

***Persea americana* Mill.: Evaluation of cytogenotoxicity and phytochemical prospection of leaf extracts**

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Persea americana Mill., belonging to the family Lauraceae, is noteworthy for the large amount of ethnopharmacological information in its regard, attributing to it many and varied medicinal properties. The tea and alcoholic extracts made from its leaves are used in folk medicine to treat various ailments. This study was designed to analyze the cytogenotoxicity and underlying chemistry of aqueous and hydroalcoholic extracts of avocado leaves, using the *Allium cepa* and micronucleus tests. The results obtained by applying the experimental models demonstrate that the extracts did not have a genotoxic effect at any of the concentrations analyzed, and even demonstrated a certain protective effect, possibly due to the presence of flavonoids and phenols, both of which are antioxidant substances. However, the extracts did present a cytotoxic effect. There were numerous karyorrhectic cells and those with nuclear alterations related to cell death. At the highest concentrations, it was possible to observe cytoplasmic alterations and binucleated cells. The extracts also caused a significant reduction in the number of cells undergoing division. These effects can be a response to the phytochemical agents present in the extracts. The results suggest that the extracts contain bioactive components that deserve further studies related to cancer therapies.

Keywords: *Persea americana*. *Allium cepa*. Micronucleus. Genotoxicity. Cytotoxicity. Phytochemical.

INTRODUCTION

Persea americana Mill., belonging to the family Lauraceae and popularly called avocado, is a tree with rounded crown that is native to Central America, and was introduced in Brazil in 1809. Not only do the fruits have high nutritive value, the species is noteworthy for the large amount of ethnopharmacological information,

attributing to it numerous and varied medicinal properties (Lorenzi, Abreu-Matos, 2008).

The tea made from its leaves is used for its diuretic, carminative, antirheumatic, antidiarrheal, analgesic and anti-inflammatory effects, as well to stimulate the biliary vesicle in the treatment of kidney and bladder ailments, among many other uses, while homemade alcoholic extracts are used as topical remedies for rheumatic pain, contusions and headaches (Adeyemi, Okpo, Ogunti, 2002; Lorenzi, Abreu-Matos, 2008; Biasi-Garbin *et al.*, 2016).

For being rich in phytochemicals, *P. americana* has attracted a large number of studies. The presence of

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substances with anticancer properties was demonstrated by Laleh *et al.* (2014) in ethanol, chloroform, ethyl acetate and petroleum ether extracts obtained from the fruits of *P. americana*. Christian *et al.* (2014) demonstrated the antidiarrheal effect of methanol and chloroform fractions obtained from the leaves. However, other researchers had previously detected chromosome aberrations in human peripheral lymphocytes caused by methanolic extracts of avocado leaves and fruits (Kulkarni, Paul, Ganesh, 2010). Then in 2017, Muhammad, Willey and Riviere reported that hydroalcoholic extracts of some medicinal plants, including *P. americana*, increase the dermal absorption of ¹⁴C-cafein, and stressed the importance of studies related to the safety of using natural extracts in cosmetics and topical pharmaceutical products.

Therefore, because of the widespread medicinal use of the aqueous and alcoholic extracts of *P. americana* to treat various ailments, and considering that many types of extracts are rich in various metabolic chemicals, tests to assess the cytotoxicity and genotoxicity of these extracts are necessary to contribute to the safe use of the target extracts to produce drugs, as well as to plan actions to inform the public regarding the use of medicines based on plants.

MATERIAL AND METHODS

Sample collection and identification

Leaves of *P. americana* at different stages of development were collected on the campus of Rio de Janeiro Federal Rural University (UFRRJ), in the municipality of Seropédica, state of Rio de Janeiro. The botanical material was recognized by the herbarium technician Thiago Azevedo Amorim of the Botany Department of UFRRJ and a voucher specimen was deposited in the university's herbarium under number RBR 37993.

Preparation of the extracts of *P. americana* leaves

Immediately after collection, the leaves were taken to the Laboratory of Plant Genotoxic Activity (LAGEP) of UFRRJ, where they were spread on a table and left at room temperature (28 °C) in an area protected from

sunlight for 72h (air drying). The naturally dried leaves were then placed in an amber flask until the moment of preparing the extracts.

Aqueous extract

Samples of dried and ground leaves weighing 1.5 g, 3.0 g and 5.0 g were placed in glass vials, into which 100 mL of warm distilled water was poured (90 °C). The vials were capped immediately and maintained in this condition for at least 10 minutes, obtaining extracts at concentrations of 15 and 30 mg mL⁻¹ (used in *Allium cepa* assay) and 50 mg mL⁻¹ (used in micronucleus assay and phytochemical analysis) respectively. After being cooled to room temperature, the crude extracts were filtered through cotton cloth to remove residues. Fresh extracts were prepared daily, just before use in the experiments.

Hydroalcoholic extract

The hydroalcoholic extract was obtained by maceration in the proportion of 60 g of the pulverized material and 700 mL of 70% ethanol, at room temperature (28 °C), for 72 hours, protected from light, with occasional agitation and without renewal of the extracting liquid. After this period, the extract was filtered and evaporated in a water bath at 60 °C, yielding dry weight of 7.65 g (Stange *et al.*, 2009 with modifications). A portion of the dried extract was weighed and redissolved in distilled water to obtain the concentration used in the experiment. Extracts at concentrations of 15 and 30 mg mL⁻¹ (*Allium cepa* assay) and 50 mg mL⁻¹ (micronucleus assay and phytochemical analysis) were prepared daily, just before use in the experiments.

Phytochemical screening of the extracts

To identify and quantify the chemical constituents present in the aqueous and hydroalcoholic extracts, phytochemical analysis was carried out with 50 mg of the extracts, through the techniques described by Trease and Evans (1989), Matos (2009), Cai, Shi and Gao (2011), Saklani *et al.* (2012) and Morsy (2014). The aqueous extract was dried by evaporation in a water bath at 50

°C. The screening was performed for reducing sugars, saponins, tannins, cardiac glycosides, polysaccharides, anthraquinones, flavonoids, alkaloids, steroids and triterpenoids. The reducing sugars were detected by reaction of Benedict's reagent. The presence of saponins was observed by the formation of froth in a test tube upon vigorous shaking using diluted samples. The cardiac glycosides were detected by using Kedde's reagent. The presence of polysaccharides was verified by using lugol. The presence of alkaloids was determined by using Dragendorff's, Mayer's, Sonnenschein's and Bouchardat's reagents. Anthraquinones were verified by using the Bornträger test. Flavonoids were detected by using the Shinoda test. The detection of tannins was realized by using gelatin solution test, ferric chloride reagent and the lead acetic test. Finally, steroids and triterpenoids were detected by using the Liebermann-Burchard test.

Analysis of total flavonoids and phenols

To determine the content of total flavonoids and phenols, the dried aqueous extract was obtained by infusing 11 g of crushed leaves in 150 mL of water, followed by heating in an oven at 50 °C until constant weight. For the hydroalcoholic extract, 3.15 g of the extract was used, prepared as described above.

The total flavonoid content was measured by the method described by Barroso *et al.* (2011) with some modifications, using as complexing agent aluminum chloride. Quercetin was the standard used to construct the calibration curve for quantification.

The determination of the total phenol concentration was performed according to the method described by Salgueiro and Castro (2016), based on a standard curve of commercial gallic acid and using the colorimetric method with Folin-Ciocalteu reagent.

Analysis of antioxidant activity

Determination of antioxidant activity (ABTS assay)

Determination of antioxidant activity was performed using the method described by Sant'Ana *et al.* (2012). An aliquot of 50 µL of each extract (1.00 mg mL⁻¹) was mixed

with 5.0 mL of ABTS solution. After 6 minutes, the absorbance readings were taken at 734 nm using absolute ethanol as a blank. A calibration curve was prepared with Trolox solution (0.00-2.400 mmol L⁻¹; $Y = -26.37778X + 0.65164$; $R = -0.9997$). The data were expressed as millimoles of Trolox equivalent per 100 milligrams of extract (mmol TE 100mg⁻¹). All measurements were performed in triplicate.

Determination of antioxidant activity (FRAP assay)

Antioxidant activity was evaluated using the method described by Sant'Ana *et al.* (2012). An aliquot of 0.5 mL of each extract (2.0 mg mL⁻¹) was mixed with 4.5 mL of the FRAP reagent. After 10 minutes of incubation at 37 °C, the absorbance was measured at 593 nm using 0.5 mL of methanol in 4.5 mL of the FRAP reagent as blank. Quantitative analyses were performed with the external standard method using ferrous sulfate (100-1000 µM; $Y = 0.0018X + 0.00107$; $R = 0.99961$) as the standard and correlating the absorbance with the concentration. The results were calculated and expressed as millimoles of Fe²⁺ equivalent per 100 milligrams of extract (mmol Fe(II) 100 mg⁻¹). All measurements were performed in triplicate.

Allium cepa assay

For these tests, organically grown onions with diameter of approximately 2.0 cm were obtained from a local produce market. The outer layer of each bulb was removed with a paring knife without damaging the root buds.

The bulbs were initially placed in a container holding distilled water for 48 hours to allow the roots to grow, with daily water exchange. Then the bulbs were separated into control groups and treatment groups, each with five onions. The negative control group remained in the distilled water, while the treatment groups were immersed in *P. americana* leaf extract solutions (aqueous and hydroalcoholic) at the concentrations of 15 mg mL⁻¹ and 30 mg mL⁻¹ for each extract. The positive control group was immersed in a solution containing ethyl methanesulfonate (EMS, 25 mM). EMS is a highly efficient mutagenic agent, which acts directly on DNA molecules through its alkylation activity. The solutions

were exchanged daily for all groups and the temperature was maintained at 25 °C.

For each treatment, root tips, between 2 and 2.5 cm in length, were removed from the bulbs after 48 hours of exposure to the respective solutions and used to prepare slides according to the method described by de Castro e Sousa *et al.* (2017), with modifications. Five root tips were removed from each bulb, fixed in an ethanol: glacial acetic acid solution at 3:1 (V/V) and stored at 4 °C until the moment of preparing the slides. Five slides were prepared for each bulb, using five different root tips (one slide for each). The root tips were washed with distilled water twice for 5 minutes, hydrolyzed in HCl 5N for 30 minutes, washed again twice in distilled water for 5 minutes and then placed on the slides with tweezers. The subapical meristems were fragmented with a scalpel, stained with 2% acetic orcein, and covered with a coverslip.

All slides were coded and evaluated under a common optical microscope with 100X magnification. The parameters used to determine the genotoxic and cytotoxic potential of the extracts were the presence of chromosome and cell alterations as well as changes in the mitotic index. A total of 1,000 cells were analyzed per bulb, or 5,000 cells for each group. With the exception of the mitotic index, expressed as a percent, the results are expressed in absolute terms. The most frequent anomalies are shown in the micrographs.

Micronucleus assay

Male and female Swiss albino mice aged 10 weeks, weighing 25 g, were provided by the Central Animal House of UFRRJ. The animals were kept in polyethylene cages, each one with ten animals, at a constant temperature of 22 ±1 °C and 12 h light/dark cycle. Standard feed pellets and water were provided *ad libitum*.

The micronucleus assay was performed according to Andrade, Perazzo and Maistro (2008), with modifications. Assays were carried out with 10 animals/group (five of each sex), and with the exception of the positive control, the treatment was performed by intragastric route. Group I: animals received a single dose of 2,000 mg/

kg of aqueous extract of *P. americana* leaves; Group II: animals received a single dose of 2,000 mg/kg of hydroalcoholic extract of *P. americana* leaves; Group III (positive control): animals received a single dose of cyclophosphamide at 50 mg/kg by intraperitoneal application; and Group IV (negative control): animals received only water. A 20 µL aliquot of peripheral blood was collected from each animal 48 h and 72 h after treatment, by terminal bleeding from the retro-orbital plexus. Samples were deposited on a slide to prepare the smears, which were fixed in methanol and stained with Leishman's solution.

For each Swiss mouse, 4,000 polychromatic erythrocytes (PCE) were scored (2,000 cells from the 48 h blood sample and 2,000 cells from the 72 h sample). For evaluation of the cytotoxic potential, 1,000 cells were analyzed per animal to determine the polychromatic/normochromatic erythrocyte (PCE/NCE) ratio. All slides were coded and evaluated under a common optical microscope with 100X magnification.

This study was approved by the Ethics Committee on Animal Use of the State University Center of the West Zone Foundation (UEZO) (protocol no. 016/2016).

Statistical analysis

The data were analyzed by the Chi-square χ^2 test with probability <0.05, using Yates correction, employing the Bioestat 5.0 program.

RESULTS

Allium cepa assay

The results of the *A. cepa* assay 48 hours after the treatment showed that the aqueous and hydroalcoholic extracts obtained from the *P. americana* leaves did not have a genotoxic effect at any of the concentrations analyzed, since no alterations of chromosomes or micronuclei were observed. Besides this, the meristematic cells treated with the extracts had a significantly lower number of large nucleoli, an alteration characteristic of the positive control (EMS) (Figure 1H) (Tables I, II and III).

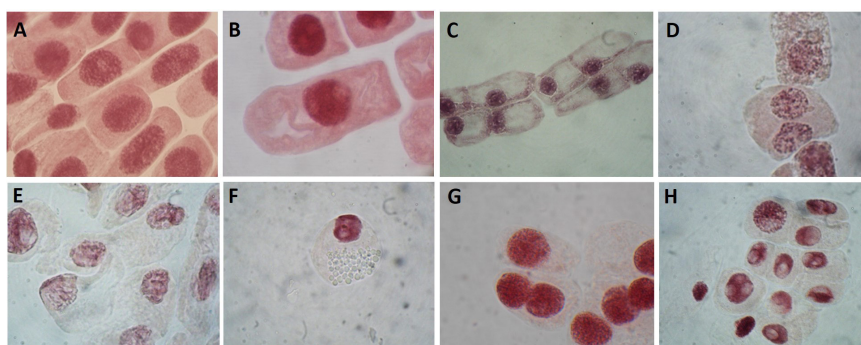


FIGURE 1 – Changes in *A. cepa* cells submitted to different treatments: A – normal cells (negative control), B – cytoplasmic alteration, C- cytoplasmic vacuole, D- karyorrhexis, E- nuclear abnormalities, F- necrosis, G- binucleated cells and H- nucleolar changes (positive control).

TABLE I - Cell changes and mitotic index in *A. cepa* submitted to different treatments

Tx	CONC	NC	NE	KR	CV	CA	NA	BC	M ^(%)
NEG	dH ₂ O	542 ^(b)	9 ^(b)	50 ^(c)	0 ^(c)	22 ^(b)	39 ^(c)	3	1.5 ^(a)
EMS	25mM	1278 ^(a)	14 ^(b)	4 ^(d)	7 ^(b)	0 ^(b)	73 ^(b)	1	1.2 ^(a)
PAQ	15 mg mL ⁻¹	350 ^(c)	13 ^(b)	252 ^(b)	0 ^(c)	16 ^(b)	95 ^(b)	0	0.3 ^(b)
PHA	15 mg mL ⁻¹	194 ^(d)	31 ^(a)	991 ^(a)	31 ^(a)	215 ^(a)	407 ^(a)	2	0.0 ^(c)

(a) (b) (c) and (d) different letters in the same column differ from each other (P<0.05) according to the χ^2 test; Tx – treatment; NEG – negative control; EMS – positive control; PAQ- *P. americana* aqueous extract; PHA- *P. americana* hydroalcoholic extract; CONC – concentration; dH₂O – distilled water; NC- nucleolar changes; NE- necrosis; KR- karyorrhexis; CV – cytoplasmic vacuole; CA – cytoplasmic alteration; NA- nuclear abnormalities; BC – binucleated cells; M- mitotic index. 5000 cells for each treatment were analyzed.

TABLE II - Cell changes and mitotic index in *A. cepa* submitted to different treatments

Tx	CONC	NC	NE	KR	CV	CA	NA	BC	M ^(%)
NEG	dH ₂ O	643 ^(a)	9	15 ^(b)	0	10 ^(b)	17 ^(b)	89 ^(b)	3.4 ^(a)
EMS	25mM	702 ^(a)	4	7 ^(b)	1	8 ^(b)	21 ^(b)	98 ^(b)	1.9 ^(b)
PAQ	30 mg mL ⁻¹	577 ^(c)	5	59 ^(a)	0	123 ^(a)	82 ^(a)	126 ^(a)	0.7 ^(c)

(a) (b) and (c) different letters in the same column differ from each other (P<0.05) according to the χ^2 test; Tx – treatment; NEG – negative control; EMS – positive control; PAQ - *P. americana* aqueous extract; CONC – concentration; dH₂O– distilled water; NC- nucleolar changes; NE- necrosis; KR- karyorrhexis; CV- cytoplasmic vacuole; CA – cytoplasmic alteration; NA – nuclear abnormalities; BC – binucleated cells; M – mitotic index. 5000 cells for each treatment were analyzed.

TABLE III - Cell changes and mitotic index in *A cepa* submitted to different treatments

Tx	CONC	NC	NE	KR	CV	CA	NA	BC	M ^(%)
NEG	dH ₂ O	264 ^(b)	19 ^(b)	8 ^(c)	0 ^(b)	0 ^(b)	30 ^(c)	3 ^(b)	2.4 ^(a)
EMS	25mM	1496 ^(a)	23 ^(a)	19 ^(b)	0 ^(b)	0 ^(b)	6 ^(b)	6 ^(b)	2.3 ^(a)
PHA	30 mg mL ⁻¹	98 ^(c)	10 ^(b)	1105 ^(a)	17 ^(a)	463 ^(a)	995 ^(a)	11 ^(a)	0.4 ^(b)

(a) (b) and (c) different letters in the same column differ from each other (P<0.05) according to the χ^2 test; Tx – treatment; NEG– negative control; EMS – positive control; PHA - *P. americana* hydroalcoholic extract; CONC – concentration; dH₂O – distilled water; NC- nucleolar changes; NE- necrosis; KR- karyorrhexis; CV- cytoplasmic vacuole; CA – cytoplasmic alteration; NA – nuclear abnormalities; BC – binucleated cells; M – mitotic index. 5000 cells for each treatment were analyzed.

The analyses also revealed the existence of a cytotoxic effect of both extract types. There were numerous karyorrhectic cells (Figure 1D) and cells with alterations related to death processes (Figure 1E), caused by both extracts at both concentrations analyzed (15 mg mL⁻¹ and 30 mg mL⁻¹) (Tables I, II and III).

The hydroalcoholic extract caused a larger number of cytotoxic alterations, since there were a substantial number of cells with vacuoles (Figure 1C) and cytoplasmic alterations (Figure 1B) at the two concentrations. Besides this, there was a greater number of cells undergoing necrosis (Figure 1F) at the concentration of 15 mg mL⁻¹, and of binucleated cells (Figure 1G) at the concentration of 30 mg mL⁻¹ compared with the negative control group (Tables I and III).

In the treatments with the aqueous extract, besides the presence of karyorrhectic cells and cells with nuclear alterations at the two concentrations, significant numbers of cells with cytoplasmic alterations and binucleated cells were observed at the concentration of 30 mg mL⁻¹ (Tables I and II).

The mitotic index results demonstrated a large reduction in the number of cells undergoing division in the treatments with both concentrations and with both

extract types in comparison with the positive and negative controls (Tables I, II and III).

Micronucleus assay

The analyses of the peripheral blood of the mice submitted to a single dose of 2000 mg/kg of the aqueous and hydroalcoholic extracts of the *P. americana* leaves demonstrated the absence of genotoxic potential of either of the extracts (Tables IV and V).

The results obtained 48 h (Table IV) and 72 h (Table V) after the treatments demonstrated no increase in the number of micronucleated polychromatic erythrocytes (MNPCEs) in the mice treated with the extracts. At 48 hours after treatment, there was a significant reduction in the number of MNPCEs in the mice treated with the extracts in relation to the positive and negative controls.

However, although a genotoxic effect of the extracts was not detected, there was a significant difference in the PCE/NCE ratio between the treatments. The animals submitted to the extract treatments showed a significant reduction in the PCE/NCE ratio compared to the negative control animals, indicating the cytotoxic activity of the extracts.

TABLE IV - Frequency of micronucleated polychromatic erythrocytes (MNPCEs) in mice 48h after the different treatments.

Treatment	Number of cells analyzed	PCEs	MNPCEs		PCE/NCE ratio
			N°	%	
Positive control	20000	19917	83 ^(a)	0.415	0.0006 ^(c)
Negative control	20000	19974	26 ^(b)	0.13	0.021 ^(a)
PAQ	20000	19990	10 ^(c)	0.05	0 ^(d)
PHA	20000	19991	9 ^(c)	0.045	0.014 ^(b)

(a) (b) (c) and (d) different letters in the same column differ from each other ($P < 0.05$) according to the χ^2 test; PAQ- *P. americana* aqueous extract; PHA- *P. americana* hydroalcoholic extract; PCEs – polychromatic erythrocytes; NCE – normochromatic erythrocytes; MNPCEs – micronucleated polychromatic erythrocytes.

TABLE V - Frequency of micronucleated polychromatic erythrocytes (MNPCEs) in mice 72 h after the different treatments

Treatment	Number of cells analyzed	PCEs	MNPCEs		PCE/NCE
			N°	%	ratio
Positive control	20000	19977	23 ^(a)	0.115	0.001 ^(c)
Negative control	20000	19992	8 ^(b)	0.04	0.015 ^(a)
PAQ	20000	19995	5 ^(b)	0.025	0.007 ^(b)
PHA	20000	19995	5 ^(b)	0.025	0.006 ^(b)

(a) (b) (c) and (d) different letters in the same column differ from each other ($P < 0.05$) according to the χ^2 test; PAQ- *P. americana* aqueous extract; PHA- *P. americana* hydroalcoholic extract; PCEs – polychromatic erythrocytes; NCE – normochromatic erythrocytes; MNPCEs – micronucleated polychromatic erythrocytes.

Phytochemical screening of the extracts

The phytochemical analysis of the aqueous and hydroalcoholic extracts detected the presence of reducing sugars, saponins, flavonoids, tannins, steroids and triterpenoids, as presented in Table VI.

TABLE VI - Phytochemical analysis of aqueous and hydroalcoholic extract of *P. americana* leaves.

Secondary compounds	Hydroalcoholic extract	Aqueous extract
Reducing sugars	+	+
Saponins	+	+
Cardiac glycosides	-	-
Polysaccharides	-	-
Alkaloids	-	-
Flavonoids	+	+
Tannins	+	+
Anthraquinones	-	-
Steroids and triterpenoids	+	-

(-) Absence or inconclusive, (+) Presence

Analysis of total contents of flavonoids and phenols

The results reported in Table VII demonstrate the presence of flavonoids and phenols in both types of extracts analyzed. The hydroalcoholic extract presented a higher level of phenols while the aqueous extract had a higher level of total flavonoids.

Analysis of antioxidant activity

The results for antioxidant activity using the ABTS and FRAP methods for the different extracts are summarized in Table VII. Both extracts presented antioxidant activities, but the hydroalcoholic extract showed the highest values for antioxidant activity by the two methods used.

TABLE VII - Total phenols (TP), flavonoids (TF) and antioxidant activities of the extracts of *P. americana* leaves^a

Samples	TP	TF	ABTS	FRAP
PHA	12.04 ±0.022	0.09 ±0.002	116.84 ±0.005	241.78 ±0.016
PAQ	4.04 ±0.009	3.07 ±0.001	13.91 ±0.007	84.89 ±0.015

^aAll data expressed on extract weight basis as means ± SD (n=3). PHA = *P. americana* hydroalcoholic extract; PAQ = *P. americana* aqueous extract. The values were expressed as TP (mgGAE 100 mg⁻¹), TF (mgQE 100 mg⁻¹), FRAP (mmolFe(II) 100 mg⁻¹), ABTS (mmolTE 100 mg⁻¹)

DISCUSSION

The results of this study demonstrate, unlike the findings of Kulkarni, Paul and Ganesh (2010) with methanolic extracts, that the aqueous and hydroalcoholic extracts obtained from the *P. americana* leaves, at both concentrations investigated, did not have a genotoxic effect, since no alterations of chromosomes or micronuclei were observed. It is also important to stress that the

extracts had a protective effect, revealed by the two test methods employed. In the micronucleus assay, 48 hours after treatment there was a significant decrease in the number of micronucleated erythrocytes in comparison with the negative control group, while the *A. cepa* test results revealed a significantly smaller number of cells with nucleolar alterations than in the negative control group. In animal cells, the analysis of alterations in the size and shape of the nucleoli is used as a tool to help distinguish between benign and malignant cells, where the presence of hypertrophied nucleoli with irregular shapes is considered an indication of malignancy (Makinen *et al.*, 1993; Kruger, Stahlhut, Muller, 2000; Montanaro, Treré, Derenzini, 2008). Similar studies involving the variation of the size and shape of nucleoli due to the action of genotoxic agents have also been conducted with other plant cells (Arkhipchuk, Malinovskaya, Garanko, 2000; Ventura-Camargo, Maltempi, Marin-Morales, 2011). Therefore, the presence of a significantly smaller number of nucleolar changes in the cells submitted to treatment with the extracts is an indication of their protective effect. Such effects can be related to the presence of substances (flavonoids and phenols) with antioxidant activity, as observed in the ABTS and FRAP assays. The phytochemical analysis demonstrated the presence of these two substances in both extract types. The hydroalcoholic extract contained a higher concentration of phenolic substances than the aqueous extract, while the latter contained more flavonoids. Considering that the hydroalcoholic extract also had a stronger antioxidant effect and caused a steeper reduction in the number of cells with nucleolar alterations, it can be concluded that the substances with protective biological activity, although also present in flavonoids, are more concentrated in the phenolic substances of the extract.

Similar results to ours were reported by Brai, Adisa and Odetola (2014), who demonstrated the hepatoprotective effect of the aqueous extracts of *P. americana* on the livers of rats submitted to carbon tetrachloride. According to the authors, that activity occurs partly due to the antioxidant effect and elimination of free radicals, attributed to the phenols and flavonoids.

Although no genotoxic effects were observed, the cytotoxic activity of the extracts was detected by both

methods used. In the micronucleus assay, there was a significant reduction of the PCE/NCE ratio in the animals submitted to the treatments. The *A. cepa* assay revealed a reduction in the mitotic index caused by both extract types and concentrations, besides a significant number of karyorrhectic cells and cells with alterations related to death processes. Karyorrhexis is characterized by irregular distribution of chromatin and is found in cells that are undergoing necrosis or apoptosis (Thomas *et al.*, 2007; Thomas *et al.*, 2009; Sulczewski *et al.*, 2014).

Toxicity of *P. americana* leaves has been observed in other studies. Kingsbury (1964), cited in Butt *et al.* (2006), reported the development of non-infectious mastitis and loss of milk production in cattle that had eaten avocado leaves and associated this effect with specific necrosis of the secretory epithelium cells of the mammary glands. Similar results obtained in mice led to the isolation of a biologically active component derived from fatty acids, called persin (Oelrichs *et al.*, 1995). In Nigeria, the decoction of avocado leaves is used to treat tumors and related diseases, with indication that components with polar characteristics are likely responsible for anticancer activity (Engel *et al.*, 2011).

Despite the existence of various reports about the biological activity of extracts obtained from different organs of *P. americana*, many active substances have not yet been structurally characterized (Ding *et al.*, 2007). Methanolic extracts from avocado leaves were not efficient to significantly inhibit the proliferation of breast cancer cell lines (Engel *et al.*, 2011), but the study performed by Bonilla-Porras *et al.* (2014) showed that ethanolic extracts from the endocarp, seeds and leaves, at low concentrations, induced apoptosis in leukemia cells, while at higher concentrations they caused necrosis. According to the authors, the cell response to the agents contained in the extracts, such as polyphenols, steroids, triterpenoids and tannins, appears to involve a sequence of events leading to apoptosis, so that the extracts act as pro-apoptotic components, with the leukemia cells being eliminated by an oxidative stress mechanism.

According to our results, the cells treated with the hydroalcoholic extract presented a larger number of changes than did the cells treated with the aqueous extract. At the concentration of 15 mg mL⁻¹, it was possible to

observe, besides the presence of karyorrhectic cells (also detected after treatment with the aqueous extract at 15 mg mL⁻¹), the presence of cells undergoing necrosis with alterations to the cytoplasm and cytoplasmic vacuoles, which can indicate cell degeneration (Franco *et al.*, 2010). The presence of necrotic cells and cells with cytoplasmic vacuoles was not significant at either concentration of the aqueous extract. The detection of these alterations can be related to the presence of steroids and triterpenoids in the hydroalcoholic extract, which can act separately or in combination with other components.

Besides the increase in the number of cells undergoing the death process, a substantial decline was noted in the mitotic index of the cells treated with the two extract types, according to the results of both assays.

Similar results have been observed for extracts of *P. americana* fruits. According to Ding *et al.* (2007), the chloroform fraction of the extract was more selective at inhibiting the growth of pre-malignant and malignant human epithelial cell lines. The authors reported that the subsequent fractioning of the extract by column chromatography indicated that the active components are widely distributed in the less polar sub-fractions. In 2013, Khalifa *et al.* found that the methanolic extract from the fruits and leaves diminished the viability of cancer cell lines, and in 2015 the same researchers reported that the aqueous extract of avocado fruits significantly reduced the mitotic index of meristematic cells from the roots of *Vicia faba*.

These results together with ours demonstrate that extracts of *P. americana* with different polarities contain bioactive components with anticancer activities. Considering that components with therapeutic activities should exhibit low toxicity to normal cells for their safe use (Ding *et al.*, 2007), the aqueous extract obtained from the avocado leaves is a promising source both to obtain isolated components and for further testing of the crude extract at different concentrations on various cancer cell lines.

CONCLUSION

According to the results, it can be concluded that the aqueous and hydroalcoholic extracts of the leaves of *P.*

americana, at the concentrations tested, are not genotoxic, and even have a protective effect, probably related to their antioxidant activity. Both extracts contain substances responsible for reducing the mitotic index and increasing the number of cells undergoing death. The aqueous extract should be tested at other concentrations on different cell lines, in an effort to obtain new components that can be used in therapy.

ACKNOWLEDGEMENTS

We gratefully acknowledge the herbarium technician Thiago Azevedo Amorim of the Botany Department of Federal Rural University of Rio de Janeiro (UFRRJ) for the recognition of botanical material.

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Received for publication on 23rd March 2019
Accepted for publication on 19th October 2019