



Cancer chemopreventive and antioxidant activities of seed, skin, and pulp of Maximo hybrid grapes (IAC 138-22) at five different ripening stages

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ABSTRACT: *This study evaluated the composition and the cancer chemopreventive and antioxidant activities of the seed, skin, and pulp of Maximo hybrid grapes (IAC 138-22) harvested at different ripening stages. The content of total phenolic compounds and tannins were determined by the Folin-Ciocalteu method, and total anthocyanins were determined using the differential pH method. Different chemical methods assessed the antioxidant activity. Cell viability (macrophage and melanoma skin cancer) and cancer chemopreventive activity were tested in cell lines by quinone reductase induction assay, TNF- α -induced NF- κ B inhibitory activity assay, and aromatase inhibitory activity assay. The hybrid grape had high phenolic contents in both seeds and skin, significantly reduced upon ripening. The results showed that fruit ripening significantly affected both the composition and the antioxidant and cancer chemopreventive activities. Except for the anthocyanin content, the most significant results were observed for green seeds, with the greenest stage showing the best results. The green seeds showed the highest antioxidant activity, even when compared to free resveratrol. In the assessment of cancer chemopreventive activity, the green seeds showed the ability to inhibit N-F κ B and aromatase activity, important factors for the early stage of cancer development. Besides, no extracts showed cytotoxicity. Therefore, this grape's green seeds showed strong antioxidant and cancer chemopreventive activities confirmed herein, suggesting beneficial health effects.*

Key words: aromatase, antioxidant, grape, cancer chemopreventive.

Atividades quimiopreventiva de câncer e antioxidante de sementes, casca e polpa de uvas híbridas Maximo (IAC 138-22) em cinco diferentes estágios de maturação

RESUMO: *O objetivo deste estudo foi avaliar a composição e as atividades quimiopreventiva de câncer e antioxidante da semente, polpa e casca de uvas híbridas Maximo (IAC 138-22) colhidas em diferentes estágios de maturação. Os teores de compostos fenólicos totais, antocianinas e taninos foram determinados a partir dos extratos etanólicos. O conteúdo de fenólicos e taninos totais foi determinado pelo método de Folin-Ciocalteu, e o teor de antocianinas totais foi determinado pelo método do pH diferencial. A atividade antioxidante foi avaliada por diferentes métodos químicos, e a viabilidade celular (macrófagos e melanomas), e atividade quimiopreventiva de câncer foi testada em culturas de células por ensaio de indução de quinona redutase, ensaio de atividade inibidora de NF- κ B induzido por TNF- α e ensaio de atividade inibidora de aromatase. A uva híbrida apresentou alto teor de fenólicos nas sementes e na casca, teor que reduziu significativamente com o amadurecimento. Os resultados mostraram que o amadurecimento dos frutos afetou significativamente a composição e as atividades antioxidantes e quimiopreventivas de câncer. Com exceção do teor de antocianinas, os resultados mais significativos foram observados para as sementes verdes, com o estágio mais verde apresentando os melhores resultados. As sementes verdes apresentaram a maior atividade antioxidante, mesmo quando comparadas ao resveratrol livre. Na avaliação da atividade quimiopreventiva do câncer, as sementes verdes mostraram capacidade de inibir a atividade do NF- κ B e da aromatase, fatores importantes para o estágio inicial do desenvolvimento do câncer. Além disso, nenhum extrato apresentou atividade citotóxica. Portanto, as sementes verdes desta uva apresentam resultados promissores dadas as fortes atividades antioxidantes e quimiopreventivas do câncer aqui confirmadas, sugerindo efeitos benéficos à saúde.*

Palavras-chave: aromatase, antioxidantes, uva, quimioprevenção de câncer.

INTRODUCTION

Grapes are popular worldwide, and their use is diverse. They can be consumed fresh, dehydrated, in wines, juices, jellies, dietary supplements, and ice cream, among other processed foods. Grapes comprise

various nutrients, such as carbohydrates, proteins, vitamins, minerals, and dietary fibers. Additionally, they contain phytochemicals, such as phenolic compounds known to have numerous biological activities, e.g., anti-inflammatory, antimicrobial, antitumor, antioxidant, and particularly cancer

chemopreventive activities (STEWART & BROWN, 2013; ACUÑA-AVILA et al., 2016;).

The use of natural, biological, or synthetic agents capable of suppressing, reversing, or preventing the early stages of carcinogenesis is known as cancer chemoprevention. These agents may also play a preventive role in the premalignant cells' progression (SPORN, 1976). It is also common knowledge that cancer is a chronic process determined by the uncontrolled growth of abnormal cells and that the global incidence of this disease has been increasing considerably, which arouses the interest for new prevention strategies (NCI, 2015).

Many studies on different grape species involved in beneficial health processes indicate that the genetic variability among species, including new hybrids, directly influences these effects since they have distinct compounds and agronomic characteristics (SILVA, et al., 2018, 2019; TECCHIO et al., 2020).

According to the Brazilian Agricultural Research Corporation (Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA), there is a predominance of hybrid or American grapes in various producing regions. However, some Brazilian regions opt for exclusive *Vitis vinifera* cultivation even if their production corresponds to a lower percentage (MELLO, 2017).

Both the Brazilian Agricultural Research Corporation (EMBRAPA) and the Agronomic Institute of Campinas (IAC) developed, through studies on genetic improvement, the Maximo hybrid grape (IAC 138-22). This hybrid results from the crossing between Syrah (*Vitis vinifera*) and Seibel 11342 (hybrid) grapes, which intends to improve the resistance to pathogens and enhance the sensory quality of these hybrid grapes byproducts to the consumers. The current literature highlights that Maximo hybrid grapes (IAC 138-22) are from recent cultivation. However, previous studies have shown that this grape has relevant sensory characteristics and comprises promising compounds, thus inciting the study of their biological properties and potential applications, such as in flour, juices, and jellies (MORELLI & PRADO, 2012; BIASOTO et al., 2014; FRAIGE; et al., 2014; MARTINS et al., 2016; SILVA, et al., 2019).

Evaluation of the Maximo hybrid grape composition has demonstrated that these grapes feature higher contents of individual and total anthocyanins, flavonols, t-resveratrol, and total phenolic compounds compared to other grape varieties (SILVA, et al., 2019). In another study on

the presence of anthocyanins, the authors reported that cyanidin, peonidin, delphinidin, petunidin, and malvidin, mainly in the forms of glycosides and acyl glycosides, were the main compounds. Moreover, malvidin-3-glycoside is the most abundant in almost all grape varieties (FRAIGE et al. 2014).

Among the non-flavonoid compounds, phenolic acids stood out not only for contributing to the sensory characteristics but also by providing beneficial effects either by consuming the fresh fruit itself or its byproducts (FARHADI et al., 2016). A study by MORELLI & PRADO (2012) indicated that grape jellies formulated with the Maximo variety (IAC 138-22) were considered good sources of antioxidant phenolic compounds. Another study on the evaluation of Maximo grape residue resulting from wine production demonstrated larger quantities of flavonoids and phenolic acids, as well as a more distinguished antioxidant activity than those reported in residues of other grape varieties (MARTINS et al., 2016).

Bearing in mind the economic importance of developing new hybrid grapes, the present study evaluated the composition, the cancer chemopreventive potential, and antioxidant activities of seed, pulp, and skin of Maximo hybrid grape (IAC 138-22) at different ripening stages.

MATERIALS AND METHODS

Reagents

Anhydrous sodium carbonate, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ), 2,2-azino bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate, peroxidase, and resveratrol (CAS n° 501-36-0; empirical formula: $C_{14}H_{12}O_3$, molecular weight: 228.24) were obtained from Sigma-Aldrich®, St. Louis, USA. Hydrochloric acid, acetic acid, monobasic and dibasic potassium phosphates, and potassium chloride were acquired from Cromoline®, Diadema, SP. Folin-Ciocalteu reagent was obtained from Merck®, Darmstadt, Germany, and 99.5% ethanol from Quimex®, Cotia, SP. Sodium acetate and ferric chloride were acquired from Dinâmica®, Diadema, SP. Hydrogen peroxide and sodium hydroxide were purchased from Neon®, São Paulo, SP. Phenol red was obtained from Synth®, Diadema, SP.

Samples

The Maximo hybrid grapes (IAC 138-22) were grown on an espalier system, harvested weekly

in a winery at the State of Espírito Santo, Brazil (19°55'03.7"S, 40°44'00.1"W) at five different ripening stages. The date of the first sampling corresponded to the unripest stage of the fruit [Seed 1 (S1), Skin 1 (C1), Pulp 1 (P1)], while the last date of sampling corresponded to the ripest one [Seed 5 (S5), Skin 5 (C5), Pulp 5 (P5)] (Figure 1). The harvest was performed on both sides of the selected rows, averting the first two vines of each row (5 kg each). The grapes were washed with tap water and then in deionized water three times; they were then carefully separated manually into the skin, pulp, and seeds. Each fraction was frozen at -80 °C in an ultra-freezer (CL580-86V, Coldlab®), freeze-dried (Enterprise I - Terroni®), milled in a knife mill, and stored under refrigeration until analyzed.

Extracts

The extraction was performed using 20 g of freeze-dried material in 200 mL of 99.5% ethanol by the ultrasound-assisted maceration method (Elmasonic P 60 Hz - Elma®), according to the protocol described by GUSS et al. (2017), with some modifications. After two cycles of 30 min, the extracts were filtered through filter paper twice. The organic fractions were pooled and evaporated in a rotary evaporator (Fisatom® 802 – 1200W) until ethanol was removed. The process was completed in the oven (TE 394/2 MP, 1500W – Tecnal®) at 40 °C for 24 h, with subsequent freeze-drying for another 24 h.

Total phenolic compounds

The content of total phenolic compounds was determined by the Folin-Ciocalteu method, according to the methodology described by GUSS et

al. (2017). Briefly, 30 µL of the extracts in ethanol at a concentration of 0.5 mg/mL and 150 µL of Folin reagent diluted in water (1/10) were used. After 5 min, 120 µL of 7% anhydrous sodium carbonate was added and allowed to react for 90 min in the dark. The reading was performed in an ELISA reader (Elisa Spectra count Packad – USA) at 740 nm. Results were expressed in milligrams of gallic acid per gram of extract.

Quantification of tannins

To analyze the chemical components, 60 mg of the skin, pulp, and seed extracts were weighed and transferred to a flat bottom flask containing 150 mL of carbon dioxide-free water after complete solubilization, according to the methodology described by KREPSKY et al. (2012), with adaptations. The flask was then kept in a water bath at 60 °C for 30 min. The solution was filtered and transferred to a 250 mL volumetric flask that had its volume completed with carbon dioxide-free water; after discarding the first 50 mL, this sample became solution A. Subsequently, 20 mL of solution A was removed, added to an Erlenmeyer flask with 0.2 g of Polyvinylpyrrolidone (PVPP), which was stirred for 60 min. Then, the solution was filtered (solution B) and 25 µL of solution A or B were transferred along with 10 µL of Folin-Ciocalteu reagent (10%) and 215 µL of sodium carbonate (10.6% w/v) into a 96-well microplate. The readings were taken at 715 nm after 3 min, using water as blank. Gallic acid was used as standard. The tannin content was determined by subtracting the total polyphenol content (solution A) from the non-adsorbed polyphenol content (solution B).



Figure 1 - Five different ripening stages of grapes in a winery in Santa Teresa - ES.

Total anthocyanins

Total anthocyanins were determined using the differential pH method described by GIUSTI & WROLSTAD (2001). Sample solutions (5 mg/mL) were prepared in two different buffers (potassium chloride 0.025 M, pH 1.0; sodium acetate 0.4 M, pH 4.5). The absorbances of these extracts were monitored according to equation 1: $A = (A_{520 \text{ nm}} - A_{700 \text{ nm}})_{\text{pH 1.0}} - (A_{520 \text{ nm}} - A_{700 \text{ nm}})_{\text{pH 4.5}}$. The concentration (mg g^{-1} extract) of anthocyanins was expressed in cyanidin-3-glucoside equivalents and calculated using equation 2: $(A \times \text{MW} \times \text{DF} \times 1000) / (\epsilon \times L)$, where A is obtained in equation 1, MW is the molecular weight of cyanidin-3-glucoside, DF is the dilution factor, ϵ is molar absorptivity (26,900), and L is the optical path (1 cm).

Antioxidant Activity

DPPH free radical scavenging

Samples' antioxidant activities were determined by the DPPH method, according to SCHERER & GODOY (2009). The radical scavenging activity was calculated as follows: $I (\%) = [(Abs_0 - Abs_1) / Abs_0] \times 100$, where Abs_0 is the absorbance of the blank and Abs_1 is the absorbance in the presence of the test compound at different concentrations. The IR_{50} (concentration capable of reducing in 50% of the free radicals) calculations demanded a linear range calibration curve by plotting the final concentration of the extract *versus* the corresponding scavenging effect. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated as follows: $AAI = \text{final concentration of DPPH } (\mu\text{g/mL}) / IC_{50} (\mu\text{g/mL})$. The AAI values were classified as: weak ($AAI < 0.5$), moderate (0.5–1.0), strong (1.0–2.0), and very strong ($AAI > 2.0$) antioxidant activities. The extracts' antioxidant activities were compared to that of the phenolic compound resveratrol.

Ferric Reducing Antioxidant Power (FRAP) assay

Antioxidant activity was based on the FRAP method according to BENZIE & STRAIN (1996), with some modifications. A mixture of 25 mL of sodium acetate trihydrate (0.3 M, pH 3.6), 2.5 mL of TPTZ solution (10 mM/L) in 40 mM HCl, and 2.5 mL of aqueous ferric chloride (20 mM) resulted in the FRAP reagent, immediately after preparation. Test aliquots of 30 μL were added to a 96-well microplate, along with a 270 μL FRAP reagent. For the blank, 30 μL of ethanol was added. After reacting for 10 min, the reading was performed at 595 nm using a microplate reader (SpectraMax 190 Microplate Reader, Molecular Devices, California,

USA). Calculations on antioxidant activity respected the following formula: $I (\%) = (Abs_1 - Abs_0) \times 100$, where Abs_0 is the blank and Abs_1 is the absorbance of the tested extract. Results were expressed as IR_{50} (concentration capable of reducing 50% of the free radicals), calculated using the linear equation obtained from the calibration curve (final concentration mg/mL *versus* $I\%$). The extracts' antioxidant activities were compared to that of the phenolic compound resveratrol.

ABTS free radical scavenging

The antioxidant activity was determined according to RE et al. (1999), with modifications. The ABTS radical cation ($ABTS^{+}$) was formed by the reaction among 7.0 mM ABTS (in 50% ethanol) and 2.45 mM potassium persulfate (in distilled water), and stored under refrigeration for at least 24 h. Before use, the reagent was diluted with 50% ethanol until the measured absorbance at 734 nm was 1.0 (± 0.02). $ABTS^{+}$ (270 μL) and the compounds (30 μL of each concentration) were added into 96-well microplates. The blank was 30 μL of ethanol. After reacting for 10 min in the dark, the reading was performed at 734 nm using a microplate reader (SpectraMax 190 Microplate Reader, Molecular Devices, California, USA). The radical scavenging activity was calculated as follows: $I (\%) = [(Abs_0 - Abs_1) / Abs_0] \times 100$, where Abs_0 is the absorbance of the blank and Abs_1 is the absorbance in the presence of the test compound at different concentrations. The results were expressed as IR_{50} (concentration capable of reducing 50% of the free radicals) and calculated using a calibration curve in the linear range by plotting the final concentration of the extract *versus* the corresponding scavenging effect. The extracts' antioxidant activities were compared to that of the phenolic compound resveratrol.

Hydrogen peroxide (H_2O_2) assay

Grapes' antioxidant activity at a concentration of 1000 $\mu\text{g/mL}$ was also evaluated by the hydrogen peroxide scavenging method according to PICK & KEISARI (1980), with modifications. In 96-well microplates, 25 μL of the samples, 50 μL of H_2O_2 , 20 μL of phenol red solution, 25 μL of peroxidase enzyme, and 130 μL phosphate buffer pH 7.0 were added. The microplates were incubated for 15 min in the dark, and the reaction ended after adding 25 μL of NaOH (1.0 M). The reading was performed at 610 nm, and the percentage of hydrogen peroxide reduction index ($IR\%$) was calculated by the following equation: $IR (\%) = [(AbsC - AbsT) / AbsC] \times 100$, where AbsC is the control mean absorbance and AbsT is the test absorbance. The control reaction

was performed in the absence of the sample, and the entire experiment was conducted in triplicate. The extracts' antioxidant activities were compared with that of the resveratrol.

Cell viability assessment

The MTT colorimetric method was used according to the methodology described by MOSMANN (1983) to investigate the cytotoxic activity. This method uses the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the enzyme succinate dehydrogenase within the cellular mitochondria, which forms a blue compound called formazan that, upon accumulation within the cell, is removed by dimethyl sulfoxide (DMSO). The method allows the determination of mitochondrial functionality of untreated cells and has been widely used to measure cell survival and functionality so that the number of living cells is proportional to the amount of produced formazan. In this study, two cell lines were used: RAW 264.7 macrophage and melanoma skin cancer (MV3). The cells were plated at a concentration of 7×10^4 cells/mL in 96-well plates and cultured with the extracts of the seeds, skin, and pulps of the Maximo hybrid grape at 100 $\mu\text{g}/\text{mL}$ for 24 h. Samples were diluted in DMSO up to 0.5%. Resveratrol was a positive control. After the 24-h incubation period, 100 μL of MTT (5 mg/mL) was added to each well, and the formed formazan crystals dissolved in dimethyl sulfoxide (DMSO). The absorbance readings of the plates were made by an ELISA reader using 595-nm filters. Cell viability was expressed as a percentage.

Cancer chemopreventive activity

In vitro quinone reductase induction assay

Quinone reductase induction assay followed the method described by PROCHASKA & SANTAMARIA (1988) and modified by GERHÄUSER et al. (1997). Rat hepatoma culture Hepa1c1c7 was used to evaluate the extracts as inducers of the enzyme quinone reductase. Grapeseed, skin, and pulp extracts were tested at 20 $\mu\text{g}/\text{mL}$. Results were expressed as percentage inhibition (IR%).

TNF- α -induced NF- κ B inhibitory activity assay

The assay was performed as described by HOMHUAL et al. (2006) with a 20 $\mu\text{g}/\text{mL}$ extract. For the NF- κ B inhibition assay, the 293-NF- κ B cell line (NF- κ B-luciferase transfected renal embryonic cells 293.12 PTA – 5554) was used after being cultured

and induced by TNF- α (5 ng/mL), and the Luc assay Promega® conveyed the reporter system. Na-tosyl-L-phenylalanine-chloromethyl ketone was used as positive control; $\text{IC}_{50} = 3.8$ nM. The experiments were performed in triplicate. Results were expressed as percentage inhibition.

Aromatase inhibitory activity assay

The aromatase inhibition assay respected PEZZUTO et al. (2005). The effect of the extracts (20 $\mu\text{g}/\text{mL}$) on the aromatase enzyme was evaluated by detecting fluorescein produced when dibenzylfluorescein was hydrolyzed by the aromatase. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission). A naringenin curve was used as a positive control (74.46 μM to 0.57 μM). Results were expressed as percentage inhibition.

Statistical analyses

Two-way analysis of variance (ANOVA) and Tukey's post hoc test were used to, respectively, determine if there was a significant difference between the means and the magnitude of this difference ($P < 0.05$). The data are parametric and expressed as mean \pm standard deviation values. Pearson's correlation coefficient was used to measure the degree of correlation between two metric variables. Biostat 5.0 software was used.

RESULTS

Yield, total phenolics, anthocyanins, and tannins

Table 1 presents extracts' total yield and the contents of total phenolics, anthocyanins, and tannins. The extract yield was higher in Skin 1 (25.5%) and lower in Pulp 3 (11.9%).

The total phenolic content was significantly higher ($P < 0.05$) in seeds than in skin and pulp. Seed samples presented a significant difference between each other ($P < 0.05$), with the ripening stage being inversely proportional to the content of total phenolics, *i.e.*, the riper the grapes, the lower the phenolic content. Skin 1 presented significantly higher phenolic content than others skin samples ($P < 0.05$). No significant difference ($P > 0.05$) in the phenolic content was reported between pulp samples.

Although, the anthocyanin content was evaluated in seeds, pulp, and skin, this class of phenolic pigments was reported only in the skin, as shown in table 1. The anthocyanin content was significantly higher in Skin 5 ($P < 0.05$), which corresponded to the ripest stage, whereas the lowest content was found in the completely unripe stage, as expected.

Table 1 - Yield and contents of total phenolics, anthocyanins, and tannins in Maximo hybrid grape (IAC 138-22) extracts.

	Yield (%)	TPC* (mg GA/g extract)	Anthocyanins (mg/g extract)	Tannins (mg GA/100g extract)
Seed 1	19.5	217.2 ± 1.9 a	ND	26.1 ± 0.22 a
Seed 2	20.1	187.0 ± 7.0 b	ND	21.0 ± 1.4 b
Seed 3	16.7	153.1 ± 2.2 c	ND	16.0 ± 0.44 c
Seed 4	15.8	143.7 ± 1.9 d	ND	15.2 ± 0.3 c
Seed 5	12.3	132.5 ± 0.5 e	ND	14.3 ± 0.1 c
Skin 1	25.5	51.0 ± 3.7 f	ND	1.6 ± 0.4 e
Skin 2	23.3	27.9 ± 1.3 g	2.9 ± 0.5 d	1.2 ± 0.4 e
Skin 3	24.3	16.7 ± 0.4 h	63.1 ± 1.2 c	1.8 ± 0.2 e
Skin 4	24.4	19.8 ± 2.0 h	81.9 ± 0.2 b	2.4 ± 0.4 d
Skin 5	23.2	18.9 ± 1.6 h	83.9 ± 0.5 a	2.3 ± 0.4 d
Pulp 1	22.6	0.4 ± 0.1 i	ND	ND
Pulp 2	17.2	1.1 ± 0.3 i	ND	ND
Pulp 3	11.9	1.2 ± 0.3 i	ND	ND
Pulp 4	18.8	1.7 ± 0.0 i	ND	ND
Pulp 5	20.8	1.6 ± 0.1 i	ND	ND

*TPC: Total phenolic content. ND: not detected. Different letters in the same column represent significant differences ($P < 0.05$). GA: gallic acid.

The tannin content was significantly higher ($P < 0.05$) in seeds than in skin, regardless of the ripening stage, and no tannin was detected in the pulp. Seed 1 had the highest tannin content, while Skin 2 had the lowest, showing no statistical difference compared to Skins 1 and 3.

Antioxidant activity

The antioxidant activity of Maximo hybrid grape (IAC 138-22) extracts and free resveratrol were evaluated by distinct methods to investigate different antioxidant characteristics of the samples. AAI (antioxidant activity index) represented the DPPH free radical scavenging activity, and the free radical sequestration methods ABTS and FRAP were represented by IR_{50} (sample quantity required to decrease the initial radical concentration by 50%). Unlike the methods mentioned above, the hydrogen peroxide scavenging method was expressed as I% (percentage of H_2O_2 inhibition index).

Seed extracts showed relevant antioxidant activity by the DPPH free radical scavenging method. Besides, the seed AAI values at all ripeness levels were significantly higher ($P < 0.05$) than that of resveratrol (Table 2). Moreover, the AAI decreased with grape ripening in both seeds and skin, indicating a possible relationship with the total phenolic content. Conversely, antioxidant activity was classified as

moderate in Skin 1 (AAI between 0.5 and 1.0) but weak in the other skin samples. That reduced the content of total phenolic compounds.

The FRAP method showed no significant difference among the activities of Seeds 1 and 2 extracts compared to resveratrol, confirming its remarkable antioxidant activity. Furthermore, riper grapes denoted a significant reduction in activity (increase in IR_{50}) (Table 2).

The antioxidant activity determined by the ABTS method showed similar results, with seed extracts presenting significantly higher antioxidant activity than skin extracts ($P < 0.05$). Moreover, the antioxidant activity decreased with grape ripening (an increase of IR_{50}) in both seeds and skin. Conversely, even though the seed extracts presented notable antioxidant activity as indicated by this method, resveratrol showed significantly higher activity.

Testing the antioxidant activity by the hydrogen peroxide (H_2O_2) method showed that the percentage of inhibition index (I%) was significantly higher in Seeds 1 and 2, confirming that green seeds have high antioxidant activity. The skin showed low activity and no significant difference ($P > 0.05$) by this method. Results also showed that, regardless of the ripening stage, grape pulp presented negligible antioxidant activity in all evaluated methods, as shown in table 3.

Table 2 - Antioxidant activities of Maximo hybrid grape (IAC 138-22) extracts determined by the DPPH, FRAP, ABTS, and H₂O₂ methods.

	DPPH (AAI ¹)	FRAP (IR ₅₀) ²	ABTS* (IR ₅₀) ²	H ₂ O ₂ (I%) ³
Seed 1	4.7 ± 0.0a	5.1 ± 0.5a	5.3 ± 0.4b	69.3 ± 1.0b
Seed 2	4.5 ± 0.1b	5.3 ± 0.1a	5.6 ± 0.1b	72.1 ± 1.3b
Seed 3	3.6 ± 0.0c	7.6 ± 0.1b	7.9 ± 0.5c	9.0 ± 0.1d
Seed 4	3.2 ± 0.0d	11.0 ± 0.2c	11.9 ± 1.0d	5.8 ± 2.4de
Seed 5	3.3 ± 0.0d	10.8 ± 0.9c	9.5 ± 0.5c	9.3 ± 3.8d
Skin 1	0.8 ± 0.0f	23.8 ± 0.6d	50.0 ± 2.9e	2.1 ± 0.6e
Skin 2	0.4 ± 0.0g	45.8 ± 0.2e	72.3 ± 0.5f	1.7 ± 0.1e
Skin 3	0.2 ± 0.0h	98.1 ± 2.3g	144.8 ± 10.1g	4.1 ± 0.5e
Skin 4	0.3 ± 0.0h	78.2 ± 1.5f	130.6 ± 3.8g	5.2 ± 1.1de
Skin 5	0.3 ± 0.0h	65.4 ± 2.1f	126.2 ± 8.9g	4.1 ± 0.2e
Pulp 1	< 0.01	729.6 ± 13h	> 1000	NA
Pulp 2	< 0.01	> 1000	> 1000	NA
Pulp 3	< 0.01	> 1000	> 1000	NA
Pulp 4	< 0.01	> 1000	> 1000	NA
Pulp 5	< 0.01	> 1000	> 1000	NA
Resveratrol	1.5 ± 0.1e	5.6 ± 0.6a	3.3 ± 0.0a	86.8 ± 2.3a

¹Antioxidant activity index. ²Results are expressed in µg/mL. Different letters in the same column represent significant differences (P < 0.05). ³ Extracts evaluated at the concentration of 1000 µg/mL and resveratrol at the concentration of 250 µg/mL. NA: no activity.

Correlation analyses were performed to assess the contribution of total phenolic compounds and anthocyanins to grapes' antioxidant activity. The total phenolic content of seed and skin extracts showed positive correlations with the antioxidant activity obtained through all methods, except for the hydrogen peroxide scavenging one, which showed a moderate correlation, as observed in table 3 and table 4. Despite the lack of significant correlation between the anthocyanin content in the skin and antioxidant activity as determined by the DPPH, ABTS, and FRAP methods, the anthocyanins present in the skin showed a strong correlation with the hydrogen peroxide inhibition method (r = 0.95) (Table 4). Thus, the anthocyanin activity could be more effective in H₂O₂ scavenging than in the other evaluated mechanisms. The correlation test for skin samples also showed a moderate negative correlation

(r = -0.79) between the total anthocyanin and phenolic contents. Conversely, a strong correlation was reported between the phenolic content and the antioxidant activity by the DPPH, ABTS, and FRAP methods, which could suggest that the antioxidant activity of the skin is strongly correlated with other phenolics reported in the skin, but not with the presence of anthocyanins.

Cell viability

To assess the effects of the extracts of Maximo hybrid grape seeds, skin, and pulp, at different ripening stages, on cell viability, *in vitro* MTT colorimetric assay with two cell lines, RAW 264.7 macrophages and skin cancer cells - MV3 (melanoma), was conducted at 100 µg/mL. None of the investigated extracts presented a cytotoxic effect

Table 3 - Correlation matrix for grape seed samples.

	TPC ¹	AAI ²	H ₂ O ₂	ABTS	FRAP
TPC	-				
AAI	0.95	-			
H ₂ O ₂	0.77	0.90	-		
ABTS	-0.82	-0.94	-0.83	-	
FRAP	-0.86	-0.95	-0.85	0.95	-

¹Total phenolic content. ²Antioxidant activity index.

Table 4 - Correlation matrix for grape skin samples.

	TPC ¹	ACY ²	AAI ³	H ₂ O ₂	ABTS	FRAP
TFC	-					
ACY	-0.79	-				
AAI	0.99	-0.71	-			
H ₂ O ₂	-0.70	0.95	-0.61	-		
ABTS	-0.92	0.92	-0.89	0.88	-	
FRAP	-0.90	0.79	-0.91	0.79	0.96	-

¹Total phenolic content. ²Total anthocyanin content. ³Antioxidant activity index.

on the tested strains (Figure 2). The discovery of novel toxicity-free agents with low side effects remains the main objective of cancer chemoprevention efforts (PEZZUTO et al., 2005). Therefore, all subsequent *in vitro* assays were performed at concentrations $\leq 100 \mu\text{g/mL}$. Resveratrol, used as a positive control, showed 96.5% and 42.6% of cell viability for the macrophage strain and MV3, respectively, at a concentration of $100 \mu\text{g/mL}$.

Cancer chemoprevention

The National Cancer Institute (NCI), based on empirical evidence, advocated that cancer chemopreventive trials should be conducted at a concentration of $20 \mu\text{g/mL}$, as higher concentrations could lead to undesirable health effects. Following

these recommendations, experiments to induce quinone reductase, TNF- α -induced NF- κ B inhibitory activity, and aromatase inhibitory activity were conducted. All extracts showed baseline inhibition of quinone reductase. However, this value should be at least twice the value reported for basal metabolism to indicate activity (PROCHASKA; SANTAMARIA, 1988; GERHÄUSER et al., 1997).

Nevertheless, the values reported in the NF- κ B inhibition assay are noteworthy: 24.1 (Skin 2), 22.7 (Seed 3), and 19.0 (Seed 2) (Table 5). The outcome is promising because, in future studies, active compounds, such as cyanidin, malvidin, delphinidin, petunidin, and other anthocyanins, can be isolated from such crude extracts, enhancing their activity. Regarding the aromatase inhibition, the

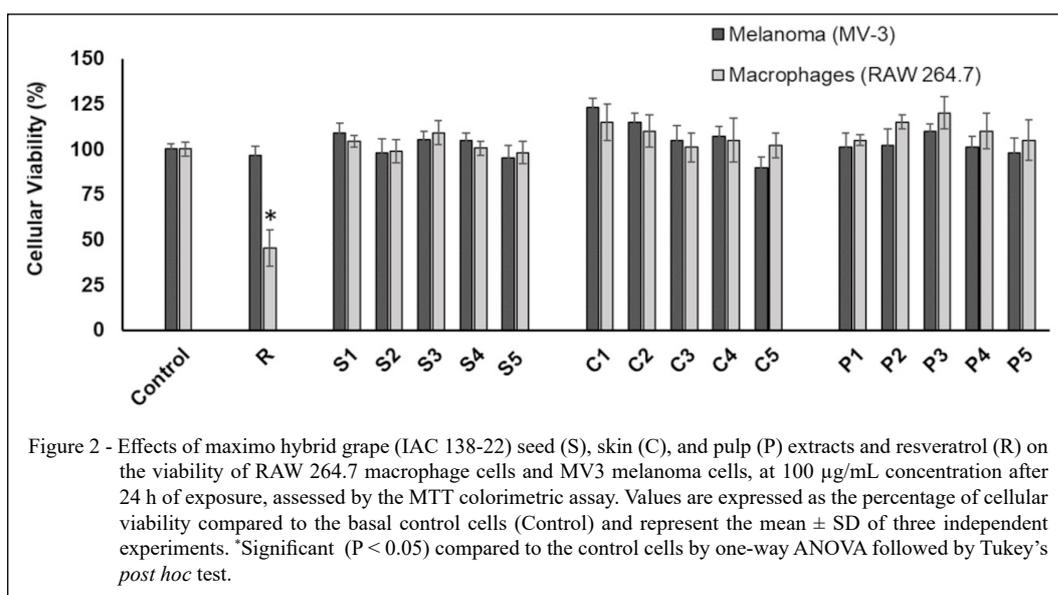


Table 5 - Cancer chemoprevention of Maximo hybrid grape (IAC 138-22) extracts.

	QR [#] Index	% NFκ-B inhibition	% Aromatase inhibition
Seed 1	1 ± 0.0	0.0	5.0 ± 0.0
Seed 2	1 ± 0.0	19.0 ± 0.0	13 ± 0.0
Seed 3	1 ± 0.0	22.7 ± 0.0	68 ± 0.0
Seed 4	1 ± 0.0	0.0	0.0
Seed 5	1 ± 0.0	0.0	0.0
Skin 1	1 ± 0.0	7.9 ± 0.0	0.0
Skin 2	1 ± 0.0	24.1 ± 0.0	0.0
Skin 3	1 ± 0.0	0.0	0.0
Skin 4	1 ± 0.0	0.0	0.0
Skin 5	1 ± 0.5	0.0	0.0
Pulp 1	1 ± 0.0	0.0	48.0 ± 0.0
Pulp 2	1 ± 0.2	0.0	0.0
Pulp 3	1 ± 0.0	3.7 ± 0.0	0.0
Pulp 4	1 ± 0.0	6.5 ± 0.0	0.0
Pulp 5	1 ± 0.0	7.7 ± 0.0	0.0

#QR: Quinone Reductase. The extract concentration was 20 µg/mL. The results were expressed in % of inhibition.

most promising results were found again for Seed 3 (68%), followed by Skin 1 (48%), Seed 2 (13%), and Seed 1 (5%). Seed 3 had a significant value of aromatase inhibition compared to other grape extracts in different ripening stages (PEZZUTO et al., 2005). As mentioned before, the outcome is promising since these are crude extracts from which active compounds could be isolated in future studies. Therefore, green grape seeds may be a possible alternative to be explored as a source of cancer chemoprevention products.

DISCUSSION

Grapes are relevant sources of polyphenols for the human diet. The occurrence and content of these bioactive compounds in grape skin, pulp, and seeds can be affected by several factors. FARHADI et al. (2016) reported different grape cultivars and parts affected both phenolic content and antioxidant properties. Previous studies have also reported that grape seed extracts had a higher content of phenolic compounds and antioxidant activity when compared to skin extracts, corroborating the present study results (SANTOS et al., 2011).

Fruit ripening is another factor that can alter the phenolic composition of grapes. XIAO et al. (2018) reported that the amount of these phenolic substances decreased with grape ripening, with the

largest accumulation of phenolic compounds being observed in the unripe grape, also corroborating the results of the present study. In other studies, grape seeds showed significantly reduced contents of catechins, procyanidins, and flavanones upon ripening (FERRER-GALLEGO et al., 2010; RODRÍGUEZ-PULIDO et al., 2012).

RODRÍGUEZ-PULIDO et al. (2012) reported that more than 20 phenolic compounds found in different grape varieties decreased in content during ripening in the seeds of Spain's *Vitis vinifera*, which is related to the polymerization reactions that occur during this process. These polymerization reactions may explain the decrease in phenolics during seed ripening (DOWNEY; et al., 2003).

As stated by GOWRISANKAR & CLARK, (2016), the relationship between reactive oxygen species (ROSs) and free radicals is well established for cellular damage and aging-related diseases, just as the effects and benefits of natural antioxidants have been extensively explored over the past years. In agreement with these data, many studies have indicated that diseases such as cancer, cardiovascular disease (CVD), and aging are associated with oxidative stress caused by the imbalance between oxidants and antioxidants in the body (FARHADI et al., 2016). The strong antioxidant activity demonstrated in the present research suggested possible beneficial health effects, mainly

arising from the seeds of Maximo grapes. It could reduce the oxidative damage that can cause certain types of cancer and degenerative diseases.

The selective inhibition of the aromatase enzyme is a relevant target trait in searching for cancer chemopreventive agents that can act against hormone-dependent cancer (PEZZUTO et al., 2005). Here, Seed 3 showed 68% of aromatase inhibition, indicating that the crude extract contains some bioactive substances that inhibit this enzyme. Therefore, it is necessary to isolate these substances to expand the investigations. A previous study has shown the efficacy of grape seed extracts combined with resveratrol (obtained from bioactive grape compounds) in preventing and/or treating colon cancer (REDDIVARI et al., 2016). Another study produced a biocompatible chitosan matrix loaded with grape seed extracts that increased the antitumor effect, suggesting a possible innovative drug (IANNONE et al., 2017). KIJIMA et al. (2006) demonstrated that grape seed extracts containing procyanidin dimers were potent inhibitors of aromatase enzymatic activity, suppressing estrogen production in breast cancer. Then, they managed to prove that the consumption of grape seed extracts suppressed the *in vivo* growth of aromatase-expressing breast tumors. The inhibitory activity over NF- κ B is also noteworthy since it activates the innate immune response. NF- κ B is a transcription factor related to the development and progression of cancer, giving resistance of pre-neoplastic cells to the mechanism of cellular apoptosis (Karin, 2006). Thus, the Skin 2, Seed 3, and Seed 2 samples showed promising cancer chemopreventive activity, acting in the stage of promotion and progression of carcinogenesis.

The seeds showed the strongest antioxidant activity, even when compared to free resveratrol. Furthermore, in the assessment of cancer chemopreventive activity, the seeds presented relevant inhibition in the NF- κ B and aromatase inhibition assay, given promising results for cancer chemopreventive, suggesting beneficial health effects. The main limitations of the present research were the incomplete characterization of grape seed and the isolation of substances, such as those present in green seeds. These substances could have significantly higher activity when isolated from the crude extract.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception of the study and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

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