to molecular aspects, melanomas have a great capacity for progression by metastasis.[10] Tumor cells also have mechanisms for not being recognized by the patient's immune system and this provokes resistance to therapeutic approaches for the treatment of the tumor.[11]

This problem brings up the need to explore new effective therapeutic practices to contain the metastatic action of melanoma and reduce the number of deaths related to the disease.[12] Since the discovery of the alkaloids vinblastine and vincristine, isolated from the species *Catharanthus roseus* G. Don. (Apocynaceae), a new era has begun for the search for new substances that are effective in

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combating cancer.[13] Natural products have brought hope to the search for efficient chemotherapeutics for the treatment of cancer, effective for the eradication of tumors and also, which reduces side effects to the patient.[14] This trend increases attention to research on medicinal plants and secondary metabolites and is called the "green anti-cancer approach".[15]

Among the classes of compounds already tested with pronounced anticancer activity, it is possible to mention the terpenes and alkaloids, which were the first to be proven with anti-tumor action; coumarins, lignin's, tannins, stilbenes, curcuminoids, polysaccharides and flavonoids.[16] Flavonoids occur as a complex mixture of components and have an ideal structure for sequestering free radicals.[17] In the literature, it is possible to find studies that describe flavonoids with anti-inflammatory, analgesic, anti-hepatotoxic, antimicrobial, antiviral, antioxidant and antitumor activities.[18]

Regarding antitumor activity, flavonoids are capable of containing the rapid growth of melanoma; among the mechanisms of action, they can induce apoptosis and stimulate the induction of antitumor immunity in the body.[19] In combination with chemotherapy, these substances have cytostatic action, that is, they limit growth and favor the rejection of the organism to melanoma tumor cells.[20]

The Malpighiaceae family is renowned for hosting many species with antioxidant activity and great therapeutic potential.[21] It is one of the ten best represented families in the cerrado and has approximately 60 genus and 1200 species of trees and lianas.[22] The *Banisteriopsis* genus, also belonging to the Malpighiaceae family, consists of 92 lianas and shrubs distributed in Mexico, Argentina and Brazil, the studied species of the genus have demonstrated great potential for use in the pharmaceutical and cosmetics industry.[23,24]

A species related to the genus, *Banisteriopsis caapi*, stands out as a liana known worldwide for producing hallucinogenic compounds with great bioactive potential and for being one of the components of Ayahuasca tea, used by indigenous tribes in detox rituals.[25] In addition to *B. caapi*, the species *B. argyrophylla* has also been described with important pharmacological potential; leaf and stem extracts of the species have anti-inflammatory and antioxidant action.[23] The extract and leaf partitions directed to *B. laevifolia* flavonoids, another species of the genus, demonstrated a relevant effect of free radical scavenging and possible antitumor action.[26]

In view of the queries made in the literature regarding the species, it is possible to notice that there are no studies related to *D. pubipetala* with regard to the biological activities of the stem and leaves. Therefore, the objective of this work was to study the antitumor potential in melanoma cell lines.

MATERIALS AND METHODS

Collection of plant material

Leaves and stems of young and healthy plants of *D. pubipetala* were collected, near the district of Nova Esperança in Montes Claros - Minas Gerais. The identification was carried out by specialists based on the manufacture of exsiccatae deposited at the Herbário Montes Claros Minas Gerais (MCMG), of the State University of Montes Claros (UNIMONTES), under voucher 4033.

Preparation of Plant Material

The collected material was washed in running water and dried in an oven (Nova Ética) under heating and air circulation at 38°C (± 2) for 7 days until it presented constant weight. Then, the botanical material was sprayed in a Willey mill, conditioned and kept refrigerated (5°C). All stages of collection and preparation of plant material were determined based on modifications to the "Manual of practice for collection and herbalization of botanical material".[27]

Liquid-Liquid Partitions for Flavonoids

Ethanol (p.a) was added to the sprayed samples of *D. pubipetala* stem and leaf in the proportion of 10 mL of ethanol for each gram of vegetable powder. The mixture was ultrasounded for two hours every day for one week and after that time, the mixture was filtered and the residue was resuspended again in ethanol in the proportion of 5 mL for each gram of residue, the mixture was passed through maceration for two weeks, was filtered again and placed in an oven at 38°C (± 2) to dry the solvent.

For the fractionation of the extract directed to flavonoids, the samples obtained in the previous procedure were resuspended in a mixture of ethanol: water (7: 3), in the proportion of 3 g of extract to 125 mL of 70% ethanol. In the first wash of the mixture, 200 mL of hexane p.a was added three times in a separatory funnel. In the residue from the first wash, 200 mL of ethyl acetate were added three times. Both partitions were taken to the greenhouse under air circulation at 38°C until the solvents were dried,[28] however only the ethyl acetate partition was used for the next stages of the study.

Classic Liquid Column (CLC)

The separation of the components of *D. pubipetala* ethyl acetate partition was performed using classical liquid chromatography with an increasing polarity gradient. The column used is 2.25 cm in diameter, was filled with silica gel 60 for column chromatography in the proportion of 100 mg of the extract to 100 g of silica in 400 mL of hexane. The mobile phases used were: 100% hexane; hexane: ethyl acetate (8: 2; 7: 3; 1: 1; 3: 7; 2: 8); 100% ethyl acetate; and 100% methanol. The fractions were collected in 10 mL test tubes with manually controlled flow.

High Performance Liquid Chromatography - DAD

The device is equipped with binary pump 1525, automatic injector 717, automatic collector of fractions III, diode array detector 2996 and SoftWare Empower 1. The column used was the C_{18} 250 x 4.6 mm 5 micron Spherisorb and 5 µm particle. The fractions eluted at a flow rate of 0.6 mL/min. The mobile phase used was a solution of acetonitrile acidified in a solution of trifluoroacetic acid 0.01% (1:99). The run was isocratic, the run time was 60 min and the peak readings were taken at a wavelength of 220 nm. The analyzes took place at a temperature of 30°C.[29]

B16F10 cell culture

The melanoma tumor cell line (B16F10) used was kindly provided by the Federal University of Minas Gerais - UFMG. The cells were cultured in RPMI medium (Gibco ™ RPMI Medium 199 Powder, 10x1L), supplemented with 10% inactivated fetal bovine serum (Gibco ™ fetal bovine serum, certified, heat inactated, US origin) and 1% antibiotic (Penicillin/Streptomycin). The cells were kept in culture bottles (75 cm^2, filter lid, transparent polystyrene, surface treated with plasma for adherent cells), in an incubator (Sterisonic ™ GxP MCO-19AIC (UVH) cell incubator CO_2 culture. SANYO Eletric Co. Ltd.) at 37°C and humidified atmosphere with 5% CO_2 and observed daily.

Cell Count

The cells were removed from the flask with trypsin solution (Gibco ™ Trypsin - EDTA - 0.05%, Phenol red). After this procedure, the solution was inactivated with complete medium, centrifuged at 1000 rpm for five minutes, then the supernatant was removed and the cells were resuspended in 1 to 2 mL of medium. To define the number of cells in culture, an aliquot of 10 µL was removed from the mixture, added to 80

µL of trypan blue dye (Lac Biotecnologia, Trypan Blue 0.4 solution in PBS 1X - 100 mL), the number of cells/mL was measured in a Neubauer chamber (Marienfeld - Depth profundeur 0.100 mm - 0.0025 mm^2) using the formula:

$$\frac{(n° \text{ total cells})}{n° \text{ counted quadrants}} \times \text{dilution factor} \times 10^4$$

Partition preparation and *D. pubipetala* fractions and Treatment

The diluents of the partition and fractions were chosen from a previous dilution test. Fractions F1, F12, F13 and F14 were excluded from the antitumor activity tests because they had a yield lower than 2 mg/column, the F7 fraction was also excluded from the tests for not having solubility for the preparation of stock solution a 500 µg/mL.

To carry out the tests for the anti-tumor activity of *D. pubipetala*, a stock solution of 1000 µg/mL of the ethyl acetate partition diluted in incomplete RPMI was prepared. The stock solution of the fractions was initially prepared at 500 µg/mL in a solution of RPMI:acetone (99:1). After dilution and homogenization of the stock solutions, the mixtures were filtered with a syringe and a 0.22 µm Millipore filter. The cells were subjected to treatments made from the serial dilution of the stock solutions.

Determination of Cell Viability (MTT method)

The cell viability test was used to measure cell proliferation and cytotoxicity and is determined by the reduction of MTT (3- (4,5 dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide), with modifications in the methodology described by Mosmann.[30] $3x10^3$ B16F10 cells/well were plated in supplemented RPMI medium. Each well was treated with 200 µL of sample. From the stock solution of the ethyl acetate partition, treatments of seven different concentrations were made (1000; 500; 250; 125; 62.5; 31.25; 15.625 µg/mL). For all fractions, treatments were performed at concentrations of 250 and 125 µg/mL, but for fractions that demonstrated in the initial test a cell viability greater than 78% at a concentration of 125 µg mL, in the 24 hr treatment, the concentration of 500 µg/mL was also tested.

After removing the medium with the treatment in 96-well plates, 150 µL of complete RPMI medium containing 10% MTT at 5 mg/mL diluted in 1x phosphate buffer in 1x concentration (PBS 1x = 137 mMNaCl, 10 mM phosphate, 2.7 mMKCl with pH 7.4). The mixture was incubated in an oven at 37°C with 5% CO_2 for 2 hr. The MTT medium was then removed and the formed precipitate dissolved in 200 µL of dimethyl sulfoxide (DMSO). The reading was performed at 24 and 48 hr, in an ELISA reader, at 540 nm.

The absorbance values were converted to the percentage of viable cells present in each well using the formula:

$$\frac{\text{sample absorbance-Blank absorbance}}{\text{Control absorbance-Blank absorbance}} \text{ x } 100$$

The cell viability assessment was performed in quadruplicate, in three different experiments.

Cell Migration Assay

In a 12-well plate, 1.8×10^5 B16F10 cells/well were seeded in supplemented RPMI medium. After acquiring 80% confluence, the cells were subjected to a scratch made with a sterile pipette tip and subsequently treated with the ethyl acetate partition and the *D. pubipetala* fractions at a concentration of 125 µg/mL diluted in incomplete medium. For fractions that were tested at a concentration of 500 µg/mL in the cell viability test (MTT), migration and cell death tests were also performed at a concentration of 250 µg/mL. The wells were photographed at the beginning of the experiment, after 24 and 48 hr of treatment. The images of the experiment were made using the SC30 camera (Olympus, Center Valley, Pennsylvania USA), in an inverted IX81 microscope (Olympus). The measurement of the cleft area and analysis of the cleft occupation by the cells were performed using the ImageJ program. The percentage of migration was calculated using the formula:

$$100 \text{ t} \frac{\text{final area}}{\text{initial area}} \text{ x } 100$$

The experiment was carried out in triplicate and in three different analyzes.

Cell Death Detection

For the cell death test, a 12-well plate was used and 1.8×10^5 B16F10 cells/well were seeded in supplemented RPMI medium, after a period of approximately 20 hr, the cells were treated with the partition and fractions in the concentration of 125 and 250 µg/mL (according to the cell viability test). The medium containing dead cells was stored and the cells still adhered to the bottom of the plate after 48 hr were removed by trypsin solution. By centrifugation at 1000 rpm for five minutes, the supernatant was discarded and the cells were resuspended in 25 µL of a mixture of 2% Acridine Orange and Ethidium Bromide (AO/EB) diluted in 1X PBS. 10 µL of the mixture was transferred to a slide protected from light and immediately, pictures of three different fields were captured using fluorescence microscopy. Acridine orange (AO) is absorbed by viable and non-viable cells and emits green fluorescence. Ethidium bromide (EB) is absorbed only by non-viable cells and emits red fluorescence. The cell death test performed in this study (AO/EB) is double stained and distinguishes four different cell types: (1) viable cells with bright green nuclei and an organized structure; (2) Early apoptotic cells that have already begun to undergo ADN cleavage; (3) red and orange late apoptotic cells with disorganized structure; and (4) red and orange necrotic cells with uniformly organized structure.[31]

The red and orange cells were considered dead and the green colored cells were considered alive. The number of cells was measured manually using the cell count of the ImageJ ® software. And the percentage of cell death was calculated using the following formula:

$$\frac{n°\text{dead cells}}{n°\text{total cells}} \text{ x } 100$$

In all tests, a control was performed containing only complete RPMI medium (CT1) for the ethyl acetate partition and a control with RPMI: 1% acetone (CT2) solution for the fractions.

Statistical analysis

Data were evaluated using one-way ANOVA analysis of variance, followed by the Tuckey *post-test*, using the GraphPadPrisma® software version 5.00 and statistical significance was accepted with a *p*-value less than 0.05.

RESULTS AND DISCUSSION

Determination of Cell Viability (MTT method)

All concentrations of the ethyl acetate partition, albeit to a lesser extent, have an effect on the cell viability of melanoma cells 24 hr: 16.625 µg/mL (95.25%); 31.26 µg/mL (70.84%); 62,5 µg/mL (58.6%); 125 µg/mL (46.11%); 250 µg/mL (17.12%). 48 hr: 16.625 µg/mL (83.60%); 31.26 µg/mL (68.49%); 62.5 µg/mL (54.20%); 125 µg/mL (40.99%); 250 µg/mL (15.23%). The percentage of cell viability decreased by more than 10% from one concentration to the other, with the exception of the concentrations of 500 (8.5 and 4.4% in 24 and 48 hr) and 1000 µg/mL (3.1 and 1.2% in 24 and 48 hr). This demonstrates that the partition causes a dose-dependent effect on the cells, that is, the more the concentration of

the ethyl acetate partition increases, the lower the cell viability of B16F10 (Figure 1).

The effect of the F2 fraction stands out in 24 hr at a concentration of 125 µg/mL (73.25%), the F3 fraction has little difference in cell viability in the same situation (75.81%). At the time of 48 hr, still at the concentration of the 125 µg/mL, F6 was the fraction that most decreased the cell viability value (51.82%). At the concentration of the 250 µg/mL, the F3 fraction was the fraction that most decreased the cell viability value of B16F10 (32.41% in 24 hrand 17.90% in 48 hr) (Figure 2).

Fractions F9, F10, F11 and F15 were not able to decrease by at least 20% of cell viability in the first 24 hr at any of the two concentrations of 125 and 250 µg/mL tested initially. No dose-dependent and time-dependent effect was observed in treatments with the F9 fraction at concentrations of 125 and 250 µg/mL. Fractions F3, F5 and F6 have both a dose-dependent effect at both concentrations and a time-dependent effect at both times. Among them, the fraction with the greatest dose-dependent effect is F3 and the fraction with the greatest time-dependent effect is F6.

Fractions F8 and F11 do not have a dose-dependent effect, but they do have a time-dependent effect, however the F11 fraction. The F4 fraction has a dose-dependent effect at 24 and 48 hr and has a time-dependent effect only at the concentration of the 125 µg/mL. The F10 fraction, on the other hand, has the greatest dose-dependent effect observed in 48 hr, but it has a time-dependent effect only at the concentration of the 250 µg/mL.

The F2 fraction has the greatest time-dependent effect observed in the tests, however, the fraction has a difference in cell viability greater than 10% only in the concentration of 250 µg/mL and in the time of 48 hr. The F15 fraction has no dose-dependent effect, but it does have a time-dependent effect at a concentration of the 250 µg/mL.

Fractions F4, F8, F9, F10, F11 and F15, with cell viability greater than 78% at a concentration of 125 µg/mL in 24 hr, were also tested at a concentration of 500 µg/mL (Figure 3). The fractions with prominence in the decrease in the cell viability value within 24 hr were F4 (13.35%), F9 (13.46%) and F11 (14.56%). Within 48 hr, F11 (1.722%), F9 (4.328%) and F4 (6.834%) also stood out.

The polarity of ethyl acetate influences the extraction of phenolic compounds, among them, mainly flavonoids.[32] Taking into account the polarity of the mobile phase and the peaks in the chromatographic profile in the first 10 min of elution, it is possible to infer that the substances extracted from the fractions in the CLC have, for the most part, medium polarity.[33]

To consider the time/dose-dependent effect of a plant extract, it is necessary to observe the difference in its effect on cell viability with increasing dose.[34] In this study, to define the positive dose/time dependent effect of the ethyl acetate partition and the *D. pubipetala* fractions, cell viability values were considered with a difference greater than 10% from one concentration to the other and from one time to the next other.

The ethyl acetate partition of *Mormodica charantia* leaves, already described in the literature as a species with anticancer activity,[35] reduces 50% of the cell viability of colorectal adenocarcinoma cells at a concentration of 350 µg/mL,[36] while the partition *D. pubipetala* ethyl acetate reduces over 50% of the cell viability of B16F10 at a concentration of 125 µg/mL. The *Scutellaria barbata* and *Catharanthus rouseus* partitions, species with flavonoids and alkaloids widely used as chemotherapeutic agents were also unable to reduce 50% of the cell viability of different cancer strains with doses lower than the 125 µg/mL.[37]

It is possible to notice that there is a difference in cell viability greater than 10% only in the concentration of 16.6 µg/mL (95.25% in 24 hr and 83.6% in 48 hr) when comparing the same concentrations in the times of 24 and 48 hr. B16F10 cells are extremely aggressive and invasive,[38]

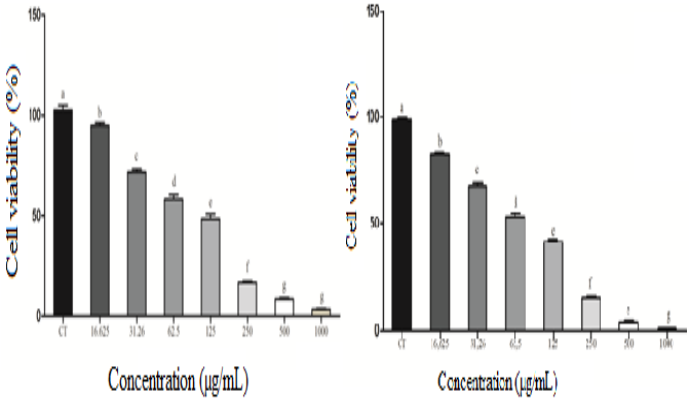

Figure 1: Cell viability of 24 hr (A) and 48 hr (B) of the ethyl acetate partition.

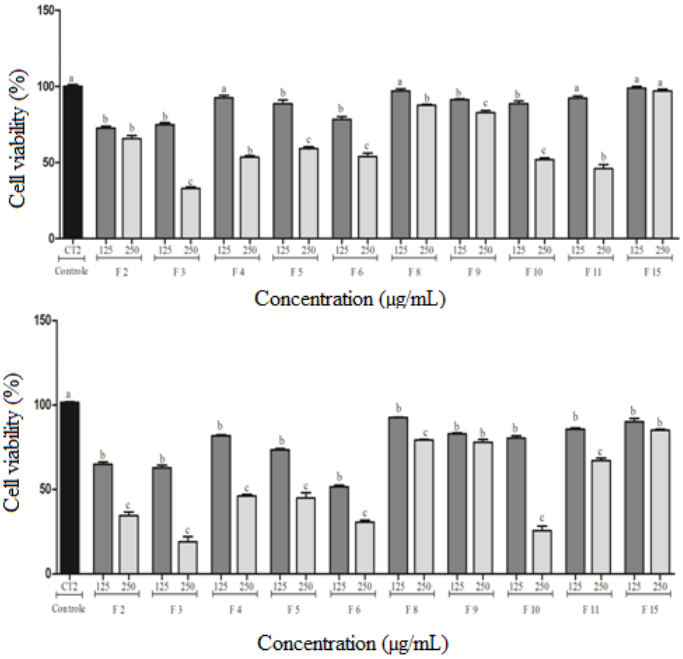

Figure 2: Cell viability of 24 hr (A) and 48 hr (B) of fractions F2, F3, F4, F5, F6, F8, F9, F10, F11 and F15 at concentrations of 125 and 250 µg/mL.

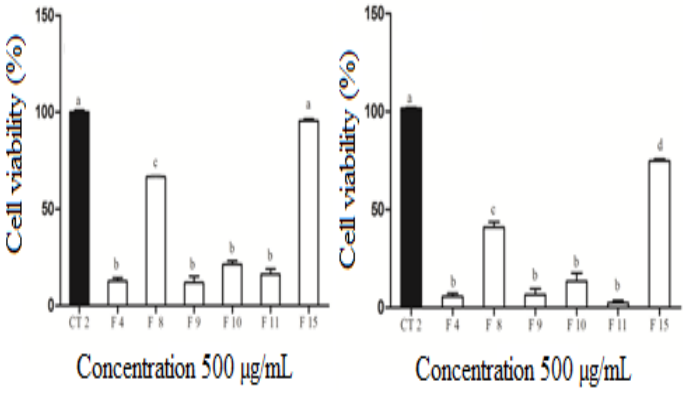

Figure 3: Cell viability of fractions F4, F8, F9, F10 and F15, in 24 hr (A) and 48 hr (B).

and even though the difference in the effect of each concentration from 24 to 48 hr is less than 5%, the effects it causes when comparing the concentration values are still very interesting.

The ability to decrease cell viability is the first indication of the antineoplastic potential of a chemotherapeutic agent.[39] The cytotoxic action of the *D. pubipetala* partition and fractions may be associated with phenolic compounds, mainly flavonoids, which represent a group of natural compounds with promising anticancer potential,[40] and are the main substances extracted in ethyl acetate partitions.[41]

In extractions made under the same conditions as this study, in *B. argyrophylla* of the same genus as *B. pubipetala* (*D. pubipetala*), the presence of catechins and five chemical variations of quercertin with cytotoxic characteristics were evidenced.[42] In *B. laevifolia*, the following compounds were identified: procyanidin B1; 4, epicatechin; 5, catechin; 6, 3-O-2-D-apiofuranosyl- (1 → 2) -galactopyranosylquercetin; 7, procyanidin B2; 8, quercetin-3-O-glucuronide and 9, rutin, which have great antioxidant potential.[26]

These compounds have received special attention due to their ability to inhibit oxidative stress, proliferation and cell cycle, they also induce detoxification, apoptosis and a better functioning of the immune system.[43] These characteristics facilitate the progress of new strategies and approaches for the treatment of cancer.[44]

Cell migration test

The ethyl acetate partition (-4.1% in 24 hr and - 59.94% in 48 hr) and the F2 fractions (-26.4% in 24 hr and -29.5% in 48 hr) F3 (-30.20% in 24 hr and - 107.0% in 48 hr) and F6 (-12.85% in 24 hr and -60.8% in 48 hr), significantly suppressed the migratory activity of the cells studied in the concentration 125 µg/mL (Figures 4). The samples were able to completely suppress the migration of B16F10 and also to increase the crack area, F3 was the fraction with the greatest effect on migratory activity (Figures 5).

The migration test was performed again for fractions F4, F8, F9, F10 and F15 at a concentration of 250 µg/mL. The F15 fraction (49.03% in 24 hr and 87.30% in 48 hr), again showed results similar to the control (50.40% in 24 hr and 99.84% in 48 hr), showing that there influences the migratory activity of B16F10 (Figures 6 and 7). For the other fractions, the increase in dose resulted in the suppression of the migratory activity of the cells and also in the increase of the crack area both within 24 hr (F4: - 6.090%; F8: - 15.93%; F9: - 25.90%; F10: - 22.9%) and within 48 hr (F4: - 19.02%; F8: - 42.09%; F9: - 42.50%; F10: - 36.70 %). In this situation, the fraction F9 stood out in 24 hr and F8 and F9 in 48 hr with the greatest effect on migratory activity.

The migration capacity of cancer cells is one of the events that cause the disease to evolve.[45,46] The potentially metastatic characteristic of cutaneous melanoma, even in its early stages, is mainly due to the innate ability to invade and spread to other tissues,[47] and this factor reduces the effectiveness of traditional therapies.[48] For a better understanding of the disease, it is of great importance that the mechanisms of invasion and migration of cancer cells are studied, for the development of new antitumor drugs.[45]

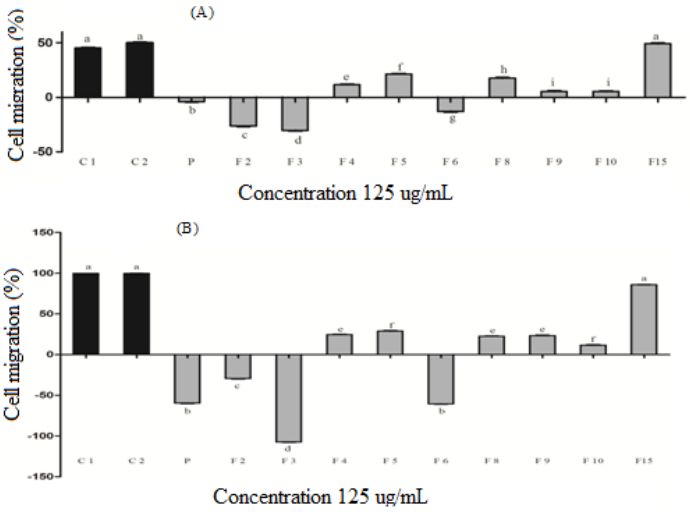

Figure 5: Migration test of the ethyl acetate partition and fractions F2, F3, F4, F5, F6, F8, F9, F10 and F15 at a concentration of 125 ug/mL, 24 hr (A) and 48 hr (B).

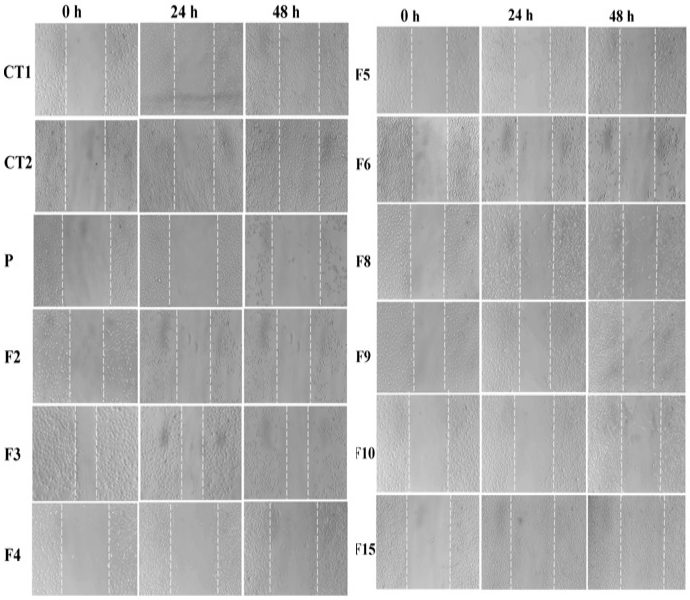

Figure 4: Migration assay of CT1 (control with complete medium), CT2 (control with medium + 1% acetone), Ethyl acetate (P) partition and fractions F2, F3, F4, F5, F6, F8, F9, F10 and F15 at a concentration of 125 µg/mL.

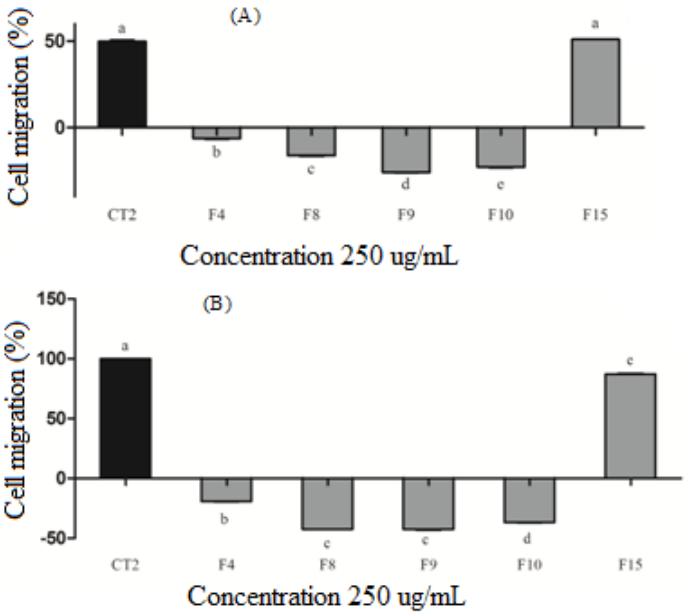

Figure 6: Migration test of fractions F4, F8, F9, F10 and F15 at a concentration of the 250 ug/mL, 24 hr (A) and 48 hr (B).

In this study, the influence on the migration and healing of *D. pubipetala* in B16F10 cells was evaluated from a crack in a confluent layer of cells made with a sterile pipette tip. With the exception of the F15 fraction, both the partition and the fractions of *B. pubipetala* significantly inhibited the migration capacity of B16F10 at concentrations of 125 and 250 µg/mL and this effect may be related to the presence of flavonoids characteristic of the species family.

Flavonols and flavones are subclasses of flavonoids with very similar chemical structures and their occurrence is extremely common in species of the Malpighiaceae family.[49] These substances are capable of interfering in the G0/G1 and S phases of the cell cycle, can cause a strong inhibition of lipid peroxidation,[50] and can also provide a negative regulation of metalloproteinase enzymes, involved in every stage of cell healing.[51] These characteristics make these substances have antiangiogenic, antimetastatic effects and are able to suppress the proliferation and invasion of cancer cells.[50]

Other studies have also shown the presence of flavonols and flavones in species of the Malpighiaceae family: in the genus *Stigmaphyllon*: *S. auriculatum*, *S. ciliatum* and *S. paralia*;[50] *Heteropteristo mentosa*,[52] *Byrsonima cinera*,[53] and also in the genus *Banisteriopsis*, in the species *B. variabilis*, *B. caapi*,[54] *B. laevifolia*[26] and *B. anisandra*.[55] Not all of the mentioned species have been tested in cutaneous melanoma cell lines, but even so, some compounds have been reported in the literature with the ability to inhibit the migration, invasion and cell adhesion of B16F10 and also other cell lines.[56,57,50]

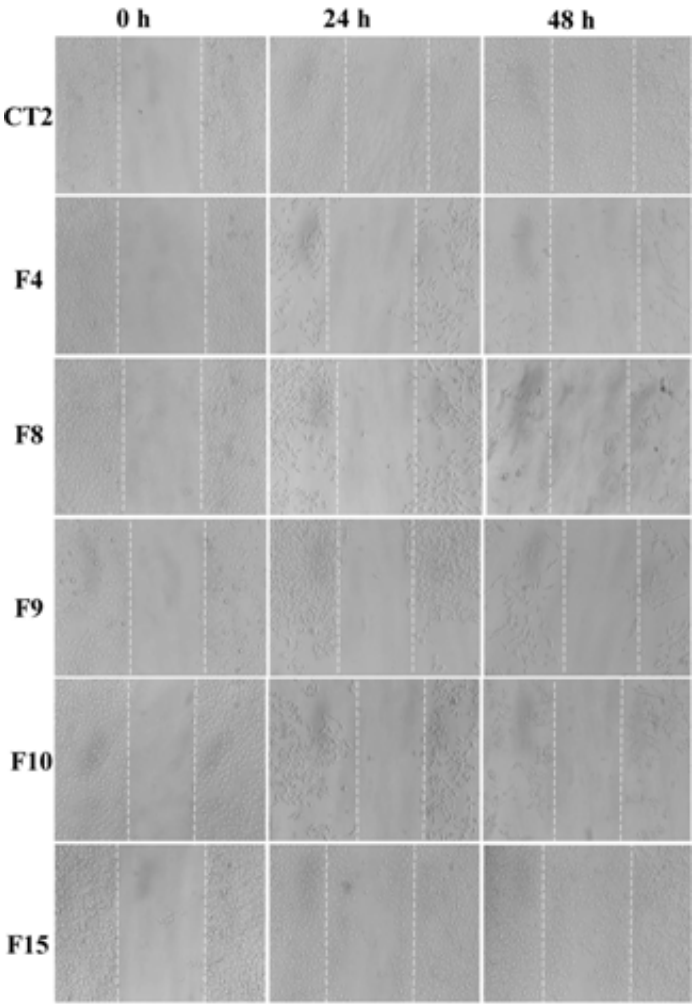

Figure 7: Migration test of fractions F4, F8, F9, F10 and F15 at a concentration of the 250 ug/mL, 24 and 48 hr.

Cell Death Test

In the treatments done with the concentration of the 125 µg/mL, the ethyl acetate partition (95.93%) and the F10 fraction (72.88%) were the samples that most influenced the cell death of melanoma cells. The fractions F2 (42.49%), F3 (39.48%) and F6 (40.28%) caused statistically similar effects in the cell death of B16F10. The fractions F4 (22.54%), F5 (31.41%), F8 (35.07%) and F9 (16.34%) also caused cell death, even though the effect was less compared to the other samples (Figures 8). The percentage values of cell death of the F15 fraction (9.932%) were not significantly different from the control values (6.58%) and thus, it is possible to state that the F15 fraction is also not capable of causing cell death in the studied cells.

The cell death assay was performed at a concentration of 250 µg/mL for fractions F4, F8, F9, F10 and F15 (Figure 9) and again the effect of the F10 fraction (91.06%) stood out among the other fractions in cell death of cutaneous melanoma cells. The F4 fraction (55.86%) had its effect

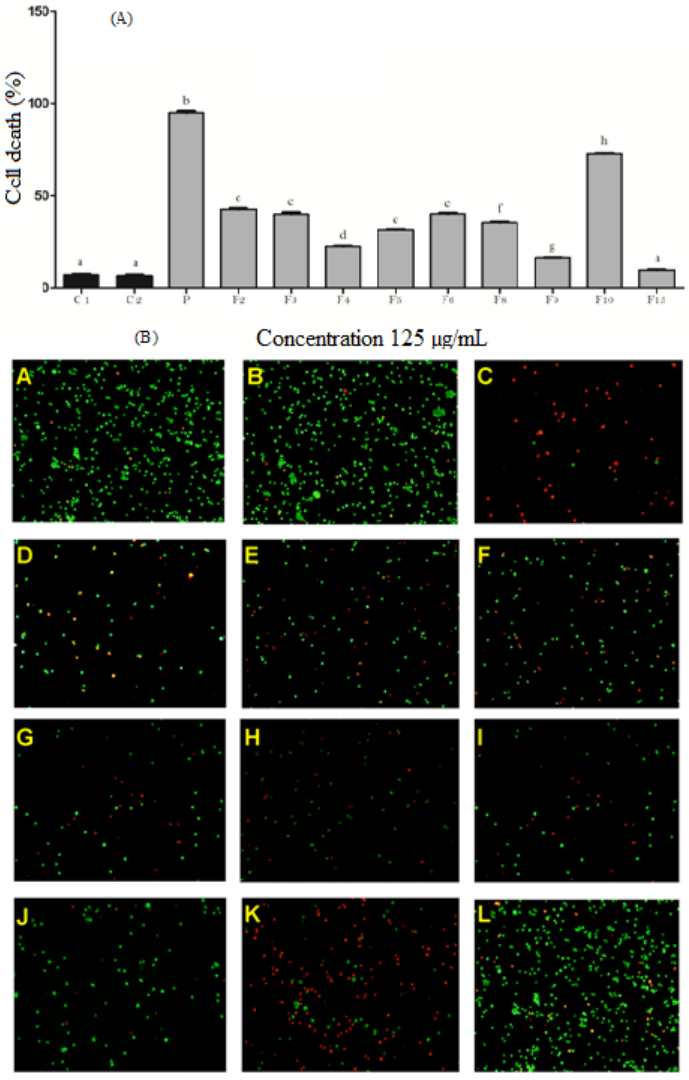

Figure 8: (A) Cell death assay performed at a concentration of the 125 µg/mL within 48 hr; (B) Plank of cell death at a concentration of the 125 µg/mL, within 48 hr. A: Control 1; B: Control 2; C: Ethyl acetate partition; D: F2; E: F3; F: F4; G: F5; H: F6; I: F8; J: F9; K: F10 and L: F15.

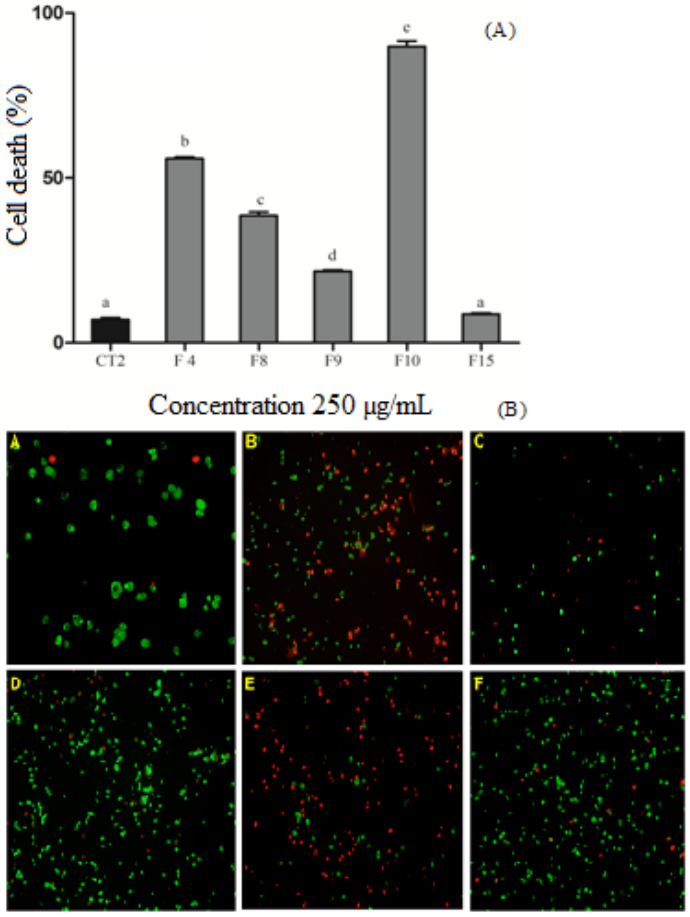

Figure 9: (A) Cell death assay performed at a concentration of the 250 µg/mL in 48 hr; (B) Cell death test board made at a concentration of the 250 ug/mL, within 48 hr. A: control 2; B: F4; C: F8; D: F9; E: F10; F: F15.

enhanced in more than 30% of cell death with the dose increase from 125 to 250 µg/mL, the F10 fraction (39.64%) increased slightly more than 4% in its effect on cell death with increasing dose, the F9 fraction (21.84%) increased by just over 5% and the F15 fraction (8.93%) once again did not influence cell death, with values statistically similar to those control values (7.35%).

The cell is considered dead when the plasma membrane loses integrity, when it fragments into apoptotic bodies, or when its fragments are taken up by other cells.[58] Death characteristics due to apoptosis, such as detachment, rounding, shrinkage and apoptotic bodies, were observed in B16F10 cells treated with the partition and fractions of *D. pubipetala* at concentrations of 125 and 250 µg/mL.

Apoptosis is a strictly organized process of programmed cell death, essential for the maintenance of the organism.[59,60] Through mechanisms still little known and studied, cancer cells are able to produce enzymes that inhibit apoptosis and as a consequence, this event causes the disease to evolve.[58] The induction of cell death and the dose-dependent effect may be the key to demonstrate the potential of *D. pubipetala* as an anticancer agent.

CONCLUSION

Through the results obtained in this study, it was possible to conclude that the samples of *D. pubipetala* extracts are promising in relation to the results of antitumor activity, as they were able to inhibit the metastatic action of melanoma cells, decrease the viability, the ability to migrate and cause the death of B16F10 tumor cells. The species may be a promising therapeutic possibility for the development of new strategies for the treatment and prevention of melanoma, for this, studies are needed to elucidate the molecular mechanisms involved in the species' antitumor action and also the identification of the substances responsible for this action.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

ABBREVIATIONS

AO: Acridine orange; **B16F10:** Murine melanoma cell line; **CLC:** Classic Liquid Chromatography; **CT (1,2):** Control; **DMSO:** dimethyl sulfoxide; **EB:** Ethidium bromide; **EDTA:** Ethylenediaminetetraacetic acid; **ELISA:** Enzyme linked immunosorbent assay; **F:** (1,2,3...) Fraction; **MTT:** 3- (4,5 dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide; **PBS:** phosphate-buffered saline; **RPMI:** Roswell Park Memorial Institute medium.

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