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ANTIOXIDANT ACTION AND CELL VIABILITY OF THE GROSS EXTRACT AND SEED FRACTIONS: *Persea Americana mill* CULTIVATED IN PIAUÍ

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Abstract: Introduction: The compounds that are concentrated in the seed and endocarp are mostly based on compounds of catechins, epicatechins, acids and proanthocyanidins, among others, antioxidants such as vitamins C and E. **Goal:** To analyze the antioxidant activity and cell viability of the crude extract and the ethyl acetate and aqueous fractions of the fruit fragments: *Persea Americana Mill:* endocarp with the seed grown in Piauí and list the main substances elucidated in the hydroethanolic extract of these associated parts. **Methodology**: The hydroethanolic crude extract (7:3) and the H2O:MeOH (7:3) partition were obtained to obtain the ethyl acetate and aqueous fractions. Then, each sample was diluted in 1:1 of MeOH and distilled H2O, to a concentration of 2.0mg/ml. To analyze the antioxidant action, the DPPH (2,2-diphenyl-1-picryl-hydrazyl) test was performed and for cell viability an in vitro colorimetric assay based on the tretazolium salt MTT (3-4,5 dimethylthiazole bromide) was used. -2-yl-2,5-diphenyl tetrazolium) of high efficiency. For the elucidation of the extract molecules: *Persea Americana Mill* used mass spectrometry (LC-MS, Esquire 2000 Plus, Bruker Daltonics) with a quadrupole ion trap analyzer in tandem mode, with ionization by electronebulization (ESI). **Results and discussion**: Substances were elucidated whose major compounds were condensed tannins, coming from flavonoids called catechins, specifically, catechin condensates (3.2/289), B1 proanthocyanidinides (3.6/1151), trimers (3.8/863) and catechin tetramers (3.6/1151). The viability level of the crude extract showed levels below 50% at concentrations from 150 µg/ml, indicating high levels of cellular toxicity. However, in the ethyl acetate and aqueous fractions, cell viability did not show worrying levels of toxicity. The analyzed extract also showed good levels of antioxidant action both in the crude extract and in its fractions. **Conclusion**: The study revealed substances with good antioxidant actions in the investigated extract and that may be beneficial to cardiovascular health. In addition to adding values to the parts discarded by the region of cultivation and subsidizing with information the pharmaceutical and research industries, among other interests.

Keywords: Antioxidant. Adverse effects. Extract.

INTRODUCTION

Persea Americana mill has been the subject of scientific investigations whose findings show nutritional functions, high amounts of vitamins, minerals, proteins, fibers and unsaturated fatty acids that contribute positively to health (TREMOCOLDI, 2015). As a result of their promising findings, parts such as leaves, stem and seed also arouse interest and demonstrate antioxidant functions and phenolic concentrations important for free radical inhibition activities and anti-inflammatory action, respectively (DABAS, 2013). Considering that the fruit grown in the municipality of José de Freitas - PI have their seeds and endocarps, generally, as discard parts, it becomes interesting to analyze the antioxidant activity and cell viability of the crude extract and the ethyl acetate and aqueous fractions. of these associated fragments.

It is known that the pulps of the fruit: *Persea Americana Mill* brings together significant concentrations of different substances that are used as a basis for productions that benefit health in different aspects. Proteins, omegas, fatty acids, phytosterols, tocopherols, squalene, fat-soluble vitamins such as type A and B, and type D and E are examples of these substances (SANTOS, 2014; DUARTE et al., 2016). The latter are being associated with human physiology in serum values, more precisely in the reduction of plasma

concentrations of total cholesterol (TC), blood pressure, triglycerides (TG), low-density lipoproteins (LDL-C-cholesterol) and maintenance of high-density lipoprotein (HDL-C-cholesterol) values (CREDIDO, 2010). Research has been intensified in recent years, whose investigations highlight the molecules of this fruit and other fragments that compose it, but which remain articulated with the lipid and glycemic profile, blood pressure and body weight, including the inhibition of free radicals, such as antioxidants, and a higher concentration of phenolic compounds in these segments.

The seed of fruits grown in regions such as the South and Southeast of Brazil, for example, is composed of values greater than 70% of these compounds mentioned (BAZZO, 2015) and the endocarp of the seed, not yet deeply investigated, aroused bioactive interests in this study. aimed at knowing its chemical profile, cell viability and antioxidant action. The discarded fragments were analyzed and may become options that help human physiology in serum values, in reducing levels of total cholesterol (TC), blood pressure, triglycerides (TG), low-density lipoproteins (LDL-cholesterol) C) and maintenance of blood values of high-density lipoproteins (HDL-C-cholesterol) (CREDIDO, 2010).

Such information, awakens for this investigation, the types of substances found in the seeds of the fruits developed in the region of José de Freitas in Piauí, which until now had not been examined. Seeds grown in other regions were studied and showed active compounds, as normally, the seeds of this fruit serve as a functional food ingredient or even as an antioxidant additive (KOSINKA et al., 2012). There is no in-depth research on the biological function of the seed coat, the endocarp, however, the chemical profile of seeds from other regions were researched and active constituents were also found (FIGUEROA et al. 2018). The compounds that are concentrated in the seed and endocarp are mostly based on compounds of catechins, epicatechins, acids and proanthocyanidins, among others, antioxidants such as vitamins C and E (BAZZO, 2015; FIGUEROA et al. 2018).

Based on the literature and on the need to provide information to support other studies, especially the biological ones and the fruit growing region, which aimed not only to analyze the antioxidant activity and the cellular viability of the crude extract and the ethyl acetate and aqueous fractions of these fragments (endocarp and seed) as to list the main substances elucidated in the hydroethanolic extract.

METHODOLOGY

This experiment followed the care obtained with the processing and extraction technique of the fruit, as they were essential to increase or decrease the concentration of phenolic compounds and determine the antioxidant action. The variety of the fruit, degree of maturation, harvest and planting are mechanisms that interfere in these concentrations, leaving it a richer and more natural source of free radicals. However, to increase the chances of success in the concentration of the desired compounds, the material needed to be matured and the harvest was carried out directly from planting to adequate storage, protected from light and heat (PIAZZA et al., 2018).

The hydroethanolic crude extract (7:3) and the H2O:MeOH (7:3) partition were obtained to obtain the ethyl acetate and aqueous fractions. Then, each sample was diluted in 1:1 of MeOH and distilled H2O, to a concentration of 2.0mg/ml. To analyze the antioxidant action, the DPPH (2,2-diphenyl-1-picryl-hydrazyl) test was performed at a wavelength of 515/517 nm (FURLAN et al.,

2015) at concentrations 150/300/500/ 700 and 1000 µg/ml. All samples were diluted in 1000µL of MeTOH and 1000µl of H20 (1:1), respecting the proportion of 2mg in 2mL. The samples were packaged in 96-well plates with gallic acid as the control substance.

For the cell viability test, an in vitro colorimetric assay based on the tretazolium salt MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) of high efficiency was used (ASSIS, 2015) at the concentrations of 75/150/300 and 600 µg/ml, where the samples were diluted in 2mL of H20 milli-Q and 10min in the sorifier, tested in 96-well plates with Raw macrophage cells, maintained in 10% v/v bovine serum, in an oven with 5% v/v CO2 and 37º C.

Mass spectrometry (LC-MS, Esquire 2000 Plus, Bruker Daltonics) was used to elucidate the molecules of the Persea Americana Mill extract with a quadrupole ion trap analyzer in tandem mode, with electronebulization ionization (ESI). The ionization conditions were as follows: electrospray voltage with ion source at 35 V, a potential of 4.0 kV, capillary voltage of -90V, tube lens of -100 V, temperature at 280°C and a of 5µL/min. Ultrapure helium (He) using as collision gas and nitrogen (N2) as nebulizing gas. Desolvation was facilitated using a flow rate of 7.0 L/min. Analyzes were performed in full scan mode with MS2, with negative ionization, in the range from 100 to 2000 m/z, and by electrospray (ESI) in positive mode up to 2000 Da, showing fragmented masses of substances.

The first scan analysis was performed taking into account the full mass spectrum to acquire enough information about ions in the pre-established m/z range. The scan then focused on experimenting with the MS/MS ratio using data dependent on the [M-H]- molecules of interest, allowing the resulting ions to undergo further fragmentation under the same conditions until no more fragments were observed. Retention time data, charge mass ratio (m/z) of the molecular ion and its fragments were used in an attempt to elucidate compounds through MassBank searches and comparative analysis with the literature.

In the results, for the differences between the groups, the two-way ANOVA Ordinary analysis of variance test was applied, followed by the post-test in multiple Newman-Keuls comparisons, using GraphPad Prism, Inc software, version 6.00 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant when $p<0.05$ (95%CI).

RESULTS AND DISCUSSION

As for the main molecules elucidated in the hydroethanolic extract: *Persea Americana Mill* (ESP) by LC-MS-ESI are analyzes that characterized the main substances identified in it. The identification occurred in a positive way, making it possible to find secondary metabolites of different classes, however, the majority were within the classes of condensed tannins (Table 1).

It was possible to observe variations in classes of secondary metabolites among the substances found in ESP. The literature reports different classes found among the elucidated constituents such as alkaloids, coumarins, triterpenes and/or steroids, saponins and polyphenols. This last class is predominant in the extract analyzed by this study.

A análise cromatográfica do extrato *Persea Americana Mil:* endocarpo com a semente por HPLC-PDA-VIS de alta eficiência – λ 254nm e o perfil por HPLC-PDA do ESP estão representados pela figura 1.

ESP spectrometry by LC-MS-ESI, represented by figure 2, shows fragmented masses of substances from electrospray analysis (ESI) in positive mode up to 2000 Da.

In figure 3, it can be seen the chemical structures of the main substances elucidated through the LC-MS-ESI of the hydroethanolic

Order number	TR	[M-H]$^-$	Ms^n	Substance
01	3,07	191	179	Chemical Acid
02	3,1	179	161	Caffeic Acid
03	3,5	163		Coumaric Acid
04	7,1	271	151	Naringenin
05	7,0	609	301	Rutine
06	6,7	433	301	Quercetin-O-deoxysoside
07	6,4	441	289	Galatocatechin (catechin+galic acid)
08	3,2	289		Catechin
09	4,8	1151	863;577;289.	catechin tetramer (proanthocyanidin)
10	5,4	1149	861;575;289	catechin tetramer (proanthocyanidin)
11	6,4	441	289	Galatocatechin (catechin+galic acid)
12	6,7	433	301	Quercetin-O-deoxysoside
13	3,6	1151	863;577;289.	catechin tetramer (proanthocyanidin B_1)

Table 1. Elucidation of the molecules of the hydroethanolic extracts of the associated part of the fruit: *Persea Americana Mill*: endocarp with the seed (ESP).

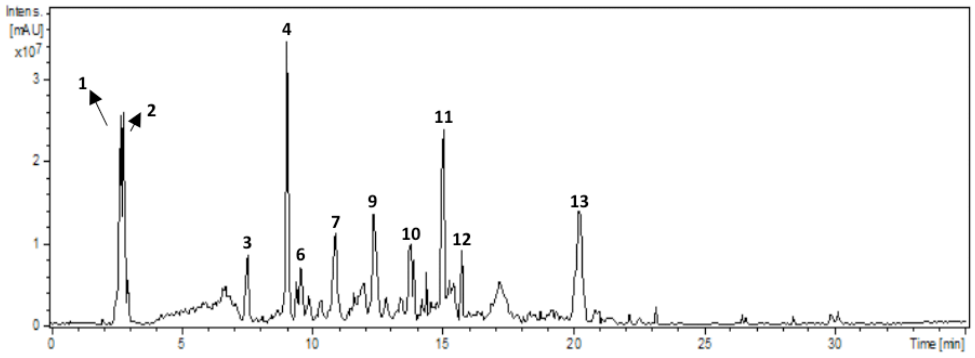

Fig. 1. Perfil da cromatografia obtido por HPLC-UV/VIS de alta eficiência – λ 254nm do extrato *Persea Americana Mill:* endocarpo com a semente (ESP).

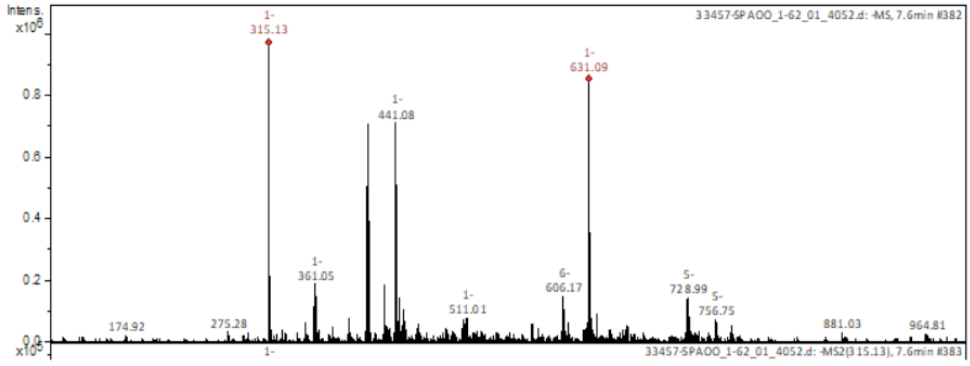

Figure 2. Mass spectrum [M-H]+ of the hydroethanolic extract (7:3) of the fruit: *Persea Americana Mil* (ESP) of the associated parts: endocarp with the seed.

extract of fruit: *Persea Americana Mill*: endocarp with seed.

The cell viability of the crude extract of the associated parts of the fruit: *Persea Americana Mill:* endocarp with the seed (ESP) can be seen in figure 4 whose revelation quantifies the cells that are viable when interacting with MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide), determining its level of cell viability.

When analyzing the results, it was noticed that the viability level of the crude extract showed levels below 50% in concentrations from 150 µg/ml, indicating high levels of cellular toxicity.

For differences between groups, the two-way ANOVA Ordinary analysis of variance test was applied, followed by the Newman-Keuls post-test. **p<0.01 and ***p<0.0001 when compared to DC (Normolipid Control). The following were evaluated by the program:GraphPadPrism 6.0 (p<0,5).

In the analyzes of the extract fractions shown in fig. 5, ethyl acetate and aqueous, cell viability did not show worrying levels of toxicity, except at the concentration of 600 µg/ml of the ethyl acetate fraction.

For differences between groups, the two-way ANOVA Ordinary analysis of variance test was applied, followed by the Newman-Keuls post-test. **p<0.01 and ***p<0.0001 when compared to DC (Normolipid Control). The following were evaluated by the program: GraphPadPrism 6.0 (p<0,5).

The antioxidant effect of the hydroethanolic extract of the associated parts of the fruit: *Persea Americana Mill:* endocarp with the seed using the free radical capture method (DPPH) it was observed that in the tested concentrations of ESP they are with values greater than 50% (p<0.0001), with the majority being around 70%, equating up to the control values (ns), gallic acid (Fig.6) (FURLAN et. al., 2015).

For differences between groups, the two-way ANOVA Ordinary analysis of variance test was applied, followed by the Newman-Keuls post-test. **** p<0.0001 and ns – not significant compared to control concentrations. They were evaluated using the GraphPadPrism 6.0 program (p<0.5). 300 C: 300µm/L concentration of the control substance; 300: 300µm/L concentration of ESP substance; 500 C: 500µm/L concentration of the control substance; 500: 500µm/L concentration of ESP substance; 700 C: 700µm/L concentration of the control substance; 700: 700µm/L concentration of ESP substance; 1000 C: 1000µm/L concentration of the control substance; 1000: 1000µm/L concentration of ESP substance;

Antioxidant activity was observed from the concentration of 150µm/mL, but it was at the concentration of 300µm/mL that it reached 50% (p<0.0001) of the captures (IC50) and it was from 500µm/mL that the captures were equivalent to the control, with no significant differences (ns).

Daiuto et. al. (2014), obtained antioxidant results from the extract made from avocado seed similar to those revealed in this research, with an IC50 of 310µm/mL, perhaps due to the concentration and polarity of the extract. The comparison also shows that ESP slightly reduced the IC50, despite not being cultivated in regions with more favorable temperatures and soils for harvesting.

When analyzing the fractions in fig.7, ethyl acetate and aqueous, through the DPPH test, there were significant levels of free radical capture in all analyzed concentrations, reaching values close to 80%, in concentrations between 100 and 500 µg/ml when compared to the control, gallic acid. This fact indicates that by concentrating the polar substances in each of the fractions, regardless of where the fruit was grown, there are significant increases in the antioxidant action.

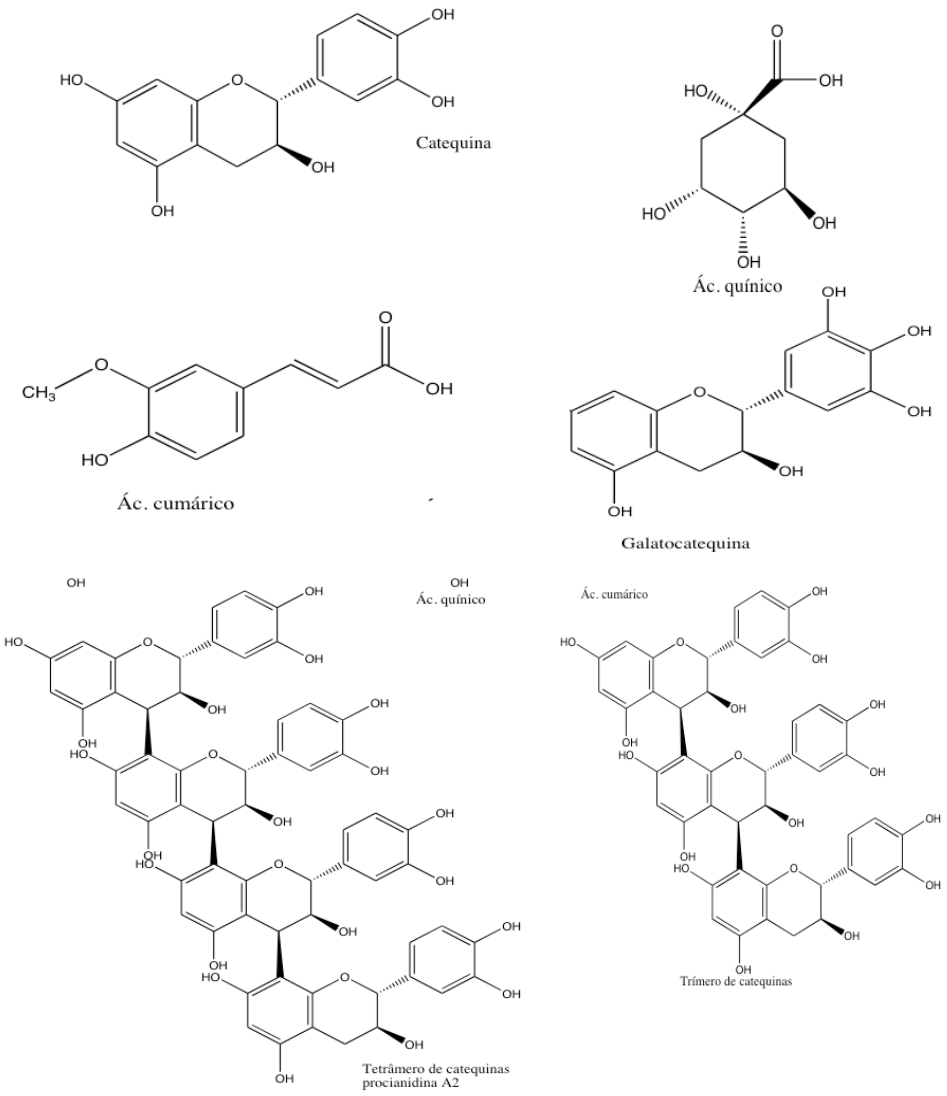

Figure 3. Chemical structures of the main molecules elucidated from the hydroethanolic extract of the fruit :*Persea Americana Mill*: endocarp with seed.

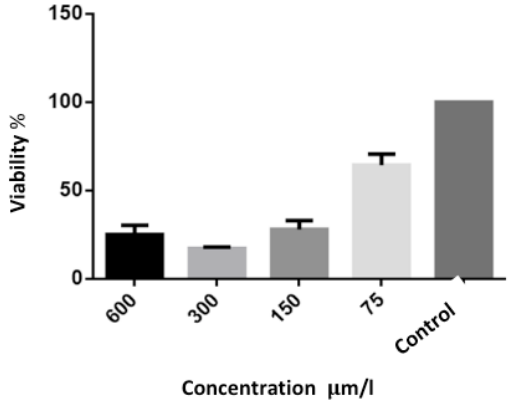

Figure 4. Cellular viability of the hydroethanolic extract of the associated parts of the fruit: *Persea Americana Mill:* endocarp with the seed through the test: *in vitro* MTT.

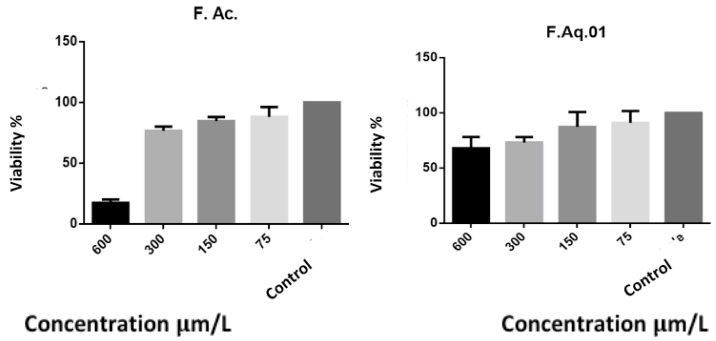

Figure 5. Cell viability of ethyl acetate (F.Ac.) and aqueous (F. aq.01) fractions using the test: *in vitro* MTT.

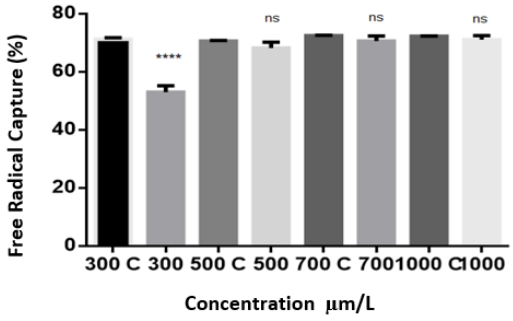

Figure 6. Antioxidant effect (DPPH) of the extract of the associated parts of the fruit: *Persea Americana Mill:* endocarp with the seed.

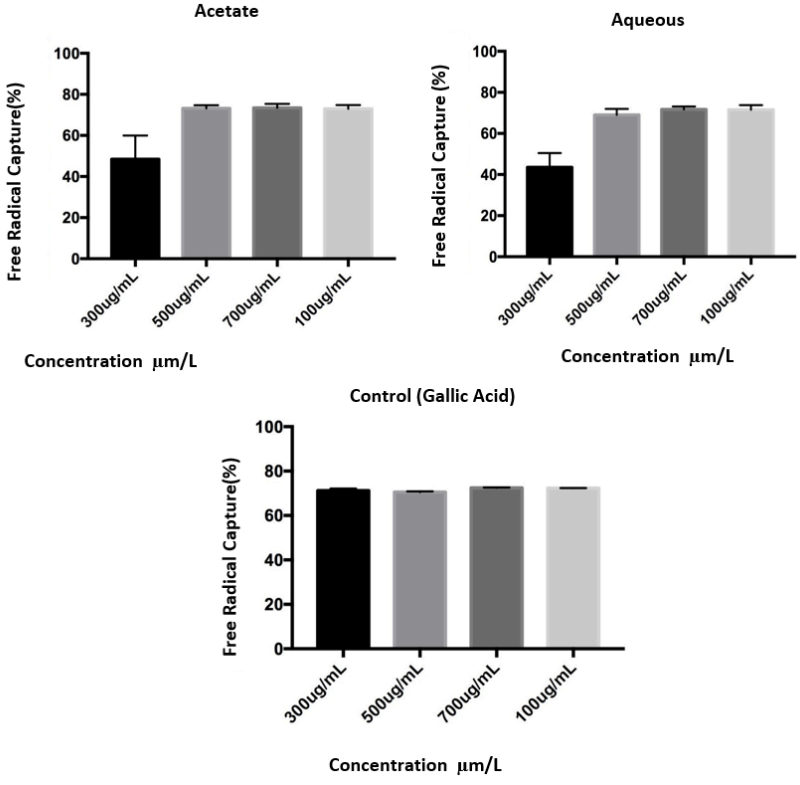

Figure 7. Antioxidant effect (DPPH) of the fractions, ethyl acetate and aqueous, of the associated parts of the fruit: *Persea Americana Mill:* endocarp with the seed.

For differences between groups, the two-way ANOVA Ordinary analysis of variance test was applied, followed by the Newman-Keuls post-test. **** $p<0.0001$ compared to control concentrations. They were evaluated using the GraphPadPrism 6.0 program ($p<0.5$).

The obtained levels of cellular viability that suggest the toxicity of the extract were reduced in the fractions, ethyl acetate and aqueous, favoring most of the evaluated concentrations. However, in the ESP crude extract, only the concentration of 75 µg/ml did not show signs of toxicity in the investigated cells. Therefore, the fractions analyzed by this study stand out in the designations of biotechnological research.

Antioxidant effects are related to several health benefits such as lipid-lowering, hypotensive, antidiabetic and cardioprotective effects (TABESHPOUR, 2017). In this investigation, the majority compounds, condensed tannins, from flavonoids called catechins, specifically, the condensates of catechins (3.2/289), proanthocyanidinides B1 (3.6/1151), trimers (3.8/863) and tetramers of catechins (3.6/1151). However, other important compounds for health were also identified in this extract, including phenolic acids: Quinic (3.07/191), Caffeic (3.1/179) and Cumaric (3.5/163). Galatocatechin (6.4/441) (catechin+gallic acid) was also found to be an excellent antioxidant. Emphasizing that it was the first time that a study delineated the chemical profile of the associated parts of the Persea America Mill fruit: endocarp and seed, cultivated in the region of José de Freitas in Piauí.

It is known that these major compounds have antioxidant activities. In the studies by Trecomoldi (2015) predominantly antioxidant compounds were identified in the skins and seeds using the HPLC-DAD-UV and LC-MS/MS techniques, proving higher amounts than those found in the pulps. In the seeds, he identified trans-5-O-caffeoyl-D-quinic acid, proanthocyanidin B1, catechin and epicatechin. The results corroborate those found by this research, as the elucidated substances can generate benefits for cardiovascular health and be a perspective to generate bioproducts that aim to control associated pathologies. In addition to offering data on the product for the region of cultivation.

CONCLUSION

The crude extract and the fractions of the associated parts of the Persea Americana Mill fruit: endocarp with seed showed levels between 70 and 80% of the antioxidant action, values equal to or greater than those of gallic acid (control). However, in the crude extract the levels of toxicity were more alarming, being in most concentrations, below 50% of cell viability, while in the fractions, ethyl acetate and aqueous, did not represent risks to the cells. Given the above, the results revealed substances with good antioxidant actions in the investigated extract and that can be healthy for cardiovascular health. In addition to adding values to the parts discarded by the region of cultivation and subsidizing pharmaceutical and research industries, among other interests.

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