RESEARCH ARTICLE

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Antiproliferative and Apoptosis Effects of Hybrid Varieties of *Vitis vinifera* L. Sweet Sapphire and Sweet Surprise on Human Prostate Cancer Cells Using *In Vitro* and *In Silico* Approaches

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Abstract

Objective: Grape hybrids are characterized by different chemical compositions; often with high hybrids are characterized by different chemical compositions, often with a high phenolic content and a specific profile of anthocyanins. The aim of study was to characterize the constituents of hybrid *Vitis vinifera* L. varieties Sweet Sapphire (SA) and Sweet Surprise (SU) extracts and their influence on apoptosis induction and antiproliferative effects on human prostate cancer cells. **Methods:** We used the MTT assay to evaluate the cytotoxic effect of extracts of SA and SU, on the prostate adenocarcinoma cell lines PC-3 and DU-145. To analyze the inhibiting impact by flow cytometry, used 24 and 48 hours. Anthocyanins were quantified by liquid chromatography and analysed by their absorption rate, hepatotoxicity, blood concentration, blood-brain barrier passage ability and maximum recommended dose by in silico approaches. **Results:** Our results showed that malvidin derivatives present the highest content in both cultivars. We identified 14.46mg/100g malvidin-3-O-glycoside in SA and 2.76 mg/100 g in SU. A reduction in cell viability of DU-145 (45 and 65%) and PC-3 (63 and 67%) cells after 48h treatment with SA and SU, respectively, was found via MTT assay. Flow cytometry showed that the treatment with extracts from SA and SU had an inhibitory impact on cell development due to G2/M arrest and caused a rise in apoptotic cells compared to control group. None of the anthocyanin presented hepatotoxicity as well as blood-brain barrier passage ability. Peonidin 3-O-glucoside had the lower maximum recommended dose as well as the highest intestinal absorption rate. However, delphinidin 3-O-glucoside had the highest blood concentration values. **Conclusion:** The findings of this study highlight the potential of hybrid *Vitis vinifera* L. varieties as an important source of natural antioxidants and their protective effect against prostate cancer cells as well as elucidate in part their anthocyanin's metabolism.

Keywords: Cancer – antioxidant – grape - hybrid – anthocyanins

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Introduction

Due to the beneficial effects on human health and its economic importance, grape is a fruit widely grown and eaten around the world. Historically, the production and export of grapes were controlled almost exclusively by traditional European countries. However, in recent years, South America has shown a significant rate of growth in production and export of grapes with two crops a year (Gutiérrez-Gamboa and Pszczólkowski, 2020). Although the practice of viticulture in Brazil is recent when compared to traditional European countries, there is an improvement in the quality of Brazilian grape cultivar composition due to the use of hybridization techniques (Olivati et al., 2019). In contrast to the almost exclusive growth of V. vinifera cultivars in traditional wine producing countries, hybrid grape cultivars represent more than 80% of the volume of grapes (1,399,262 tons) processed in Brazil (De Rosso

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et al., 2012; Nicolini et al., 2020).

Hybrid grapes are obtained from the crossing of two or more species of Vitis that allows the selection of characteristics of interest, such as high resistance to diseases and pathogens. Interspecific hybrids are also characterized by different chemical compositions, are especially known to exhibit high content of phenolic compounds and a specific profile of anthocyanins, and are highlighted for their potential in the production of quality red wines (Samoticha et al., 2017). However, the hybrid varieties are currently scarcely studied. Phenolic compounds have been extensively studied due to their potentially beneficial antioxidant, anti-inflammatory, and anti-carcinogenic properties, which are spurring the interest of both industry and consumers for phenolic-rich foods (Gorzynik-Debicka et al., 2018).

A wide range of biological activities have been attributed to these compounds, indicating that some fruit sources can provide more than nourishment (Kelly et al., 2018). Natural foods of high nutritional quality play an important role in maintaining human health (Gülçin et al., 2002; Cory et al, 2018). As a result, much attention has been focused on the use of exogenous antioxidants, especially natural antioxidants to inhibit the oxidation of cellular components, thereby protecting from damage caused by free radicals (Liu et al., 2019).

The administration of phytochemicals into diets or the possibility of applying chemo preventive agents has been demonstrated in the literature to be effective against allergies, hypertension, viruses, inflammations, arthritis, mutations, and carcinogenesis (Hamza et al., 2018). Cancer is a group of diseases characterized by the growth and multiplication of abnormal cells, and if not controlled, can easily lead to death. Among the types of cancer affecting the world's male population, prostate cancer is the second most common (De Martel et al., 2020). The number of incident cases of prostate cancer has increased more than any other malignancy, regardless of development status (Russo et al., 2017). Animal studies have shown that phenolic compounds can prevent and/or delay the progression of initiation of different types of cancer (Basli et al., 2017), prostate cancer among them (Singh et al, 2004; Darweesh et al., 2020).

In the last decade there has been increasing interest in the determination of suitable dietary sources of antioxidant phenolic compounds (Delgado et al., 2019). However, there is little knowledge about the phenolic compounds of hybrid grape cultivars reported in literature: few reports describe only the anthocyanins for hybrid species (Nixdorf et al., 2010; Fujita et al., 2020).

Anthocyanins are phenol compounds present in grape skin and are responsible for the red colour of both grapes and wines (Shahab et al., 2020; Pereira et al., 2020). These compounds have been widely studied in V. vinifera varieties because they play a key role in the organoleptic characteristics of grapes (Dumitru et al., 2019). They also have antioxidant, antimicrobial and anti-cancerogenic activities, showing a protective effect on the cardiovascular system (Demirbas et al., 2017; Qin et al, 2019). Anthocyanins are studied for grape variety characterization and represent an important resource for the natural food colorant industry (Albuquerque et al., 2020; Chatham et al., 2020).

The chemistry of grape cultivars, especially varietal aroma, has a significant impact on the character of grapes, their sensory perception, and quality, influencing consumer acceptance. Varietal aroma can relate to a specific compound or to a small group of odoriferous molecules but is usually attributable to the contribution of several volatile compounds occurring in grapes, in proportions that differ from one variety to another (Slegers et al., 2015). Those aromas comprise hundreds of volatile organic compounds (VOCs) made up of different chemical groups, including alcohols, esters, aldehydes, ketones, monoterpenoids, and others. Methods for extracting VOCs often include liquid–liquid extraction, simultaneous distillation and extraction, headspace solid-phase microextraction (HS-SPME), and stir bar sorptive extraction techniques, among others (Lee et al., 2016).

Hence, the aim of this study was to identify and compare the extracts of two Hybrid grapes fruits obtained by water extracts. These extracts were additionally investigated for their antiproliferative effect and apoptotic induction in prostate (DU-145 and PC-3) cancer cells. The differential of the article was to address issues not yet discussed and identified the main phenolic compounds (anthocyanins) in two-selected species of hybrid grapes and their interaction between the antioxidant proprieties and antiproliferative activity in prostate cancer.

Materials and Methods

Samples

Two grape cultivars, Sweet Sapphire (SA) and Sweet Surprise (SU), were provided by Labrunier farm, located in Petrolina (Pernambuco, Brazil). These new hybrid cultivars are classified as table grapes. The grapes were harvested ripe and after the fruits were completely developed and then were transported under refrigeration to the laboratory. Initially, the grapes were then separated into three types of samples - whole, skin and pulp - and immediately frozen and stored at -80◦C in an ultrafreezer without undergoing any type of mechanical action, extraction, or homogenization.

For the preparation of extracts of Sweet Sapphire (SA) and Sweet Surprise (SU), the fruits were cleaned, and the peel was manually isolated from the pulp. Approximately 250 g of pulp of SA and SU was extracted with 80 mL of distilled water and then shaken for 2 h. After the pulp maceration period, the aqueous SA and SU extracts were filtered through Whatman #1 filter paper. The extracts were then frozen at -80◦C in an ultra-freezer and lyophilized (Terroni® LD 300, São Carlos, SP, Brazil) for 24 h. After this process, extracts were frozen at -20◦C until use in the experiments (Vizzotto et al., 2011).

Total phenolic content assays

Total phenolic content (TPC) analysis was performed using the Folin–Ciocalteu method. A 1 mL of sample (1 mg/mL) was mixed with 1 mL of Folin–Ciocalteu's phenol reagent. After 5 min at room temperature, 10 mL of a 7% Na2CO3 solution was added to the mixture followed

by the addition of 13 mL of distilled water and mixed thoroughly. The mixture was kept in the dark for 90 min at 23◦C, after which the absorbance was read at 750 nm. The TPC was determined by extrapolation from the calibration curve, which was made by preparing gallic acid solution (1 mg/ml). The estimation of the total content of phenolic compounds was performed in triplicate. The TPC was expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fresh weight (Saedd et al., 2012).

Anthocyanin analysis

Whole grape samples were cut into pieces with a scalpel and weighed until 1 g on an analytical scale. The grape was macerated with 1 mL of methanolic / formic acid (90:10; v/v) in a mortar and then placed in the ultrasound bath and subsequently subject to centrifugation until discoloration of the solution was achieved. A high-performance liquid chromatograph (HPLC) (Alliance 2695, Waters) equipped with photodiodes arrangement detector and a column C18 BDS (100 mm × 4.6 mm, 2.4 µm, ThermoScientific) utilising a gradient elution mode with acetonitrile and formic acid was used for the chromatography separation. The quantification of anthocyanins was made by external standardisation using isolated patterns. The results were expressed in mg of cyanidin-3-O-glucoside equivalents /100 g of fresh weight (Lves et al., 2007).

Cell assays

Prostate adenocarcinoma cell lines PC-3 and DU-145 were kindly provided by the Cell Interactions Laboratory, Federal University of Rio de Janeiro (UFRJ). PC-3 is a prostate cancer bone metastasis cell line, while DU-145 is a prostate cancer brain metastasis cell line (Sobel et al., 2005). The prostate cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% amphotericin, pH 7.4, under 5% CO2 atmosphere and 37◦C temperature. Controls used in cell assays were the cell lines in the same medium without any extract.

MTT cell viability assay

Cell viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay (Mosmann, 1983). The cells were plated in 96-well microplates with 1.0×10^4 cells/well. After incubation with the extract, the culture medium is removed and added 200 µL of MTT. After incubation for 4 h, the MTT was removed and 200 µL of dimethyl sulfoxide (DMSO) were added to solubilize the formazan. Samples were read in an ELISA reader (Bio-Rad iMARK) at 570 nm. Cell viability was calculated in comparison with the control (100%).

Cell cycle analysis

Cells were plated in 6-well microplates with 5.0×10^5 cells/well. Prostate cancer cells incubated for 48 h in the presence and in absence of the four samples at different concentrations (500 µg/mL and 1,000 µg/mL) were detached using trypsin solution at 25◦C. The cell suspension was analysed for DNA content by a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Vindlov's reagent was used to read 30,000 cells for each replicate (Pozarowawski and Darzynkiewicz, 2004). Cells with lower DNA content than G1 in the cell cycle distribution were considered hypodiploid cells (subG1). Relative proportions of diploid G0/G1 (2n), S (>2n, but < 4n), and G2/M (4n) indicative of DNA content were acquired using Cell Quest iPro. The percentage of cell population in each specific phase was estimated with FlowJo v 10.0.6 software and compared to the control.

Apoptosis assays

Phosphatidylserine externalization was observed through the Annexin-V assay using the flow cytometry technique (Van Engeland et al., 1998) to indicate the percentage of cells that were likely viable in apoptosis or nonapoptotic cell death. Prostate adenocarcinoma cells were incubated in a 6-well microplate using 5.0×10^5 cells/well with the extracts for 48 h. The cells were detached using a trypsin solution, and subsequently the propidium iodide and annexin markers were added. Detection was carried out with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) using Cell Quest iPro software to count 30,000 cells units/replicate. The cell populations analysed were recognized by their forward scatter (FSC)/side scatter (SSC) properties. Fluorescein isothiocyanate (FITC) green fluorescence was measured at 530 ± 30 nm (FL1 detector) and propidium iodide red fluorescence was measured at 585 ± 42 nm (FL2). The percentage of viable cells and cells in early or late apoptosis or non-apoptotic death was calculated using FlowJo v 10.0.6 software.

In silico approaches

The anthocyanins tentatively identified were submitted to in silico approaches as proposed by Galvão (2021). Molecular structure was obtained with the SMILES strings at PubChem (https://pubchem.ncbi.nlm.nih.gov/). For their recommended maximum dose (MRD, expressed as mg/kg of body weight/day) the QSAR framework algorithm was applied (Lazar in silico toxicology, https://lazar.in-silico.ch/predict) based on similarity structural alerts, The pkCSM algorithm (http://bleoberis.bioc.cam.ac.uk/pkcsm/prediction) was used for intestinal absorption rate (IAR) and hepatotoxicity. The blood brain barrier (BBB) permeability was estimated with SwissADME algorithm (http://www.swissadme.ch/). Additionally, the blood concentration (BC, expressed as mg/kg of body weight) of each anthocyanin was estimated based on the maximum recommended dose and the intestinal absorption rate.

Statistical analyses

All assays were performed in triplicate and the results are expressed by mean ± standard deviation. Data were analysed using GraphPad Prism (version 5.04, GraphPad Software, San Diego, CA, USA). Results were compared by one-way analysis of variance (ANOVA) together with the Tukey post-test with a confidence level of 95%. Cell cycle and apoptosis assays tested 99% and 99.9%

confidence levels.

Results

The composition of the extracts used in this work was previously presented and globally, a total of 87 phenolic compounds were tentatively identified, and among these the main classes were flavonoids, phenolic acids. Flavonoids presented the highest abundance, followed by the phenolics acids, other polyphenols, stilbenes and lignans (Pascoal et al., 2022).

Cyanidin derivatives showed the lowest concentration in SA with 0.63 mg/100 g. In SU, peonidin derivatives showed the lowest concentration with 0.77 mg/100 g. The SA sample showed high values of total anthocyanins (23.04 mg/100 g) compared to the SU sample (9.43 mg/100 g). In the determination of total phenolic compounds, the SU extract sample had the highest mean value of 55.67 mg AGE/100 g fresh weight, followed by the SA sample (Figure 1).

In the cell culture assays (cell viability, cell cycle, and apoptosis), tests were performed to guarantee 3 replicates of sample results for each concentration, the results are discussed based on the most used tests- extract of hybrid grapes in liquid and secondary metabolites alone or accompanied by grape extract. According to Camby et al. (Camby et al.,1994), PC-3 cell line appears to maintain a higher degree of differentiation than the DU-145 cell line, both are insensitive to androgens, and growth is similar because they grow as colonies. The two cell lines are similar in that they present themselves in the form of islands containing polygonal cells predominantly adorned in monolayers with central elements stacking on top of each other. The PC-3 cell line is more differentiated than the DU-145 cell line (Cristofani et al., 2018)

In this study, the cell proliferation was evaluated by the MTT (3-(4,5 dimethylthiazol2-yl) -2,5-diphenyl tetrazolium bromide) assay, which is one of the most used methods to access the action and interaction of natural products such as fruits and plants on cell proliferation, viability, and cytotoxicity. This assay is based on the reduction of a tetrazolium salt to a purple insoluble formazan by metabolically active cells. The absorbance of the solubilized formazan is taken as a measure of the number of living cells. To evaluate the effect of SA and SU on the viability of the two prostate cancer cell lines, DU-145 and PC-3, they were treated with 10-1000 µg/mL of each extract for 24h and 48h. A reduction in cell viability of DU-145 (45 and 65%) and PC-3 (63 and 67%) cells after 48h treatment with SA and SU, respectively, was found via MTT assay. These results have been supported by epidemiological studies in which the consumption of grape was associated with decreased risk of several types of cancer, including lung, prostate, and colon cancer.

Through the cell viability assessment with the MTT assay it was possible to analyse the cytotoxic effect of Sweet Sapphire and Sweet Surprise as shown in figure 2 referring to the DU-145 cells and figure 3 for the PC-3 cells at incubation times of 24h and 48h. For both cells, cell culture without the presence of the extract was used as a negative control. Both grape extracts showed viable DU-145 and PC-3 cell reduction. The extracts began to show a decrease in this viability after 24h of treatment in low concentrations, presenting a better effect on the concentration of 1000 µg/ml. On the other hand, for the PC-3 cell, no significant difference was seen between the concentrations of 500 and 1000 µg/ml. Regarding the incubation period of 24 and 48 h, no significant difference was seen in the reduction capacity obtained at the different concentrations for DU-145. However, for PC-3, the action was seen to take place more optimally in the period of 24 hours because in 48 hours the number of viable cells increases, leading to suggest that the cells have a mechanism for proliferating. Based on the MTT results, two of the six tested concentrations were selected for the cell cycle and apoptosis exams.

The cell cycle analyses showed a decrease in the two lines of the G0/G1 phase, as well as in the S and G2/M phases, regardless of treatment. This result shows that the treatments with the extracts of SA and SU showed positive results, since there was a decrease in proliferation in the different phases, but for both, there was no statistically significant difference (Figuras 4 e 5). The concentrations of 500 µg/mL and 1000 µg/mL were tested at 48h of incubation time to visualize the moment when cell viability decreased during cell growth, followed by the evaluation of the percentage of viable cells in the different phases of the cell cycle.

Table 1 shows the decrease in the percentage of cells in phase G0/G1, and cell decrease in phase S and G2/M, observed in comparison with the control at the concentration of 1000 and 500 µg/mL for DU-145, both

Table 1. Effect of SA and SU Extracts (500-1000 µg/ml) on Cell Cycle Progression in DU-145 and PC-3 Cells after 48 hours.

	DU-145			PC-3		
	G0/G1	S	G2/M	G0/G1	S	G2/M
Control	72.69+1.25	11.12+2.57	13.92+1.28	83.84+4.06	7.00+2.65	15.42+2.61
SA 500 µg/ml	63.00+1.23*	21.35+1.20*	12.60+1.95	56.86+14.79*	17.92+7.85*	10.07+4.08*
SA 1000 µg/ml	65.57+2.11*	19.42+3.64*	10.02+3.35	63.09+11.63*	11.05+3.55*	10.94+3.46*
Control	72.69+1.25	11.12+2.57	13.92+1.28	83.84+4.06	7.00+2.65	15.42+2.61
SU 500 µg/ml	65.36+0.89*	21.53+1.07*	10.65+2.08	72.88+1.51*	11.91+3.18*	13.34+1.77
SU 1000 µg/ml	64.58+0.62*	22.81+1.70*	10.21+2.78	68.67+3.91**	9.71+1.10*	14.80+0.96

Results are expressed as a percentage of total cells. Significant differences between untreated cells (Control) and cells treated with grape extracts were compared (* $p < 0.05$; ** $p < 0.01$;). Abbreviations: SA, Sweet sapphire; SU, Sweet surprise.

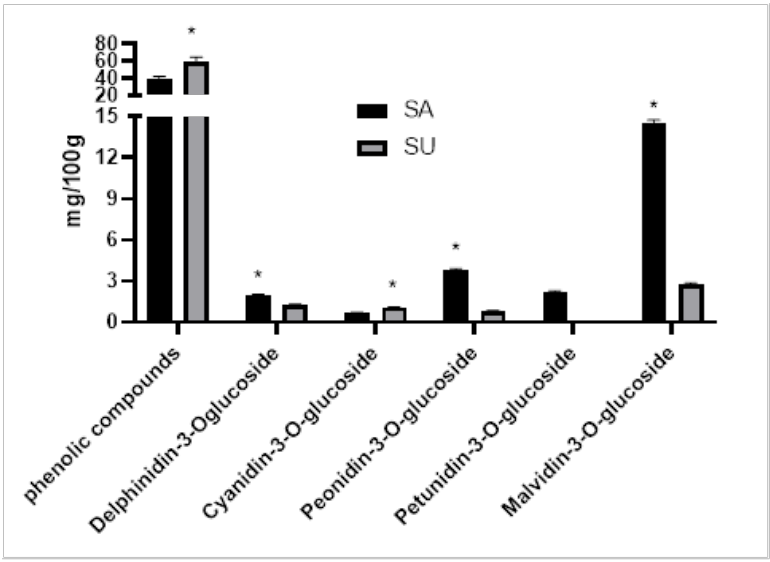


Figure 1. Total Phenolic Compounds and Total Anthocyanins Present in the Cultivars Sweet Sapphire (SA) and Sweet Surprise (SU).

Table 2. Apoptosis Rate of the DU-145 Cell Line Treated with SA (500-1000 µg/ml) and SU (500-1000 µg/ml) Extracts.

Treatment	Extract concentration	Viable cell DU-145	Initial apoptosis	Late apoptosis	Necrosis
Control	--	89.72 + 0.49	1.01 + 0.38	1.29 + 0.22	7.75 + 0.11
SA	500µg/ml	89.22+1.97	3.83 + 1.30*	2.18 + 0.34	6.51 + 0.62
	1000µg/ml	84.00+2.33*	3.83 + 1.30*	5.63 + 0.63*	4.36 + 0.54*
SU	500µg/ml	87.02+2.41	4.59 + 1.86*	3.73 + 0.73	4.62 + 0.19
	1000µg/ml	81.60+0.91*	8.14 + 1.00**	6.91 + 0.82*	3.32 + 0.60

Results are expressed as a percentage of total cells. Significant differences between untreated cells (Control) and cells treated with grape extracts were compared (* $p < 0.05$; ** $p < 0.01$;). Abbreviations: SA, Sweet sapphire; SU, Sweet surprise.

for the extracts of SA and SU. For the PC-3 cell line, the same cellular behaviour as the DU-145 scan was observed. The treatment showed a positive result for both, demonstrating to affect their proliferation.

For both concentrations, no evidence was found to prove statistical difference between the two. In the cell cycle and apoptosis experiments, in a study with DU-145 in treatments of 24 and 48 hours, the extract induced the phase G0/G1 stop and cell death (subG1 phase), and these effects occurred in a time-dependent manner. Proteins that had increased were associated with the stop cycle cell in phase G0/G1 (Table 1). The extract also significantly induced cell death by apoptosis leading to morphological and biochemical marks, such as cell shrinkage, membrane bubbles and condensation of nuclear chromatin.

In the apoptosis assay, the type of cell death caused by the bioactive compounds of the grape was evaluated by flow cytometry, and whether it would be able to trigger the apoptotic process without necrosis. For the cell line of brain metastasis (DU-145), we noticed that both concentrations (500 and 1000 µg/mL) showed statistical difference in relation to their control with a higher percentage of initial and late apoptosis (Table 2 and Figure 4). For the PC-3 cell line, the difference between treated cells and control is noticeably clear due to having several cells concentrated in the regions of early and late apoptosis, while the control is concentrated in viable cells (Table 3 and Figure 5).

In the apoptosis assay, the type of cell death caused by the bioactive compounds of the grape was evaluated by flow cytometry, and whether it would be able to trigger the apoptotic process without necrosis. For the cerebral

Table 3. Apoptosis Rate of the PC-3 Cell Line Treated with SA (500-1000 µg/ml) and SU (500-1000 µg/ml) Extracts.

Treatment	Extract concentration	Viable cells PC-3	Initial apoptosis	Late apoptosis	Necrosis
Control	--	98.66+0.68	0.26+0.13	0.36+0.034	0.06+0.013
SA	500µg/ml	92.92+3.39*	0.34+0.11	2.12+0.13*	6.49+0.79**
	1000µg/ml	93.15+1.17*	0.35+0.07	2.22+0.45*	4.49+1.20**
SU	500µg/ml	86.77+4.32**	10.2+0.70**	4.36+0.41**	3.72+1.32**
	1000µg/ml	88.72+1.23**	1.45+0.54**	3.85+0.50**	5.72+1.26**

Results are expressed as a percentage of total cells. Significant differences between untreated cells (Control) and cells treated with grape extracts were compared (* $p < 0.05$; ** $p < 0.01$;). Abbreviations: SA, Sweet sapphire; SU, Sweet surprise.

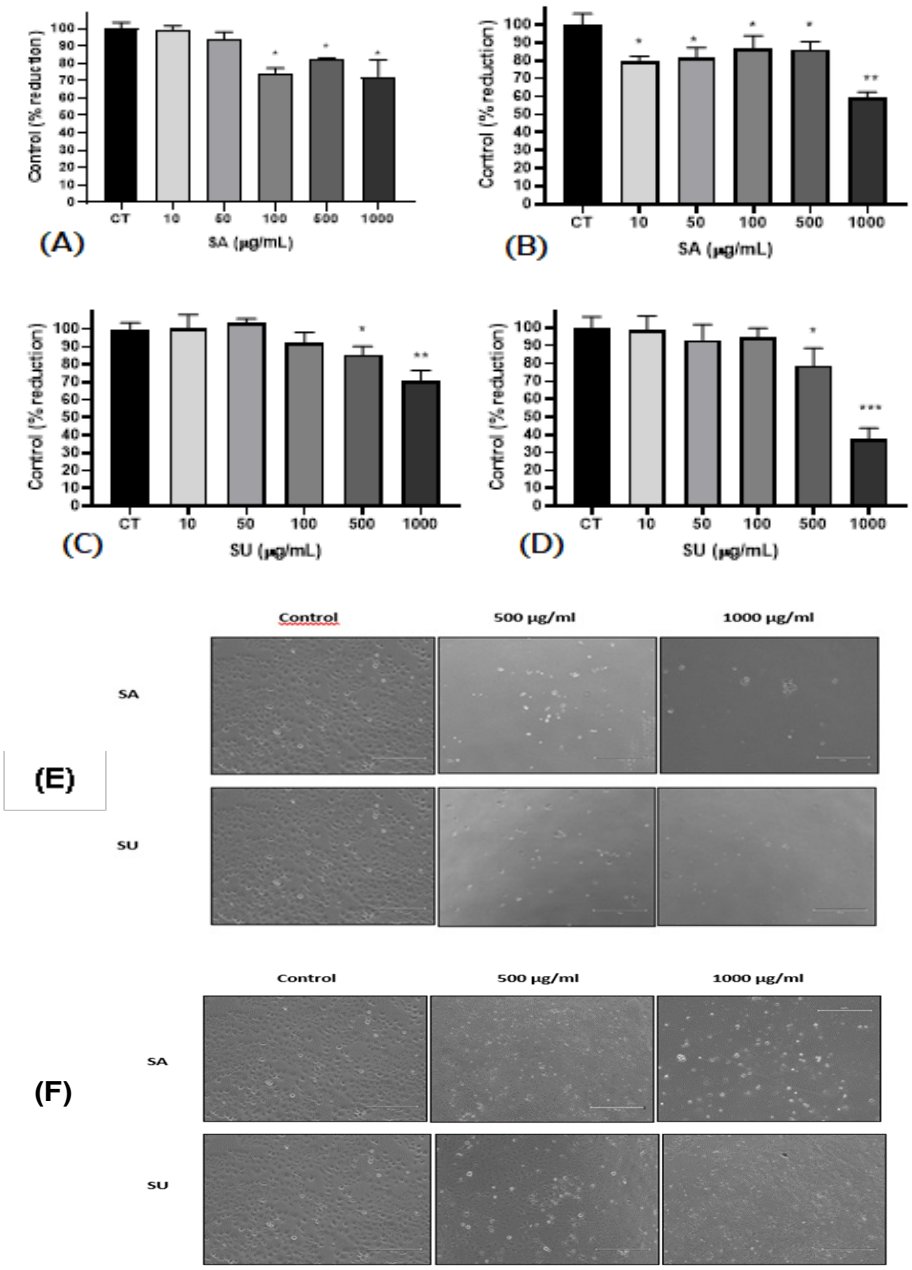


Figure 2. Effect of Treatment of Sweet Sapphire (SA) and Sweet Surprise (SU) Extracts under the Viability of DU-145 Prostate Cancer Cells after 24h ((A), (B), and 48h ((C)., (D) and (F)) of incubation. ($p< 0.05$). CT – control.

metastasis cell line (DU-145), we noticed that both concentrations (1000 and 500 µg/mL) showed statistical difference ($p<0.05$) in relation to their control with a higher percentage of initial and late apoptosis (Tables 2 and 3). Different algorithms are used in this approach, which contributes to increase the range of studied parameters. In this study, the recommended maximum dose, the intestinal absorption rate, hepatotoxicity, and BBB permeability were predicted.

The Table 4 presents the results of the in-silico approach. The compounds with the least recommended maximum dose were peonidin 3-O-glucoside and petunidin 3-O-glucoside, with 59.5 and 60.2 mg/kg of body weight/day, respectively. However, these compounds had the higher absorption rate (50% for peonidin and 42% for petunidin). Cyanidin 3-O-glucoside, delphinidin 3-O-glucoside and malvidin 3-O-glucoside both had around 30% of intestinal absorption rate. However, the compound with the higher estimated blood concentration after intestinal absorption was delphinidin 3-O-glucoside due to the higher maximum recommended dose between the five compounds. None of the compounds were predicted as hepatotoxic. On the other hand, none of the compounds were predicted to pass the blood brain barrier.

Discussion

In a study of Rosa Niagara grape cultivars, values from 208 to 214 mg AGE/100 g fresh weight were quantified, which shows values high to those found in the present

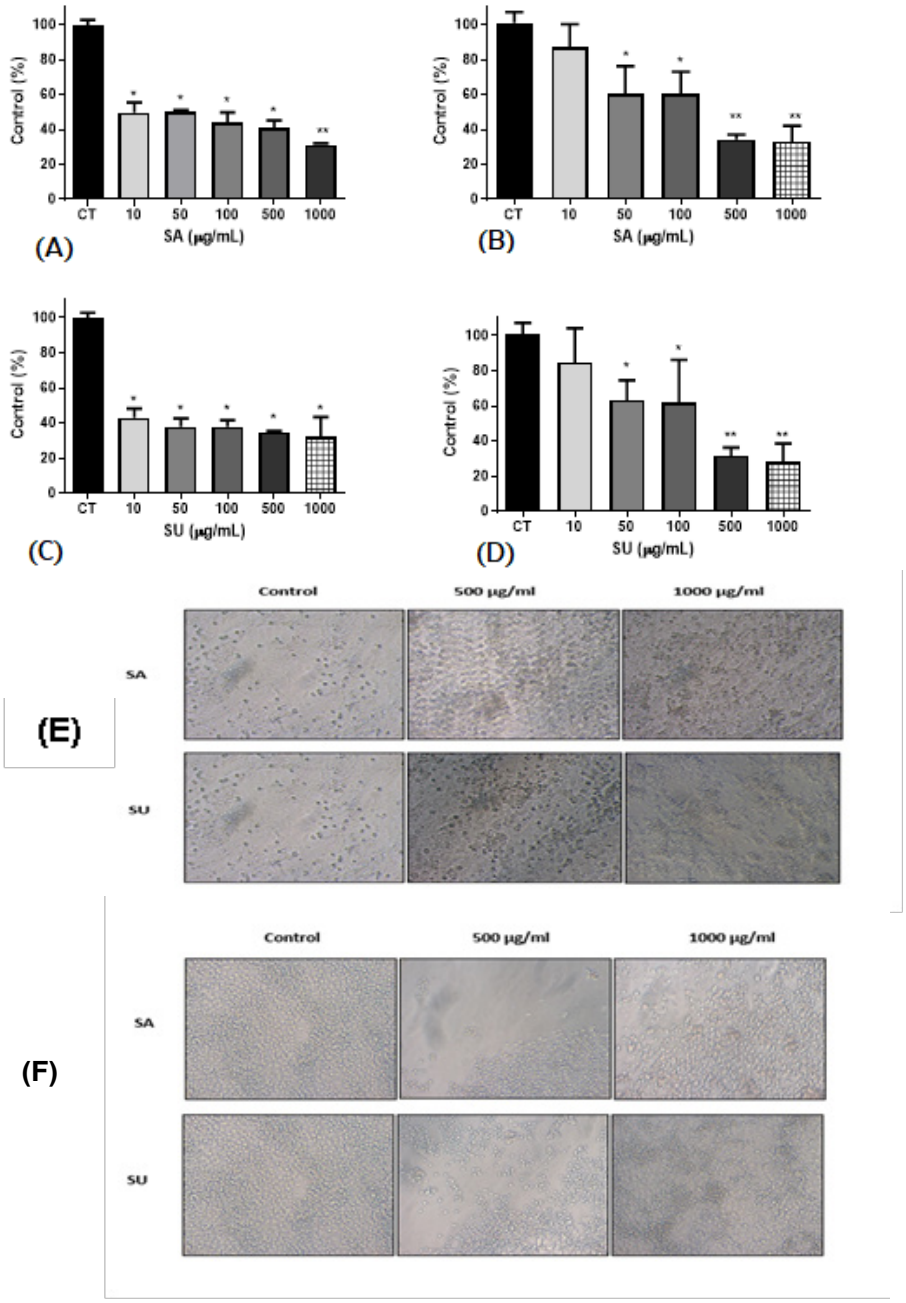


Figure 3. Effect of Treatment of Sweet Sapphire (SA) and Sweet Surprise (SU) Extracts under the Viability of PC-3 Prostate Cancer Cells after 24h ((A), (B), and E) and 48h ((C)., (D) and (F)) of incubation. ($p< 0.05$). CT – control.

study (Soares et al., 2008). In this study, we used only pulp, which may explain the difference for other studies that study the whole grape. Grape seeds are richer sources of total phenolic content than peel and pulp and peel was found to be richer than pulp for all nine Karaerik grape clones (Xia et al., 2018; Kupe et al., 2021).

Among the phenolic compounds present in grapes, the main ones are anthocyanins (Xia et al., 2018). In grapes of V. vinifera L., five coloured aglycons commonly occur, namely cyanidin, peonidin, delphinidin, petunidin and malvidin (Abe et al, 2007). Anthocyanins are in the berry skin and are the compounds mainly responsible for the red colour of grapes and wines (Lingua et al., 2016; Spada et al., 2022). Our results showed that malvidin derivatives present the highest content in both cultivars. We recorded 14.46 mg/100 g malvidin 3-O glycoside in Sweet Sapphire and 2.76 mg/100 g in Sweet Surprise, in accordance with Balik (2013) in more recent years.

Phenolic compounds were found in the extracts 94%, 85% and 66%, respectively, for flavonoids, other polyphenols, and phenolic acids (Pascoal et al., 2022).

The extract also significantly induced cell death by apoptosis leading to morphological and biochemical marks, such as cell shrinkage, membrane bubbles and condensation of nuclear chromatin being time-dependent effects (Lin et al., 2018). Regarding cell viability and cell cycle results, the lack of cell cycle regulation is a fundamental aspect in cancer development. Normal cells only proliferate in response to cell development or signals that occur during mitosis (Oshima and Campisi, 1991).

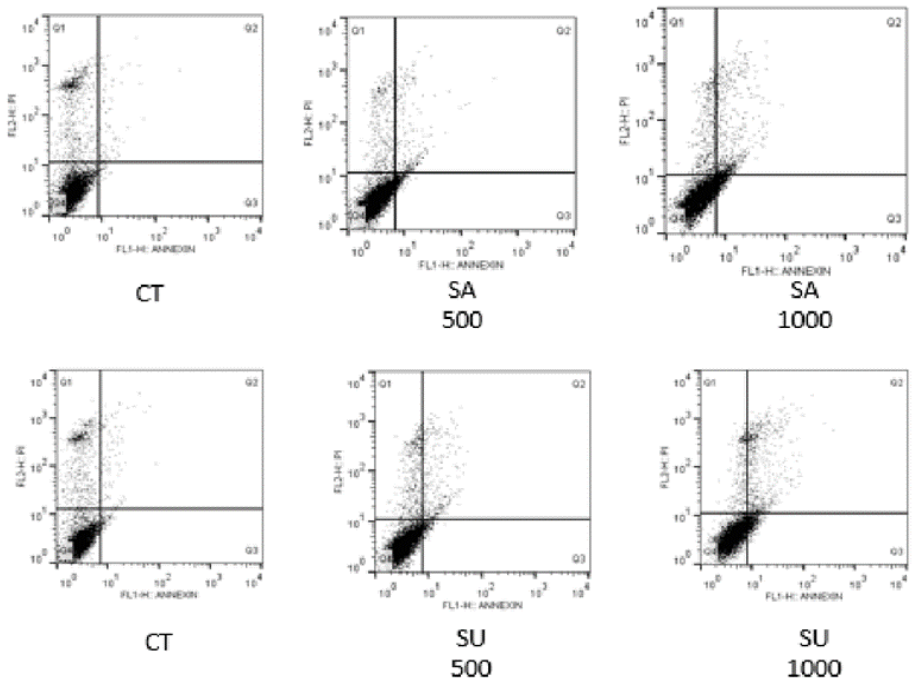
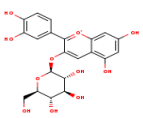
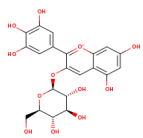
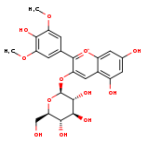
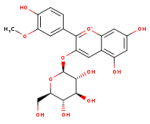
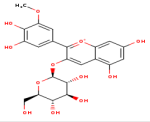


Figure 4. Apoptosis Induction in 48h in DU-145. Control Cells (CT) and cells treated with grape extracts of SA (Sweet Saphire) e SU (Sweet Surprise) (500 µg/mL) and (1000 µg/mL). The top-down quadrants clockwise: initial apoptosis (Annexin V+ PI-), late apoptosis (Annexin V+ PI+), non-apoptotic death (Annexin V- PI+) and viable cells (Annexin- PI-).

The cell cycle consists of distinct phases of events that occur in a cell in preparation for cell division: Phase G0 is seen as an extended G1, (gap 1, or G1, stage), copies its DNA (synthesis, or S, stage), prepares to divide (gap 2, or G2, stage), and divides (mitosis, or M, stage). Steps G1, S and G2 make up an interphase, which explains the gap

Table 4. Results of the in-silico Approach of Anthocyanins Identified in the Extracts Studied

Anthocyanin	PUBChem Molecular structure	Formula and molecular weight	LAZAR MRD	pkCSM IAR (%)	SwissADME Hepatotoxicity	SwissADME BBB permeability	BC
Cyanidin 3-O-glucoside		$C_{21}H_{21}ClO_{11}$ 484.84 g/mol	99.9	29,927	No	No	29.9
Delphinidin 3-O-glucoside		$C_{21}H_{21}O_{12}^{+}$ 465.38 g/mol	129.0	32,504	No	No	41.9
Malvidin 3-O-glucoside		$C_{23}H_{25}ClO_{12}$ 528.89 g/mol	92.0	31,346	No	No	28.8
Peonidin 3-O-glucoside		$C_{22}H_{23}O_{11}^{+}$ 463.41 g/mol	59.5	50,098	No	No	30.3
Petunidin 3-O-glucoside		$C_{22}H_{23}O_{12}+$ 479.41 g/mol	60.2	42,394	No	No	25.5

Abbreviations: MRD, maximum recommended dose in mg/kg of body weight/kg; IAR, Intestinal absorption rate; BBB, blood brain barrier; BC, blood concentration after intestinal absorption expressed as mg/kg of body weight.

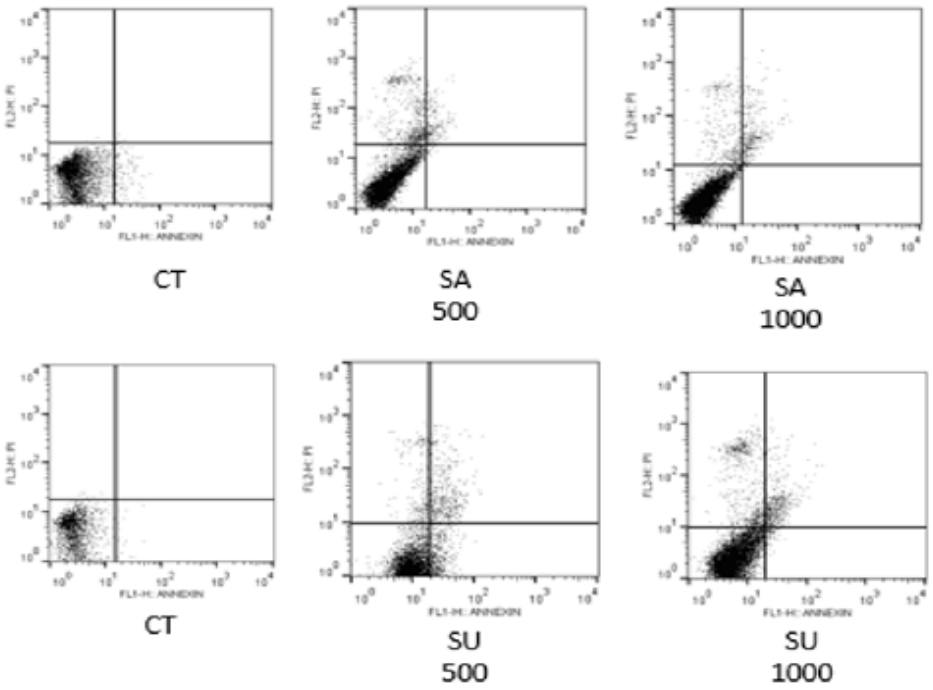


Figure 5. Apoptosis Induction in 48h in PC-3. Control Cells (CT) and cells treated with grape extracts of SA (Sweet Saphire) e SU (Sweet Surprise) (500 µg/mL) and (1000 µg/mL). The top-down quadrants clockwise: initial apoptosis (Annexin V+ PI-), late apoptosis (Annexin V+ PI+), non-apoptotic death (Annexin V- PI+) and viable cells (Annexin- PI-).

between cell division (Prakash et al., 2001). Each phase of the cell cycle is heavily regulated and there are checkpoints to detect potential DNA damage and allow it to be repaired before a cell split. If the damage cannot be repaired, a cell becomes the target of apoptosis, which is a programmed cell death process that occurs in multicellular organisms in different circumstances and involves different stages (Obermuller-jevic Uc et al., 2003).

The morphological and biochemical marks presented in the induction, by extract, of cell death by apoptosis may have been time-dependent effects (Lin et al., 2018).

Other studies have shown that the extract of grape seed powder negatively regulated Cyclin D1 and caused a slight positive regulation of PTEN (a tumour suppressor that modulates apoptosis, cell cycle, and migration) in DU-145 cells. The pAkt levels, which promote cell survival and prevent apoptosis, were affected by treatment with the extract in PC-3 cells. The p21 expression was induced in a dose-dependent manner after treatment with extract in PC-3 (Agarwal et al., 2000; Kumar et al., 2018).

Assays in an isolated polyphenolic fraction of procyanidin-rich grape seeds tested in the DU-145 cells presented results suggestive that this treatment possibly involves the modulation of mitogenic signalling and cell cycle 52 regulators and G1 stop induction, cell growth inhibition, and apoptotic death (Agarwal et al., 2000). The differential activity in selective targeting of cancer cells, preserving normal cells acting as pro-oxidants, occurs by inducing and degrading DNA in the presence of metal ions such as copper. This mechanism for polyphenols that involves the mobilization of chromatin-bound copper and the consequent pro-oxidant action that leads to cell death has anticancer activity and apoptosis inducers. As it is known that copper levels in tissues and cells are significantly elevated in several malignant diseases. Cancer cells are more subject to redox cycles between copper ions and polyphenols to generate reactive oxygen species (ROS) responsible for DNA breakdown. In other types of cancer, such as colorectal (Ravindranathan et al., 2019) the use of oligomeric proanthocyanins from grape seed extracts demonstrated cell cycle stoppage, double tape breaks and p53 protein accumulation in cells. In lung cancer (Mao et al., 2016), the procyanidin extract inhibited dose-dependent proliferation, induced apoptosis, and negatively regulated microRNA known as oncomirs that mediate pro or antitumor effects. Grape extract containing a mixture of bioactive compounds may have pharmacokinetics and pharmacological potency superior to isolated metabolites, presenting higher potential as a drug for natural products (Kumar et al., 2018).

The previous metabolomics results corroborate with our antioxidant analysis results and we can suggest the tentatively identified phenolic compounds play this role according to the literature, depending on the quantity found in each food matrix. Indeed, flavonoid compounds have been pinpointed as effective compounds in the control of cell cycle and apoptosis (Pascoal et al., 2022).

In cancer research, it is extremely relevant to ensure the safety of the compounds that are studied, even if these were from edible fruits as grapes. The in silico approaches are recently being used as a tool for the study of safety, as well as predictor of compounds toxicity (Galvão et al., 2021).

The in-silico studies were conducted, considering that, since, it may be metastatic cerebral prostate cancer (Sobel and Sadar, 2005), passing the BBB is relevant for the

exposure of metastatic cells to compounds that are capable of deterred their cell cycle and/or lead to apoptosis).

Even though cancer is among the most studied human diseases in a systemic way, its significant challenges remain close to the great potential of cancer biology in silico, preventing full realization (Edelman et al., 2010).

In conclusion, the results of this study, both extracts of hybrid grapes, Sweet sapphire and Sweet surprise, were able to reduce the viability of prostate cancer cells in the two cell lines presented, as they were able to cause a cytotoxic effect, as demonstrated in the results of cellular viability. Their compounds inhibited cell growth and proliferation in PC-3 and DU-145 as well as inhibited growth and apoptosis death by cell parade in phase S. In addition, the extract was considered non-toxic due to absence of hepatotoxicity and the maximum recommended dose for further possible assays was stablished.

Author Contribution Statement

Marta Angela de Almeida Sousa: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft, Writing - review & editing. Gabriela de Freitas Laiber Pascoal: Formal analysis, Validation. Maria Eduarda De Souza Jacintho: Formal analysis. Maria Luísa Barambo Wagner: Methodology, formal analysis. Pedro Paulo Saldanha Coimbra :Formal analysis, Writing - review & editing. Carlos Fernando de Araujo-Lima: Formal analysis, Writing - review & editing. Antonio Palumbo Junior: Methodology, Conceptualization. Anderson Junger Teodoro: Methodology, Conceptualization, Supervision, Project administration, Funding acquisition, Writing - review & editing.

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Approval

This study makes a lot of the doctoral thesis of Marta Angela de Almeida Sousa Cruz.

Conflict of Interest

The authors declare no conflict of interest.

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