Preliminary evaluation of antioxidant, antiproliferative and antimutagenic activities of pitomba (*Talisia esculenta*)

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Iramaia Angélica Neri-Numa [a,*], Luciano Bruno de Carvalho-Silva [b],
Jose Emilson Macedo Ferreira [a], Ana Rita Tomazela Machado [b], Luciana Gomes Malta [a],
Ana Lucia Tasca Gois Ruiz [c], João Ernesto de Carvalho [c], Glaucia Maria Pastore [a]

[a] *Department of Food Science, Faculty of Food Engineering, University of Campinas, Brazil*
[b] *School of Nutrition, Federal University of Alfenas, Brazil*
[c] *Center for Chemistry, Biology and Agriculture, State University of Campinas, Brazil*

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ABSTRACT

The present study provides preliminary data about the total polyphenols and flavonoids content, anti-oxidant, antiproliferative, and antimutagenic activities of crude extract of pitomba (*Talisia esculenta*). Levels of flavonoids aglycons obtained after acid hydrolysis to myricetin and quercetin by HPLC were 89.90 mg/100 g and 30.20 mg/100 g, respectively. Total polyphenols and flavonoid contents found were 105.84 ± 2 .05 mg GAE/g and 88.05 ± 0.04 mg CE/g for the extract, respectively. Average values for the $DPPH_{IC50}$, TEAC using ABTS and T-ORAC assays were 9.56 ± 6.75 µg/mL, 115.69 ± 4.23 TE µg/g and 1692.96 µmol TE/100 g, respectively. Regarding the ability to inhibit the growth of tumor cell lines, we observed an antiproliferative activity against UACC-62 (2.6 µg/mL), NCI-ADR/RES (3.1 µg/mL), and OVCAR-3 (3.1 µg/mL) cell lines. By micronucleus test, our extract showed anticlastogenic activity at all concentration level tested. These results suggest that *T. esculenta* extract has great antioxidant potential that could be used as a preventive agent for cancer. However, additional studies are needed to evaluate the mechanism of action of these compounds as well as the adverse effects in other bioassay systems and animal models.

1. Introduction

Talisia esculenta- ST. HIL (RADLK- SAPINDACEAE)) knowed as pitomba is a member of the Sapindaceae family which occurring from temperate to tropical regions throughout the world. This family include edible species, e.g. lychee (*Litchi chinensis*) and guaraná (*Paullinia cupana*) (Díaz & Rossini, 2012; Majhenič, Škerget, & Knez, 2007). Nevertheless, some species this family produce in different phenological stages (including fruits in some cases), bioactive compounds with medicinal or toxicological properties.

Otherwise, crude extracts, as well as isolated flavonoids from plants belonging to this family, have been investigated for their antioxidant, anti-diabetic, anti-inflammatory and anti-viral properties in many parts of the world (Díaz & Rossini, 2012; Napolitano et al., 2005; Tsuzuki et al., 2007). These capabilities are in some cases accounted for isolated phenolic compounds such as preny-lated flavonoids, but in many cases, it is still unknown which are the active principles (Acevedo-Rodríguez, Van Welzen, Adema, & van Der Ham, 2011; Díaz & Rossini, 2012).

P. cupana seeds powder for example, have many positive effects on human health, such as stimulating effects on the nervous and cardiovascular system (de Lima Portella et al., 2013; Majhenič et al., 2007). Similarly, some studies have demonstrated that phenolic compounds exist in the aril and pericarp of *L. chinensis* can quench reactive free radicals, and act as antioxidants or agents of other mechanisms, which contribute to their anti-carcinogenic, anti-in-flammatory and cardio-protective effects, and prevention of degenerative diseases (Liu, Lin, Wang, Chen, & Yang, 2009).

However *T. esculenta* still underexploited may have potential bioactive compounds and biological effects. Based on the hypothesis that allied species may contain similar antioxidant compounds the aim of this study was to provides preliminary data which could be used as evidence of antioxidant, antiproliferative and antimutagenic activities. Thus, effects of extract as part of a screening effort for Amazon fruits with biological activity for future work can be further extended to use them for possible applications as natural antioxidant for supplements and functional ingredients for food products.

2. Materials and methods

2.1. Chemicals

All chemical used in this study were either analytical reagent or HPLC grade and were given as: 2,2-diphenyl-1-picrylhydrazil (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,20-azobis(2-methylamidinopropane)-dihydrochloride (AAPH), sodium fluorescein, cyclophosphamide monohydrate, gentamicin, doxorubicin, fetal bovine serum and RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO, USA); absolute ethanol, methanol and dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany).

2.2. Extraction method

The extraction was performed according to Roesler, Malta, Carrasco, and Pastore (2006). The whole fruits were homogenized with aqueous ethanol (5:95 v/v, water, ethanol) for 20 min, and after the sample was concentrated using a vacuum rotary evaporator under reduced pressure at 40 °C, lyophilized, pulverized and stored at −20 °C in dark glass bottle. For the lipophilic fraction was homogenized with hexane (1:3 w/v) using a blender for 20 min. Residual hexane was evaporated and the residue was pulverized and kept in the dark at −20 °C until analysis.

2.3. Flavonoid extraction and hydrolysis by HPLC analysis

The simultaneous extraction, hydrolysis and HPLC analysis were based on the method described by Hoffman-Ribani, Huber, and Rodrigues Amaya (2009). This analysis was performed in Waters system, equipped with a Rheodyne injection valve with a 5 μL fixed loop, a quaternary pump (Waters model 600) and a UV–VIS photodiode array detector (Waters model 996) controlled by a Millennium workstation (version 32). A Symmetry C-18 (2.1 mm × 150 mm, 3.5 μm) Waters column was used, the mobile phase consisting of methanol and water, both acidified with 0.3% formic acid, and the flow rate was 0.2 mL per minute. A multilinear gradient was used and the analytes were monitored from 200 to 600 nm and detection at 370 nm was used for quantification, which was done by external standardization.

The linearity of the HPLC method was checked for flavonoids in the 0.03–4.8 mg/100 mL range (0.06–2.3, 0.03–40.8 mg/100 mL for myricetin and quercetin, respectively). Calibration was performed by injecting the standard working solution in triplicate at five different concentrations for each flavonoid, based on the expected flavonoid content ranges in the samples. All standard curves passed through the origin, were linear in the concentration ranges expected in the samples, with coefficients of determination ranging from 0.99 for quercetin.

2.4. Determination of total phenolics and flavonoids contents

TP were determined using the Folin–Ciocalteu method (Roesler, Catharino, Malta, Eberlin, & Pastore, 2007), and the results were expressed as mg GAE/g dried extract. Standard concentrations of gallic acid (0.625–30 μg/mL) were used to prepare a calibration curve. Furthermore, FC were measured using a colorimetric assay (Zhishen, Mengcheng, & Jianming, 1999) and used catechin as a standard. The results were expressed as mg CE/g dried extract.

2.5. Antioxidant activity

2.5.1. DPPH• scavenging assay

Free radical-scavenging activity was measured using a method adapted by Brand-Williams, Cuvelier, and Berset (1995). In brief, 50 μL of extract in different concentrations (0.09–100 mg/mL) was mixed with 250 μL of DPPH (0.004% w/v). After 30 min of the reaction, the absorbance of the remaining DPPH was measured at 517 nm against blank.

2.5.2. ABTS•+ assay

The radical cation ABTS•+ was chemically generated with potassium persulfate from ABTS, as described by Re et al. (1999). The AA of extract, was measured against a Trolox standard at 734 nm and expressed as TEAC.

2.5.3. ORAC assay

The $ORAC_{FL}$ assay was described by Ou, Huang, Hampsch-Woodill, Flanagan, and Deemer (2002) and modified by Dávalos, Gómez-Cordovés, and Bartolomé (2004). The automated ORAC assay was performed on a NovoStar Microplate reader (BMG Labtech, Germany) with fluorescence filters (excitation, λ 485 nm; emission λ 520 nm). The experiment was conducted at 37 °C under pH 7.4 condition with a blank sample in parallel. The final results were calculated using the differences of areas under FL decay curves between the blank (net AUC) and sample and were expressed as μmol/TE/g of extract.

2.6. Antiproliferative activity

Human tumor cell lines U251 (glioma, CNS) UACC-62 (melanoma), MCF-7 (human breast adenocarcinoma), NCI-ADR/RES (breast expressing phenotype multiple drug resistance), 786-0 (kidney), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (human colon adenocarcinoma) and Vero (kidney, non cancer cell line, African green monkey), were kindly provided by the National Cancer Institute (NCI-USA). Stock cultures were grown in a medium containing 5 mL of RPMI 1640 (GIBCO BRL) supplemented with 5% of fetal bovine serum. Gentamicin (50 μg/mL) was added to experimental cultures. Cells in 96 well plates (100 μL cells/well) were exposed to sample concentrations in DMSO/RPMI (0.25, 2.5, 25, and 250 μg/mL) at 37 °C, 5% of CO_2 in air for 48 h. Final DMSO concentration did not affect cell viability. Afterwards, cells were fixed with 50% trichloroacetic acid and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content, employing sulforhodamine B assay (Monks et al., 1991). Using concentration–response curve for each cell line, TGI (concentration that produces total growth inhibition) was determined through nonlinear regression analysis, utilizing software ORIGIN 7.5 (OriginLab Corporation) (Shoemaker, 2006).

2.7. Animals and in vivo study

2.7.1. Animals

Newly weaned male Swiss albino mice were obtained and maintained under controlled conditions of temperature (22–24 °C), light (12 h light/12 h dark), and humidity (45–65%), with food and water *ad libitum*. All mice used for experimental research had body
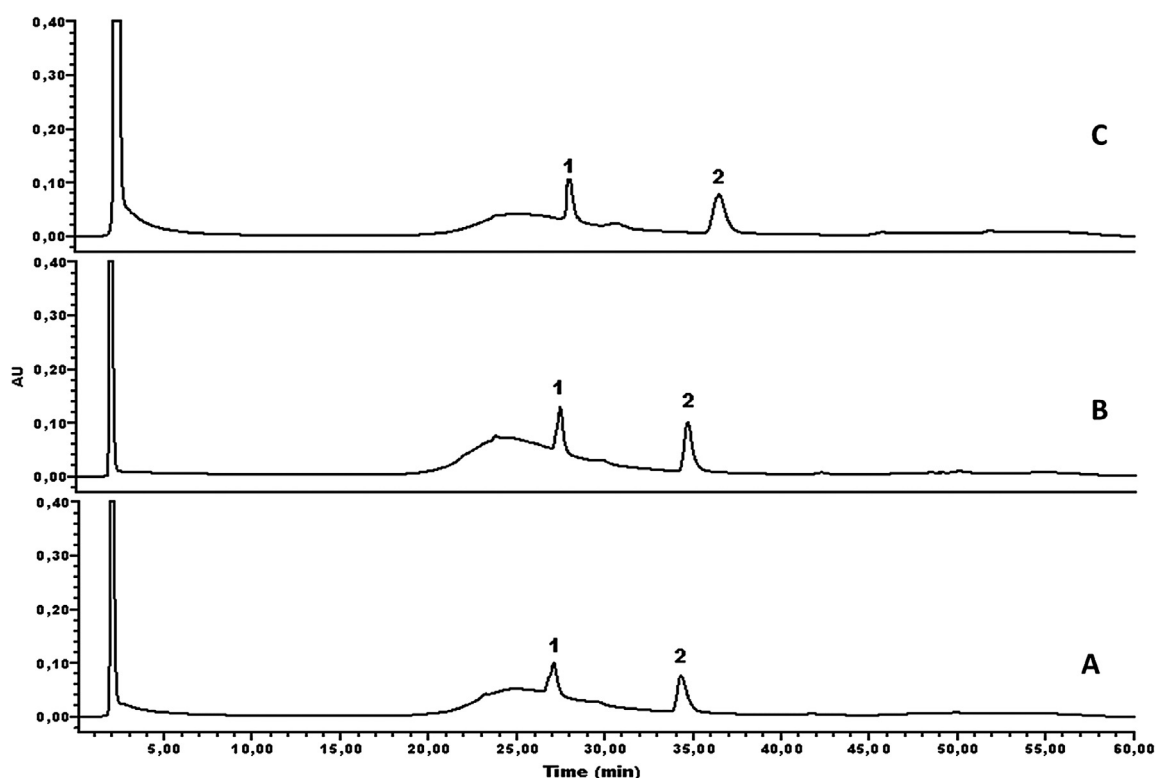
Fig. 1. Separation of Pitomba (*T. esculenta*), by HPLC with diode array detection (370 nm). 1. Myricetin; 2. quercetin after hydrolysis to obtain the flavonoid aglycone forms in A) 35.0 min of hydrolysis; B) 37.5 min of hydrolysis; C) 40.0 min of hydrolysis. The levels of myricetin and quercetin obtained were 89.90 ± 4.81 mg/100 g and 30.20 ± 0.25 mg/100 g, respectively. Range (mean ± standard deviation), in mg/100 g for the fruit pulp analyzed in triplicate.

weight between 25 and 35 g. The UNICAMP Animal Bioethical Committee approved this study under protocol number 2362-1 in accordance with the Brazilian Society of Science in laboratory animals.

2.7.2. Micronucleus test (MN)

The MN test was performed according MacGregor et al. (1987). To investigate the protective effect of *T. esculenta* extract against the clastogenicity induced by cyclophosphamide (CP). Fruit extract was administered at dose of 100 μL by orogastric gavage for 15 consecutive days, at concentrations of 30, 100 and 300 mg/kg body weight, selected on the basis of our acute toxicity studies in mice, which was higher than 1000 mg/kg. The negative control group received 0.9% NaCl. The positive control group received (CP) at a dose of 50 mg/kg body weight. The bone marrow cells were collected with bovine serum fetal with the help of a syringe and cell suspension were prepared on clean glass slide and stained with Leishman's stain. All the slides were analyzed in blind test by light

microscope at 1000× magnification. The frequency of micro-nucleated polychromated erythrocytes (MNPCEs) in each mice was used as the experimental unit. The number of micronucleated normochromatic erythrocytes (MNNCEs) was registered in a total of 1000 normochromatic erythrocytes (NCEs) per animal. Toxicity to bone marrow was estimated by the relationship between the frequency of PCEs and NCEs. The ratio of PCEs to NCEs was determined in the first 1000 erythrocytes scored per coded slide by two independent microscopists.

2.7.3. Statistical analysis

SPSS for Windows version 15.0 was used for data analysis (Norussis, 2006). Statistically significant differences between groups were calculated by the application of the one way analysis of variance (ANOVA) for unpaired observations between controls and experimental samples. Tukey test was used for multiple comparisons: p values of 0.05 or less were considered statistically significant. All tests were performed in triplicate. Correlations between

Table 1
Total phenolics. flavonoids content and antioxidant capacity of *Talisia esculenta versus* antioxidant standard.

Sample	TP (mg GAE/g)	FC (mg CE/g)	DPPH$_{IC50}$ (μg/mL m/v)	ABTS$^{•+}$ (TE μg/g)	H-ORAC$_{FL}$ (μmol TE/g)	L-ORAC$_{FL}$ (μmol TE/g)	T-ORAC$_{FL}$ (μmol TE/g)
T. esculenta	105.84 ± 2.05	88.05 ± 0.4	9.56 ± 6.75[a]	115.69 ± 4.23	180.23 ± 2.06[a]	1510.01 ± 0.75	1692.96
Ascorbic acid	n.a	n.a	10.46 ± 0.17[a]	n.a	6.81 ± 0.53[b]	n.a	n.a
Kaempferol	n.a	n.a	7.66 ± 0.36[b]	n.a	16.11 ± 0.48[b]	n.a	n.a
Gallic acid	n.a	n.a	5.18 ± 0.26[cd]	n.a	9.15 ± 0.79[b]	n.a	n.a
Myricetin	n.a	n.a	6.07 ± 0.52[c]	n.a	9.57 ± 1.24[b]	n.a	n.a
Quercetin	n.a	n.a	4.33 ± 0.28[d]	n.a	18.61 ± 1.74[b]	n.a	n.a

Data was expressed as mean of triplicate ± standard deviation measurements. n.a = not available; CE = catechin equivalents; GAE = gallic acid equivalents; FC = flavonol contents; TP = total phenols; DPPH$_{IC50}$ = radical scavenging activity expressed as final concentration of extract in the cuvettes defined as μg/mL of dried extract required to decrease the initial DPPH concentration by 50%; H-ORAC$_{FL}$ = hydrophilic; L-ORAC$_{FL}$ = lipophilic. T-ORAC$_{FL}$ = total antioxidant capacity; calculated as the sum of H-ORAC$_{FL}$ and L-ORAC$_{FL}$. Equal letters in the same column are statistically equals if $p < 0.05$ by Tukey test.

Table 2
Comparative total growth inhibition (TGI) in human tumoral cell lines.

	TGI_{50} (mg/mL)									
	2	u	m	a	7	4	p	o	h	v
Doxorubicin	0.040	0.30	0.025	0.24	0.028	<0.025	0.15	0.31	0.14	1.0
Talisia esculenta	30.8	2.6	23.8	3.1	29.9	26.1	44.8	3.1	35.6	29.2

2 = U251 (glioma, SNC); u = UACC-62 (melanoma); m = MCF-7 (breast adenocarcinoma); a = NCI-ADR/RES (ovary, multidrug resistance phenotype); 7 = 786-0 (kidney); 4 = NCI-H460 (lung, non-small cell adenocarcinoma); p = PC-3 (prostate); o = OVCAR-3 (ovary); h = HT-29 (colon); v = VERO (kidney, normal cell of green monkey).

various parameters were also investigated. Significance was determined at $p < 0.05$. All data were reported as the mean ± standard deviation (SD) for three replications.

3. Results and discussion

3.1. Flavonoid extraction and hydrolysis by HPLC analysis

The levels of myricetin and quercetin in *T. esculenta* obtained were 89.90 ± 4.81 mg/100 g and 30.20 ± 0.25 mg/100 g, respectively (Fig. 1). According to the existing literature, the investigation of this fruit is limited and nothing appears to have been published about the flavonoid contents. Although other species have been investigated and detected flavonoid aglycones as myricetin, quercetin and luteolin (Chen, Lin, Liu, Lu, & Yang, 2011; Rashed et al., 2013).

3.2. Total phenolics, flavonoids contents and antioxidant activity

TP, FC content and AC of *T. esculenta* are reported in Table 1.
TP and FC were 105.84 ± 2.05 mg GAE/g and 88.05 ± 0.04 mg CE/g for the extract, respectively. Previous studies reported the amount of TP of *P. cupana* seed extracts ranged from 119 to 186 mg GAE/g extract and the free and bound phenolic contents of 13 litchi varieties ranged from 66.17 to 226.03 mg GAE/100 g, respectively (Majhenič et al., 2007; Zhang et al., 2013). According Vasco, Ruales, and Kamal-Eldin (2008) TP varied among fruit species and were classified into three categories: low (<100 mg GAE/100 g), medium (100–500 mg GAE/100 g) and high (>500 mg GAE/100 g) for samples based on fresh matter. So, according this classification *T. esculenta* fits in the medium category.

About the free radicals scavenging assays *T. esculenta* showed 9.56 ± 6.75 μg/mL and 77.3 μg/mL to $DPPH_{IC50}$ and ABTS, respectively. We also observe that our results were similar the antioxidant capacity to *Dimocarpus longan* and *Cardiospermum canescens* (Rangkadilok et al., 2007; Udhayasankar, Danya, Punitha & Arumugasamy, 2013).

The total $ORAC_{FL}$ was 1692.96 μmol TE/100 g, but a previous study reported better results in longan flower methanolic extract at 6.19 ± 0.05 mmol TE/mL (Hsieh, Shen, Kuo, & Hwang, 2008). However the highest ORAC value are still attributed to green tea, where range from 1239 to 1686 TE/g tea (Henning et al., 2003). Probably, our method of extraction may influence the results and in the future will be required multiple extractions to increase the efficiency of extraction of compounds of interest.

3.3. Antiproliferative activity

The IC_{50} values of the *T. esculenta* extracts are shown in Table 2. The percentage of growth for each cell in the presence of different concentrations of positive control doxorubicin (A) and *T. esculenta* (B). A horizontal line at 0% was traced to visualize total growth inhibition (TGI), *i.e.* the concentration required to completely inhibit cell growth (total cytostatic effect), which is show in Table 2.

The negative values represent the percentage of cell death. It can be seen that great part of the cell lines are sensitive to doxorubicin, with TGI in a concentration range of <0.025–0.3 μM.

In order to consider the extract as active, *i.e.*, antiproliferative, it must present growth inhibition higher than 50% and show a dose-dependent pattern, showing selectivity for the cell types (Fig. 2).
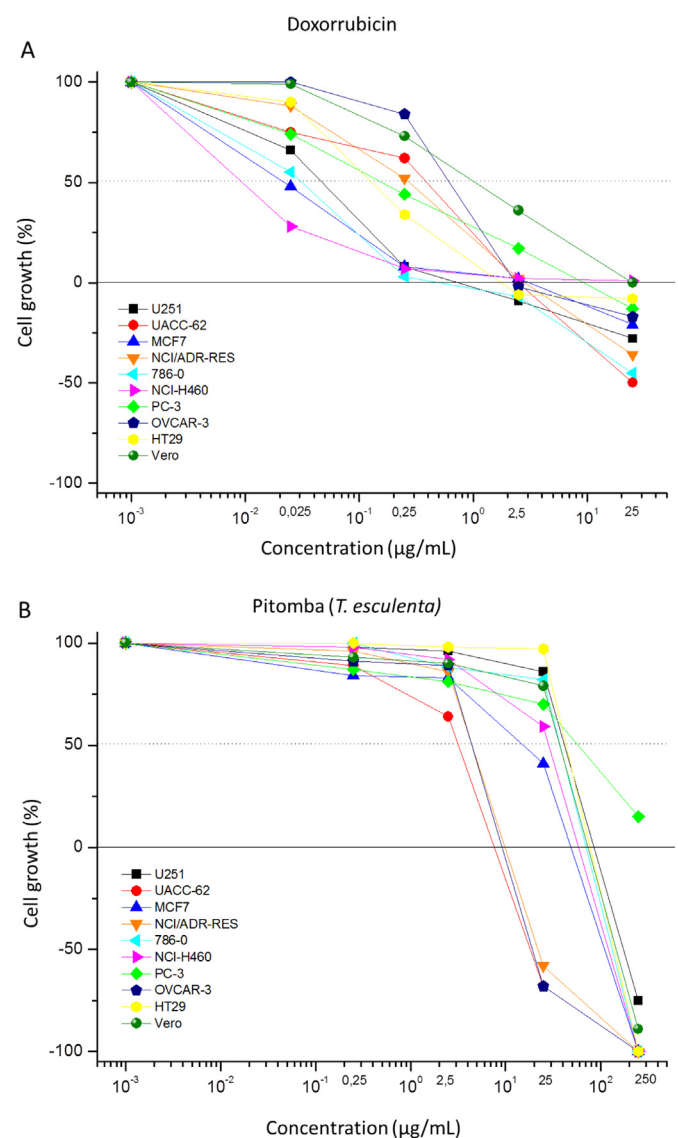

Fig. 2. Antiproliferative activity of doxorubicin (A) and (B) pitomba (*T. esculenta*), against nine cancerous cell lines: 2 = U251 (glioma, SNC); u = UACC-62 (melanoma); m = MCF-7 (breast adenocarcinoma); a = NCI-ADR/RES (ovary, multidrug resistance phenotype); 7 = 786-0 (kidney); 4 = NCI-H460 (lung, non-small cell adenocarcinoma); p = PC-3 (prostate); o = OVCAR-3 (ovary); h = HT-29 (colon); v = VERO (kidney, normal cell of green monkey).

Table 3
Effect of treatment with *Talisia esculenta* on micronucleus test induced by cyclophosphamide in bone marrow cells of Swiss mice.

Talisia esculenta Group	Treatment	Number of analyzed cells	MNPCEs No.	MNPCEs %*	% Reduction
G1	NaCl + CP	12.000	122	71.0	
G2	NaCl + NaCl	12,000	20	71.0	73.26
		24,000	142		
G3	30 mg/kg extract + CP	10000	9	59.5	
G1	NaCl + CP	12,000	122	71.4	78.66
		22,000	131		
G4	30 mg/kg extract + NaCl	12,000	24	22.0	
G2	NaCl + NaCl	12,000	20	22.0	0.36
		24,000	44		
G5	100 mg/kg extract + CP	10,000	33	70.45	
G1	NaCl + CP	12,000	122	84.55	36.49
		22,000	155		
G6	100 mg/kg extract + NaCl	12,000	29	24.5	
G2	NaCl + NaCl	12,000	20	24.5	1.65
		24,000	38		
G7	300 mg extract + CP	12,000	10	66.0	
G1	NaCl + CP	12,000	122	66.0	95.03
		24,000	132		
G8	300 mg/kg extract + NaCl	12,000	23	21.5	
G2	NaCl + NaCl	12,000	20	21.5	0.21
		24,000	43		
G3	30 mg/kg extract + CP	10,000	9	21.0	
G5	100 mg/kg extract + CP	10,000	33	21.0	13.71
		20,000	42		
G3	30 m/kg extract + CP	10,000	9	8.64	
G7	300 mg/kg extract + CP	12,000	10	10.36	0.03
		22,000	19		
G5	100 mg/kg extract + CP	10,000	33	19.55	
G7	300 mg/kg extract + CP	12,000	10	23.45	16.9
		22,000	43		

MNPCEs = micronucleated polychromatic erythrocytes. CP = cyclophosphamide.

Doxorubicin has growth inhibition higher than 50% and is dose-dependent.

At the concentration tested (250 μg/mL), *T. esculenta* showed significant antiproliferative activity against all cell line, mainly to UACC-62 (2.6 μg/mL), NCI-ADR/RES (3.1 μg/mL), and OVCAR-3 (3.1 μg/mL) cell lines, under the experimental conditions. Previous reports also revealed that *P. cupana* and *D. longan* can control melanoma and colorectal carcinoma cells growth (Chung, Lin, Chou, & Hsu, 2010; Fukumasu et al., 2008).

Probably, levels of myricetin and quercetin are responsible to antiproliferative activity from *T. esculenta*. According Neto (2007), quercetin inhibits the growth of MCF-7,HT-29 and K562 cell lines with GI$_{50}$ in the range of 15–60 mg/L. However, other polyphenols or compounds should also be investigated in our fruit to confirm their biological effects.

3.4. Anticlastogenic activity by micronucleus test in vivo

Administration of a *T. esculenta* extract led to an important reduction of micronucleus in PCEs. Thus, positive control (CP + NaCl) caused a significant ($p = 0.05$) increase in MNPCE compared to the negative control, confirming the sensitivity of the test. All concentrations analyzed of *T. esculenta* extract when compared with positive control were significant different ($p < 0.05$), showing anticlastogenic activity.

A combination of different doses (30, 100 and 300 mg/kg b.w.) of *T. esculenta* reduced significantly the MNPCES induced by CP. The dose of 300 mg/kg b.w. showed the higher reduction (95.03%) (Table 3). In contrast, *P. cupana* increased the frequency of MNPCE and induced testicular chromosomal aberrations in mice (Al-Majed, 2006).

4. Conclusion

The results suggest that *T. esculenta* can be explored as antioxidant source and as a potential chemoprevention and treatment agent cancer. However, future studies will be conducted in order to investigate the toxicity, and steps of isolation, characterization of other compounds and elucidating the mechanism of action of these compounds by complex biological assays.

Acknowledgments

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