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Original Article

Cytotoxic/antiproliferative and nutraceutical activity of aqueous and ethanolic extracts of green and mature *Averrhoa carambola*

Atividade citotóxica/antiproliferativa e nutracêutica de extratos aquosos e etanólicos de *Averrhoa carambola* verde e madura

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Abstract

Averrhoa carambola L. presents in its composition diversity of nutrients and vitamins. The present study aimed to extract water and fat-soluble compounds from this fruit at different stages of maturation (green and mature), perform the physical-chemical characterization as well as evaluate its cytotoxicity against hepatoma cells of *Rattus norvegicus* (HTC). The physicochemical results showed that the pH and molar acidity is influenced by the fruit maturation state. The fruit presented high percentage of moisture, while the percentage of total minerals (ash) increased according to its maturation stage. The results of the phytochemical screening showed that star fruits present phenolic compounds. The antioxidant activity showed greater potential for the ethanolic extracts of the green and mature star fruit. For HTC cells treated with ethanolic extract of green and mature star fruit the data show absence of cytotoxic effect. The tests with the aqueous extract showed cytotoxic/antiproliferative effect of green and mature star fruit extract, in 24, 48 and 72 hours. The presence of nutraceutical compounds and the cytotoxic/antiproliferative activity were more expressive in the aqueous extract, being an option of easily accessible solvent economic and not harmful to organisms.

Keywords: antitumor, DDPH, phenolic compounds, MTT, star fruit, cytotoxicity.

Resumo

A *Averrhoa carambola* L. apresenta em sua composição diversidade de nutrientes e vitaminas. O presente estudo teve como objetivo extrair compostos hidrossolúveis e lipossolúveis deste fruto em diferentes estádios de maturação (verde e maduro), realizar a caracterização físico-química, bem como avaliar sua citotoxicidade contra células de hepatoma de *Rattus norvegicus* (HTC). Os resultados físico-químicos mostraram que o pH e a acidez molar são influenciados pelo estado de maturação dos frutos. O fruto apresentou alto percentual de umidade, enquanto o percentual de minerais totais (cinzas) aumentou de acordo com seu estádio de maturação. Os resultados da triagem fitoquímica mostraram que as carambolas apresentam compostos fenólicos. A atividade antioxidante apresentou maior potencial para os extratos etanólicos da carambola verde e madura. Para células HTC tratadas com extrato etanólico de carambola verde e madura os dados mostram ausência de efeito citotóxico. Os testes com o extrato aquoso mostraram efeito citotóxico/antiproliferativo do extrato de carambola verde e madura, em 24, 48 e 72 horas. A presença de compostos nutracêuticos e a atividade citotóxica/antiproliferativa foram mais expressivas no extrato aquoso, sendo uma opção de solvente de fácil acesso econômico e não prejudicial aos organismos.

Palavras-chave: antitumoral, DDPH, compostos fenólicos, MTT, carambola, citotoxicidade.

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1. Introduction

A diet composed of fruits and vegetables beneficially influences the homeostasis of the body, ensuring the well-being and health of the individual, with ability to prevent chronic diseases, cardiovascular, diabetes and even cancer. This is due to the existence of bioactive compounds that have functional properties capable of influencing biological responses of the organism, such as phytochemicals and vitamins (Fernandes et al., 2020; Hassimotto et al., 2009). This is why in recent years popular medicine has gained prominence in the development of the pharmaceutical industry, especially in the search for herbal drugs (Silva et al., 2021a).

In particular, studies have been developed to identify more effective and safe chemical compounds for cancer treatment (Xue-Dong et al., 2016) mainly from natural sources that are less aggressive to normal cells (Costa-Lotufo et al., 2010).

The star fruit (*Averrhoa carambola* L.), belonging to the family Oxalidaceae, is one of the species of economic interest and medicinal potential. The plant is native to tropical and subtropical regions, being widely consumed and cultivated in the Asian continent (Vargas-Madriz et al., 2021). In Brazil, the culture of star fruit is expanding to present itself as a profitable option for diversification of cultivation to fruit grower, since the domestic market of exotic fruits has grown. In addition, the "caramboleira" plant has rapid development, high productivity and easy adaptation to the soil (Almeida et al., 2021).

The fruit can be divided into two main groups, the acids and sweets, with the bittersweet taste and the characteristic star shape. Its color varies between green and intense yellow and the peel is smooth and shiny, being its pulp generally firm (Almeida et al., 2021; Luan et al., 2021). It is often consumed in fresh form, but can be found in salads, jams, juices, teas, especially in Asian cuisine (Luan et al., 2021). In the context of traditional medicine, different parts of star fruit, such as pulp, leaves and flowers, are used in the treatment of fevers, indigestion, hemorrhoids, headaches, vomiting, diarrhea, cough, scurvy, disintegration, and act as a diuretic and appetite stimulant (Almeida et al., 2021; Ramadan et al., 2020).

Phytochemical analysis of star fruit has indicated the presence of flavonoids, tannins, saponins and alkaloids (Thomas et al., 2008). The following were isolated and identified: 28 flavonoids by Jia et al. (2018) and Yang et al. (2015), 29 terpenes by Jia et al. (2019), Gunawardena et al. (2015) and Yang et al. (2012), 8 phenolics by Yang et al. (2014), Jia et al. (2017) Shui and Leong (2004) and 9 phenylpropanoids by Yang et al. (2014), Jia et al. (2017) and Sritharan et al. (2019).

Several studies prove the antioxidant power of star fruit, mainly by presenting in its composition polyphenolics, carotenoids, proanthocyanidins, epicatechin and vitamin C (Thomas et al., 2008; Gunawardena et al., 2015; Shui and Leong, 2004; Shofifian et al., 2011; Zainudin et al., 2014; Setiawan et al., 2002; Silva et al., 2021b). This means that the fruit has the ability to help in the treatment of diseases related to oxidative stress, such as cancer. Anti-inflammatory and antimicrobial activities were also studied, and the hypoglycemic effect is already explored in traditional medicine in the treatment of diabetes (Vargas-Madriz et al., 2021; Luan et al., 2021). The antitumor effect, in turn, was studied by Siddika et al. (2020) and Gao et al. (2015), using tumor cells *in vivo* and *in vitro*, in which was perceived a significant inhibition of cell growth using crude extracts and compounds isolated from *A. carambola* (Luan et al., 2021). Singh et al. (2014) demonstrated that the extract from star fruit can be regarded as a prophylactic for hepatocellular carcinoma in mice.

The maturation stage can influence the composition of the bioactive compounds of a fruit. The star fruit has oxalic acid in its composition, which is responsible for its characteristic acidity. As the fruit matures the pH becomes less acidic, tannin and reducing sugars levels vary (Lakmal et al., 2021; Ferrara et al., 2022). Physical and biochemical changes directly influence the concentration of phenolic compounds as maturation, and may even induce different biological responses of the organism (Vargas-Madriz et al., 2021). Liposoluble and water-soluble compounds, with different actions, can be extracted through the variation of solvents. Thus, the aim of the present study was to characterize ethanolic and aqueous extracts of green and mature star fruits and to evaluate their cytotoxic/antiproliferative activity for hepatic tumor cells of *Rattus norvegicus* (HTC).

2. Materials and Methods

2.1. Solution treatment

The star fruits, green and mature, were acquired in the fruit retail trade in the municipality of Francisco Beltrão – Paraná, Brazil. They were sanitized in running water and frozen in portions in a domestic freezer (-6°C) until the moment of use (approximate one month).

2.2. Preparation of extracts

The ethanol and aqueous extracts were prepared with 100 g of *in natura* pulp of green or mature star fruit (crushed in a blender) in 500 mL of each solvent extractor: 95% ethanol (CH_3CH_2OH) and distilled water. These were kept in magnetic agitation for 2 hours under light at room temperature (Palioto et al., 2015). The extracts were filtered and diluted in 1000 mL volumetric flask, with the solvent (ethanol/water), to obtain the final concentration of 0.1 g mL^{-1} of each extract.

The crude ethanolic extract was obtained after vacuum evaporation of the solvent at a maximum temperature of 40 ºC and the crude aqueous extract was obtained after lyophilization process.

2.3. Physical and chemical analysis of fruits

The physicochemical analyzes of the green and mature star fruit were performed following the techniques recommended in the manual of Physicochemical Methods for food analysis of the Adolfo Lutz Institute (IAL, 2008), determining: pH, molar acidity, moisture, ash and soluble solids. The statistical analysis was performed by the ANOVA

variance test, followed by the Tukey test (α=0.05; n=4), for comparison of the quadruplicates performed.

2.4. Phytochemical analysis of extracts

Phytochemical screening was performed using manual methodology of phytochemical analysis methods of plant extracts (Barbosa et al., 2004) using at a concentration of 0.1 g mL^{-1}. For the analysis of flavonoids and tannins, 2.0 mL of previously prepared extracts and 0.5 mL of ferric chloride were added to a test tube. The presence of flavonoids was confirmed with the colour between brown green and green and the presence of hydrolysable tannins was confirmed with the formation of a blue precipitate. The presence of green precipitate confirms the presence of condensed tannins. The determination of steroids/triterpenoids was carried out by adding 2.0 mL of extracts, 2.0 mL of chloroform and 1.0 mL of acetic anhydride, and three drops of concentrated sulfuric acid to a test tube. The presence of evanescent blue colors followed by green indicates the presence of steroid and triterpenoid compounds, respectively. The saponin test was performed by adding 2.0 mL of distilled water to 2.0 mL of extracts and three drops of hydrochloric acid. After three minutes of continuous agitation, the formation of persistent and abundant foam indicates the presence of saponins. The alkaloids were determined by adding 1.0 mL of hydrochloric acid and 4 likes of Dragendorff's reagent to 2.0 mL of the extracts. The formation of insoluble and flocculent precipitates confirms the presence of alkaloids.

2.5. Total phenolic content

The total phenolic content of the ethanolic and aqueous extracts prepared (C = 0.1 g mL-1) were quantified using the Folin-Ciocalteau reagent according to the methodology described by Singleton and Rossi Junior (1965), with modifications. To a tube were added 250.0 µL of extract, 250.0 µL of Folin-Ciocalteau reagent (diluted 1:1 (v/v) in distilled water), 500.0 µL of a saturated solution of sodium carbonate and 4.0 mL of distilled water. After one hour keeping the tubes in the dark, readings were performed at 760 nm on a spectrophotometer (Agilent Technologies, model Cary 60 UV-VIS). For the quantification of total phenolics, a calibration curve was constructed with an aqueous solution of gallic acid at concentrations of 100, 80, 60, 40, 20 and 10 µg mL-1 (y = 0.004x + 0.013; R2 = 0.997). The results were expressed in mg GAE 100 g-1 of pulp, where GAE represents the equivalent in gallic acid.

2.6. Total flavonoid content

The total flavonoid content of the ethanolic and aqueous extract (C = 0.1 g mL-1) were determined according to the methodology proposed by Woisky and Salatino (1998), with modifications. To a tube were added 500.0 µL of the extract, 250.0 µL of a 5% aluminum chloride ($AlCl_3$) methanolic solution and 4.25 mL of methanol. The mixture was stirred and remained at room temperature for 30 min. After this time, reading was performed in a spectrophotometer (Agilent Technologies, model Cary 60 UV-VIS) at 420 nm. Analysis was performed in triplicate. A calibration curve was constructed with a methanolic solution of rutin. The concentrations were 200, 150, 125, 100, 75, 50 and 25 µg mL-1 (y = 0.001x – 0.013; R^2 = 0.994). The results were expressed in mg of RUE 100 g-1 of pulp, where RUE represents the rutin equivalent.

2.7. Determination of antioxidant activity by the DPPH free radical scavenging method

The determination of antioxidant activity of the ethanolic and aqueous extract (C = 0.1 g mL-1) by DPPH free radical scavenging method was carried out according to Brand-Williams et al. (1995) with modifications. 1.0 mL of each extract were mixed with 2.0 L DPPH solution (C = 0.0034 g mL-1) and incubated in the dark at room temperature for 30 min. The absorbance of the mixture was then measured at 517 nm. The antioxidant activity was expressed in relation to the control (% AA) (Equation 1), in which (Ac) represents the absorbance of the control, (Ab) the absorbance of the sample blank and (Aa) represents the absorbance of the sample.

$$\%AA = \frac{Ac - (Aa - Ab)}{Ac} \times 100 \quad (1)$$

2.8. Determination of antioxidant activity by ABTS+ radical cation scavenging method

The methodology used for the ABTS radical cation capture test was described by Rufino et al. (2007). The readings were obtained after 6 minutes of reaction in a spectrophotometer (Agilent Technologies, model Cary 60 UV-VIS) at 734 nm. The antioxidant activity was expressed in relationship to the control (% AA). The analysis was performed in triplicate.

2.9. Cytotoxic/antiproliferative activity of the extracts

The hepatoma cells of *Rattus norvegicus* (HTC), obtained from the Cell Bank of Rio de Janeiro, were grown in 25 cm^2 culture bottles containing 10.0 mL of DMEM culture medium supplemented with 15% fetal bovine serum (Invitrogen - Carlsbad, CA, USA) incubated in a BOD-type oven at 37 °C.

To perform the cytotoxic/antitumor activity test, the MTT [3-(4,5-Dimethilthiazol-2-il)-2,5-diphenyl tetrazolium bromide] assay was used, according Mosmann (1983) with modifications. Cultivation plates of 96 wells were used where, in each well, $2.0x10^4$ HTC cells were sown, except for the control well without cell (white). After 24 hours, the culture medium of each well was discarded and 100.0 µL of complete medium was added to the groups: negative control (CO-) (culture medium), positive control (CO+) with the cytotoxic agent methyl methanesulfonate (MMS 500 µM) and treatments with concentrations of 5, 50, 100, 200, 300, 400, 500 and 1000 µg of extracts (ethanolic and aqueous, green and mature star fruit) per mL of culture medium supplemented with fetal bovine serum.

The cells were incubated for 24, 48 and 72 hours and, after this time, the culture medium was replaced by 100.0 µL of culture medium not supplemented, plus MTT (0.167 mg mL-1). The plate was incubated again for another 4 hours, then the medium containing MTT was discarded

and 100 µL dimethylsulfoxide (DMSO) wells were added for solubilization of formazan crystals. The absorbance reading was performed in a microplate reader (Thermo Plate) at 560 nm.

The results were presented as mean and standard deviation of absorbances and submitted to the normality test, analysis of variance (one way ANOVA), followed by the Dunnet mean comparison test. The differences were considered to be statistically significant when the p-value was less than 0.05 ($p<0.05$).

The percentage values of cell viability (VC) were estimated by the mean absorbance ratio of the treatment by the mean absorbance of the negative control.

3. Results and Discussion

3.1. Physical and chemical analysis of fruits

The physicochemical results of the green and mature star fruit (Table 1), through the pH values indicate acid character of these fruits, showing no statistical difference ($p>0.05$) between the stages of green and mature maturation. Lower pH values were presented by Torres et al. (2003) (mature: 3.69 and green: 3.52) and Patil et al. (2010) (mature: 4.82 and green: 3.43), possibly due to the variation of the species evaluated.

As well as pH, the degree of acidity did not differ statistically ($p>0.05$) between the fruits with the different stages of maturation. Lower acidity values (0.17%) in mature fruits were found by Torres et al. (2003) and Almeida et al. (2021), which may be due to different analytical methods adopted.

High percentage of moisture was found in green and mature star fruit ($p>0.05$), which corroborates with the report by Torres et al. (2003), which shows high water content in the fruit. These results corroborate with those presented by Torres et al. (2003) which obtained moisture of 90.57% in samples of star fruit without differentiation of maturation and Patil et al. (2010) which found moisture of 95.60% for the green star fruit and 95.71% for the mature star fruit.

It was found that the ash content (Table 1) also did not differ statistically ($p>0.05$) between the different maturation stages of the star fruit. Similar values in mature star fruits (0.41%) were found by Almeida et al. (2021) and lower values were reported by the same authors (0.19%) for green fruits.

The analysis of soluble solids showed that the highest contents were presented by the mature fruits ($p<0.05$) which can be justified by the maturation process, because the more mature the fruit, the greater the accumulation of sugars. Similar values of Brix degrees were presented by Torres et al. (2003) (6.57 and 7.45 °Brix) and Almeida et al. (2021) (6.42 and 7.98 °Brix).

3.2. Phytochemical analysis and determination of total phenolic compounds, flavonoids and antioxidant activity of extracts of star fruit

The results of the phytochemical screening of the ethanolic and aqueous extracts of green and mature star fruit are shown in Table 2. The presence of flavonoids and tannins at the concentration tested was confirmed. According to Huber and Rodriguez-Amaya (2008) and Souza et al. (2004), characteristics such as the degree of maturation of the fruits, climatic and soil factors, the presence of pesticides, processing, storage and the genetics of the plant can influence the amount of antioxidant compounds in fruits.

Solvents ethanol and water were used to analyze their effects on extraction of bioactive antioxidants compounds of green and mature star fruit. The results of quantification of phenolic compounds and flavonoids (Table 3) showed that the ethanolic extract of green star fruit had the highest amount of these compounds, followed by the ethanolic extract of mature star fruit.

Khanam et al. (2015), also demonstrated that the ethanolic extract of star fruit showed higher levels of phenolic compounds and flavonoids than the aqueous extract, suggesting that this may have happened because the treatment with ethanol resulted in the precipitation of non-phenolic compounds or the presence of greater concentration of less polar phenolic compounds, contributing to the higher content of flavonoids.

Studies of Lim et al. (2007) with the mix of 50% ethanol:H2O, in extract of star fruit grown in Malaysia, they found a value of 131 ± 54 mg GAE 100 g-1 for total phenolics, which is in agreement with what was found in this work. Comparing the maturation stage for the ethanolic extracts of star fruit, there was a decrease of 38% and 34% for the total number of phenols and flavonoids, respectively. Our results are in agreement with the study conducted by Das (2012) which showed that mature star fruit, grown in India, has lower amounts of phenolics compared to the green stage. Zainudin et al. (2014) also observed a decrease in phenolics (44%) and flavonoids (65%) with maturation, for methanolic extracts of star fruit grown in Malaysia.

Possible contributions to the phenolic and flavonoid content decreasing value are increasing in polyphenol oxidizing enzyme activity and decreasing phenylalanine

Table 1. Physical-chemical parameters of green and mature star fruit.

Sample	Parameters				
	pH	Acidity Molar (%)	Moisture (%)	Ashes (%)	Soluble Solids (°Brix)
Green star fruit	3.22 ± 0.88[a]	0.44 ± 0.00[a]	90.30 ± 0.34[a]	0.28 ± 0.04[a]	6.25 ± 0.28[a]
Mature star fruit	3.51 ± 0.02[a]	0.25 ± 0.00[a]	90.66 ± 0.13[a]	0.42 ± 0.13[a]	7.87 ± 0.25[b]

Columns with the same letter do not differ statistically from each other, by the Tukey test, with a significance level of 5%.

Table 2. Result of phytochemical screening of ethanolic and aqueous extracts of green and mature star fruit.

Classes of compounds	Green star fruit		Mature star fruit	
	Aqueous	Ethanolic	Aqueous	Ethanolic
Flavonoids/tannins	+	+	+	+
Steroids/triterpenoids	-	-	-	-
Saponins	-	-	-	-
Alkaloids	-	-	-	-

(+) present; (-) absent.

Table 3. Total phenolics (TF), flavonoids (FLV) and antioxidant activity (%AA) by DPPH and ABTS methods of ethanolic and aqueous extracts of green and mature star fruit.

Assay	Green star fruit		Mature star fruit	
	Aqueous	Ethanolic	Aqueous	Ethanolic
TF (mg GAE 100 g^{-1})	60.73 ± 0.37^{c}	134.43 ± 0.96^{a}	53.93 ± 0.81^{d}	83.94 ± 0.07^{b}
FLV (mg RUE 100 g^{-1})	36.07 ± 2.40^{d}	156.03 ± 6.12^{a}	24.87 ± 1.56^{b}	102.93 ± 1.00^{c}
%AA – DPPH	73.21 ± 0.97^{b}	93.89 ± 0.62^{a}	30.08 ± 0.68^{c}	92.54 ± 0.18^{a}
%AA – ABTS	15.83 ± 0.10^{d}	58.94 ± 0.24^{a}	27.39 ± 0.21^{c}	44.75 ± 0.24^{b}

The same letter does not differ statistically from each other, by the Tukey test, with a significance level of 5%.

ammonia lyase activity (Ortega-Garcia and Peragon, 2009; Ayaz et al., 2008) and/or the decrease of internal antioxidant enzymes such as superoxide dismutase, catalase and guaiacol peroxidase, making the cells, to maintaining cellular integrity turn to other reserved antioxidant compounds such as phenolics (Huang et al., 2007).

Polyphenols in plants play an important role in scavenging and neutralizing the free radicals and contribute significantly to the antioxidant activity due to their redox properties (Watson, 2014; Pereira et al., 2013). Phenolic compounds were found to be the major antioxidants in star fruit, attributed to singly-linked proanthocyanidins that existed as dimers, trimers, tetramers and pentamers of catechin or epicatechin (Shui and Leong, 2004).

The antioxidant activity evaluated by the DPPH free radical scavenging method showed no significant difference ($p < 0.05$) for the ethanolic extracts of green (93.89%) and mature (92.54%) star fruit, presenting high antioxidant potential. By the ABTS radical capture method, the ethanolic extract of green star fruit stood out (58.94%), followed by the ethanolic extract of mature star fruit (44.75%). Ethanol proved to be the best extracting solvent for the bioactive compounds of star fruit, when compared to water.

No significant difference was observed for the DPPH radical scavenging assay for the ethanolic extracts of green and mature star fruit, which can be attributed to the high concentration tested, since for the ABTS radical capture assay, a decrease in the percentage was observed with maturation, as observed by Zainudin et al. (2014).

Leong and Shui (2002) and Lim et al. (2007) studied star fruit grown in Malaysia, Luximon-Ramma et al. (2003) analyzed the fruits grown in Mauritius and also demonstrated high antioxidant potential for fruit, attributed to its high phenolic compounds content.

Pang et al. (2016) demonstrated the correlation between the phenolic content and the antioxidant activity of acetone extract (80%) of star fruit from Guangdong Province, and reported of dominant constituents as gallic acid, syringic acid, p-coumaric acid, epicatechin, procyanidin B2 and isoquercitrin.

Jia et al. (2017) isolated eleven non-flavonoid phenolic compounds, and Jia et al. (2018) isolated thirteen flavonoids present in 95% aqueous ethanol extract of fresh sweet star fruit and demonstrated the potent antioxidant of these compounds. It could be concluded that these non-flavonoid phenolics and flavonoids were important contributors to the antioxidant and anti-tumor activities of star fruit.

A positive correlation was observed between the antioxidant activity and the content of total phenolics and flavonoids in star fruit. The results revealed the green fruits of star fruit with a promising antioxidant agent.

3.3. Cytotoxic/antiproliferative activity

3.3.1. Ethanolic extract

Table 4 presents the results of the cytotoxicity test of the treatment with the ethanolic extracts of the green and mature star fruits.

At 24 hours, all concentrations of green star fruit showed cell viability greater than 100%, but only the concentrations of 5, 200, 400 and 1000 µg mL-1 were statistically different from the negative control, with stimulation of cell proliferation. At 48 hours, similar effect was observed for the concentrations of 300 and 500 µg mL-1 of green star fruit. Due to the variances of the experiment, which interfere in statistics, the intermediate concentrations, which also showed high cell viability, did not show statistical proliferative effect.

Table 4. Percentage of viability of tumor cells (VC) of rat liver, treated with different concentrations (µg mL^{-1}) of ethanolic extracts of green and mature star fruit, and controls negative (Co-) and positive (Co+) for 24, 48 and 72 hours, by the MTT test.

Groups		VC (%) Tumor Cells of Rat Liver		
		24 h	48 h	72 h
Green star fruit	**Co-**	100.00	100.00	100.00
	Co+	88.51	58.78*	58.92*
	5	121.08*	98.92	94.51
	50	103.85	85.55	90.60
	100	111.65	88.84	83.90
	200	133.96*	111.62	107.93
	300	119.11	121.76*	91.26
	400	137.01*	115.98	105.14
	500	114.65	125.94*	80.12
	1000	127.58*	104.01	87.99
Mature star fruit	**Co-**	100.00	100.00	100.00
	Co+	101.17	70.81*	63.84*
	5	125.79	142.04*	109.68
	50	138.39	147.98*	100.35
	100	123.61	144.57*	113.07
	200	150.50*	175.53*	109.75
	300	134.19	159.13*	104.31
	400	142.27	161.63*	109.91
	500	148.51	164.95*	104.68
	1000	250.81*	167.49*	107.53

* Results statistically different from negative control, by the Dunnet test ($p<0.05$).

According to studies by Guéant et al. (2013) and Wang et al. (2014), the presence of vitamin C is related to cell growth and multiplication. The stimulation of cell divisions of cells treated with fruit extracts may be associated with the presence of bioactive compounds such as vitamins, phenolic compounds and antioxidants (Rizzon and Meneguzzo, 2007; Rizzon and Miele, 2012; Farahpour et al., 2016; Moghadam et al., 2017) which may explain the increased cell viability for green star fruit extract. Similar results were obtained by Düsman et al. (2014), using as treatment the full and natural juice of conventional and organic grapes, and Rocha (2018), using extracts of grape bagasse, in which cell viability increased in the time of 48 hours of treatment in HTC cells.

For the mature star fruit extract, effect similar to green star fruit extract was observed. At 24 hours, all concentrations showed cell viability greater than 123%, but only the concentrations of 200 and 1000 µg mL-1 were statistically different from the negative control. Again, due to the variances of the experiment, the intermediate concentrations showed no statistical proliferative effect. However, at 48 hours, all concentrations of mature star fruit extract were statistically different to the negative control, showing proliferative effect, with cellular viability above of 142%. The extract of the mature fruit showed greater stimulation of increased cell division compared to the green extract. According to Table 1, this can be proven by the greater amount of ash and soluble solids that attest to a higher concentration of nutraceutical compounds in the mature fruit.

When analyzing the mean absorbance values obtained with HTC cells in the time of 72 hours (Table 4), it can be observed that there were no statistical differences ($p>0.05$) between the different concentrations of ethanolic extracts of green and mature star fruit and negative control, showing no cytotoxic or proliferative effect. Considering the evident stimulation of cell proliferation observed in 24 and 48 hours, it is possible that the decrease of cell viability in 72 hours is due to cell death caused by the lack of free area within the culture well for cell growth, since this cell line grows adhered to the bottom of the well. Or, the substances present in these extracts, which resulted in the stimulation of cell proliferation, were used by cells, in their metabolic processes, or degraded, and thus in long time (72 hours) do not interfere with cell divisions of HTC cells.

3.3.2. Aqueous extract

Table 5 presents the results of the cytotoxicity test of the treatment with the aqueous extracts of the green and mature star fruits.

Table 5. Percentage of viability of tumor cells (VC) of rat liver, treated with different concentrations (µg mL^{-1}) of aqueous extracts of green and mature star fruit, and controls negative (Co-) and positive (Co+) for 24, 48 and 72 hours, by the MTT.

Groups		VC (%) Tumor Cells of Rat Liver		
		24 h	48 h	72 h
Green star fruit	**Co-**	100.00	100.00	100.00
	Co+	77.67*	26.82*	11.03*
	5	85.95*	77.69	65.90
	50	83.96*	28.46*	46.38*
	100	86.90*	72.05	64.72
	200	86.06*	85.13	38.16*
	300	53.14*	121.03	47.24*
	400	67.82*	121.30	33.73*
	500	96.44*	130.52	44.24*
	1000	70.44*	105.58	42.76*
Mature star fruit	**Co-**	100.00	100.00	100.00
	Co+	6.81*	22.49*	11.74*
	5	66.38	95.11	34.12*
	50	65.47	125.20	46.43*
	100	77.13	105.09	41.26*
	200	26.79*	130.36	50.20*
	300	58.89*	109.75	38.08*
	400	77.58	64.52	50.10*
	500	95.67	129.54	52.66*
	1000	11.88*	33.28*	13.12*

*Results statistically different from negative control, by the Dunnet test ($p<0.05$).

In 24 hours, the statistical analysis showed that all concentrations of the green star fruit extract presented mean absorbances statistically lower than that of the negative control, that is, they presented cytotoxic/antiproliferative effect on the HTC tumor cells. At 48 hours, it was observed only in the concentration of 50 µg mL-1 and, at 72 hours, the concentrations of 50 µg mL-1 and above of 200 µg mL-1 presented cytotoxic/antitumor effect on tumor cells. The lowest cell viability was 53.14% (24 hours), 28.46% (48 hours) and 33.73% (72 hours). In general, the lowest cell viability was observed at the highest concentrations of the extract (especially at 72 hours), indicating a dose-dependent effect on cytotoxicity. And in general, the longer the exposure time (72 hours) the lower the cell viability.

For the mature star fruit extract, the concentrations of 200, 300 and 1000 µg mL-1 showed mean absorbances statistically lower than the negative control, that is, they showed cytotoxicity in tumor cells, reaching cell viability of 11.88% at the highest concentration assessed [1000 µg mL^{-1}]. At 48 hours, only the highest concentration (1000 µg mL-1) was cytotoxic/antitumor effect on tumor cells. And, at 72 hours, all concentrations present these effects, with cell viability below 52.66%. Thus, the most evident antiproliferative effect was at 72 hours. In addition, only the highest concentration that maintained cytotoxicity in the three evaluation times, indicating, as well as the observed for the extract of the green star fruit, dose-dependent effect and exposure time.

The cytotoxic/antitumor effect observed in aqueous extracts of green and mature star fruit can be explained by the presence of phenolic compounds (flavonoids/tannins/phenols) in these fruits, as evidenced by phytochemical analysis (Table 2). Phenolic compounds are important contributors to anti-inflammatory, antimicrobial and antitumor activities (Jia et al., 2017). According to Luan et al. (2021) star fruit has several activities, such as antioxidant, anti-hyperglycemic, antiobesity, anti-hyperlipidemic, antitumor, anti-inflammatory, hepatoprotective, cardioprotective, antihypertensive, neuroprotective and others.

In addition, another study has shown that star fruit had a prophylactic role against hepatocellular carcinoma in mice, thus being considered a good chemopreventive against cancer (Singh et al., 2014). This result corroborates with this study, which analyzed cytotoxic/antiproliferative activity of mature and green star fruit extract in rat liver tumor cells.

According to Gao et al. (2015) 2-Dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione (DMDD) is a cyclohexanedione found in the roots of the star fruit. DMDD inhibited the growth of breast carcinoma cells by inducing cell cycle arrest in phase G1, oxidative stress and

apoptosis, events that may have occurred in the present study and result in cytotoxic/antiproliferative activity of star fruit extracts.

Through this study it was possible to confirm the antioxidant activity of *Averrhoa carambola* L. using the DPPH free radical sequestration assay for ethanolic and aqueous extracts, validating the literature that guarantees a good antioxidant potential of star fruit. Phytochemical analysis confirmed the presence of flavonoids, tannins and phenols. The cytotoxic/antiproliferative activity against liver tumor cells was observed more expressively in the treatment with aqueous extract, being an advantageous solvent option since water is a cheaper solvent and non-toxic to the environment and the body.

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