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Antiproliferative potential of *Plinia edulis* leaves

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ABSTRACT. *Plinia edulis*, popularly known as 'cambucá', is used in Brazilian folk medicine to treat several conditions. The aim of this study was to evaluate the mutagenicity and cytotoxicity of cambucá leaf extract, as well as its antioxidant activity. The 'Ames' test and Orac assay were used to evaluate the mutagenicity and antioxidant activity, respectively, and the phytochemical analysis of the ethanol leaf extract (LE) was performed by chromatographic and spectrometric methods. The *in vitro* cytotoxicity was tested on CHO (Chinese Hamster Ovary) cells and on nine human tumor cell lines. Phytochemical analysis of LE has revealed the presence of triterpenes and flavonoids. The LE did not show mutagenicity at the concentration of 20 mg plate^{-1} and exhibited high antioxidant activity, with Orac value of 3948 µmol TE g^{-1}. The LE and its fractions (ethyl acetate; methanol) showed antiproliferative activity against cancer cell lines and proliferative activity in normal cells. The ethyl acetate fraction was the most active extract, presenting antiproliferative activity against strains of breast cancer, prostate cancer, colon cancer, lung cancer, melanoma and leukemia (GI_{50} 0.01-8.57 µg mL^{-1}), being more effective than the reference drug (doxorubicin) against human breast adenocarcinoma MCF-7 cell line. These results suggest *P. edulis* as a potential adjuvant in cancer therapy and chemoprevention.

Keywords: medicinal plants; cambucá; Myrtaceae; mutagenicity; anticancer; antioxidant.

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Introduction

Plinia edulis (Vell.) Sobral (Myrtaceae) is endemic of the Brazilian Atlantic Rain Forest, popularly known as cambucá, and it was found to be a species of high of conservation priority for sustainable use (Lima, Marchioro, Joner, Steege, & Siddique, 2020; Rodrigues et al., 2020). The fruits and leaf infusions of this plant species are used as food and to treat countless conditions in Brazilian folk medicine, such as diabetes, gastric and inflammatory diseases, bronchitis, and diarrhea (Ishikawa et al., 2008; Rosa et al., 2018).

In previous assays, this species exhibited gastroprotective (Ishikawa et al., 2014; Rosa et al., 2018), anti-inflammatory and antinociceptive activities (Azevedo et al. 2016; Nesello, Campos, Capistrano, Buzzi, & Cechinel Filho, 2018), which could be related with the oleanane- and ursane-type triterpenoids and flavonol glycosides identified in the crude extracts (Azevedo et al., 2016; Nesello et al., 2018; Santos et al., 2020).

The preliminary toxicological evaluation of *P. edulis* has not shown acute toxic effects in mice at oral doses of 5 g kg^{-1} (Ishikawa et al., 2008). However, the genetic damage provoked by medicinal plants have been evidenced in different models and the safety on using plants with potential toxic substances must be better evaluated (Yang et al., 2019; Anywar, Kakudidi, Oryem-Origa, Schubert, & Jassoy, 2022). In this context, *in vitro* assays for evaluation of medicinal plants safety are recommended and, aiming to reduce the number of animal tests, the International Organization for Standardization prescribes the cytotoxicity assay as the method of choice (Mafioleti, Silva Junior, Colodel, Flach, & Martins, 2013).

Therefore, based on the lack of toxicological information regarding the safety of *P. edulis*, the mutagenicity and cytotoxicity of the leaf extract and fractions of this species were investigated. Besides, previous assays have indicated the aqueous extract as a good candidate for chemoprevention and antineoplastic drug development, since it has shown to be antioxidant and cytotoxic against human breast adenocarcinoma MCF-

7 cell line (Carvalho, Ishikawa, & Gouvêa, 2012). Thus, the present study also reports the antiproliferative assay of leaf extracts and fractions on nine Human tumor cell lines, the phytochemical analysis and the antioxidant activity evaluated by Orac assay.

Material and methods

Plant material

The leaves of *P. edulis* were collected in Trindade, Rio de Janeiro State, Brazil, in the summer. The voucher specimen has been deposited in the Herbarium of the Botanical Institute of São Paulo, São Paulo State, Brazil (SP 356.472).

Extraction and phytochemical analysis

The crude extract (LE, 1.0 kg) was obtained by percolation of air-dried powdered leaves (2.9 kg) with 70% ethanol (elution rate: 3 mL $min.^{-1}$; 25 ºC), concentration (40 ºC; low pressure), and lyophilization to yield the dried crude extract (LE) (1.0 kg). One hundred grams of LE was dissolved in aqueous methanol 50% (1.0 L) and subsequently partitioned as previously described, to yield, after evaporation of solvents (25 ºC; reduced pressure) and lyophilization, the ethyl acetate fraction (EF, 15.9%) and the methanol fraction (MF, 73%) (Ishikawa et al., 2014). After chromatographic analysis of LE, the isolated substances were identified by comparison of observed spectral data (NMR) with those reported in the literature and confirmed by HPLC-MS co-elution with authentic samples (Azevedo et al., 2016).

Oxygen radical absorbance capacity – Orac assay

The Orac assay has been performed as previously reported by Noriega et al. (2012). In microplates, the LE (5–20 μg mL^{-1}) or the reference drug Trolox (Sigma, 6.25–100 μg mL^{-1}) was added to fluorescein solution (40 nM in PBS) in triplicate. Blank and negative control consisted of solvent, and solvent plus fluorescein. After incubation (37 °C, 30 min.), 2.2'-azobis(2-amidino-propane) dihydrochloride (AAPH, 153 mM in PBS) was added to all the wells except in the blank one. The plate reader (Synergy HT Multi-Detection Microplate Reader) with 485 nm 20 nm^{-1} bandpass excitation filter and 528 nm 20 nm^{-1} bandpass emission filter was programmed to record the fluorescence at every minute. Trolox was used as standard and the results were expressed as μmol Trolox equivalents per gram of LE (μmol TE g^{-1}).

CHO cytotoxicity assay

The determination of the cytotoxicity of LE, EF and MF was carried out following the method previously reported by Santos et al. (2006). Microplates with Chinese hamster ovary cells (ATCC CHO K1) (~3000 cells) were exposed to the diluted sample (0.98-125.0 μg mL^{-1} in DMSO) for 72 hours at 37 ºC in a 5% CO_2 humidified atmosphere. Cells were exposed to maximum 0.1% (v v^{-1}) DMSO concentration, which was shown to be nontoxic. Afterwards cells were submitted to 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium bromide (MTT) assay for cell viability quantification at 490 nm. The IC_{50} values (concentration to provoke 50% inhibition of cell viability) were calculated by linear regression, and data were reported as mean in quadruplicates ± standard error of the mean (S.E.M.). The comparison between groups was performed by one-way analysis of variance (ANOVA) followed by Tukey's contrast, considering p-values less than 0.05 significant.

Cancer cells cytotoxicity assay

The antiproliferative assay of LE, EF and MF was carried out according to Cabral et al. (2020). Human tumor cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-460 (lung, non-small cells), Ovcar-03 (ovarian), PC-3 (prostate), HT-29 (colon), 786-0 (renal), NCI-ADR (breast expressing phenotype multiple drugs resistance) and K-562 (leukemia) were kindly provided by the National Cancer Institute (NCI). In microplates, cancer cells (100 μL cells $well^{-1}$) were exposed to diluted sample (0.25-250 μg mL^{-1}) or to the reference drug doxorubicin (Boehringer-Ingelheim) in DMSO/RPMI for 48 hours at 37 ºC in a 5% CO_2 humidified atmosphere. The DMSO used in dilution did not affect cell viability. Cells were then fixed with trichloroacetic acid 50%, and cell proliferation quantification was determined by sulforhodamine B (SRB) assay at 540 nm. Through non-linear regression analysis using software Origin 8.0 (OriginLab Corporation), the concentration of sample that has

inhibited cell growth by 50% (GI_{50}) was determined for each cell line. The results represent data from two independent experiments, each done in triplicate.

Salmonella mutagenicity assay

The *Ames* test was performed according to Morandim-Giannetti et al. (2011) using *S. typhimurium* strains TA97a, TA98, TA100 and TA102, kindly provided by Dr. B.N. Ames (University of California, Berkeley, CA), with (+) and without (-) metabolic activation (S9, Aroclor-1254-induced rat liver fraction, Moltox).

The extracts were dissolved in DMSO, and the concentrations were selected according to a preliminary toxicity test (2.5-20 mg plate^{-1}). The mutagenic substances used as positive controls in the experiments without S9 mix were 4-nitro-*o*-phenylenediamine (Sigma, 10 μg plate^{-1}) for TA97a and TA98, sodium azide (Sigma, 1.25 μg plate^{-1}) for TA100 and daunomycin (Sigma, 3 μg plate^{-1}) for TA102. In the experiments with metabolic activation the positive control consisted of 2-anthramine (Sigma, 1.25 μg plate^{-1}). DMSO was used as negative (solvent) control.

We added either the samples, DMSO or the positive controls to PBS (0.2M, pH 7.4) or S9 mix. The bacterial culture was added and then incubated at 37ºC for 20–30 min. Afterwards top agar was added, and the mixture was transferred to a plate containing minimum agar. The His+ revertant colonies were manually counted after incubation at 37°C for 48 hours. All the experiments were performed in triplicate. The statistical analysis was performed with the Salanal software, and the mutagenic index (MI), that is, the ratio between the number of revertants per plate by the number of revertants per plate in the negative control (solvent) was calculated for each dose. A sample was considered mutagenic when its MI was ≥ 2.

Results and discussion

The determination of the cytotoxicity was carried in the CHO non-neoplastic cell line and the LE, EF and MF of *P. edulis* have shown cytotoxicity in a concentration-dependent way, with IC_{50} values of 6.3± 0.2, 10.6±0.3 and 8.8±0.3 μg mL^{-1} respectively (Table 1). Further, at the concentration of 1.0 μg mL^{-1}, the extract and fractions have shown significant proliferative activity compared to the control ($p < 0.001$). The same was observed at the concentration of 3.9 μg mL^{-1} of EF.

According to the protocol of National Cancer Institute (NCI) for antiproliferative screening of new anticancer drugs, the cancer cells cytotoxicity assay of LE, EF and MF was carried out against nine Human tumor cell lines. The cytostatic effect of each sample was calculated as GI50 (concentration that inhibits 50% of cell growth) and it was considered weak (1.1 < log GI50 < 1.5), moderate (0 < log GI50 < 1.1) and potent (log GI50 < 0), following NCI's criteria (Cabral et al., 2020).

The MCF-7 was the most sensitive cancer cell line to the *P. edulis* tested extracts (GI50 < 5 μg mL^{-1}) (Table 2). The EF was the most active extract, presenting potent to moderate antiproliferative activity against the strains of breast cancer (MCF-7), prostate cancer (PC-03), colon cancer (HT-29), non-small cell lung cancer (NCI-460), melanoma (UACC-62) and leukemia (K-562) (GI50 0.01-8.57 μg mL^{-1}), and was roughly as potent as doxorubicin against MCF-7 cells. The LE displayed selectivity against breast cancer expressing phenotype multiple drugs resistance (NCI-ADR), and MF was selective against melanoma cells (UACC-62).

In accordance with these results, Carvalho et al. (2012) have also observed that the infusion of *P. edulis* promoted cytotoxicity to MCF-7 neoplastic cells with a mechanism of action that seemed to include the induction of apoptosis, changes in cell rounding-up, shrinkage, nuclear condensation and reduction of colony and cell diameter with a maximum effect at 5 μg mL^{-1}.

Table 1. Cytotoxicity evaluation of 0.1% DMSO (control), crude extract (LE), ethyl acetate fraction (EF) and methanolic fraction (MF) of *Plinia edulis* in Chinese hamster ovary cells (CHO) and concentration to cause 50% decrease of cell viability (IC_{50} in μg mL^{-1}).

Concentration (μg mL^{-1})	Cell Viability (%)		
	LE	EF	MF
1.0	124.31±1.49*	128.41±3.06*	156.69±1.96*
3.9	60.28±1.67	124.79±3.28*	90.67±2.52
7.8	16.34±1.00	55.95±2.10	22.66±2.05
15.6	14.56±0.66	15.79±0.19	14.37±0.51
31.3	1.44±0.38	0.66±0.18	4.00±0.95
IC_{50} (μg mL^{-1})	6.28±0.22	10.62±0.26	8.75±0.27
0.1% DMSO	100.75±1.09		

The results were obtained from two independent experiments, each done in quadruplicates, and they are presented as mean ± SD values. Asterisks indicate significant difference from control (100% cell viability) (ANOVA followed by Tukey test): $p < 0.001$.

Table 2. Concentration of doxorubicin (reference drug), crude extract (LE), ethyl acetate fraction (EF) and methanolic fraction (MF) of *Plinia edulis* that elicits cancer cell growth inhibition by 50% (GI_{50} in μg mL^{-1}).

Cancer cell lines*	LE	EF	MF	doxorubicin
UACC-62	21.77	2.88	5.45	0.05
MCF-7	0.02	0.01	4.58	0.05
NCI-ADR	7.27	18.71	28.72	1.20
786-0	29.09	23.53	30.85	0.23
NCI-460	> 250.0	5.14	31.72	2.20
PC-03	25.54	0.77	32.38	0.25
Ovcar-03	29.68	44.62	33.22	4.59
HT-29	26.12	8.57	27.40	0.92
K-562	47.68	3.37	249.95	0.07

GI_{50} values were obtained through non-linear regression analysis from two independent experiments, each done in triplicate. Dose range tested: 0.25 to 250.00 μg mL^{-1}. *UACC-62, melanoma; MCF-7, breast; NCI-ADR, breast, multidrug- resistant; 786-0, kidney; NCI-460, lung, non-small cells; PC-03, prostate; Ovcar-03, ovary; HT-29, colon; K-562, leukemia.

Phytochemical analysis of LE has revealed the presence of triterpenes (β-amyrin, lupeol, ursolic acid, maslinic acid and corosolic acid) and flavonoids (quercitrin, myricitrin and quercetin) as previously reported for others, *P. edulis* extracts from leaves and fruit peels and for medicinal species of the same genus (Azevedo et al., 2016; Nesello et al., 2018; Santos et al., 2020).

Both flavonols and triterpenoids are substances with pharmacological interest as chemo preventive agents. Quercetin, for instance, is considered a natural antioxidant chemo preventer and can control many tumor-related events, such as angiogenesis, cell cycle, proliferation, metastasis, and apoptosis (Almatroodi et al., 2021). Pentacyclic triterpenoids, such as ursolic acid, are considered anticancer leads from higher plants, since they can modulate the human immune system, induct autophagy, and regulate many inflammation-related signaling pathways (Ren & Kinghorn, 2019).

At lower concentrations, LE and EF of *P. edulis* showed no evidence of cytotoxicity against CHO non-neoplastic cells, but significant proliferative activity compared with the control while, in tumor cell lines, at these concentrations, both extracts were able to inhibit the growth of MCF-7 cells. EF has also showed antiproliferative activity to strains of prostate cancer (PC-03), melanoma (UACC-62) and leukemia (K-562) at concentrations lower than that capable of causing the growth of non-neoplastic cells, demonstrating a possible selective cytotoxicity for cancer cells.

In fact, compounds present in the LE, such as quercetin, lupane-, oleanane- and ursane-type triterpenoids, have shown *in vitro* selective cytotoxicity against cancer cells, without alteration of normal tissues (Ren & Kinghorn, 2019; Mokhtari, Pérez-Jiménez, García-Salguero, Lupiáñez, & Rufino-Palomares, 2020; Almatroodi et al., 2021). Besides, many pentacyclic triterpenes, such as lupeol, improve fibroblast proliferation *in vitro* and *in vivo*, which may be related to the healing of gastric ulcers (Ghiulai et al., 2020), and, consequently, can be associated to the traditional use of *P. edulis* as a gastroprotector.

Since mutagenesis and carcinogenesis are closely related, the mutagenicity evaluation of medicinal plants should be recommended for detecting potential genotoxic compounds, and the *Ames* assay is a widely accepted method for that purpose (Dantas, Castilho, Almeida-Apolonio, Araújo, & Oliveira, 2020). Analysis of mutagenic activity of *P. edulis* showed a mutagenicity index < 2 for all tested doses, revealing that the extract did not show potential to cause base pair substitution mutations and frameshift (Table 3) (Palozi et al., 2020).

The acute toxicological evaluation of *P. edulis* has not shown toxic effects in mice at oral doses of 5 g kg^{-1} (Ishikawa et al., 2008) and, considering the present results of *Ames* assay, it may corroborate the safety in the traditional use of this species, although LE has significantly increased the number of revertants at TA97a and TA98, with and without metabolic activation, which reinforces caution in the long-term usage of this species.

Oxidative stress has been related to the accumulation of mutations in the genome and thus it is closely associated with carcinogenesis (Sharifi-Rad et al., 2020). Indeed, ROS overproduction plays crucial role in cancer development, leading to cell damage, metastatic potential and it contributes with invasiveness and cancer cell migration through many different mechanisms (Sharifi-Rad et al., 2020).

The Oxygen Radical Absorbance Capacity (Orac) assay is a widely accepted method, since it mimics the antioxidant activity *in vivo*, using an important biological radical source and analyzing degree and time of inhibition (Barros et al., 2019; Schmidt et al., 2020).

The LE showed Orac value of 3948±225 μmol TE g^{-1}, higher antioxidant activity than *P. edulis* fruits (28.11±4.60 μmol TE g^{-1} dry weight) (Silva et al., 2019) and others *Plinia* species like *P. peruviana* (1941±718 μmol TE g^{-1} of leaf extract) (Moraes et al., 2021) and *P. cauliflora* (841±21 μmol TE g^{-1} of peel extract) (Barros et al., 2019).

Table 3. Mutagenic activity of *Plinia edulis* hydroalcoholic extract in the presence (+) and absence (-) of metabolic activation (S9).

Treatment (mg plate^{-1})	Number of revertants/plate in *Salmonella typhimurium* strains							
	TA97a		TA98		TA100		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	107±1	214±13	25±2	77±6	144±5	127±7	244±24	363±9
2.5	175±10 (1.6)*	301±8 (1.3)**	25±2 (1.0)	69±1 (0.9)	126±4 (0.9)	124±5 (1.0)	313±12 (1.3)	354±13 (1.0)
5.0	166±7 (1.6)**	328±6 (1.5)**	30±2 (1.2)	88±1 (1.1)	131± 7 (0.9)	137±6 (1.1)	373±4 (1.5)	353±17 (1.0)
10.0	180±7 (1.7)**	263±8 (1.2)*	42±2 (1.7)**	89±2 (1.2)	121±1 (0.8)	133±4 (1.1)	296±18 (1.2)	363±15 (1.0)
15.0	195±13 (1.8)*	295±4 (1.4)**	48±2 (1.9)**	90±4 (1.2)	156±11 (1.1)	133±8 (1.1)	280±8 (1.1)	320±13 (0.9)
20.0	192±3 (1.8)**	209±9 (1.0)	31±5 (1.3)	101±2 (1.3)*	115±7 (0.8)	136±7 (1.1)	237±9 (1.0)	380±8 (1.0)
Control +	1716±69	1547±27	2509±46	1352±66	784±7	1696±62	2324±119	1637±20

Data are mean of triplicates ± SD values (mutagenic index). The negative control (0) was dimethyl sulfoxide (100.0 μL plate^{-1}). In the presence of metabolic activation, the positive control was 2-anthramine (1.25 μg plate^{-1}) for all the strains. In the absence of metabolic activation, the positive control was 4-nitro-*o*-phenylenediamine (10.0 μg plate^{-1}) for TA97a and TA98, sodium azide (1.25 μg plate^{-1}) for TA100 and daunomycin (3.0 μg plate^{-1}) for TA102. Statistically different values by ANOVA are indicated: *p < 0.05; **p < 0.01.

The flavonoids and triterpenoids present in the LE, are probably related with the high antioxidant activity, since compounds such as maslinic acid, quercetin and quercitrin have potent antioxidant capacity and free radical scavenging properties, being capable to decrease intracellular Reactive Oxygen Species (ROS) and to protect the cells against oxidative damage (Mokhtari et al., 2020; Almatroodi et al., 2021; Xiong et al., 2021).

Consistent data show that the concurrent use of antioxidants in radiation and chemotherapy, not only increases the effectiveness of conventional treatments, but also decreases their side effects (Fernández et al., 2021). Therefore, plant extracts, such as LE from *P. edulis*, which has both oxygen radical absorbance capacity and anticancer activity, could be a potential adjuvant in cancer therapy and chemoprevention.

Conclusion

Plinia edulis leaf extract and fractions do not have shown signs of mutagenicity at the *Ames* test and have exhibited proliferative activity in non-neoplastic cells and antiproliferative activity against strains of breast cancer, prostate cancer, colon cancer, lung cancer, melanoma, and leukemia. Besides, the present results have revealed high antioxidant activity of LE and the presence of substances with pharmacological interest, such as flavonoids and triterpenes. This study suggests a potential use of 'cambucá' in chemoprevention and the necessity of further studies to investigate the mechanisms involved in the possible selective cytotoxicity for neoplastic cells.

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