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# Phenolic-rich apple extracts have photoprotective and anti-cancer effect in dermal cells

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ABSTRACT

*Background:* Skin cancer is the most common type of malignancy in light-skinned populations and phenolics are promising anticarcinogenic agents.
*Purpose:* To characterise and evaluate the protective potential of apple extracts against the DNA damage caused by UV-radiation in culture of human fibroblasts as well as to verify the anticarcinogenic effect of these extracts in murine and human melanoma cells.
*Methods:* Acetone-ethanol extracts of apple were purified and fractionated by solid phase extraction. Four phenolic fractions (PF-I, PF-II, PF-III, PF-IV) and one Unfractionated Phenolics (UFP) were obtained. The presence of amygdalin, hydroxycinnamic acid, flavonols, flavanols and dihydrochalcones was confirmed by High-Performance Liquid Chromatography–Mass Spectrometry.
*Results:* Following 48 h exposure to UV-radiation, UFP, PF-III and PF-IV protected (14–34 %) fibroblast DNA against UV-radiation; the UFP and PF-IV, both at 31.25 $\mu g.mL^{-1}$, showed high antiproliferative effect in murine melanoma cells (B16F10); UFP and PF-IV, both at 125 $\mu g.mL^{-1}$, and PF-II, at 250 $\mu g.mL^{-1}$, were effective against human melanoma cells (SK-Mel-103).
*Conclusions:* The results indicate that phenolic extracts from apple (*Malus domestica* Borkh cv. Gala) have potential for use in the formulation of cosmetic products and/or medicines for the protection of cellular DNA against UV radiation and for the treatment of melanoma.

*Abbreviations*

| | |
|---|---|
| AA | Antioxidant activity |
| CAE | Chlorogenic Acid Equivalent |
| DMEM | Dulbecco's Modified Eagle's medium |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| DPPH | 2,2-Diphenyl-1-picrylhydrazyl |
| FBS | Foetal Bovine Serum |
| $IC_{50}$ | 50% inhibitory concentration |
| LC | Liquid Chromatography |
| MS | Mass spectrometry |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| PF-I | Phenolic fraction I |
| PF-II | Phenolic fraction II |
| PF-III | Phenolic fraction III |
| PF-IV | Phenolic fraction IV |
| RASIP | Randon Agrosilvopastoril S.A. |
| ROS | Reactive Oxygen Species |
| SPE | Solid phase extraction |
| TA | Total Anthocyanins |

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|---|---|
| TPC | Total Phenolic Content |
| UFP | Unfractionated phenolics |
| UV | Ultra-Violet |

## 1. Introduction

Skin cancer is classified based on the origin of the cell type as melanoma and non-melanoma. The melanoma, is the most aggressive type of skin cancer, originates from cutaneous, mucous and uveal melanocytes (Chandra Pal et al., 2016), and non-melanoma originates from epithelial cells (Cives et al., 2020; de Silva and Tencomnao, 2018). Melanoma is a complex disease that involves several genetic and environmental risk factors and has a high mutation rate, the ability to metastasize and high lethality (Bray et al., 2018; Narayanan et al., 2010).

Worldwide, the incidence of cutaneous melanoma has risen rapidly. It is estimated that about 1.7 % (232,100 cases) of all newly diagnosed primary malignant tumours, as well as 0.7 % (55,500 deaths) of all cancer deaths each year, are caused by cutaneous melanoma (Bray et al., 2018).

Fair-skinned populations living in low-latitude regions, with greater exposure to ultraviolet light and without adequate sun protection, are more affected by cutaneous melanoma (Chen et al., 2017; Gado et al., 2021; Islami et al., 2020). Sunburn and intermittent exposure to the sun seem to increase the risk of developing melanoma (Laikova et al., 2019) because the radiation penetrates the epidermis, generating oxidative stress, immunosuppression and the development of melanoma (Hyson, 2011; Pavithra et al., 2019).

The integrity of the cellular genome can be affected by solar radiation because the rays are mutagenic and cancerous. Exposure of the skin to ultraviolet A radiation can induce inflammation, promotion of the angiogenesis, production of free radicals and DNA damage, developing a series of skin diseases, including cancer (D'Orazio et al., 2013; Sample and He, 2018). Type B ultraviolet radiation is one of the most powerful agents that can induce a variety of mutagenic and cytotoxic DNA lesions, with direct damage through photoproducts or indirect on the skin as increased free radicals (Mintie et al., 2020).

The high aggressiveness of melanoma requires fast, accessible and multiple treatment options, both preventive and curative (Sample and He, 2018). Some secondary plant metabolites have the potential to be used in complementary therapy, for chemotherapy or immunotherapy (Pavithra et al., 2019), because their antiangiogenic action (Rajasekar et al., 2019; Vazhappilly et al., 2021).

The antimelanoma effects of natural compounds, such as flavonoids, carotenoids, terpenoids, vitamins, sulforaphane, polyphenols, include potentiation of apoptosis, inhibition of cell proliferation and inhibition of metastasis (Raphaelli et al., 2020; Russo et al., 2006). They act in different ways to promote caspase activity, inhibiting the effects of tumour-promoting proteins such as PI3-K, Bcl-2, STAT3 and MMPs (Chinembiri et al., 2014). In addition, these compounds can positively interfere with DNA methylation and histones modification, as well as regulating the expression of non-coding miRNAs (Arora et al., 2019). As an example, the polyphenols can induce senescence in cancer cell through various pathways, including changes in the redox balance (Bian et al., 2020; Liu et al., 2019; Prieto et al., 2019). Polyphenols act on the redox balance (Azqueta and Collins, 2013) resulting in the prevention or attenuation of symptoms of skin diseases and reduction in healing time (Karim et al., 2014). Several actions of polyphenols against skin cancer have been discovered thought *in vitro* studies (Dai and Mumper, 2010; Liu et al., 2019).

The apple (*Malus domestica* Borkh.), in addition to being a good source of polyphenols for human consumption, is produced on a large scale worldwide, mainly in temperate regions, being accessible to a significant proportion of the world population (D'Orazio et al., 2013). It is consumed fresh and/or processed in the form of cider, juice and puree and little used in the pharmaceutical industry (Francini and Sebastiani, 2013). This fruit has phenolic compounds with potential application in skin protection. However, there are no published studies on the potential of these compounds in preventing or controlling skin cancer (Liaudanskas et al., 2014; Montané et al., 2020). Thus, extracts of this fruit have great potential for application as an anticarcinogenic agent, being a promising source of bioactive compounds for the pharmaceutical and cosmetic industries (Liaudanskas et al., 2014; Morais et al., 2020).

Therefore, this study aimed to characterise and evaluate the protective potential of apple extracts against the DNA damage caused by UV-radiation in culture of human fibroblasts as well as to verify the anticarcinogenic effect of these extracts in murine and human melanoma cells.

## 2. Material and methods

### 2.1. Standards and reagents

Chemicals such as methanol, acetonitrile, hydrochloric acid, sodium hydroxide, acetone, ethanol, hydrochloric acid, buffer solutions pH 4.0 and pH 7.0, Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, chlorogenic acid, cyanidin3-*O*-glucoside and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Merck KGaA, Darmstadt, Germany. Trypsin-EDTA solution, penicillin, streptomycin, dimethyl sulfoxide (DMSO), agarose (Agargen-Cat. 0508), ethyl acetate and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) for cells assays were purchased from Sigma-Aldrich (St Louis, MO, EUA). Dulbecco's modified Eagle's medium (DMEN) containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate was purchased from Sigma-Aldrich and Fetal Bovine Serum (FSB) from Cultilab (Campinas, SP, Brazil). For HPLC a bidentate C18 column (100 mm x 2.1 mm ID 2.2 μm) manufactured by MicroSolv Technology Corporation (Eatontown, NJ, USA) was used. The ultrapure water was produced using a Megapurity Water Purification System (Mega Purity, Billerica, MA, USA). Acetonitrile and formic acid were purchased from Panreac Química S.L.U. (Castellar del Vallès, CT, Spain) and Sigma-Aldrich (St. Louis, MO, USA), respectively, being both HPLC grade. Procyanidin B1 and B2, catechin, epicatechin, chlorogenic acid, rutin, luteolin, and quercetin standards, with the highest purity available, were purchased from Sigma-Aldrich.

### 2.2. Apples, phenolic extraction and purification

The apples (*Malus domestica* Borkh cv. Gala) were harvested in the year 2014 from a commercial orchard (Randon Agrosilvopastoril Company - RASIP), located in the municipality of Vacaria, South Brazil (50° 56′ 02'' W, 28° 30′ 44'' S).

To obtain the crude extract, sliced apples (50 g) containing peel, pulp and seeds; were mixed with 150 mL acetone/ethanol (1:3), homogenized (T25 Ultra-Turrax®) and centrifuged (1792 *g*) for 25 min at 0°C. Previously, several tests with methanol, acetone and ethanol, pure or mixed in different proportions, were carried out to obtain the ideal extracting solvent. The supernatant was concentrated at 40°C for 90 min under vacuum, protected from light and stored at 0°C. The crude extract was purified and fractionated adapted according previous methods (Oszmianski et al., 1988; Vizzotto et al., 2014). Briefly, this extract was diluted 1: 4 (50 mL of extract in 200 mL of ultrapure water) to perform the solid phase extraction, at 10 mm Hg of pressure in a Sep-Pak C-18 Vac cartridge of 35 $cm^3$ (Part n°. WAT043345-Waters Association, Milford, MA, USA) containing 10 g of silica. After the process, 5 extracts were obtained: Phenolic Fraction I (PF-I) eluted with methanol (100%, pH 7,0), Phenolic Fraction II (PF-II) eluted with acetonitrile (16 %, pH 2); Phenolic Fraction III (PF-III) eluted with ethyl acetate 100%, pH 7); Phenolic Fraction IV (PF-IV) eluted with methanol (100%, pH 7) and the Unfractionated Phenolics (UFP) eluted with methanol (100%, pH 7). These extracts were concentrated in a rotary-evaporator, lyophilized,

and stored at 0°C until analysis.

### 2.3. Total phenolic content, total anthocyanins and antioxidant activity

All extracts were weighed and resuspended in 5 mL of ultrapure water for analysis. The total phenolic contents were quantified by the previously adapted Folin-Ciocalteu method (Swain and Hillis, 1959). Spectrophotometric readings were done at 725 nm wavelength (SpectraMax 190 Microplate Reader, Molecular Devices); chlorogenic acid was dissolved in 95 % methanol (0 mg.mL$^{-1}$ to 400 mg.mL$^{-1}$) to obtain a standard curve ($R = 0.9993$). Samples were diluted in methanol 95 %, and results of total phenolics were expressed as equivalents of chlorogenic acid (CAE) (mg CAE.g$^{-1}$ of lyophilized extract (LE)).

Total anthocyanins were measured using a previously adapted method (Lees and Francis, 1972). Samples were diluted in acidified ethanol solution pH 1.0 (95 % ethanol and 1.5 M HCl 85:15 v/v) before measuring the absorbance at 535 nm (SpectraMax 190 Microplate Reader, Molecular Devices). Results were expressed as cyanidin 3-*O*-glucoside (CGE) (μg.100 g$^{-1}$ of LE).

To measure the antioxidant activity of extracts, the DPPH (2,2-diphenyl-1-picrylhydrazyl) method Brand-Williams et al., 1995 was used with slight modification. Briefly, all extracts were diluted in methanol at previously tested concentrations, added to DPPH solution, vortexed and allowed to react for 4 h at room temperature. The absorbance was measured at 515 nm (SpectraMax 190 Microplate Reader, Molecular Devices). Pure methanol added to DPPH solution was used as blank. The antioxidant activity was expressed as Trolox equivalents (TE) (μg.mg$^{-1}$ of LE). Sample's antioxidant activity was obtained by comparison to a standard curve of DPPH (R = 0.9882) with dilutions of Trolox in methanol ranging from 0 μg.mL$^{-1}$ to 300 μg.mL$^{-1}$, in triplicate.

### 2.4. Quantification of phenolics using HPLC−QTOF-MS

First, the identification and quantification of compounds by LC-MS were passed out in a Bidentate C18 column in a liquid chromatography equipment (UFLC, Shimadzu, Japan) coupled to a high-resolution quadrupole-time-of-flight mass spectrometer (Maxis Impact, Bruker Daltonics, Bremen, Germany), according to conditions described in the literature (Hoffmann et al., 2018). The flow was 0.2 mL.min$^{-1}$, column temperature 40 °C and the mobile phases were water acidified with 0.1 % formic acid (eluent A) and acetonitrile with 0.1 % formic acid (eluent B). The gradient program was started at 10 % B and increased linearly to 90 % B at 15 min and was maintained for 6 min at 90 % B; returned to 10 % B in 2 min and was maintained at 10 % B for 7 min. The injection volume was 10 μL.

Mass spectra (MS) were obtained over a mass range of m/z 50–1200. MS parameters were: ESI operated in negative mode for extracts from apple, font temperature of 200 °C, capillary voltage of +3.5 kV, nebulizer gas, nitrogen, four bars; dry gas, nitrogen, 9 L min$^{-1}$, RF of collision 150 Vpp; transfer time 70 μs and pre-pulse storage of 5 μs. Then, this equipment was adjusted with 10 mM sodium format, covering the entire acquisition range (*m/z* 50 to 1200). Automatic MS/MS experimentations were done by adjusting collision energy values: *m/z* 100, 15 eV; *m/z* 500, 35 eV; *m/z* 1000, 50 eV, using nitrogen as the collision gas.

The MS data were processed through Data Analysis 4.0 software (Bruker Daltonics, Bermen, Germany), which provided a list of possible elemental formulas exploitation the Smart Formula editor by combining accurate mass and isotopic distribution reflected in their error (ppm). Phenolic compounds were identified by comparison with the retention times, UV-visible spectral data and molecular weights of standard compounds reported in the literature to apple fruits. Quantification was done based on standard curves of catechin, chlorogenic acid, quercetin and rutin in the range of 39–10.000 mg.mL$^{-1}$. Results were expressed as μg.mg$^{-1}$ of dry extract.

### 2.5. Ultraviolet radiation and DNA damage protection

#### 2.5.1. Cell culture

Cells (CC-2511 from Clonetics, Cambrex/Lonza, USA) were seeded in 75 cm$^2$ bottles, grown and expanded in a humidified atmosphere of 5 % $CO_2$, 95 % air, at 37 °C and 70 % - 80% confluence, using Fibroblast Cell Basal Medium and the growth supplements: hFGF-B, 0.5 mL; insulin, 0.5 mL; FBS, 10 mL; GA-1000, 0.5 mL. Then trypsinized and counted cells ($1 \times 10^5$ cells/well) were incubated in 96-well plates with the fractions.

#### 2.5.2. Cytotoxic assay

To assess cytotoxicity of the extracts, metabolically active cells were quantified by incubating human dermal fibroblast assays with MTT (Mosmann, 1983). $1 \times 10^5$ cells/well were incubated, for 24 h, using the following concentrations of fractions: PF-I, PF-II and UFP (0.38, 0.76, 1.53, 3.10, 6.10, 12.20, 24, 49, 98, 200, 390, 780, 1560, 3130, 6250, 12500, 25000 and 50000) μg.mL$^{-1}$, PF-III and PF-IV (0.25, 0.50, 1, 2, 4, 8, 16, 32, 64, 130, 260, 510, 1030, 2060, 4110, 8220, 16450 and 32890) μg.mL$^{-1}$. After exposure to extracts, cells were incubated in 96-well plates for 48 h at 37°C, in a humidified atmosphere (5% $CO_2$). After the incubation period, 50 μL of MTT (5 mg.mL$^{-1}$), dissolved in DMEM high glucose, were added to each well, and incubated for 4 h. At the end of the experiment the medium was removed and the purple insoluble formazan, produced by active metabolic cells, was dissolved in 100 μL DMSO and quantified by measuring the absorbance at 570 nm (SpectraMax 190 Microplate Reader, Molecular Devices). The cytotoxic potential was expressed in percentage of non-viable cells in comparison to control (DMEN) using the equation:

$$\text{non viable cells}\,(\%) = 100 - \frac{\text{absorbance of treated sample}}{\text{absorbance of control}} \times 100$$

Concentrations that showed ≤ 25 % non-viable cells (low cytotoxicity) were selected for the comet assay combined with UV-irradiation.

### 2.6. DNA protection against UV-radiation

Fibroblasts were incubated for 24 h in the presence of apple extracts alone (non-toxic concentrations), and cell cultures were UV irradiated (SOL-500, Honle) with UV-A/UV-B radiation (3 J.cm$^2$) for 3 min (Ferrucio et al., 2017). The UV-C radiation was reduced with an appropriate filter, and the equipment was stabilised for at least 15 min prior emission. After irradiation, the fibroblasts were incubated for 24 h and measured using the method of Ladeira et al. (2015) . The cell lysate was collected, at a density of $1.2 \times 10^6$, from each condition evaluated for analysis of the DNA protection profile by the Comet assay. An alkaline electrophoresis was performed as follows: an aliquot of cells ($1.2 \times 10^6$) was diluted in 1.2 mL of a 0.75 % agarose solution with low melting point (Agargen – Cat. 0508, Spain), an aliquot of 360 μL was separated from this solution, and 120 μL was pipetted and spread over each slide, in a total of three slides previously coated with 1 % agarose solution with normal melting point (Agargen – Cat. 6108, Spain). After solidification, the plates were immersed for 24 h in lysis solution (2.5 M NaCl, 100 mM disodium ethylenediaminetetraacetic acid hydrated and 10 mM Tris base, pH 10) added with Triton X-100 (1 %) and DMSO (10 %) immediately before the plates were immersed. After the lysis period, the plates were incubated for 5 min in cold PBS ($Ca^{2+}$ and $Mg^{2+}$ free) followed by 40 min incubation in alkaline medium (300 mM NaOH and 1 mM EDTA, pH > 13). The cells were subjected to alkaline electrophoresis (300 mA, 25V) for 30 min. After that, the plates were neutralised with three consecutive washes of 5 min using a neutralisation buffer (0.4 M Tris-HCl, pH 7.5) and immersed in absolute ethanol for DNA precipitation. Positive controls were incubated for 2 h at 37°C with 10 μL of hydrogen peroxide, for the evaluation of electrophoresis conditions and efficiency. Fifty cells from each plate had their DNA stained with 50 μL of SYBR Gold (Invitrogen-Cat. S11494) in the proportion of 1:10.000, were subjected to fluorescence microscopy (Leica DM 1000). The comet

tail length was measured using an ocular micrometre and the DNA damage was calculated as follows: Class 1 presents a minor migration of chromosomal DNA towards the anode, presenting a small tail (minimal DNA damage). Class 2, 3 and 4 present progressive increases in the length of the tail, being the Class 4 the major DNA damage. The final score of each apple extract, in the respective concentration, was obtained by the equation: $\sum$ ($Class_{1\text{-}4}$ x n° cells).

The results are expressed as mean values of three independent experiments run twice and are revealed as the percentage of the control.

### 2.7. Apple extracts antiproliferative effect in fibroblasts and melanoma cells

#### 2.7.1. Cell cultures

Human melanoma cells (SK-Mel-103), murine melanoma cells (B16-F10) and human fibroblasts (MRC5) were donated by Dr Silvya Stuchi Maria Engler. Cells were maintained in a humidified atmosphere of 5% $CO_2$, at 37°C, and used until the tenth pass. The cells were grown and maintained in high glucose DMEM, containing 0.5 $U.mL^{-1}$ penicillin/streptomycin antibiotics and supplemented with 10 % FBS. Cells were kept at a humidified atmosphere of 5 % $CO_2$, with a minimum relative humidity of 95 % at 37°C, before being seeded on 96 well plate.

### 2.8. Antiproliferative effect

Melanoma cells, human or murine, and fibroblasts were exposed to a range of concentrations, (31.25; 62.5; 125; 250 and 500) $\mu g.mL^{-1}$ of apple extracts dissolved in DMEN or DMEN with 5 % of DMSO as control group. Cells were treated and incubated for 24 h, 48 h or 72 h to analyse the effect of the compounds after a different time of exposition. The MRC5 cells were exposed to the same extracts and concentrations, however the treatment was performed for 72 h.

The test was carried out using the MTT assay methodology previously described (Mosmann, 1983). Briefly, $1 \times 10^3$ cells per well were seeded in 96-well plates and incubated with DMEM (containing 10 % FBS) at 37°C in 5 % $CO_2$ for 24 h. After 24 h incubation, the medium was replaced with culture medium containing 100 µL of test extracts, in the concentrations described above, dissolved in DMEM (except PF-III dissolved in 5 % DMSO). As positive alive control was used DMEM and as a control to perform the basal result of viability was used DMEN with 5 % of DMSO. After the time of treatment, medium with extracts were removed and 50 µL of MTT (5 $mg.mL^{-1}$), dissolved in DMEM high glucose, were added to each well, and incubated for 4 h. The absorbance was read (SpectraMax 190 Microplate Reader, Molecular Devices) at 570 nm for fibroblast and 490 nm for melanoma cells. The absorbance was linearly proportional to the number of cells with active mitochondria. The per cent growth inhibition was calculated by the equation:

$$\text{Viable cells } \% = 100 - \frac{\text{Mean absorbance of treated cell} \times 100}{\text{Mean absorbance of control}}$$

Results are expressed as mean values of three independent experiments run in duplicate and are shown as the percentage of the control.

### 2.9. Ethical aspects and statistical analysis

Phenolic extraction, fractionation and purification of apple samples were carried out at Embrapa Clima Temperado, a government research institute located in the municipality of Pelotas, Brazil. The assays with melanoma cells and MRC-5 cells were carried out at the Graduate Program in Biosciences at the Federal University of Health Sciences of Porto Alegre, Brazil. Primary Normal Human Dermal Fibroblasts assays (CC-2511) were carried out at the Chemyunion Química LTDA, São Paulo, Brazil. Data were expressed as means ± standard deviations (SD) from at least three independent experiments and analysed by two-way ANOVA followed by post-tests (Dunnet) using Stata Winstat 2.0 ($p < 0.01$). Results from cells were done using GraphPad Prism software for Windows (version 7.00, GraphPad Software, San Diego, CA).

## 3. Results and discussion

### 3.1. Total phenolic content, total anthocyanins and antioxidant activity

The TPC of apple extracts cv. Gala varied considerably, (47.4 ± 16.1–620.6 ± 39.8) µg $CAE.mg^{-1}$ of LE (Fig. 1). PF-IV showed the highest concentration (620.6 ± 39.8) µg $CAE.mg^{-1}$ LE followed by PF-III (459.3 ± 19.8) µg $CAE.mg^{-1}$ LE and UFP (390.8 ± 12.2) µg $CAE.mg^{-1}$ LE. Anthocyanins were present in all samples at concentrations of (145–271) µg $CGE.100g^{-1}$ LE. These results are higher than those reported to unpurified Brazilian Gala apple extracts, especially in PF- III and PF-IV and UFP (Alberti et al., 2017). Total antioxidant activity was higher in PF-IV (1226.8 µg $TE.mg^{-1}$), followed by UFP (814.2 µg $TE.mg^{-1}$) and PF -III (763.3 µg $TE.mg^{-1}$) and was correlated to the total content of phenolic compounds ($r = 0.92$). This antioxidant activity values were higher than those described in the literature for other apple cultivars (Han et al., 2019). The positive correlation between TPC and total antioxidant activity was expected and has been reported for different species. The TPC are known as antioxidant compounds (Wu et al., 2006).

### 3.2. Quantification of phenolics by HPLC-MS

Fifteen phenolic compounds were identified and quantified in apple extracts (Table 1). Chromatographic and spectral data are shown in supplementary material 1 and chromatograms are shown in supplementary material 2. The concentration of total phenolic compounds ranged from (1.30 to 31.64) $\mu g.mg^{-1}$ dry weight (dw) (Table 1). PF-II showed the highest total amount of phenolic compounds, being chlorogenic acid the major compound. This polyphenol seems to be the most abundant in the apple peel (Lončarić et al., 2020). The UFP, containing a pool of phenolic compounds, also showed high amounts of chlorogenic acid, which represents almost 58 % of the phenolic composition, in addition to significant amounts of flavanols, flavonols and dihydrochalcones. Besides, the UFP showed triple the amount of procyanidin (2.93 $\mu g.mg^{-1}$ dw) when compared to methanol crude extracts of Gala apples (0.98 $\mu g.mg^{-1}$ dw) (Petkovska et al., 2017). The PF-III showed the highest amount of amygdalin and flavonols and had the highest concentrations of quercetin. Flavonols as quercetin was reported before in apples fruits (Francini and Sebastiani, 2013). PF-IV had small amounts of all classes of phenolic compounds while PF-I, containing phenolic acids, was composed mainly of chlorogenic acid. The phenolic profile obtained in the study was similar to those reported by Alberti et al. (2017) for Fuji Suprema, Gala and Eva varieties. Among the compounds measured, the flavonol luteolin, observed in PF-III (Table 1), was reported in apple cider vinegar (Kelebek et al., 2017), apple leaves (Sowa et al., 2016) but not in fruit of apple (Hoffmann-Ribani et al., 2009). Rutin, a glycoside comprising flavonolic aglycone quercetin along with the disaccharide rutinose, present in PF-II, PF-I, PF-IV and UFP (Table 1), was reported in apples and a number of pharmacological effects is described for this compound (Ganeshpurkar and Saluja, 2017). The procyanidin fractions were also measured in the apple extracts, with exception of PF-I. These compounds have several therapeutic properties (Ganeshpurkar and Saluja, 2017).

Although apple contains phenolic compounds that confer health benefits, the seeds contain amygdalin, a potentially toxigenic compound (Bolarinwa et al., 2015). This compound is present in all extracts, being more expressive in PF-III. The dihydrochalcones present in PF-III, PF-IV and UFP are common in apples and are the main glycoside found in shoots, roots, leaves, apple peel and apple seeds (Jugdé et al., 2008; Rana and Bhushan, 2016). Due to these results, which show diversity in phenolic compounds and richness in compounds that are known for putative functional properties, PF-III, PF-IV and UFP supposedly have the potential to provide the best responses in DNA protection and

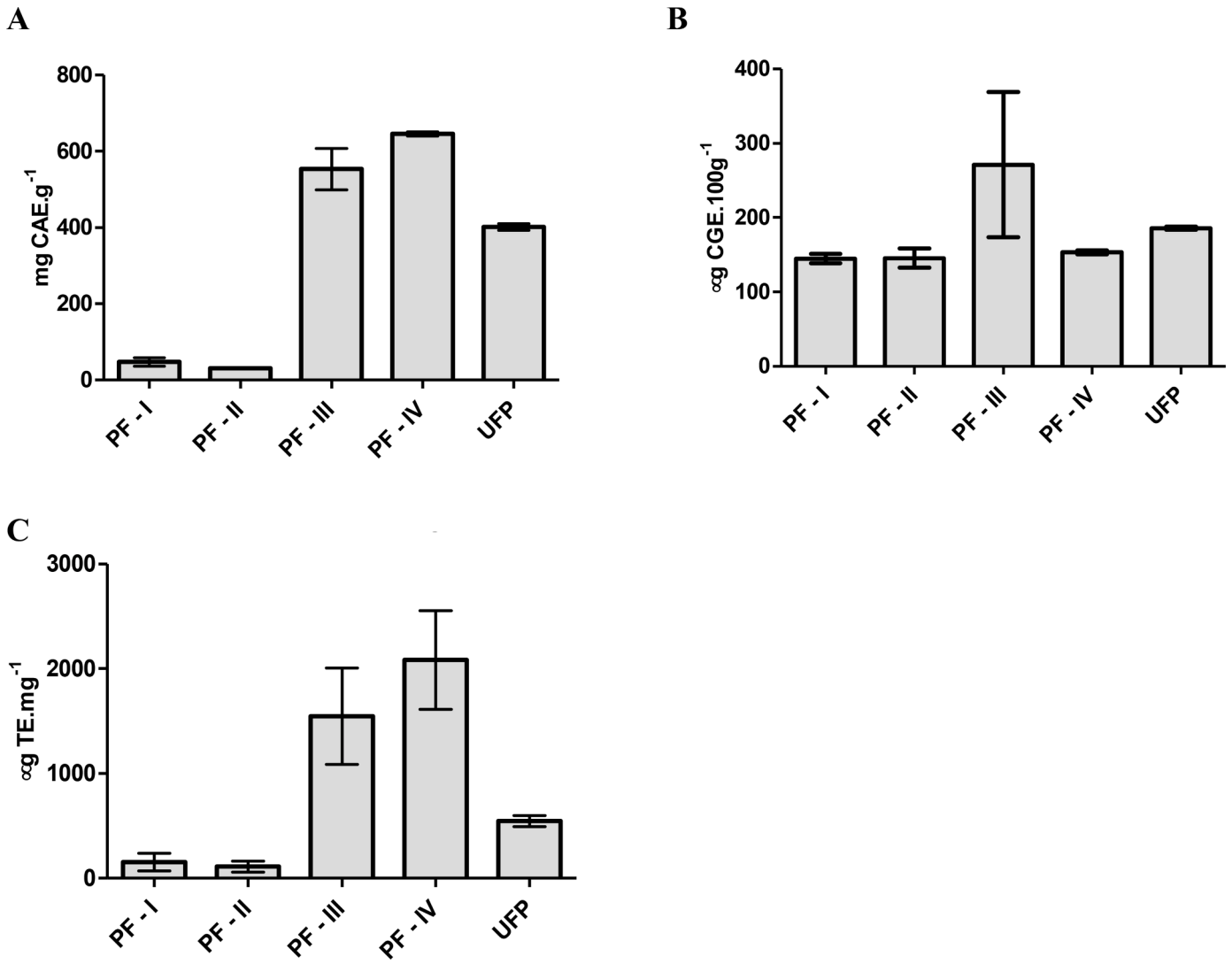


**Fig. 1.** Total phenolic content (A), total anthocyanins (B) and antioxidant activity (C) of apple (*Malus domestica* Borkh cv. Gala) phenolic fractions.

antiproliferative activity.

### 3.3. Ultraviolet radiation and DNA protection

DNA damage by ultraviolet radiation, due to its relationship with mutagenesis, carcinogenesis and aging; has become one of the most important themes in the areas of DNA research (D'Orazio et al., 2013; Pavithra et al., 2019). The results of DNA protection against the damage induced by UV exposure after fibroblasts incubation with apple extracts are shown in Figure 2. The apple extracts UFP, PF-III and PF-IV were the most promising in protecting fibroblasts DNA against the damage after UV-radiation exposure, with reductions in the range of 14 % to 34 %. The UFP showed significant DNA protection for all tested concentrations and was able to decrease the formation of DNA damage by up to 34 % (at 0.0006 mg.mL$^{-1}$) when compared to the UV Control group (Fig. 2).

UFP contains a mixture of phenolic acids (4-*p*-coumaroylquinic, chlorogenic acid), flavonols (epicatechin, procyanidins B1 and B2) and dihydrochalcones (phloretins). Epicatechin, as well as other UFP flavonols studied in photoprotection, contributed to the viability of cultured fibroblasts after irradiation. This fact reveals the potential of these compounds in reducing cell damage and blocking reactive oxygen species, facilitating the healing of radiation-induced cytotoxicity wounds (Shin et al., 2014). The *p*-coumaric acid, a hydroxycinnamic acid, did not alter the cell viability of human fibroblast (A375). However, irradiation followed by treatment with this compound reduced melanoma cells viability (SK-MEL-37) (Kianmehr et al., 2020).

Phlorentin-2'-*O*-glucoside (phlorizin), a dihydrochalcone present in apples, described as having high antioxidant activity and acting as a UV filter in plant leaves, can help protect the skin (Baldisserotto et al., 2012; Zielinska et al., 2019). Furthermore, it has been shown that phlorizin and its derivatives have high antioxidant activity and stability in cosmetic formulations (Baldisserotto et al., 2012). Epicatechin and phloretins were also present in considerable amounts in PF-III in addition to the flavonol quercetin. Quercetin is a potential agent against UVB-induced skin damage to keratinocyte cells (Zhu et al., 2017), has a high antioxidant activity (Solomon Josiah et al., 2021) and is also effective in topical formulations to inhibit UVB-induced skin damage. Even at low concentrations, quercetin inhibited damage to genetic material, induced by reactive oxygen and nitrogen species in T-lymphocytes, and this action was associated with its anti-radical activity (Johnson and Loo, 2000). This compound also protected human hepatoma cell line (HepG2) against *tert*-butyl hydroperoxide (*t*-BHP)-induced DNA damage. Quercetin's mechanism of action seems to be due to both direct effects on *t*-BHP toxicity and indirect cell-mediated effects on antioxidant defences (Johnson and Loo, 2000; Zhu et al., 2017). The antioxidant activity and the concentration of phenolic compounds were maximum in PF-IV (Fig. 1), but the diversity of phenolic compounds was lower than in other fractions. Even so, it protected the fibroblasts against

**Table 1**
Compounds identified and quantified by HPLC-MS in different apple (*Malus domestica* Borkh cv. Gala) phenolic fractions.

| Peak number | Compound | Concentration in μg $mg^{-1}$ dry weight | | | | |
|---|---|---|---|---|---|---|
| | | PF-I | PF-II | PF-III | PF- IV | UFP |
| | Non-phenolic[a] | | | | | |
| 4 | Amygdalin (cyanogenic glucoside) | – | 0.30 ± 0.00 | 0.55 ± 0.00 | 0.19 ± 0.00 | 0.39 ± 0.01 |
| | Hydroxycinnamic acid[b] | | | | | |
| 7 | 4-*p*-coumaroylquinic acid | – | 4.92 ± 0.14 | 1.31 ± 0.03 | 0.02 ± 0.00 | 1.69 ± 0.02 |
| 3 | Chlorogenic acid | 1.29 ± 0.04 | 24.97 ± 0.09 | 0.10 ± 0.00 | 0.05 ± 0.00 | 13.72 ± 0.07 |
| | Flavonols[c] | | | | | |
| 14 | Luteolin | – | – | 0.02 ± 0.00 | – | – |
| 15 | Quercetin | – | 0.01 ± 0.00 | 0.88 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 |
| 10 | Quercetin hexoside | – | – | 0.18 ± 0.00 | 0.01 ± 0.00 | 0.19 ± 0.00 |
| 12 | Quercetin-O-α-L-arabinofuranoside | – | – | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.11 ± 0.00 |
| 9 | Rutin | – | – | 0.13 ± 0.00 | 0.12 ± 0.01 | 0.16 ± 0.00 |
| | Flavanols[a] | | | | | |
| 2 | Catechin | – | 0.07 ± 0.00 | 0.30 ± 0.01 | 0.08 ± 0.00 | 0.53 ± 0.02 |
| 6 | Epicatechin | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.96 ± 0.05 | 0.34 ± 0.01 | 2.37 ± 0.13 |
| 8 | Procyanidin B trimer | – | – | 0.04 ± 0.00 | 0.05 ± 0.00 | 0.12 ± 0.00 |
| 1 | Procyanidin B1 | – | 1.02 ± 0.02 | 0.04 ± 0.00 | 0.09 ± 0.00 | 1.13 ± 0.01 |
| 5 | Procyanidin B2 | – | 0.33 ± 0.01 | 0.43 ± 0.01 | 0.60 ± 0.00 | 1.68 ± 0.01 |
| | Dihidrochalconas[d] | | | | | |
| 13 | Phlorentin-2'-O-glucoside | – | – | 1.13 ± 0.02 | 0.05 ± 0.00 | 2.36 ± 0.03 |
| 11 | Phloretin-2′-O-(2″-O-xylosyl)-glucoside | – | – | 1.20 ± 0.01 | 0.50 ± 0.02 | 1.50 ± 0.01 |

Phenolic fraction I (PF - I) eluted with methanol (100%, pH 7,0), Phenolic fraction II (PF - II) eluted with acetonitrile (16 %, pH 2); Phenolic fraction III (PF - III) eluted with ethyl acetate (100%, pH 7); Phenolic fraction IV (PF - IV) eluted with methanol (100%, pH 7,0) and Unfractionated Phenolics (UFP) eluted with methanol (100%, pH 7).

[a] Quantified by catechin
[b] Quantified by chlorogenic acid
[c] Quantified by quercetin
[d] Quantified by rutin. The values correspond to the mean ($n$ =3 ) ± standard deviation.

the DNA damage after UV radiation. The presence of phenolic compounds has been linked to protection against DNA damage, including quercetin, that seems to protect the DNA in fibroblasts, probably due to its anti-inflammatory, radioprotective and antiproliferative effects (Fischer et al., 2018; Johnson and Loo, 2000; Prasad and Katiyar, 2015). In another study, it was also verified that the extracts with the greatest anti-inflammatory activity were those that contained epicatechin, catechin, phlorizin and quercetin glycosides, or those that contained procyanidin polymers (Lauren et al., 2009).

### 3.4. Anticarcinogenic effect in melanoma cells

Bioactive compounds such as phenolics with a high antioxidant capacity neutralize free radicals produced by cancer and may act to reduce cell cancer growth and cell viability (Chandra Pal et al., 2016). The effect of apple extracts on murine melanoma cells (B16-F10 cells) are shown in Fig. 3. The UFP and PF-IV, even at the lowest concentration (31.25 μg.$mL^{-1}$), showed the highest antiproliferative effect on murine melanoma cells after 48 h. PF-III showed a reduction of the cellular viability but at a high concentration (125 μg.$mL^{-1}$). PF-III and PF-IV and the UFP have a similar phenolic profile (Table 1), and all showed high antioxidant activity (Fig. 1) and protective effect of DNA fibroblast against UV-induced damage (Fig. 2). However, the UFP and PF-III showed concentrations of phenolic compounds that have already been studied as anticancer agents such as 4-*p*-coumaroylquinic acid, chlorogenic acid, quercetin, epicatechin, procyanidin B1 and B2, phlorentin-2'-*O*-glucoside and phloretin-2′-*O*-(2″-*O*-xylosyl)-glucoside. Studies have shown that phenolic compounds inhibit murine melanoma cells (B16-F10 cells), but generally, the result is reported for isolated compounds and does not take advantage of the synergy between different phenolic compounds, which can have a more effective action on cancer cells (Uscanga-Palomeque et al., 2019). For example, quercetin showed significant effect on B16F10 cell death (Martínez et al., 2003) and have act in different ways. Signal transduction in the development of melanoma occurs through several pathways such as MAP kinases and phosphatidylinositol-3-kinase (PI3K) and the hyperactivation of these pathways occurs through mutations and gene deletion (Albuquerque et al., 2018). Flavonoids are reported to significantly reduce metastasis, decrease cell replication through cell cycle markers such as PCNA (proliferating cell nuclear antigen), cyclin D, and 3H-thymidine and reduce the number of vessels after cells treatment with flavonoids extracts. Dihydrochalcones, phenolic acids and flavanols induce apoptosis (Albuquerque et al., 2018). Amygdalin, found in all extracts, except PF-I, may increase the expression of Bax (pro-apoptotic protein) and caspase-3 and decrease the expression of Bcl-2 (anti-apoptotic protein) (Saleem et al., 2018).

Apple peel is rich in anthocyanins, which are recognized as essential in the redox balance and also as anticancer agents (Wolfe and Liu, 2003). Anthocyanins from red pitaya reduced the growth of melanoma cells (B16-F10) (Wu et al., 2006) and apple juice procyanidins were cytotoxic to HCT116 colon cancer cells (Zessner et al., 2008). Proanthocyanidins, (+) - catechin, (-) - epicatechin, appear to invade melanoma cells promoting different anticancer mechanisms, such as decreasing COX-2 expression, PGE2 production, cell migration, ERK1 / 2 phosphorylation induced by 12- O-tetradecanoylforbol-13-acetate and the activation of NFκBp65 (Chandra Pal et al., 2016).

The UFP, PF-III and PF-IV showed considerable amounts of epicatechin (Table 1). This compound has been already tested as an adjuvant to treat metastatic melanoma in mice and showed insignificant systemic toxic effects in healthy cells and led to a significant reduction in melanoma cell viability by controlling metabolic pathways (Li et al., 2019). Regarding the antiproliferative effect of apple extracts in human melanoma cells (Sk-Mel-103 cells), the PF-IV reduced cells viability after 48 h of exposure for all concentrations tested, showing 35 % cells death at the lowest concentration tested (125 μg.$mL^{-1}$) and 38 % at 250 μg.$mL^{-1}$ (Figure 4). PF-IV showed high concentrations of compounds (rutin, epicatechin, procyanidin and phlorentin-2′-*O*-(2″-*O*-xylosyl)-glucoside) reported to have anticancer activity, being procyanidin B2 and phloretin-2′-*O*-(2″-*O*-xylosyl)-glucoside the most significant compounds in this fraction. For example, apple procyanidins were effective in inhibiting cell proliferation and inducing *in vitro* apoptosis of transplanted B16 mouse melanoma cells, increasing mitochondrial membrane permeability and mitochondrial cytochrome C release, and activating caspase-3 and caspase-9 within the tumor cells

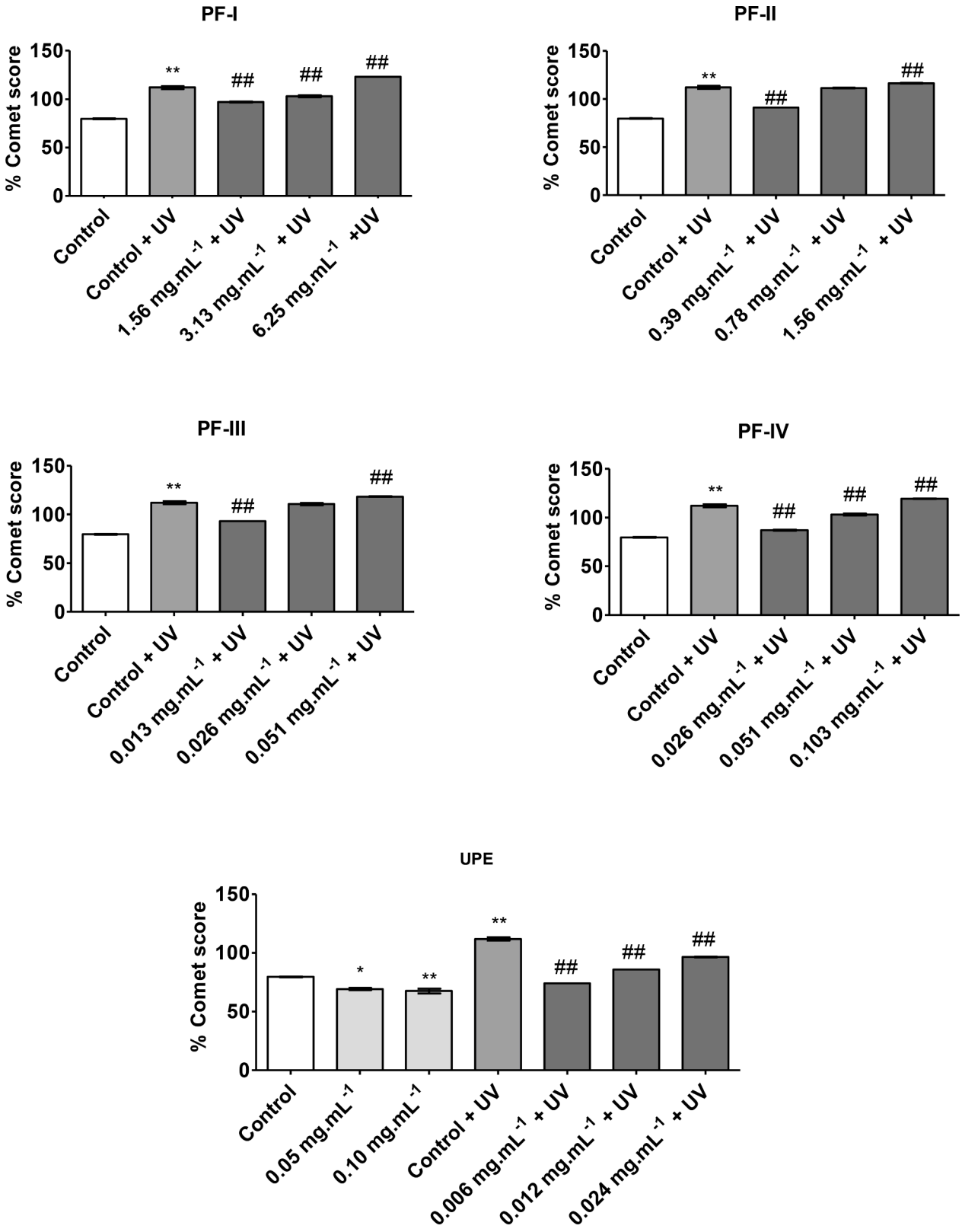


**Fig. 2.** Comet Score in human fibroblasts cells (CC-2511) subjected to ultraviolet radiation and incubated with apple (*Malus domestica* Borkh cv. Gala) phenolic fractions.

(Miura et al., 2008).

The PF-II (250 μg.mL$^{-1}$), with high concentrations of 4-*p*-coumaroylquinic and chlorogenic acid, reduced by 38 % the viability of SK-Mel28 cells after 48 h (Figure 4). Chlorogenic acid has been shown to have anticancer action on A549 cells (human adenocarcinoma basal alveolar epithelial cells) and HepG2 (human liver cancer) (Tan et al., 2019). This compound is also involved in inhibiting the activity of the tyrosinase enzyme, by suppressing melanogenesis in B16 melanoma

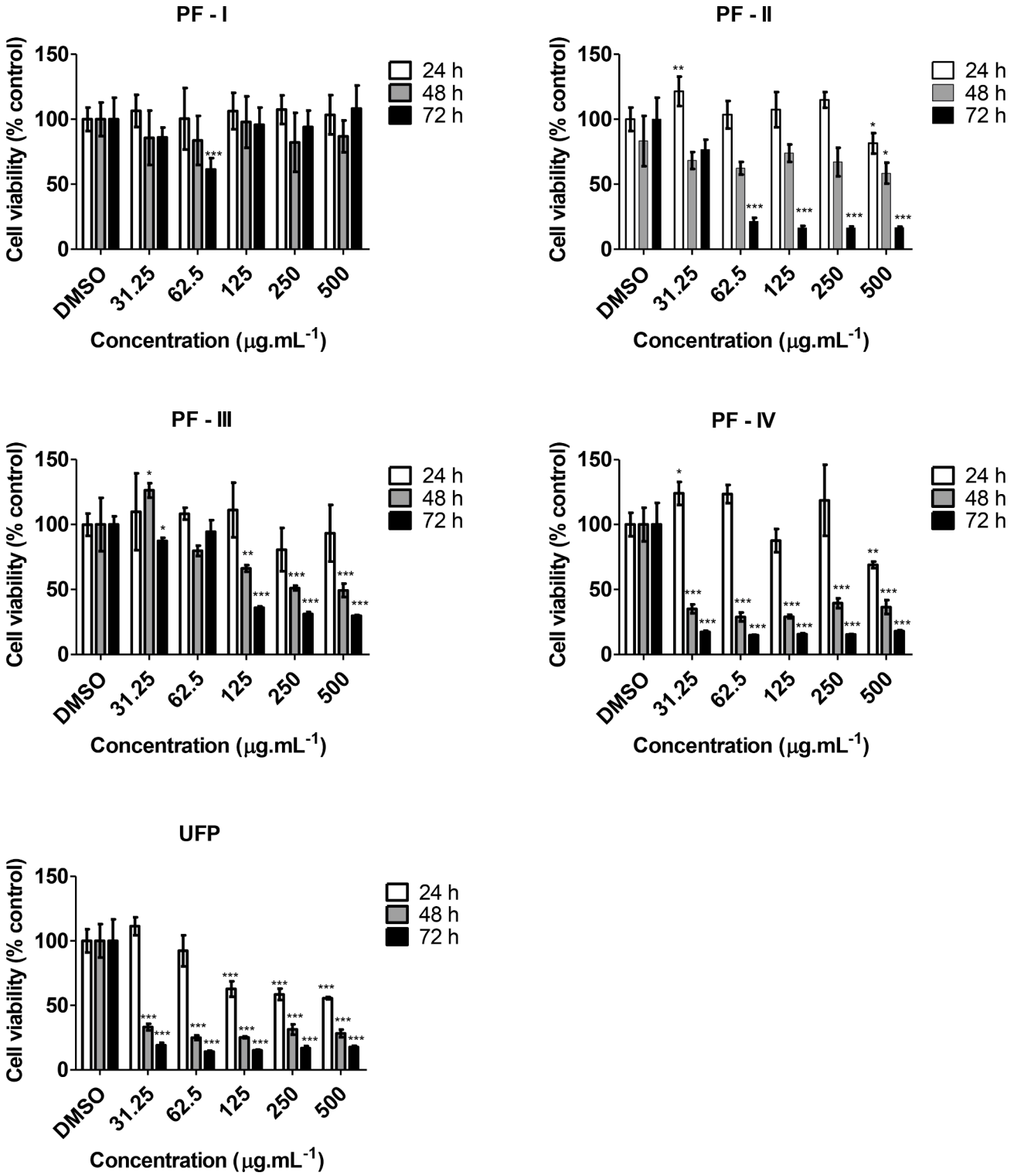


**Fig. 3.** Cytotoxicity of apple (*Malus domestica* Borkh cv. Gala) phenolic fractions on murine melanoma cells (B-16F10 cells) after 24, 48 and 72 h, using MTT assay.

cells, inhibiting melanin production and skin darkening (Li et al., 2014).

The UFP, high in 4-*p*-coumaroylquinic acid, chlorogenic acid, procyanidin B1 and B2, phlorentin-2'-*O*-glucoside, phloretin-2′-*O*-(2″-*O*-xylosyl)-glucoside and epicatechin showed high inhibition human melanoma cells viability after 48 h, with 42 % reduction at 125 μg.mL$^{-1}$. This extract is composed by a pool of phenolic compounds including epicatechins. A study reported that the epicatechins (-)-epigallocatechin-3-gallate, (-)-epigallocatechin, (-)-epicatechingallate and (-)-epicatechin at low concentrations (10 μg.mL$^{-1}$ - 60 μg.mL$^{-1}$) reduced the viability of SK-Mel28 cells after 24 h (7 % to 29 %) and after 48 h (9–50 %) (Prasad and Katiyar, 2015), showing lower percentages of reduction than the UFP. Thus, it seems that the combination of epicatechin with other apple phenolics may increase the antiproliferative effect in human melanoma.

Rodriguez et al. (2002) measured the response of B16-F10 and SK-MEL-1 cell lines towards treatment with different flavonoids.

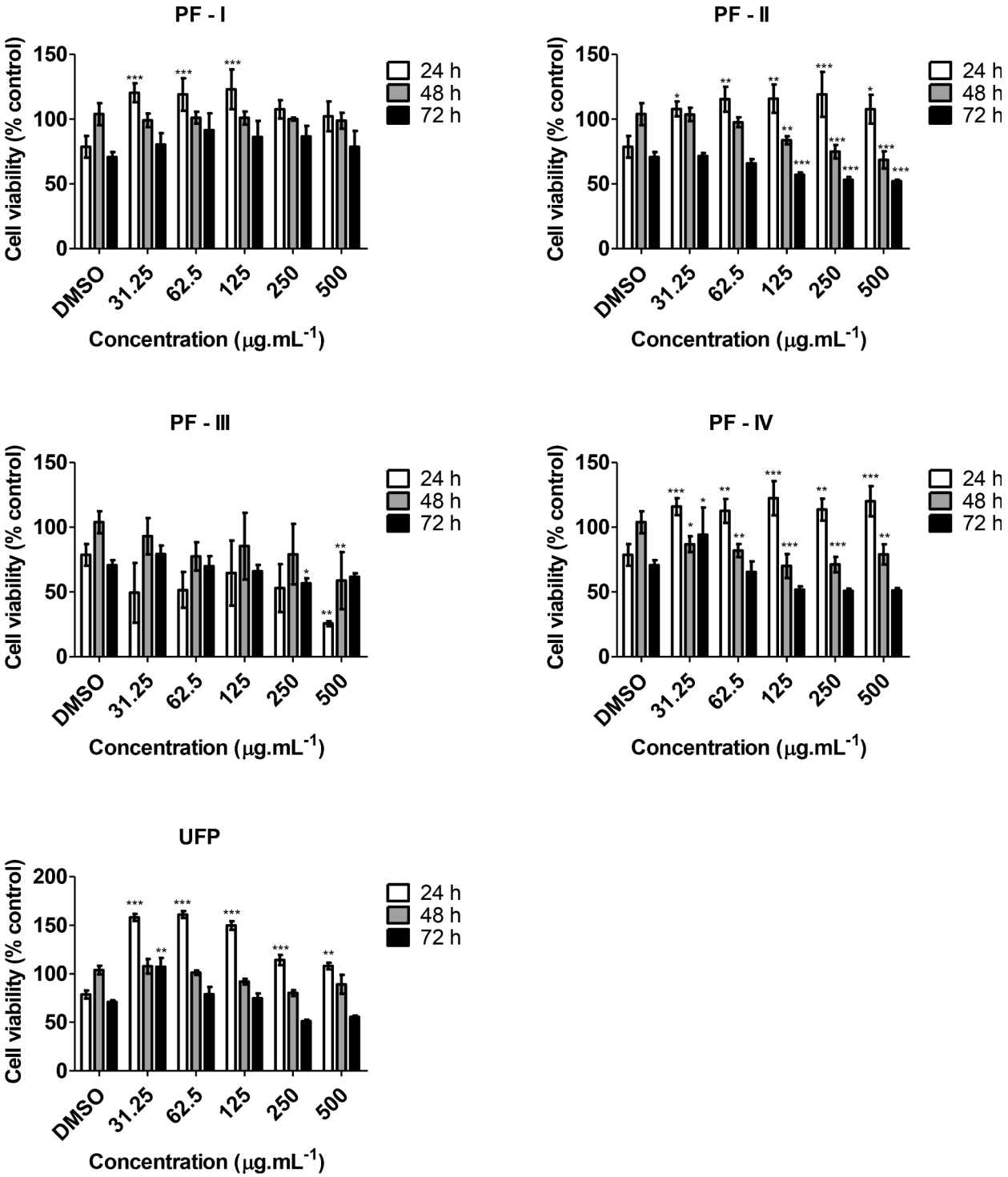


**Fig. 4.** Cytotoxicity of apple (*Malus domestica* Borkh cv. Gala) phenolic fractions on human melanoma cells (SKMel-103 cells) after 24, 48 and 72 h, using MTT assay.

Tangeretin and luteolin, at concentrations of 25 μM and 50 μM, slight inhibited B16-F10 and SK-MEL-1 cells after treatment for 24 h, being more sensitivity to the former (Rodriguez et al., 2002).

The UFP, PF-III and PF-IV were more effective in protecting fibroblasts cell DNA against radiation and also in reducing melanoma cell viability than other fractions. UFP, PF-III and PF-IV showed a similar profile; however, their concentrations differ considerably. These extracts contain flavonoids with different properties, such as antimetastatic, antiproliferative and immunomodulatory (Albuquerque et al., 2018; Chandra Pal et al., 2016). Furthermore, these extracts also contain dihydrochalcones and phenolics which are known to protect the skin against UV radiation (D'Orazio et al., 2013; Narayanan et al., 2010). Another study showed that apple polyphenol phloretin could potentially suppress human triple-negative breast cancer tumor cell growth and metastasis (Wu et al., 2018). It is known that the combined use of polyphenols or use of them as an auxiliary therapy in anticancer treatment may improve therapy (Bian et al., 2020).

Thus, the present study reinforces the role of polyphenols as antiproliferative agents in the prevention and treatment of melanoma skin cancer. Furthermore, it shows that the synergism of compounds within

each fraction appears to be more important than the concentration or effect of each compound alone. Johnson and Loo (2000) showed that low concentrations of epigallocatechin gallate and quercetin inhibited DNA damage induced by reactive oxygen and nitrogen species, however, high concentrations of these two phytochemicals led to cellular DNA damage. The red bean tunic extract contains phenolic compounds that are known to exhibit antimelanoma activity *in vitro* and appear to induce apoptosis and vacuolization through regulation PI3K-AKT-FOXO, MDM2-p53 pathway and increased the expression of Bcl-xl in B16-F10 cells (Nie et al., 2020).

The search for natural compounds that act as an adjuvant in the treatment of melanoma is of fundamental importance to reduce treatment rejection and find alternative compounds that are less aggressive to healthy body cells. One of the advantages of natural extracts obtained from fruits is their lower *in vivo* toxicity due to the low relative concentration of molecules with cytotoxic activity. In Fig. 5 it is shown that phenolic apple extracts PF-I, PF-III and UFP are safe for human fibroblasts cells (MRC5 cells) up to a concentration of 500 μg.mL$^{-1}$, while PF-II and PF-IV were slightly cytotoxic at this concentration. Nevertheless, both extracts showed biological effects at concentrations much lower than 500 μg mL$^{-1}$. Anti-cancer therapies using natural compounds have shown fewer side effects and less toxicity, being more tolerable when

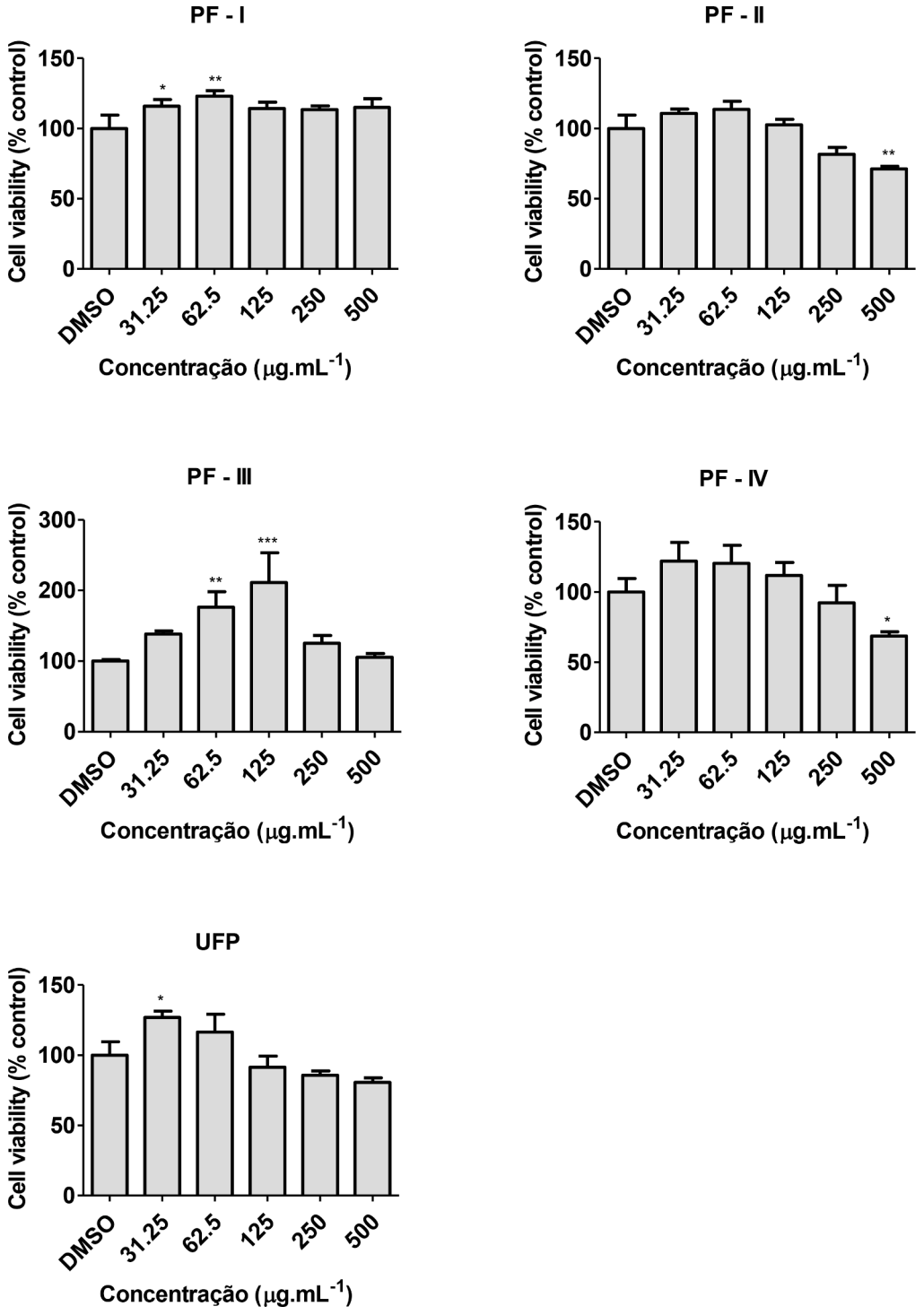


**Fig. 5.** Cytotoxicity of apple (*Malus domestica* Borkh cv. Gala) phenolic fractions on MRC5 cells after 72 h, using MTT assay.

compared to conventional antitumour drugs (Prieto et al., 2019).

## 4. Conclusion

Fifteen phenolic compounds were identified in the apple unfractionated and fractioned phenolic fractions of apples and were divided into four classes: hydroxycinnamic acid, flavanols, flavonols and dihydrochalcones. UFP, PF-III and PF-IV were the most promising in protecting fibroblasts DNA against damage after exposure to ultraviolet radiation with reductions in the range of 14 % to 34 %. These results seem to be related to samples with high polyphenols content and high antioxidant activity.

The UFP and PF-IV at 31.25 $\mu g.mL^{-1}$ showed high antiproliferative effect in murine melanoma cells after 48 h of exposure, while UFP and PF-IV at 125 $\mu g.mL^{-1}$ and PF-II at 250 $\mu g.mL^{-1}$ were effective against human melanoma at the same time of exposure.

The apple extracts evaluated have potential for use in pharmaceutical formulations for sun protection and also as anti-proliferative agents. Nevertheless, future studies should focus on determining the UV protection mechanism of these extracts. Investigation of new therapeutic approaches to treat aggressive and drug resistant cancers such as melanoma is still necessary. The results presented in this study show that apple extracts are promising candidates to be evaluated in preclinical models, alone or in association with standard chemotherapy.

## Chemical compounds studied in this article

4-*p*-coumaroylquinic (PubChem CID: 6441280); Amygdalin (PubChem CID: 656516); Catechin (PubChem CID: 9064); Chlorogenic acid (PubChem CID: 1794427); Epicatechin (PubChem CID: 72276); Luteolin (PubChem CID: 5280445); Phlorentin-2`-*O*-glucoside (PubChem CID: 6072); Phloretin-2′-*O*-(2″-*O*-xylosyl)-glucoside (PubChem CID: 442412); Procyanidin B trimer (PubChem CID: 107876); Procyanidin B1 (PubChem CID: 11250133); Procyanidin B2 (PubChem CID: 122738); Quercetin (PubChem CID: 5280343); Quercetin hexoside (PubChem CID: 25203368); Quercetin-*O*-α-L-arabinofuranoside (PubChem CID: 5484066); Rutin (PubChem CID: 5280805).

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2021.100112.

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