Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry xx (2014) xxx–xxx

Journal of Nutritional Biochemistry

Oil rich in carotenoids instead of vitamins C and E as a better option to reduce doxorubicin-induced damage to normal cells of Ehrlich tumor-bearing mice: hematological, toxicological and histopathological evaluations☆

Ana Luisa Miranda-Vilela[a,b,*], Cesar K. Grisolia[a], João Paulo F. Longo[a], Raphael C.A. Peixoto[c], Marcos Célio de Almeida[a], Lilian Carla P. Barbosa[c], Mariana M. Roll[a], Flávia A. Portilho[a], Luciana L.C. Estevanato[a], Anamélia L. Bocca[c], Sônia N. Báo[c], Zulmira G.M. Lacava[a,*]

[a]*Department of Genetics and Morphology, Institute of Biological Sciences, University of Brasília, Brasilia, Brazil*
[b]*Faculty of Medicine, Faciplac, Campus Gama/DF, Brazil*
[c]*Department of Cell Biology, Institute of Biological Sciences, University of Brasilia, Brasília, Brazil*

Received 14 January 2014; received in revised form 5 June 2014; accepted 6 June 2014

Abstract

The development of therapeutic strategies to attenuate chemotherapy toxicity represents an area of great interest in cancer research, and among them is nutritional therapy based on antioxidants. As research on this topic is still controversial and scarce, we aim to investigate the effects of antioxidant supplementation with vitamin C, vitamin E or pequi oil, a carotenoid-rich oil extracted from pequi (*Caryocar brasiliense*), on doxorubicin (DX)-induced oxidative damage to normal cells in Ehrlich solid tumor-bearing mice. Tumor weight and volume, histopathology, morphometry and immunohistochemistry were used to assess the treatments' efficacy in containing tumor aggressiveness and regression, while possible toxicity of treatments was assessed by animals' weight, morphological analysis of the heart, liver and kidneys, hemogram, and serum levels of total bilirubin, direct bilirubin, indirect bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase, creatinine and urea. Although all the chemotherapeutic treatments increased internal necrosis area and reduced the positive Ki-67 cells compared to non-treated tumors, the treatments with pequi oil provided before tumor inoculation (PTDX) or in continuous and concurrent administration with doxorubicin (PTPDX) were more effective in containing tumor growth, besides increasing lymphocyte-dependent immunity and reducing the adverse side effects associated with DX-induced oxidative damage to normal cells, mainly the PTDX treatment. Vitamins C and E given before tumor inoculation and chemotherapy were not successful against doxorubicin-induced cardiotoxicity, besides increasing doxorubicin-induced nephrotoxicity, indicating that, at least for doxorubicin, pequi oil instead of vitamins C and E would be the best option to reduce its adverse effects.

Keywords: Doxorubicin-induced damage; Pequi oil (*Caryocar brasiliense*); Chemotherapy; Breast cancer; Animal model

1. Introduction

Breast cancer is the second most common type of cancer in the world and the most common among women both in the developed and the developing world. It accounts globally for 23% of all cancers in women and 14% of cancer deaths, being the commonest cause of death for cancer in women worldwide [1–5]. Although the emergence and evolution of new therapeutic approaches such as chemotherapy, radiotherapy, hormonal therapy, immunotherapy and conservative surgery have occurred in the last half century [6,7], breast cancer

Abbreviations: DX, doxorubicin; ROS, reactive oxygen species; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; WBC, total white blood cells; PLT, platelet count; MPV, mean platelet volume; P-LCR, platelet large cell ratio; PDW, platelet distribution width; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyl transferase; MN-NCE, micronucleus frequency for normochromatic erythrocytes; MN-PCE, micronucleus frequency for polychromatic erythrocytes; %PCE, frequency of polychromatic erythrocytes.

☆ Funding sources: The Brazilian National Council for Technological and Scientific Development (CNPq), the Foundation to Support Research in the Federal District (FAPDF), the Coordination for Further Training of Graduate Staff (CAPES), the CAPES-Network CON-NANO (CAPES), the INCT-Nanobiotecnologia (MCT, CNPq, CAPES), CNANO-UnB, and DPP-UnB.

* Corresponding author. Department of Genetics and Morphology, Institute of Biological Sciences, University of Brasília, Brasilia, Brazil. Tel.: +55 61 3107 3087; fax: +55 61 3107 2923.
E-mail addresses: mirandavilela@unb.br (A.L. Miranda-Vilela), zulmira@unb.br (Z.G.M. Lacava).

http://dx.doi.org/10.1016/j.jnutbio.2014.06.005
0955-2863/

remains one of the world's most devastating diseases, with more than 458,000 deaths and 1.38 million new cases each year [8]. Thus, research in this topic remains essential and the use of mouse models for preclinical testing is a fundamental component for the study and design of new regimens for cancer treatment [9].

Many chemotherapeutic agents can induce favorable response in a substantial number of patients [1]; among them, doxorubicin (DX), an anthracyclin antibiotic, is considered one of the most effective antitumor agents and an essential component in treatment of breast cancer [10]. However, its adverse side effects such as myelosuppression, hepatotoxicity, nephrotoxicity and most notably cardiomyopathy [10,11], associated with oxidative damage to normal cells, can limit its clinical use [10,12,13]. Consequently, the development of therapeutic strategies to attenuate chemotherapy toxicity represents an area of great interest in cancer research, and among them is nutritional therapy based on antioxidants [13–15]. However, research on this topic is still controversial [16–18] and scarce, mainly if the strong possibility of combinations between these nutrients and antineoplastic agents is considered [14].

Due to the limited number and size of clinical studies on the effect of antioxidants in modulating cancer treatment, and the absence of randomized controlled trials to prove the efficacy and applicability of antioxidants during chemotherapy [19,20], many oncologists believe that concurrent use of supplemental antioxidants should be avoided during conventional cancer treatment, because antioxidants could interfere with chemotherapy, preventing cancer cells from being killed by reactive oxygen species (ROS) [16]. However, in defense of antioxidants, experimental studies have shown that antioxidant vitamins and some phytochemicals selectively induce apoptosis in cancer cells but not in normal cells and prevent angiogenesis and metastatic spread [19]. Further, increased efficacy of many chemotherapeutic agents has been demonstrated, as well as a decrease in toxic adverse effects when administered concurrently with antioxidants [16], which suggests a potential role for antioxidants as adjuvant in cancer therapy [19].

Although it is already considered that, independently of the employed treatment, antioxidants would protect normal tissues from the toxic effects of free radicals [15], enhancing the effects of chemotherapy and preventing ROS-induced adverse side effects [16], this topic needs to be further investigated. This is mainly because potential antioxidant effects on the treatment's efficacy can depend on the nature of the antioxidant (and its dose), the chemotherapy drugs being used, the type and stage of cancer being treated [15,19,16], and the prevailing oxygen partial pressure (PO_2) in the tumor [19,21,22]. Therefore, all these points must be taken into consideration for antioxidant intervention, and studies that evaluate the effect of different antioxidants on the prevention and adjuvant treatment of a single tumor type in the same stage of cancer, treated with the same chemotherapeutic drug, can contribute to a better understanding of the potential effect of these substances on the treatment's efficacy. Additionally, previous reports have suggested that the efficiency and applicability of antioxidants in the medical clinic may also depend on the type of antioxidant therapy chosen, where antioxidants administrated before tumor appearance may effectively inhibit its growth, but with the tumor already installed, they could accelerate tumor growth and favor metastases [22].

Thus, the aim of this study was to investigate the effects of antioxidant supplementation with vitamin C (ascorbic acid), vitamin E (DL-alpha-tocopheryl acetate) or pequi oil, a carotenoid-rich oil extracted from pequi (*Caryocar brasiliense* Camb.), a typical fruit of the Brazilian Cerrado, on DX-induced oxidative damage to normal cells in Ehrlich solid tumor-bearing mice, according to the antioxidant protocols previously tested [22]. Since the pequi oil in continuous administration (before and after tumor inoculation) was the only treatment which inhibited tumor growth, while those with vitamins C and E in the same protocol had the opposite effect, favoring metastasis, the continuous pequi treatment was the only one chosen here to be tested during DX chemotherapy.

2. Materials and methods

2.1. Chemicals

Doxorubicin, ketamin and xylazine were obtained as chloridrate. Lyophilized Doxorubicin, sold as Doxofil 50 mg, was obtained from Ítaca Laboratórios LTDA (Rio de Janeiro, Brazil); ketamin, sold as Dopalen 100 mg/ml, was obtained from Ceva Animal Health Ltd (São Paulo, Brazil); and xylazine (Coopazine® 20 mg/ml) came from Coopers (São Paulo, Brazil). Vitamin C (ascorbic acid) sold as Citroplex® 200 mg/ml, was obtained from Neo Química Laboratory (Goiás, Brazil), and vitamin E 400 mg per capsule was obtained as DL-alpha-tocopheryl acetate from Sandoz® Pharmaceutical Industry (Paraná, Brazil).

2.2. Plant material

Pequi fruit was obtained *in natura* from the local markets of Brasília/DF (Brazil) and surrounding areas. The internal mesocarp was peeled or grated to obtain the pulp, which was packed in a covered pot and frozen at −86 °C. Pequi pulp oil extraction and its relative fatty acid composition have been previously reported [22–24]. Its total carotenoid content was analyzed using the methodology described by Higby (1962) [25]; macronutrients and micronutrients were quantified by the Laboratory of Agrochemistry and Environment of the State University of Maringá (UEM, Brazil) using atomic absorption spectrometry (K, Ca, Mg, Cu, Fe, Mn and Zn) or UV-Vis spectrometry (P); and its antioxidant activity by the capture of the free radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) was carried out according to Brand-William et al. (1995) [26], with adaptations from Razali et al. (2008) [27] and Atmani et al. (2009) [28]. Total phenol content was also measured using the Folin-Ciocalteu method as described by Swain and Hillis (1959) [29]. A calibration curve of gallic acid (ranging from 0.02 to 0.14 mg/ml) was prepared and the results, determined from regression equation of the calibration curve ($y=0.0008x - 0.0198$; $R^2=0.998$), were expressed as mg gallic acid equivalents (GAE) per gram of the sample.

A voucher of the pequi specimen (*Caryocar brasiliense* Camb.) was deposited in the herbarium of the University of Brasilia (UnB) by Professor Cassia Munhoz (PhD), collection number 7402, registration number 165.857.

2.3. Ehrlich tumor maintenance

The Ehrlich ascitic tumor, derived from a spontaneous murine mammary adenocarcinoma, was maintained in the ascitic form by intraperitoneal passages in Swiss mice according to procedures previously reported [22,23].

2.4. Tumor induction

The animals were anesthetized by an intraperitoneal administration of ketamine (80 mg/kg) and xylazine (10 mg/kg). A volume of 40 μl (5.5×10^6 viable cells) of the Ehrlich ascitic fluid freshly collected was injected subcutaneously in the upper region of the head as previously reported [22,23] for the solid-form implantation. Euthanasia was carried out 15 days after tumor implantation by cervical dislocation, according to the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia (2007) [30].

2.5. Animals and treatments

Female Swiss albino mice purchased from the animal facility of the University of São Paulo (Ribeirão Preto/SP, Brazil) were housed in plastic cages with ventilation and air circulation, under standard conditions of 12 h dark/light cycle, controlled temperature (20 °C±2 °C) and free access to food and water. After a period of acclimatization of twenty days, the animals, aged 11–12 weeks and weighing 28.7±2.8 g, were randomly distributed in treatment groups ($N=6$ per group).

Mice without tumor received orally (*per os*, p.o.) the following treatments for ten consecutive days: (1) filtered water (group 1, "Control"); (2) pequi oil (group 2, "Pequi"); (3) vitamin C (group 3, "Vit. C"); or vitamin E (group 4, "Vit. E"), where daily dose of the antioxidants was calculated using the dose translation formula developed by Reagan-Shaw et al. (2007) [31], following our previous protocols [22], and according to the maximum levels of vitamin security recommended by the National Agency for Sanitary Surveillance (ANVISA), which are 25 mg β-carotene, 1.000 mg vitamin C, and 1.200 UI vitamin E (1 UI of vitamin E=0.67 mg alpha-tocopherol or 1 mg of tocopherol acetate). In the tumor control groups, tumor was inoculated without any previous antioxidant administration (group 5, "Tumor") or animals received pequi oil (group 7, "PT") or vitamin C (group 10, "VCT") or vitamin E (group 12, "VET") administered orally (*per os*, p.o.) for ten days before tumor inoculation. For the chemotherapeutic treatments, the antioxidant protocols previously tested [22] were used, as follows: (1) tumor inoculation and, after 48 hours, intravenous administration in the tail vein of weekly doses of 20 mg/m^2 of DX to produce a lower incidence of cardiotoxicity, according to instructions for use of the medication, without any previous antioxidant administration (group 6, "TDX"); (2) oral daily dose of antioxidants (pequi oil or vitamin C or vitamin E) for 10 consecutive days, followed by tumor inoculation and the same DX treatment (groups 8, "PTDX"; 11, "CTDX"; and 13, "ETDX"); and (3) pequi oil p.o. daily for 10 days, followed by tumor implantation and continued daily antioxidant

Table 1
Experimental group description

Controls without tumor				Tumor control groups				Chemotherapeutic treatment groups				
Group 1 Control	Following treatments for 10 consecutive days			Group 5 Tumor	Following treatments for 10 consecutive days before tumor inoculation			48 hours after tumor inoculation	Following treatments for 10 consecutive days before tumor inoculation and DX treatment as in Group 6			
	Group 2 Pequi	Group 3 Vit. C	Group 4 Vit. E		Group 7 PT	Group 10 VCT	Group 12 VET	Group 6 TDX	Group 8 PTDX	Group 9 PTPDX	Group 11 CTDX	Group 13 ETDX
Filtered water	Pequi oil	Vitamin C	Vitamin E	Without previous antioxidant	Pequi oil	Vitamin C	Vitamin E	DX – Without previous antioxidant	Pequi oil	Pequi oil 15 more days from tumor inoculation	Vitamin C	Vitamin E

Table 2
Relative composition of pequi (*Caryocar brasiliense* Camb.) pulp oil

Fatty Acids [23–25]* (%)									
Saturated				Mono-unsaturated				Poly-unsaturated	
Palmitic	41.78			Oleic	54.28			Linoleic	1.36
Stearic	1.28			Palmitoleic	0.67			Linolenic	0.51
Araquidic	0.12								

Total carotenoids* ($\mu g.g^{-1}$)	Total phenol content* ($mg.GAE.g^{-1}$)	Macronutrients* ($g.kg^{-1}$)				Micronutrients* ($mg.kg^{-1}$)			
		Mg	Ca	K	P	Fe	Cu	Mn	Zn
499.90±9.85	n.d.	0.07	0.66	0.75	0.14	n.d.	n.d.	n.d.	n.d.

* Present study. n.d.=not detected. Data of total carotenoids are expressed as mean±standard deviation (SD) of three absorbance readings.

administration for 15 more consecutive days together with DX treatment (group 9, "PTPDX"). Treated mice were continuously monitored in respect to weight and hair loss, skin ulcers, hematuria and diarrhea (Table 1).

All procedures described here were reviewed and approved by the institutional Ethics Committee for Animal Research (Institute of Biological Science, University of Brasília), number 107748/2009.

2.6. Procedures and measurements

Before euthanasia, the animals were anesthetized with xylazine and ketamine according to the procedure described above. Blood samples (1 ml) collected by cardiac puncture were used to carry out hemogram, biochemical dosages of total bilirubin, direct bilirubin, indirect bilirubin, aspartate aminotransferase (AST), alanine aminotransferase

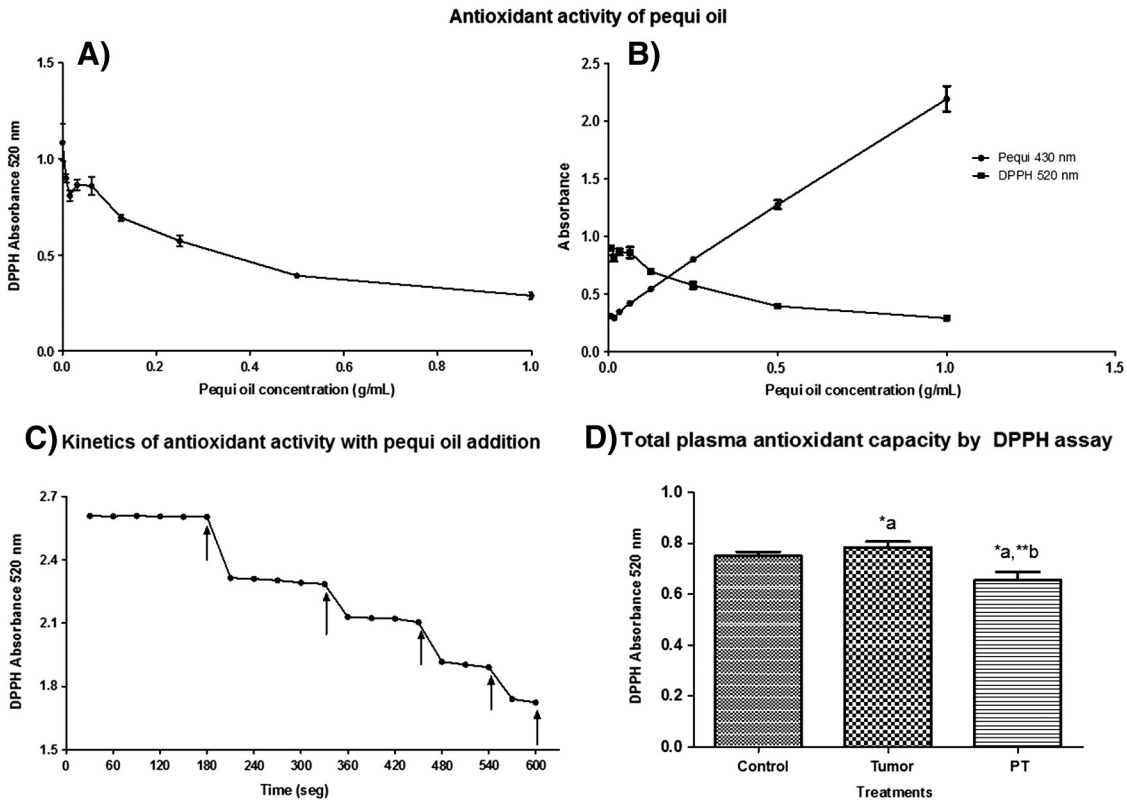
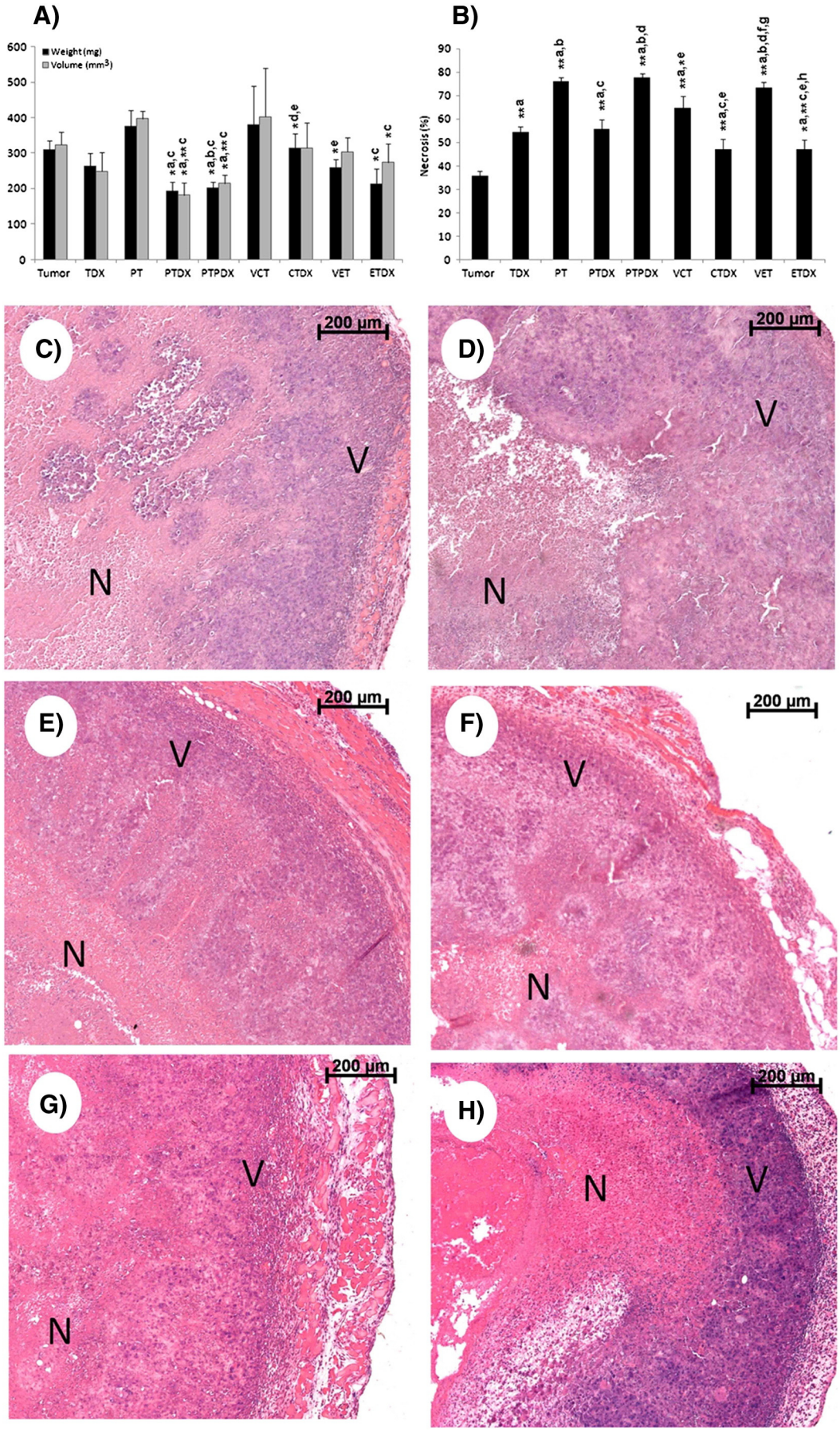


Fig. 1. Antioxidant activity of pequi oil by the capture of the free radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) in in vitro (A–C) and in vivo assays. In Panel C, arrows indicate addition of the pequi oil; in Panel D, bar graphs were expressed as SEM (standard error of mean), and the lowercase letters indicate significant differences detected by the Mann–Whitney *U* test with respect to the groups (a) Control, (b) Tumor. Asterisks indicate significant (*$P<.05$) and highly significant (**$P<.01$) differences.



(ALT), gamma glutamyl transferase (GGT), alkaline phosphatase, creatinine and urea, as well as the comet assay. After euthanasia, the tumor, heart, liver and kidneys were surgically removed for histopathological evaluations, and the bone marrow was extracted to perform the micronucleus (MN) test. Tumors were weighed on an analytical balance (Shimadzu of Brazil, São Paulo) and measured using a digital pachymeter. Their volume was calculated according to Yanase et al. (1998) [32], while organs were macroscopically examined *in situ* in respect to their color, consistency and size. Afterwards, tumors and organs were fixed with 10% formalin for 24 h, transferred to 70% ethanol, included in paraffin using an automatic tissue processor (OMA® DM-40, São Paulo, Brazil), cut to 5 μm of thickness in a Leica RM2235 manual microtome (Leica Microsystems, Nussloch, Germany) and stained with hematoxilin-eosin (HE).

2.7. Tumor histology and morphometry

Histological sections of tumors were examined to quantify the necrosis areas. A total of five histological sections with 100 μm distance between sections were analyzed per tumor sample. All sections were photographed with an MC 80 DX camera coupled to a Zeiss Axiophot light microscope, and tumor/necrotic areas were quantified using Image ProPlus 5.1 software.

2.8. Immunohistochemical analyses of tumor

In order to analyze cell proliferation in the peripheral areas of the tumors, immunohistochemical analyses by Ki-67 of the non-treated tumors and those treated with DX were carried out. For this, after paraffin removal and hydration, histological sections of the slides were immersed in citrate buffer (3 mM, pH 6.0) for 10 minutes at 120 °C for antigen retrieval. Subsequently, non-specific binding sites were blocked with 3% normal serum or BSA. Afterwards, the sections were incubated with anti-Ki-67 (1:200 Abcam, ab15580) antibodies (1:200 DAKO K4067) for 2 h at room temperature, washed and then incubated with biotinylated secondary antibodies for 20 min followed by avidin-biotin complex (LSAB-HRP Kit, K0690, Dako, Glostrup, Denmark). After washing, sections were incubated with diaminobenzidine substrate and counterstained with Mayer's hematoxylin [33]. Quantitative analyses were performed using a binocular microscope (Olympus BX50) coupled with an eyepiece with reticle (graticule) of 0.0625 mm^2 area (Olympus WH10X/22). As Ki-67 positive cells were counted in ten microscopic high power fields (1000x), a total area of 0.625 mm^2 was analyzed in each slide. Sections were photographed using a Carl Zeiss AxioVision SE64 photo documentation system (ZEN Imaging Software) coupled to a Zeiss Axioskop 2 microscope (Carl Zeiss). For better systematizing, results were then classified into the two categories proposed by Lazăr et al. (2010) [34] in tumors with high marking index Ki-67 (MI Ki-67 ≥45%) and tumors with low marking index (MI Ki-67 <45%).

2.9. Hemogram and biochemical analyses

Hemogram was processed in a multiple automated hematology analyzer for veterinary use, Sysmex pocH-100iV Diff (Curitiba/Paraná, Brazil) calibrated for mice in microtubes containing EDTA as anticoagulant. Blood smear slides were also prepared and stained with Giemsa for visual assessments of anisocytosis (variation in size), poikilocytosis (abnormally shaped red blood cells – RBCs), hypochromia (RBCs paler than normal), hemagglutination and erythrocyte rouleaux.

For the biochemical analyses, blood samples collected without anticoagulant were transferred to Vacutainer tubes for serum and centrifuged at 3000 rpm for 5 minutes. Serum samples were run on the automated chemistry analyzer ADVIA 2400 (Siemens), using the appropriate Advia chemistry reagents, protocols and controls. Total bilirubin, bilirubin fractions and creatinine were measured by colorimetric assays; GGT by a colorimetric kinetic method; urea by an enzymatic colorimetric method; and AST, ALT and alkaline phosphatase by optimized kinetic methods.

2.10. Macroscopic examination and histopathological analyses of heart, liver and kidneys

Heart, liver and kidneys were first macroscopically examined in respect to their color, consistency and size. Next, their histological sections were examined for the general aspects of organ preservation, as well as evaluations of presence of inflammatory infiltrate (all organs), nuclear pyknosis (all organs), myofibrolysis (heart), cell desquamation (kidneys) and other histological pathologies. Sections were photographed with an MC 80 DX camera coupled to a Zeiss Axiophot light microscope (Carl Zeiss).

2.11. Single-cell-gel electrophoresis (Comet assay) and Micronucleus (MN) test

Anthracyclines are potent DNA cleavage-enhancing drugs but myelosuppression and DNA damage-induced cardiotoxicity are limiting factors for their use [11,35], and antioxidant compounds can have antioxidant or pro-oxidant effects that are dose-dependent [22]. Therefore, comet assay and MN test were carried out to evaluate the genotoxicity (comet assay and MN test) and the cytotoxicity for the bone marrow cells (MN test, through the frequency of polychromatic erythrocytes – %PCE).

Comet assay (alkali method) was performed according to the method developed by Singh et al. (1988) [36], with a few modifications as previously described [22]. Briefly, 20 μl total blood of each animal was mixed with 120 μl of 0.5% low-melting-point agarose (LMA) in phosphate-buffered saline (PBS) at 37 °C and pipetted onto eight microscope slides pre-coated with a layer of 1.5% normal-melting-point agarose prepared in PBS. Slides were then immersed in a freshly prepared cold (4 °C) lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0–10.5, 1% lauroyl sarcosine, with 1% Triton X-100 and 10% dimethyl sulfoxide added fresh) for 1 hour at 4 °C. Then, slides were placed in a horizontal gel electrophoresis tank with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH >13.0), left in the solution for 40 minutes at 4 °C, and then proceeding to electrophoresis at 4 °C for 30 minutes at 25 V and 300 mA. Subsequently, slides were immersed three times (3×5 min), in neutralizing solution (0.4 M Tris, pH 7.5), fixed for 5 minutes in 100% ethanol, stained with EtBr (20 μg/ml) and analyzed with a Zeiss Axioskop 2 fluorescence microscope (filter 510–560 nm, barrier filter 590 nm) with a total magnification of 400×. One hundred comets on each slide were scored visually by a trained professional as belonging to classes 0–4, as proposed by Collins et al. (1995) [37], and the DNA damage was calculated according to Jaloszynski et al. (1997) [38] (giving a maximum possible score of 400, corresponding to 100 cells in class 4). For each animal, the slides were prepared in duplicate.

The MN test followed Estevanato et al. (2011) [39]. The assay was performed with bone marrow erythrocytes, collected from bone marrow of the femur, using insulin syringes containing 1 ml of fetal bovine serum (FBS). Cells were centrifuged at 160×*g* for 5 min, supernatant was discarded and 50 μl of FBS was used to resuspend the pellet. Subsequently, 10 μl of suspension was smeared on glass slides, fixed by methanol for 5 min and stained with Giemsa dye. Four thousand erythrocytes were counted per mouse (2000 normochromatic erythrocytes, NCE, and 2000 polychromatic erythrocytes, PCE), and the frequency of micronuclei (MN) in PCE and NCE, and the percentage of polychromatic erythrocytes (%PCE) were calculated.

2.12. Statistical analyses

Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 17.0. Data were expressed as mean±SEM (standard error of mean) and values of $P<.05$ were considered statistically significant. The continuous variables were tested for normal distribution with Shapiro-Wilk. Differences among the analyzed groups were investigated through ANOVA or Kruskal-Wallis test (when the data were not normally distributed). For significant ANOVA results, Tukey's post hoc test was chosen to carry out 2-to-2 comparisons between the treatments. For significant Kruskal-Wallis results, Mann–Whitney *U* test was performed to verify differences between the treatments (2-to-2 comparisons).

3. Results

3.1. Plant material analyses

The relative composition of pequi pulp oil is shown in Table 2, while its antioxidant activity by the capture of the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) in in vitro and in vivo assays is showed in Fig. 1.

3.2. General animal health status

Although there were no weight losses, almost all the experimental groups showed significant differences in body weight gain before and after treatments, except for the controls without tumor treated with vitamins C (Vit. C, Group 3) and E (Vit. E, Group 4), and the chemotherapeutic treatment group previously treated with pequi oil (PTDX, Group 8). There were no deaths and no evident signs of toxicity, except in two animals of the group treated with continuous and concurrent administration with doxorubicin (PTPDX, Group 9), which presented poor coat condition, such as piloerection.

After euthanasia, although splenomegaly was normally observed in animals carrying Ehrlich tumor, this was increased in one animal

Fig. 2. Dimensions, morphometry and histology of Ehrlich tumor tissue sections of the non-treated and DX-treated groups. (A) Tumor weight and volume. (B) Percentage of necrosis area (morphometry). (C–H) Histopathological aspects by HE staining at 400× magnification, showing a central area of necrotic tissue (N) surrounded by viable tumor cells (V) in the peripheral areas of tumors without treatment (C, Tumor group) and those of the chemotherapeutic treatment groups TDX (D), PTDX (E), PTPDX (F), CTDX (G), and ETDX (H).In Panels A and B, bar graphs were expressed as SEM (standard error of mean). The lowercase letters indicate significant differences detected by the Mann–Whitney *U* test with respect to the groups (a) Tumor, (b) TDX, (c) PT, (d) PTDX, (e) PTPDX, (f) VCT, (g) CTDX, and (h) VET. Asterisks indicate significant (*$P<.05$) and highly significant (**$P<.01$) differences.

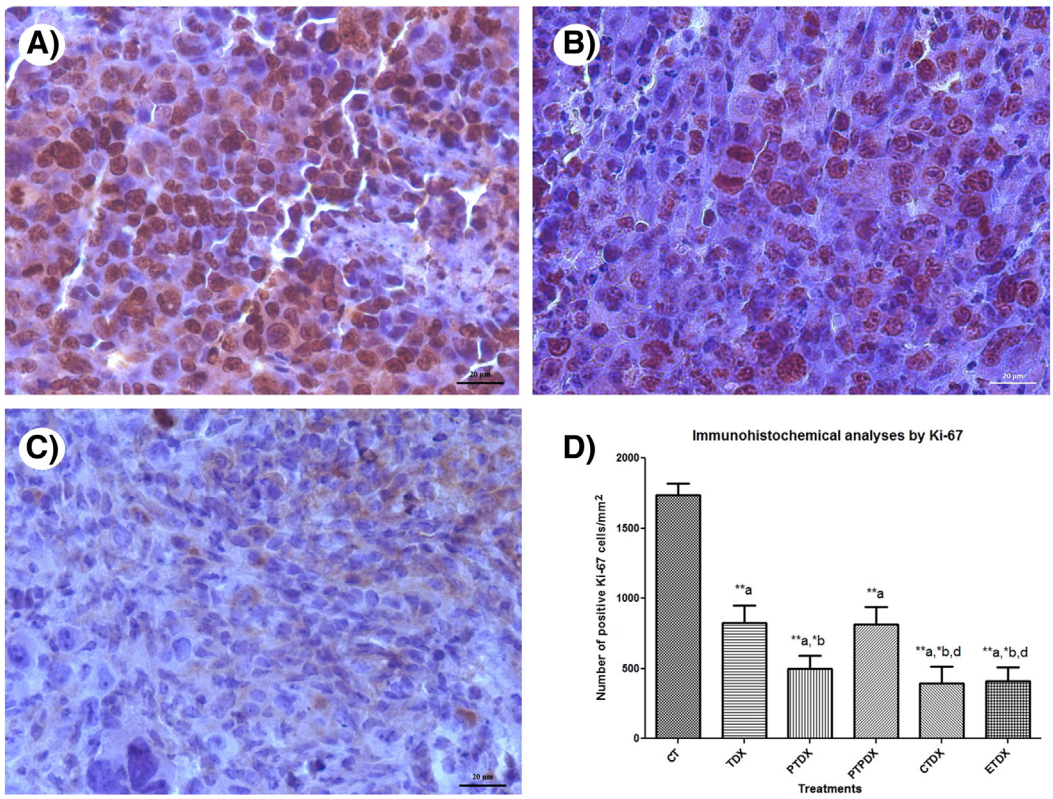


Fig. 3. Immunohistochemical staining for Ki-67 nuclear antigen in sections of the peripheral areas of Ehrlich solid tumor of the non-treated and DX-treated groups. A–C: Representative photomicrography of the Tumor group (A), and the chemotherapeutic treatment groups TDX and PTPDX (B), PTDX, CTDX and ETDX (C), showing the presence of Ki-67 antigen identified in brown nuclear staining, and its absence, in blue. D: Quantification of number of Ki-67 positive cells per mm^2.In Panel D, bar graphs were expressed as SEM (standard error of mean). The lowercase letters indicate significant differences detected by the Mann-Whitney U Test with respect to the groups (a) Tumor, (b) TDX, (c) PTDX, (d) PTPDX and (e) CTDX. Asterisks indicate significant (*P<.05) and highly significant (**P<.01) differences.

from the chemotherapeutic treatment group, which received vitamin C before tumor inoculation (CTDX, Group 11), and in three animals from the chemotherapeutic treatment receiving vitamin E before tumor inoculation (ETDX, Group 13).

3.3. Tumor dimensions, morphometry, histology and immunohistochemistry

Only the chemotherapeutic treatment groups PTDX (Group 8) and PTPDX (Group 9) presented a significantly decreased tumor weight and volume compared to the Tumor group, but the necrosis area was significantly increased in all treatment groups compared to this control (Fig. 2A and B).

Histologically, both tumor controls and treatment groups presented the typical pattern of Ehrlich solid tumor, with a central necrosis area surrounded by viable tumor cells, where large solid tumors had extensive regions of internal necrosis, while in small tumors, these regions were smaller (Fig. 2C–H). Islands of viable cells immersed in necrotic area were observed mainly in the Tumor group (Fig. 2C), while in the treatment groups, islands of necrosis area spread in peripheral regions were more frequent, but presented different patterns (Fig. 2D–H). To differentiate the chemotherapeutic treatment groups, the tumor cells were considered positive Ki-67 in the presence of brown nuclear staining, where different proliferative activities were also observed. All treatments significantly reduced positive Ki-67 cells compared to the Tumor group (Group 5, without any previous antioxidant or DX treatment), but the chemotherapeutic treatment groups PTDX (Group 8), CTDX (Group 11) and ETDX (Group 13) also had a significant decrease in positive Ki-67 cells compared to TDX group (Group 6), while TDX (Group 6) and PTPDX (Group 9) groups were similar (Fig. 3). When results were classified into the two categories proposed by Lazăr et al 2010 [34], the Tumor group was classified into the category with high marking index (MI) Ki-67 (78.19±13.10%), the Ki-67 scores varied for the TDX (37.05±17.42%) and PTPDX (36.69±21.67%) groups, and all the other groups pre-treated with antioxidants and treated with DX were classified into the category with low MI Ki-67: PTDX (22.47±19.39%), CTDX (17.84±16.60%), and ETDX (18.38±14.55%).

3.4. Hematology

Compared to the negative control, although some significant differences were observed in the erythrogram (Table 3), the main alterations were those promoted by the tumor implantation, which primarily caused significant reduction in red blood cells (RBC) and hemoglobin (HGB), as well as significantly increased red cell distribution width (RDW); no treatment significantly improved these. However, all RBC and HGB values were inside the reference intervals for mice, which are, respectively, in the range of 6.5–10.1×10^6/μl and 10.1–16.1 g/dl, according to Thrall (2007) [40]. For the visually assessed slides, poikilocytosis (abnormally shaped RBCs) was observed for the Tumor (Group 5) and all the chemotherapeutic treatment groups (Groups 6, 8, 9, 11 and 13), with some echinocytes being observed in the Tumor group, a few acanthocytes and echinocytes in the chemotherapeutic treatment groups TDX, PTDX and ETDX (there was also one animal in the ETDX group with spherocytes), and some acanthocytes in the PTPDX and CTDX groups. Discrete hypochromia was observed in one

Table 3
Erythrogram of healthy and Ehrlich solid tumor-bearing mice before and after treatments with antioxidants and/or Doxorubicin

	Treatment	G	RBC (x 10^6/μl)	HGB (g/dl)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	RDW (%)
Without tumor	Control	1	8.91±0.15	13.11±0.19	32.44±0.54	36.42±0.27	14.72±0.16	40.46±0.24	13.24±0.14
	Pequi	2	8.84±0.25	12.82±0.43	32.22±1.14	36.45±0.31	14.50±0.12	39.82±0.29	13.05±0.30
	Vit. C	3	9.70±0.16$^{*\ a,b}$	13.53±0.13	34.30±0.32	35.37±0.29$^{*\ a,b}$	13.93±0.21$^{*\ a}$	39.45±0.31	13.60±0.19
	Vit. E	4	9.18±0.19$^{*\ c}$	13.54±0.18	33.86±0.64	36.94±0.36$^{*\ c}$	14.76±0.12$^{*\ c}$	40.02±0.31	12.62±0.15$^{*\ a,c}$
With tumor	Tumor	5	8.19±0.19$^{*\ a,d,**\ c}$	11.64±0.19$^{**\ a,c,d}$	29.76±0.57	36.34±0.53	14.22±0.18$^{*\ d}$	39.10±0.22	14.78±0.73$^{*\ a,b,**\ d}$
	TDX	6	7.87±0.33$^{*\ a,d,**\ c}$	11.23±0.35$^{**\ a,c,d,*\ b}$	28.37±0.91$^{*\ c}$	36.13±0.32	14.30±0.16$^{*\ d}$	39.62±0.26$^{*\ e}$	15.33±0.66$^{*\ a,b,d}$
Pequi	PT	7	8.23±0.36	12.13±0.30$^{*\ a}$	29.45±0.82	35.93±1.16	14.80±0.72	41.20±0.71$^{*\ e}$	15.68±0.40$^{**\ a}$
	PTDX	8	7.47±0.36$^{**\ a,*\ b}$	11.03±0.48$^{**\ a,*\ b}$	26.90±1.18$^{*\ a}$	36.00±0.35	14.80±0.13$^{*\ e,f}$	41.03±0.14$^{**\ e}$	14.02±0.34$^{*\ a,b,g}$
	PTPDX	9	6.68±0.54$^{**\ a,b,e,*\ g}$	10.47±0.86$^{**\ a,*\ b}$	25.21±2.11$^{**\ a,b}$	37.69±0.35$^{*\ a,b,f,h}$	15.67±0.18$^{**\ a,b,e,f,*\ h}$	41.56±0.21$^{**\ e,f,*\ b}$	14.21±0.27$^{*\ a,b,g}$
Vit. C	VCT	10	7.70±0.84$^{*\ a,**\ c}$	10.88±1.17$^{**\ a,c}$	27.26±3.05$^{*\ c}$	35.30±0.32$^{*\ a}$	14.16±0.20$^{*\ a}$	40.04±0.43	15.90±0.57$^{**\ a,c}$
	CTDX	11	7.65±0.14$^{*\ a,c}$	11.10±0.27$^{**\ a,c}$	27.86±0.62$^{*\ c}$	36.46±0.79	14.54±0.37$^{*\ i}$	39.82±0.36$^{*\ i}$	17.86±1.06$^{**\ a,c,h,i,*\ e}$
Vit. E	VET	12	9.02±0.29$^{*\ e,f,j}$	12.66±0.27$^{*\ d,e,f}$	31.50±0.75	34.98±0.59$^{*\ d}$	14.08±0.35	40.20±0.34	16.54±0.23$^{**\ a,d}$
	ETDX	13	7.57±0.09$^{**\ a,d,l,*\ e}$	11.58±0.22$^{**\ a,d,l}$	28.34±0.31	37.44±0.19$^{*\ f,h,l}$	15.28±0.16$^{*\ e,f,h,l}$	40.86±0.38$^{*\ e}$	15.56±0.49$^{**\ a,d,h,*\ i,k}$
	P values		0.000	0.000	0.000	0.002	0.001	0.000	0.000

Data were expressed as mean±SEM (standard error of mean). G=Group; RBC=Red Blood Cells; HGB=Hemoglobin; HCT=Hematocrit; MCV=Mean Corpuscular Volume; MCH=Mean Corpuscular hemoglobin; MCHC=Mean corpuscular hemoglobin concentration; RDW=Red cell distribution width (represents an indication of the amount of variation – anisocytosis – in cell size); g/dl=grams per deciliter; fl=fentoliters; pg=picograms. *P* values of HCT and MCHC were generated by ANOVA, while *P* values of the other variables were generated by the Kruskal–Wallis test. The superscript letters indicate significant differences detected by the Tukey (HCT and MCHC) or the Mann–Whitney *U* (other variables) tests in the 2-to-2 comparisons, with a=significant compared to group 1; b=significant compared to group 2; c=significant compared to group 3; d=significant compared to group 4; e=significant compared to group 5; f=significant compared to group 6; g=significant compared to group 7; h=significant compared to group 8; i=significant compared to group 9; j=significant compared to group 10; k=significant compared to group 11; l=significant compared to group 12. Asterisks indicate significant (*P<.05) and highly significant (**P<.01) differences.

animal from the Tumor group, one from the TDX group and one from the CTDX group.

In respect to the leukogram (Table 4), the control PT (Group 7) and the PTDX (Group 8) treatment groups significantly increased the number of white blood cells (WBC), inside the reference intervals (2–10×10^3/μl) [41], compared to the negative control. These increases were related to neutrophils+monocytes (PT group), and lymphocytes and neutrophils+monocytes (PTDX group), where the number of neutrophils+monocytes was slightly larger than the reference interval reported for mice [40]. A significant increase in the number of lymphocytes was also observed for the control PT group, while the number of neutrophils+monocytes significantly increased in the controls PT (Group 7) and VET (Group 12), and in the chemotherapeutic treatment groups TDX (Group 6), CTDX (Group 11) and ETDX (Group 13); all of them inside the reference values.

In the plateletgram (Table 5), although some significant differences were observed, the main alterations were presented for the chemotherapeutic treatment group CTDX, which showed significant increased platelet (PLT) count, mean platelet volume (MPV) and platelet large cell ratio (P-LCR) compared to the negative control.

3.5. Biochemical analyses

Compared to the negative control, the tumor implantation promoted significant increase above the reference values for the total, direct and indirect bilirubin, and the treatments improved this situation or made them return to normal. Vitamin E (Group 4, without tumor) and all tumor controls and chemotherapeutic treatment groups significantly increased values of AST, while pequi (Group 2, without tumor) significantly increased ALT, and pequi (Group 2), tumor (Group 5) and the chemotherapeutic treatment groups TDX (Group 6) and ETDX (Group 13) significantly increased GGT. For alkaline phosphatase, there were significantly decreased values from the tumor implantation, while the control VCT (Group 10) and the chemotherapeutic treatment groups PTDX (Group 8), PTPDX (Group 9), CTDX (Group 11) and ETDX (Group 13) had a significant increase in

Table 4
Leukogram of healthy and Ehrlich solid tumor-bearing mice before and after treatments with antioxidants and/or Doxorubicin

	Treatment	G	WBC (×10^3/μl)	Lymphocytes (×10^3/μl)	Neutrophils+monocytes (×10^3/μl)	Eosinophils (×10^3/μl)
Without tumor	Control	1	3.02±0.27	2.35±0.21	0.63±0.10	0.04±0.03
	Pequi	2	4.05±0.70	3.40±0.57$^{*\ a}$	0.63±0.14	0.02±0.02
	Vit. C	3	2.78±0.32	2.38±0.29	0.40±0.06	0.00±0.00
	Vit. E	4	2.36±0.37	1.94±0.32	0.40±0.05	0.02±0.02
With tumor	Tumor	5	3.08±0.63	1.86±0.32$^{*\ b}$	1.20±0.33$^{*\ c,d}$	0.02±0.02
	TDX	6	3.67±0.41	2.02±0.35	1.65±0.25$^{**\ a,b,c,d}$	0.02±0.02
Pequi	PT	7	5.10±0.85$^{*\ a}$	2.68±0.51	2.23±0.26$^{**\ a,*\ b}$	0.20±0.12
	PTDX	8	6.03±0.26$^{**\ a,e,*\ b,f}$	3.43±0.25$^{*\ a,e,f}$	2.57±0.31$^{**\ a,b,*\ e}$	0.03±0.02
	PTPDX	9	6.06±1.41	3.41±0.77	2.59±0.65	0.06±0.02
Vit. C	VCT	10	3.56±0.84	1.70±0.36	1.78±0.48	0.08±0.04$^{*\ c}$
	CTDX	11	3.52±0.50$^{*\ h}$	1.56±0.28$^{*\ h}$	1.92±0.24$^{**\ a,c}$	0.04±0.04
Vit. E	VET	12	4.66±0.83$^{*\ d}$	2.90±0.56	1.70±0.28$^{**\ a,d}$	0.06±0.04
	ETDX	13	3.18±0.64$^{**\ h}$	1.48±0.28$^{**\ h,*\ l}$	1.70±0.39$^{**\ a,d}$	0.00±0.00$^{*\ h}$
	P values		0.011	0.010	0.000	0.372

Data were expressed as mean±SEM (standard error of mean). G=group; WBC=total white blood cells. *P* values of lymphocytes (%) were generated by ANOVA, while other *P* values were generated by the Kruskal-Wallis test. The superscript letters indicate significant differences detected by the Tukey (lymphocytes %) or the Mann–Whitney *U* (other variables) tests in the 2-to-2 comparisons, with a=significant compared to group 1; b=significant compared to group 2; c=significant compared to group 3; d=significant compared to group 4; e=significant compared to group 5; f=significant compared to group 6; g=significant compared to group 7; h=significant compared to group 8; i=significant compared to group 9; j=significant compared to group 10; k=significant compared to group 11; l=significant compared to group 12. Asterisks indicate significant (*P<.05) and highly significant (**P<.01) differences.

Table 5
Plateletgram of healthy and Ehrlich solid tumor-bearing mice before and after treatments with antioxidants and/or Doxorubicin

	Treatment	G	PLT ($\times 10^3$/μl)	MPV (fL)	P-LCR (%)	PDW (fL)
Without tumor	Control	1	1247.91±42.62	6.39±0.12	7.18±0.87	6.85±0.10
	Pequi	2	1251.40±11.26	6.23±0.09	6.08±0.43	6.65±0.09
	Vit. C	3	1337.17±57.21	6.70±0.17	10.60±1.07$^{*\,a,b}$	6.77±0.15
	Vit. E	4	1223.50±140.51	6.33±0.13	7.27±0.54	6.80±0.21
With tumor	Tumor	5	1462.80±117.93	6.13±0.17	5.68±1.16$^{*\,c}$	6.48±0.10$^{*\,a}$
	TDX	6	1595.17±88.01	6.24±0.14	7.08±1.01	6.44±0.09$^{*\,a}$
Pequi	PT	7	1395.00±122.83	6.70±0.12	7.30±0.27	7.00±0.11
	PTDX	8	1117.33±43.20$^{*\,f}$	6.66±0.11$^{*\,b}$	8.54±1.20	6.66±0.10
	PTPDX	9	1038.00±65.55$^{**\,f}$	6.56±0.11	8.48±0.95	6.66±0.05$^{*\,f}$
Vit. C	VCT	10	1681.25±143.84$^{*\,a}$	6.43±0.12	8.53±1.02	6.67±0.07$^{*\,f}$
	CTDX	11	1564.00±133.73$^{*\,a,**\,i}$	7.02±0.12$^{*\,a,c,e,f,h,i}$	11.76±1.19$^{*\,a,e,f,i}$	6.82±0.15$^{*\,f}$
Vit. E	VET	12	1761.33±207.62$^{*\,a}$	7.03±0.1	9.93±0.64	7.07±0.14
	ETDX	13	1312.60±90.59	6.70±0.11$^{*\,e,f}$	8.66±0.89	6.86±0.06$^{*\,e,f,i}$
	P values		0.000	0.013	0.040	0.101

Data were expressed as mean±SEM (standard error of mean). G=Group. Platelet indices: platelet count (PLT), mean platelet volume (MPV), platelet large cell ratio (P-LCR) and platelet distribution width (PDW); fl=fentoliters. *P*-value of PLT was generated by ANOVA, while *p*-values of other variables were generated by the Kruskal–Wallis test. The superscript letters indicate significant differences detected by the Tukey (PLT) or the Mann–Whitney *U* (other variables) tests in the 2-to-2 comparisons, with a=significant compared to group 1; b=significant compared to group 2; c=significant compared to group 3; d=significant compared to group 4; e=significant compared to group 5; f=significant compared to group 6; g=significant compared to group 7; h=significant compared to group8; i=significant compared to group 9. Asterisks indicate significant (*$P<.05$) and highly significant (**$P<.01$) differences.

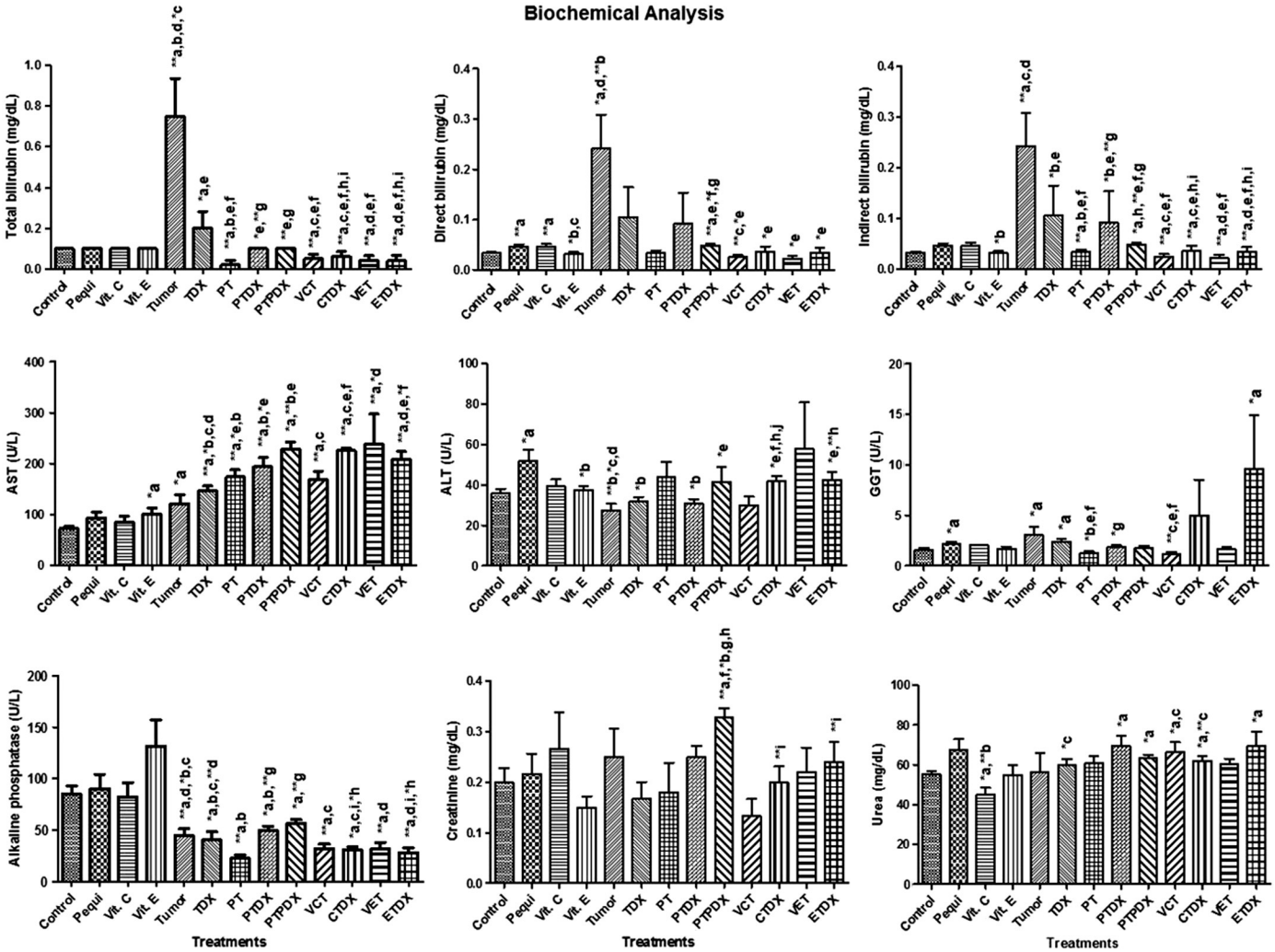


Fig. 4. Biochemical dosages of total bilirubin, direct bilirubin, indirect bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase, creatinine and urea of healthy and Ehrlich solid tumor-bearing mice without and after treatments.Bar graphs were expressed as SEM (standard error of mean). The lowercase letters indicate significant differences detected by the Mann–Whitney *U* test with respect to the groups (a) Control, (b) Pequi, (c) Vitamin C, (d) Vitamin E, (e) Tumor, (f) TDX, (g) PT, (h) PTDX, (i) PTPDX, (j) VCT, (k) CTDX, and (l) VET. Asterisks indicate significant (*$P<.05$) and highly significant (**$P<.01$) differences.

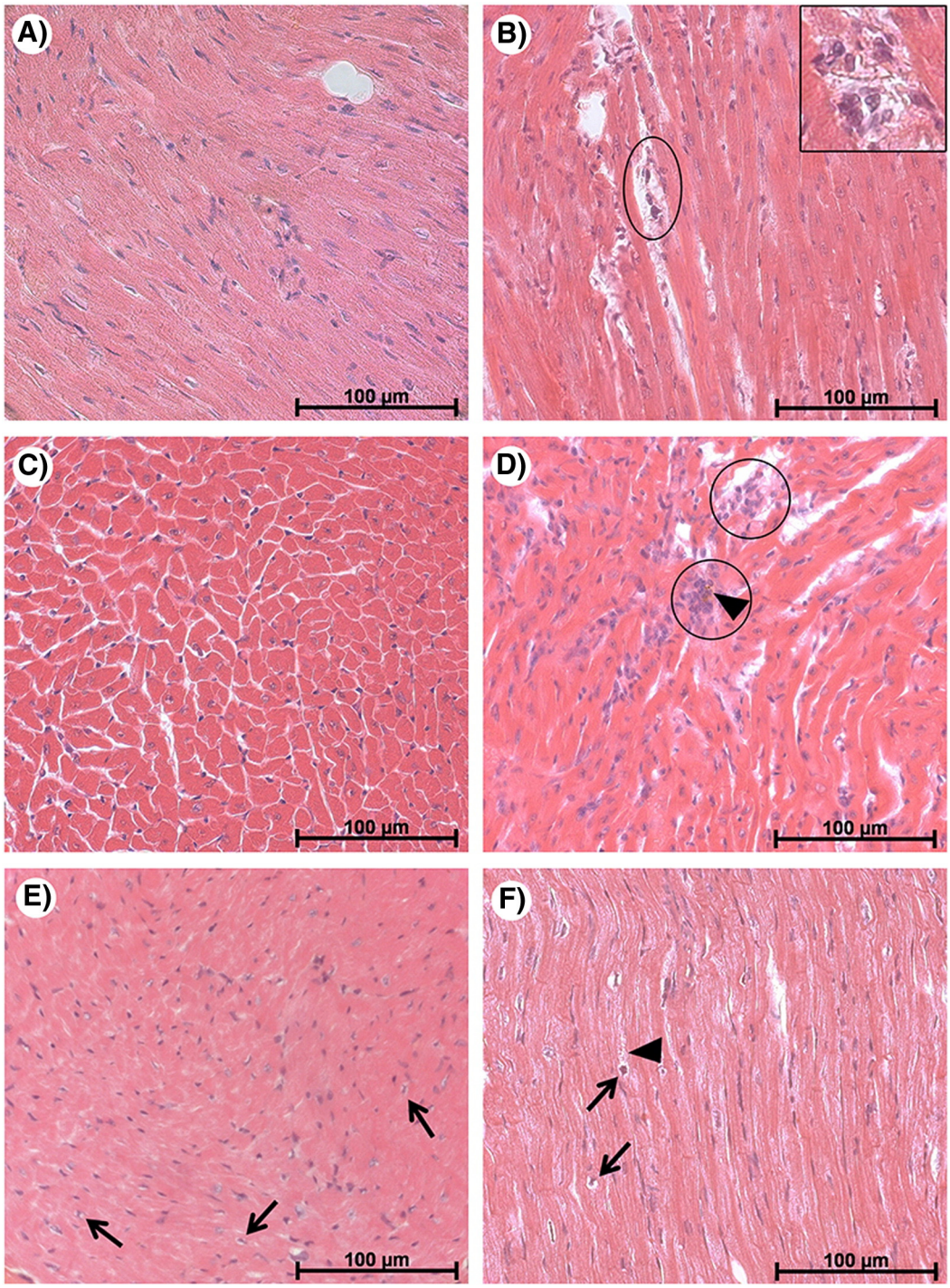


Fig. 5. Representative histopathology of the heart of Ehrlich solid tumor-bearing mice without and after treatments. (A) Tumor group: longitudinal section with normal myofibrillar structure and striations. (B) TDX group: longitudinal section showing two areas of mononuclear inflammatory infiltrate with focal myocyte destruction (ellipse and upper right corner square). (C) PTDX group: cross section of normal cardiomyocytes. (D) PTPDX group: longitudinal section showing focal areas of inflammatory infiltrate (circle) and hemosiderin (arrowhead). (E) CTDX group: longitudinal section showing focal areas with nuclear pyknosis (arrows). (F) ETDX group: longitudinal section showing cells with nuclear pyknosis (arrow) and myofibrolysis (arrowhead). HE staining (400× magnification).

urea values, the same occurring for creatinine in the PTPDX (Group 9) chemotherapeutic treatment group (Fig. 4).

3.6. Macroscopic and histopathological analyses of heart, liver and kidneys

Macroscopic examination of the heart showed a normal general aspect, except for cardiomegaly presented by one animal of the chemotherapeutic treatment group ETDX (not related to the other animals, which presented splenomegaly). In the histological sections, the heart of uninoculated mice and non-treated Ehrlich solid tumor-bearing mice showed a normal myofibrillar structure, while the TDX group showed some areas with more cellularity and focal areas of mononuclear inflammatory infiltrate with myocyte destruction. Furthermore, three animals from the PTPDX group presented focal mild inflammatory infiltrate, and the ETDX group showed focal areas

with nuclear pyknosis and myofibrolysis. No histopathological alterations were observed for the PTDX group, while the CTDX group showed a general normal aspect, although some focal areas with nuclear pyknosis were also observed (Fig. 5).

Macroscopically, the liver of all groups showed normal aspect, reddish brown and firm consistency, with no animal presenting hepatomegaly. Microscopically, livers of uninoculated mice showed a normal hepatic structure, with preserved hepatocytes and centrilobular veins (data not showed). However, from the tumor implantation, the histopathological analysis of the livers showed focal areas of mononuclear inflammatory infiltrate in portal and intralobular areas, where in the TDX group this was more intense. Occasional necrotic cells were seen near the inflammatory infiltrates, particularly in the TDX group (Fig. 6).

Kidneys of almost all animals presented a normal macroscopic appearance with reddish-pink color and firm consistency, except in one of the animals from the ETDX group, which not only presented splenomegaly but also had whitish colored kidneys. Microscopically, the tumor implantation caused histopathological alterations, with presence of inflammatory infiltrates, few focal areas with sclerotic glomeruli and tubules with nuclear pyknosis and rare cell desquamation. Although the chemotherapeutic treatments diminished or removed the inflammatory infiltrate, cell desquamation inside the tubules and nuclear pyknosis were frequent in the TDX group, and more exacerbated when these animals had been previously treated with vitamins C and E (CTDX and ETDX groups), which also presented cells with vacuolization, nuclear fragmentation and atypical mitosis, mainly the ETDX group (Fig. 7).

3.7. Genotoxicity assays by the Comet and Micronucleus (MN) tests

Compared to negative control, there was a significant increase in the percentage of DNA damage (Comet assay) for the TDX and CTDX groups (Fig. 8), but in the MN test, only the control groups without tumor treated with pequi and vitamin C showed a significant increase in the micronucleus frequency for normochromatic erythrocytes (MN-NCE), and no significant difference was showed for the micronucleus frequency for polychromatic erythrocytes (MN-PCE) or frequency of polychromatic erythrocytes (%PCE) in respect to this control (Fig. 9).

4. Discussion

Growing evidence suggests that cancer cells are under increased oxidative stress compared to normal cells, and this is associated with oncogen-induced transformation, increased metabolic activity, mitochondrial malfunction and increased ROS generation [15]. This would justify those positive results observed with antioxidant vitamins and some phytochemicals in selectively inducing apoptosis in cancer cells and preventing angiogenesis and metastatic spread [19]. On the other hand, different levels of oxidative stress appear to induce different outcomes in cancer cells [15]. Mild oxidative stress activates cell signaling mechanisms, such as proliferation, migration, and invasion, but high oxidative stress can induce cell death [15,42], also justifying the belief that concurrent use of supplemental antioxidants could interfere with chemotherapy, preventing cancer cells from being killed by ROS [16]. Because as well as these controversies there are situations in which antioxidant supplementation would be undesirable for cancer patients [16], an immunocompetent mouse model of cancer could help us to approximate the use of a more effective model system, even though it may still fail to model the human population accurately in this respect [43]. Although it is a fundamental principle of pharmacokinetics that the nature, dose, and mode of administration of a drug can have a profound impact on its physiological effect, including any potential interaction with other treatments such as chemotherapy [20], clear pharmacological responses should not be expected from antioxidants, because these compounds have a multitude of actions [44]. Thus, our study can contribute to a better understanding of the conflicting views on antioxidants appearing to swing from healthy to toxic [44], as well as their potential effects on the efficacy of DX treatment.

Doxorubicin was chosen in the present study because is it the most studied anthracycline and a chemotherapeutic drug that generates high levels of ROS [15]. The choice of antioxidants was based on the following facts: (a) pequi oil is rich in various carotenoids, several of them with pro-vitamin A activity, and all of them presenting very effective antioxidant properties under low PO_2 [23], being used in folk medicine to treat several diseases, including tumor control [22], (b) although vitamin C has been proposed as a chemotherapeutic agent since 1952, studies about its effects on cancer are still controversial [17,45]; (c) vitamin E is an efficient antioxidant under high PO_2 and has been reported to effectively protect cancer patients against systemic toxicity induced by cisplatin without interfering in its antitumor activity [16], besides having an inhibitory effect on a variety of cancer cells [21].

Ehrlich tumor is a neoplasm of epithelial malignant origin, corresponding to murine mammary adenocarcinoma [22]. It is a very aggressive tumor which readily grows more quickly from the second week, reaching large sizes in short periods [23], and under favorable conditions, such as those provided by some antioxidant interventions, Ehrlich tumor readily forms metastasis [22]. Thus, considering the injection of 5.5×10^6 viable cells instead of previously reported 5.5×10^4 viable cells [22] to produce a more clinically advanced tumor, two weeks after tumor implantation was a crucial period for investigation of the proposed treatments, mainly for ethical reasons because its fast growth impairs the use of this tumor model for long periods due to the general state of the experimental animals [23]. Thus, bearing these considerations in mind and recalling that not only increased ROS production but also reduced ROS detoxification have been reported in conjunction with tumor growth and metastasis [42], our results suggest that pequi oil provided before tumor inoculation (PTDX) or in continuous and concurrent administration with doxorubicin (PTPDX) were more effective treatments to contain tumor growth. As large solid tumors have extensive regions of internal necrosis, while in small tumors these regions are lesser [22], a smaller necrosis area would be expected for these treatments, since they promoted a significantly decreased tumor weight and volume compared to the Tumor group. However, although all treatments increased necrosis area compared to this Tumor group, the PTPDX treatment also increased the necrosis area compared to the TDX group, maintaining positive Ki-67 cells at a level similar to that found in TDX, while the PTDX treatment promoted a similar necrosis area to the TDX group but had a significant decrease in positive Ki-67 cells compared to TDX. Additionally, the PTDX and PTPDX treatments were the only ones that increased lymphocyte number compared to the Control, Tumor and TDX groups, although it was statistically significant only for the PTDX treatment. As lymphocytes are a heterogeneous population of cells of critical importance in immune responses [40], and reduced lymphocyte-dependent immunity can favor carcinogenesis [23], the increase in this type of immunity was probably responsible for the more effective inhibition of the tumor growth promoted by the pequi oil. As neutrophils and monocytes are responsible for modulation of the immune response and monocytes are primarily responsible for presentation of antigens to lymphocytes and for modulation of the immune response [41], this could justify our findings of a significantly increased number of neutrophils+monocytes for almost all treatments, especially for PTDX.

It has been reported that the main anticancer effects of doxorubicin are due to its ability to react with cancer cell DNA, intercalating within DNA, inhibiting topoisomerase II and modifying

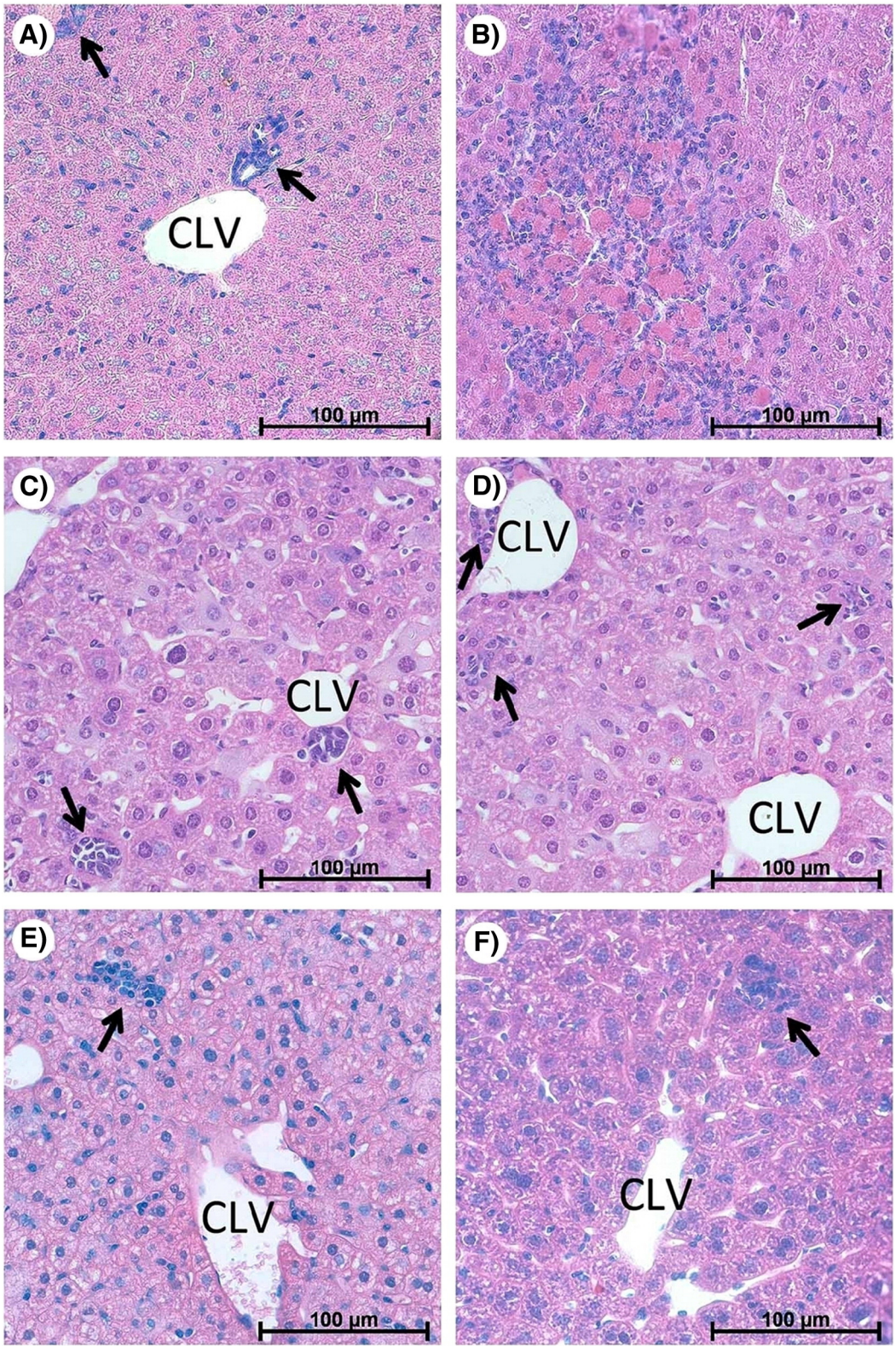


Fig. 6. Representative histopathology of the liver of Ehrlich solid tumor-bearing mice without and after treatments. Label: centrilobular vein (CLV). (A) Tumor group: presence of mononuclear inflammatory infiltrate in peripheral area and surrounding the bile duct (arrows). (B) TDX group: intense lymphomonocytic inflammatory infiltrate with necrotic hepatocytes. (C) PTDX and (D) PTPDX groups: presence of inflammatory infiltrate surrounding the centrilobular veins (CLV) and in peripheral areas (arrows); (E) CTDX and (F) ETDX groups: mononuclear inflammatory infiltrate in peripheral areas (arrows). HE staining (400× magnification).

helicases to dissociate duplex DNA into single stranded DNA, thus preventing DNA replication [15,46,47], while the oxidative damage to membrane lipids and other cellular components is a major factor in its toxicity [48]. In this respect, and because it has been proposed that independently of the employed treatment and even when the mechanism of the chemotherapeutic drug is independent of ROS

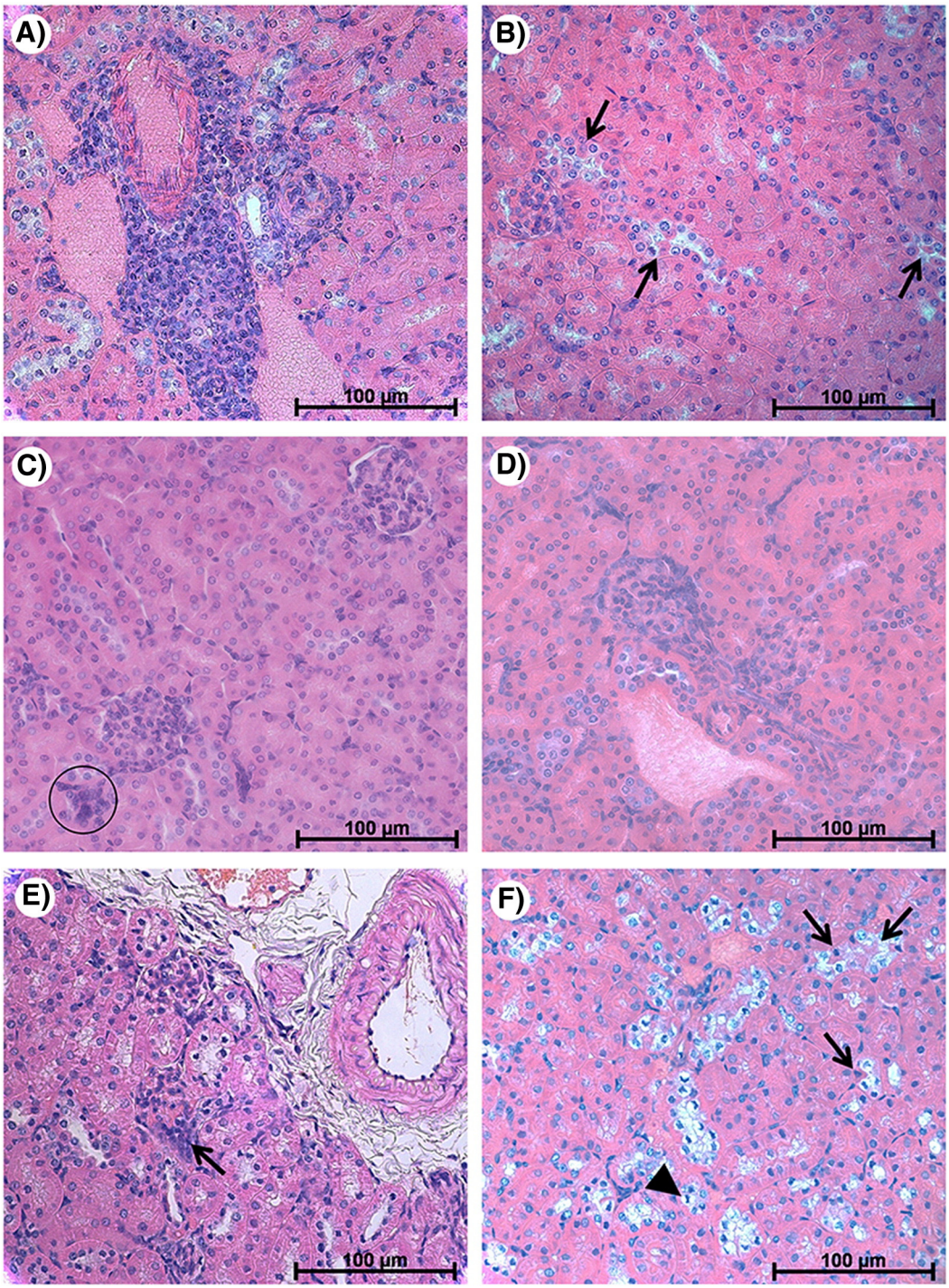


Fig. 7. Representative histopathology of the kidneys of Ehrlich solid tumor-bearing mice without and after treatments. (A) Tumor group: presence of intense periarteriolar inflammatory infiltrate. (B) TDX group: cell desquamation inside the tubules and nuclear pyknosis, signaling necrosis (arrows). (C) PTDX group: renal cortex showing an inflammatory infiltrate (arrow). (D) PTPDX group: apparently normal renal cortex. (E) CTDX and ETDX groups: presence of a muscular artery of small caliber and an interstitial inflammatory infiltrate (arrow). (F) CTDX and ETDX groups: intense nuclear pyknosis in the distal tubules, cell desquamation, eosinophilic cytoplasm and focal tubular necrosis in tubular epithelium (arrows). HE staining (400× magnification).

generation, antioxidants would protect normal tissues from the toxic effects of free radicals [15], it was expected that all preventive antioxidant treatments could at least attenuate the adverse side-effects associated with DX-induced free radical damage to normal cells. However, this did not happen as expected, mainly with the previous treatments with vitamin C or vitamin E (CTDX and ETDX groups), as will be discussed below.

When analyzing hematologic data from mice, not only concurrent controls should be used as the primary comparison for interpretation of treatment-related changes, but also comparisons with reference intervals, which are desirable to put hematologic changes in perspective [41]. In this respect, although no treatment appeared to be hematotoxic by the hemogram and %PCE, the more pronounced splenomegaly presented by some animals of the CTDX and ETDX

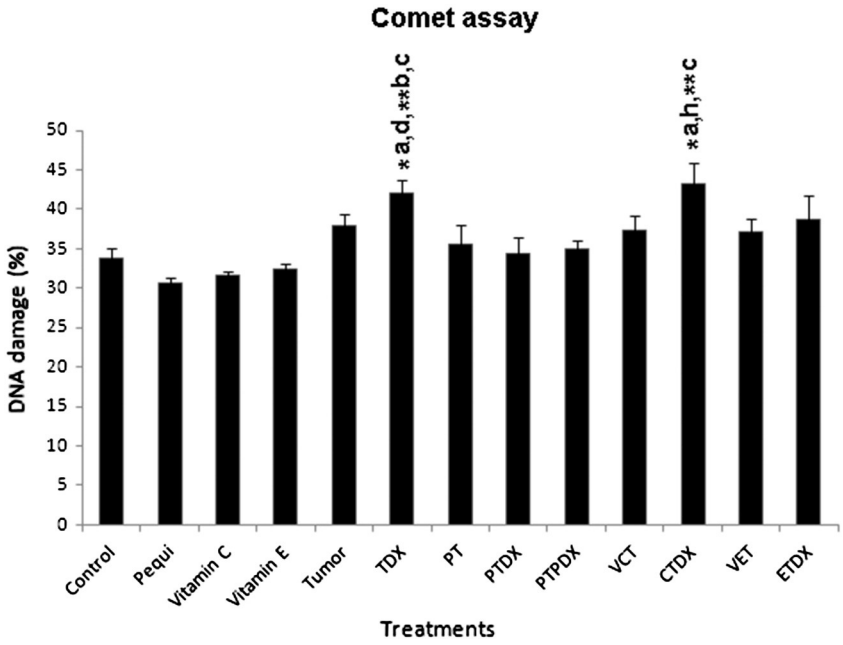


Fig. 8. Frequency of DNA damage (%) in peripheral leukocytes by Comet assay of healthy and Ehrlich solid tumor-bearing mice without and after treatments. Bar graphs are expressed as SEM (standard error of mean). The lowercase letters indicate significant differences detected by the Tukey test with respect to the groups (a) Control, (b) Pequi, (c) Vitamin C, (d) Vitamin E, (e) Tumor, (f) TDX, (g) PT, (h) PTDX, (i) PTPDX, (j) VCT, (k) CTDX, and (l) VET. Asterisks indicate significant (*P<.05) and highly significant (**P<.01) differences.

groups indicated some toxicity of these treatments for the bone marrow erythroid lineage. This is because unlike humans and many other animals, the murine spleen is a major site of normal juvenile and adult hematopoiesis. The output of splenic erythropoiesis is about half that of bone marrow during normal hematopoietic conditions, and serves as a reserve site for accelerated hematopoiesis under conditions of hematopoietic stress, such as those that occur in hemorrhage or hypoxia. Under these conditions of erythropoietic stimulation, a few extra mitoses occur in the bone marrow, but up to five or six extra divisions occur in spleen [41], justifying the splenomegaly. Additionally, normal platelet counts in mice range generally from 900–1600×10^3/μl, with MPV varying between 4 and 6 fL [41], and there were significantly increased PLT, MPV and P-LCR for the CTDX treatment. As increased platelets are generally due to increased platelet production, which in turn is often associated with the presence of giant platelets (P-LCR) in the peripheral blood [41], results corroborate the above suggestion of some toxicity of this treatment for the erythroid lineage, since the upregulation of hematopoiesis in response to erythropoietic stress can cause increases in platelet counts [41].

As along with the hemogram, clinical enzymology is also an important research tool which leads to the diagnosis of diseases in animals [49]. Thus, serum levels of bilirubin, AST, ALT, GGT and alkaline phosphatase were measured to assess possible dysfunctions in the hepatobiliary system [49,50] arising from the tumor implantation and/or treatments, while serum creatinine and urea were used as kidney function tests [49], and the results were compared to the histopathological analyses of the organs.

Bilirubin is a pigment produced by hemoglobin degradation by the mononuclear phagocyte system. Hepatocytes uptake this bilirubin and conjugate with glucuronic acid to produce a water-soluble form, known as unconjugated bilirubin, which is subsequently excreted via the hepatobiliary system in the bile [40,49]. The direct dosage measures the total and conjugated bilirubin, while the indirect dosage measures unconjugated bilirubin [49]. Since as well as in diseases where the uptake, conjugation and secretion of bilirubin are decreased, increased levels of unconjugated bilirubin (indirect) are usually observed in situations in which there is an increase in the hemolysis rate, while high levels of conjugated bilirubin (direct) are associated with intrahepatic cholestasis or extrahepatic bile duct obstruction [40], our results indicate that the antioxidant treatments improved liver response to injury caused by the tumor implantation and the TDX treatment. This is because, besides significantly reducing total, direct and indirect bilirubin compared to the Tumor group, the PTDX, PTPDX, CTDX and ETDX treatments also reduced the mononuclear inflammatory infiltrates to mild. These inflammatory infiltrates are known as inflammatory pseudotumors (IPTs) or inflammatory myofibroblastic tumor and are the expression of diverse inflammatory processes of unknown etiology that may be accompanied by a tumorlike mass [51–53]; it is generally accepted that IPTs may correspond to an unusual tissue response to injury [51]. On the other hand, results of GGT suggest that the treatments with pequi oil (PTDX and PTPDX groups) were effective to contain the oxidative stress caused by doxorubicin on the liver, while vitamins C and E (CTDX and ETDX groups) could have increased it in this organ, since they caused increased GGT. Although the most universal application of serum GGT assay is in diagnosis of hepatobiliary dysfunctions, which does not seem to be the case in view of the results of the bilirubins and histopathological analyses of the liver, GGT is closely linked to the synthesis and metabolism of glutathione through the gamma-glutamyl cycle [54]. Thus, elevated serum GGT may be a marker of depleted glutathione, since extracellular GGT can hydrolyze glutathione causing depletion of this very important endogenous antioxidant [55].

Inflammatory pseudotumor (IPT) of the heart is a rare condition, which is diagnostically troublesome in this organ because of its infrequency in this location and its mimicry of malignant processes [53,56]. In the heart IPTs consist of cellular spindle cell proliferation with an associated inflammatory cell infiltration, where the spindle cells have immunohistochemical and ultrastructural features of myofibroblasts, and may demonstrate moderate cytologic atypia and mitotic activity [56]. Thus, our results suggest that IPTs found in the heart could be related to an initial cardiac fibrosis caused by the doxorubicin treatment. Since it is well documented in animal and

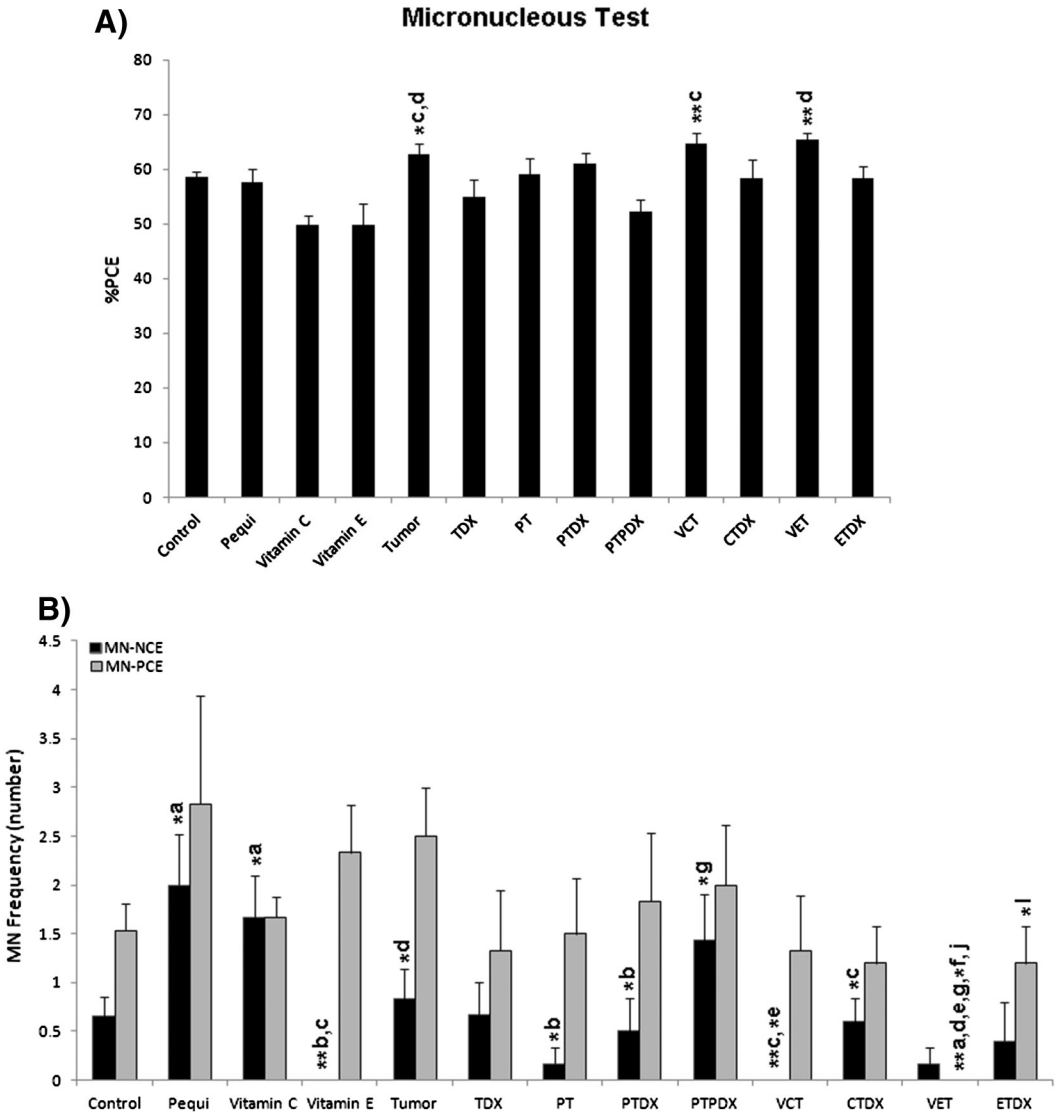


Fig. 9. Frequencies of polychromatic erythrocytes (% PCE) (A) and micronucleus (MN) evaluation (B) of bone marrow cells of healthy and Ehrlich solid tumor-bearing mice without and after treatments. Bar graphs are expressed as SEM (standard error of mean). MN-NCE and MN-PCE=micronucleus frequency for normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE), respectively. The lowercase letters indicate significant differences detected by the Tukey (%PCE) or Mann–Whitney *U* test (MN-NCE and MN-PCE) with respect to the groups (a) Control, (b) Pequi, (c) Vitamin C, (d) Vitamin E, (e) Tumor, (f) TDX, (g) PT, (h) PTDX, (i) PTPDX, (j) VCT, (k) CTDX, and (l) VET. Asterisks indicate significant (*P<.05) and highly significant (**P<.01) differences.

human studies that therapy with DX induces fibrosis, cardiac dysfunction, and cell death [57], results demonstrated that, although the PTPDX, CTDX and ETDX (except in one animal from the ETDX group, which presented cardiomegaly) treatments appeared to ameliorate this condition, the only treatment apparently without any sequelae was the PTDX. DX cardiotoxicity results in disruption of myocyte structure, including damage to the microtubules, vacuolization, sarcomere disruption, dilation of the sarcoplasmic reticulum, mitochondrial injury and loss of myofibrils [48]. Further focal areas of IPTs were also found in slides from the PTPDX group, pyknosis in CTDX and ETDX groups, and myofibrolysis in the ETDX group. Because the mechanism of this cardiotoxicity is thought to be related to generation of oxidative stress, causing lethal injury to cardiac myocytes [35], results indicate that the PTDX treatment was more effective in containing the DX-induced cardiotoxicity. Moreover, DNA damage also appears to play an important early role in DX-induced lethal cardiac myocyte injury through a pathway involving p53 and the mitochondria [35]. Because ROS acts systemically, although the DNA damage was measured only in the peripheral leukocytes, this can be used to evaluate the antioxidant status of the whole organism and, consequently, to evaluate if this state had improved after treatment [23]. In this way, results demonstrated increased DNA damage for the TDX and CTDX treatments, corroborating our above suggestion that vitamin C given before tumor inoculation and chemotherapy (CTDX treatment) may have increased the oxidative stress induced by doxorubicin. Although the biochemical results of AST did not corroborate this, in mice this enzyme is found in a variety of tissues, including skeletal muscle, myocardium, erythrocytes, blood vessels, brain, intestine, kidney, lung and testes [49]. Thus, AST is not the most suitable biochemical marker to evaluate specific myocardium lesions, although its highest activity is found in mouse cardiac muscle [49].

Urea is produced in the liver as a breakdown product of ammonia and, traditionally, its concentrations are measured in terms of the amount of nitrogen contained within urea. Elevated urea nitrogen levels can be caused by prerenal, renal and postrenal conditions. While prerenal causes are related to increased protein catabolism,

renal causes are usually associated with conditions that compromise 70–75% of functional renal mass, and postrenal include any cause that results in obstruction of the lower urinary system [49]. In the histopathological analysis of kidneys, the pequi oil treated group (PTPDX) was the only one which apparently showed a normal renal cortex, without presence of IPTs, sclerotic glomeruli or tubules with nuclear pyknosis. It therefore appears that the significantly elevated serum urea nitrogen presented by the doxorubicin treated groups (PTDX, PTPDX, CTDX and ETDX) compared to the negative control is more likely to have been a result of the catabolic processes and necrosis related to the tumor treatment than due to renal or postrenal causes. Results of creatinine corroborate this suggestion, since the PTPDX group presented the highest necrosis area together with higher creatinine levels, and pathologically, elevated serum creatinine is also caused by the same prerenal, renal and postrenal causes that elevate urea nitrogen in serum [49]. On the other hand, although the treatments diminished or removed the IPTs caused by the tumor implantation, the treatments with vitamins C and E given before tumor inoculation and chemotherapy (CTDX and ETDX groups) exacerbated the effect of pyknosis induced by doxorubicin, also presenting vacuolization, nuclear fragmentation and atypical mitosis, suggesting that these treatments could also have increased the oxidative stress in the kidneys.

There is sufficient evidence that exogenous antioxidants produce beneficial effects in various cancers, but whether an antioxidant supplement would be helpful, harmful or neutral depends in part, as previously mentioned, on the specific antioxidant (and its dose), the chemotherapy drugs being used, and the type and stage of cancer being treated [16]. Vitamin C, for example, is an antioxidant vitamin that has been hypothesized to antagonize the effects of ROS-generating antineoplastic drugs. However, it has been reported that, if given before some antineoplastic agents, it can antagonize therapeutic efficacy by preserving mitochondrial membrane potential. It is suggested that vitamin C supplementation during cancer treatment may detrimentally affect therapeutic response [58], and our results corroborate this and also another report which demonstrated that although vitamin E reduced doxorubicin-induced hepatotoxicity in rats, it was not successful against doxorubicin-induced cardiotoxicity [59]. As in our previous study vitamins C and E provided before tumor inoculation or in continuous administration (before and after tumor inoculation) stimulated tumor growth, favoring metastasis [22], results corroborate others indicating that carotenoids can reduce the risk of breast cancer [19], besides indicating that, at least for doxorubicin, pequi oil instead of vitamins C and E would be the best option to reduce its adverse effects.

The protective effect of the pequi oil observed in the present study could be related not only to its carotenoid content, but also to the fact that it has a higher concentration of oleic acid (54.28%) than palmitic, stearic and polyunsaturated fatty acids (44.93% in the total). While antitumor effect of oleic acid has been reported [60], palmitic, stearic (saturated) and linolenic (polyunsaturated) fatty acids, also present in the pequi oil composition, have been associated with prostate cancer risk [61], and dietary polyunsaturated fatty acids with breast and colorectum cancers (stimulating an increase in oxidative DNA damage and free estrogen levels for hormonal catabolism) [62].

5. Conclusions

In conclusion, although all treatments with doxorubicin increased internal necrosis area and reduced the positive Ki-67 cells compared to non-treated tumors, the treatments with pequi oil provided before tumor inoculation (PTDX) or in continuous and concurrent administration with doxorubicin (PTPDX) were more effective in containing tumor growth, besides increasing lymphocyte-dependent immunity and reducing the adverse side effects associated with doxorubicin-induced oxidative damage to normal cells, indicating that at least for doxorubicin, pequi oil instead of vitamins C and E would be the best option to reduce its adverse effects.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors have contributed to this work. ALMV was the principal investigator and takes primary responsibility for the paper. ALMV and ZGML participated in the design of the study and ZGML coordinated the research; ALMV, RCAP, LCPB, MMR, FAP and LLCE performed the laboratory work for this study; MMR was responsible for the chemical analyses of the pequi oil; LCPB for the visual analyses of the blood smear; MCA, SNB and RCAP conducted the histopathological analyses of the organs; JPFL and ALB conducted morphometry and immunohistochemical analyses; CKG did the analyses of the micronucleus test; ALMV was responsible for statistical analysis, interpretation of data and writing the manuscript; and all authors revised the manuscript.

Acknowledgments

We are grateful to Sabin Institute/Sabin Laboratories for technical support in the biochemical dosages and the Brazilian National Council for Technological and Scientific Development (CNPq), the Foundation to Support Research in the Federal District (FAPDF), the Coordination for Further Training of Graduate Staff (CAPES), the CAPES-Network CON-NANO (CAPES), the INCT-Nanobiotecnologia (MCT, CNPq, CAPES), CNANO-UnB, and DPP-UnB for financial support.

References

[1] Brufman G, Colajori E, Ghilezan N, Lassus M, Martoni M, Perevodchikova N, et al. Doubling epirubicin dose intensity (100 mg/m2 versus 50 mg/m2) in the FEC regimen significantly increases response rates. An international randomised phase III study in metastatic breast cancer. Ann Oncol 1997;8:155–62.
[2] Ingvarsson S. Breast cancer: introduction. Semin Cancer Biol 2001;11:323–6.
[3] Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer. Lancet Oncol 2001; 2:133–40.
[4] McPherson K, Steel C, Dixon J. ABC of breast diseases: breast cancer – epidemiology, risk factors, and genetics. BMJ 2000;321:624–8.
[5] Irvin JRW, Muss HB, Mayer DK. Symptom management in metastatic breast cancer. Oncologist 2011;16:1203–14.
[6] Verrill M. Chemotherapy for early-stage breast cancer: a brief history. Br J Cancer 2009;101:S2–5.
[7] Dhankhar R, Vyas SP, Jain AK, Arora S, Rath G, Goyal AK. Advances in novel drug delivery strategies for breast cancer therapy. Artif Cells Blood Substit Immobil Biotechnol 2010;38:230–49.
[8] World Health Organization (WHO). Breast Cancer Awareness Month in October, 2014.
[9] Francia G, Cruz-Munoz W, Man S, Xu P, Kerbel RS. Mouse models of advanced spontaneous metastasis for experimental therapeutics. Nat Rev Cancer 2011; 11:135–41.
[10] Ibrahim SS, Barakat MA, Helmy HTS. Modulating effect of carvedilol on doxorubicin-induced cardiomyopathy and hepatic damage. J Am Sci 2010;6:20–32.
[11] Hofland KF, Thougaard AV, Sehested M, Jensen PB. Dexrazoxane protects against myelosuppression from the DNA cleavage-enhancing drugs etoposide and daunorubicin but not doxorubicin. Clin Cancer Res 2005;11:3915–24.
[12] Xu X, Persson HL, Richardson DR. Molecular pharmacology of the interaction of anthracyclines with iron. Mol Pharmacol 2005;68:261–71.
[13] Quiles JL, Huertas JR, Battino M, Mataix J, Ramírez-Tortosa MC. Antioxidant nutrients and adriamycin toxicity. Toxicology 2002;180:79–95.
[14] Santos HS, Cruz WMS. A terapia nutricional com vitaminas antioxidantes e o tratamento quimioterápico oncológico. Rev Bras Cancerol 2001;47:303–8.
[15] Ozben T. Oxidative stress and apoptosis: impact on cancer therapy. J Pharm Sci 2007;96:2181–96.
[16] Akbas HS, Timur M, Ozben T. Concurrent use of antioxidants in cancer therapy: an update. Expert Rev Clin Immunol 2006;2:931–9.
[17] González MJ, Miranda-Massari JR, Mora EM, Guzmán A, Riordan NH, Riordan HD, et al. Orthomolecular oncology review: ascorbic acid and cancer 25 years later. Integr Cancer Ther 2005;4:32–44.
[18] Gonzalez MJ, Miranda-Massari JR. Advances in vitamin C research. Integr Cancer Ther 2006;5:7–8.

[19] Borek C. Dietary antioxidants and human cancer. Integr Cancer Ther 2004;3:333–41.
[20] Moss RW. Should patients undergoing chemotherapy and radiotherapy be prescribed antioxidants? Integr Cancer Ther 2006;5:63–82.
[21] Borek C. Antioxidants and radiation therapy. J Nutr 2004;134:3207S–9S.
[22] Miranda-Vilela AL, Portilho FA, de Araujo VG, Estevanato LL, Mezzomo BP, Almeida Santos MF, et al. The protective effects of nutritional antioxidant therapy on Ehrlich solid tumor-bearing mice depend on the type of antioxidant therapy chosen: histology, genotoxicity and hematology evaluations. J Nutr Biochem 2011;22:1091–8.
[23] Miranda-Vilela AL, Peixoto RCA, Longo JPF, Cintra DOS, Portilho FA, Miranda KLC, et al. Dextran-functionalized magnetic fluid mediating magnetohyperthermia combined with preventive antioxidant pequi-oil supplementation: potential Use against cancer. J Biomed Nanotechnol 2013;9:1261–71.
[24] Miranda-Vilela AL, Grisolia CK, Resck IS, Mendonça MA. Characterization of the major nutritional components of *Caryocar brasiliense* fruit pulp by nmr spectroscopy. Quim Nova 2009;32:2310–3.
[25] Higby WK. A simplified method for determination of some aspects of the carotenoid distribution in natural and carotene-fortified orange juice. J Food Sci 1962;27:42–9.
[26] Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT – Food Sci Technol 1995;28:25–30.
[27] Razali N, Razab R, Junit SM, Aziz AA. Radical scavenging and reducing properties of extracts of cashew shoots (Anacardium occidentale). Food Chem 2008;111:38–44.
[28] Atmani D, Chaher N, Berboucha M, Ayouni K, Lounis H, Boudaoud H, et al. Antioxidant capacity and phenol content of selected Algerian medicinal plants. Food Chem 2009;112:303–9.
[29] Swain T, Hillis WE. The phenolic constituents of Prunus domestica. I. – The quantitative analysis of phenolic constituents. J Sci Food Agric 1959;10:63–8.
[30] AVMA. AVMA guidelines on euthanasia; 2007.
[31] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J 2008;22:659–61.
[32] Yanase M, Shinkai M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: ex vivo study. Cancer Sci 1997;88:630–2.
[33] Carneiro MLB, Peixoto RCA, Joanitti GA, Oliveira RGS, Telles L, Miranda-Vilela AL, et al. Antitumor effect and toxicity of free rhodium (II) citrate and rhodium (II) citrate-loaded maghemite nanoparticles in mice bearing breast cancer. J Nanotechnol 2013; 11:4.
[34] Lazăr D, Tăban S, Sporea I, Dema A, Cornianu M, Lazăr E, et al. Ki-67 expression in gastric cancer. Results from a prospective study with long-term follow-up. Rom J Morphol Embryol 2010;51:655–61.
[35] L'Ecuyer T, Sanjeev S, Thomas R, Novak R, Das L, Campbell W, et al. DNA damage is an early event in doxorubicin-induced cardiac myocyte death. Am J Physiol Heart Circ Physiol 2006;291:H1273–80.
[36] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91.
[37] Collins A, Ai-guo M, Duthie S. The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mutat Res 1995;336:69–77.
[38] Jaloszynski P, Kujawski M, Czub-Swierczek M, Markowska J, Szyfter K. Bleomicin-induced DNA damage and its removal in lymphocytes of breast cancer patients studied by comet assay. Mutat Res 1997;385:223–33.
[39] Estevanato L, Cintra D, Baldini N, Portilho F, Barbosa L, Martins O, et al. Preliminary biocompatibility investigation of magnetic albumin nanosphere designed as a potential versatile drug delivery system. Int J Nanomedicine 2011;6:1709–17.
[40] Thrall MA, Baker DC, Campbell TW, DeNicola D, Fettman MJ, Lassen ED, et al. Hematologia e Bioquímica Clínica Veterinária. 1st ed. São Paulo, Brazil: Editora Roca; 2007.
[41] Everds NE. Hematology of the laboratory mouse. In: Fox JG, Barthold SW, Davisson MT, Newcomer CE, Quimby FW, Smith AL, editors. The mouse in biomedical research: normative biology, husbandry, and models. San Diego, California, USA: Elsevier; 2007. p. 133–70.
[42] Nishikawa M. Reactive oxygen species in tumor metastasis. Cancer Lett 2008; 266:53–9.
[43] Hunter KW. Mouse models of cancer: does the strain matter? Nat Rev Cancer 2012;12:144–9.
[44] Bast A, Haenen GR. Ten misconceptions about antioxidants. Trends Pharmacol Sci 2013;34:430–6.
[45] Parsons KK, Maeda N, Yamauchi M, Banes AJ, Koller BH. Ascorbic acid-independent synthesis of collagen in mice. Am J Physiol Endocrinol Metab 2006;290:E1131–9.
[46] Danesi R, Fogli S, Gennari A, Conte P, Tacca M. Pharmacokinetic-pharmacodynamic relationships of the anthracycline anticancer drugs. Clin Pharmacokinet 2002; 41:431–44.
[47] Hande KR. Clinical applications of anticancer drugs targeted to topoisomerase II. Biochim Biophys Acta 1998;1400:173–84.
[48] Fadillioglu E, Oztas E, Erdogan H, Yagmurca M, Sogut S, Ucar M, et al. Protective effects of caffeic acid phenethyl ester on doxorubicin-induced cardiotoxicity in rats. J Appl Toxicol 2004;24:47–52.
[49] Quimby FW, Luong RH. Clinical chemistry of the laboratory mouse. In: Fox JG, Barthold SW, Davisson MT, Newcomer CE, Quimby FW, Smith AL, editors. The mouse in biomedical research: normative biology, husbandry, and models. San Diego, California, USA: Elsevier; 2007. p. 171–216.
[50] Sutherland RJ. Biochemical evaluation of the hepatobiliary system in dogs and cats. Vet Clin North Am Small Anim Pract 1989;19:899–927.
[51] Miras-Parra FJ, Parra-Ruiz J, Gómez-Morales M, Gómez-Jiménez FJ, de la Higuera-Torres-Puchol J. Inflammatory pseudotumor of lymph nodes with focal infiltration in liver and spleen. Dig Dis Sci 2003;48:2003–4.
[52] Faraj W, Ajouz H, Mukherji D, Kealy G, Shamseddine A, Khalife M. Inflammatory pseudo-tumor of the liver: a rare pathological entity. World J Surg Oncol 2011;9:5.
[53] Li L, Cerilli LA, Wick MR. Inflammatory pseudotumor (myofibroblastic tumor) of the heart. Ann Diagn Pathol 2002;6:116–21.
[54] Goldberg DM. Structural, functional, and clinical aspects of gamma-glutamyltransferase. CRC Crit Rev Clin Lab Sci 1980;12:1–58.
[55] Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. J Amino Acids 2012;2012:1–26.
[56] Kelly SJ, Lambie NK, Singh HP. Inflammatory myofibroblastic tumor of the left ventricle in an older adult. Ann Thorac Surg 2003;75:1971–3.
[57] Schunke KJ, Coyle L, Merrill GF, Denhardt DT. Acetaminophen attenuates doxorubicin-induced cardiac fibrosis via osteopontin and GATA4 regulation: Reduction of oxidant levels. J Cell Physiol 2013;228:2006–14.
[58] Heaney ML, Gardner JR, Karasavvas N, Golde DW, Scheinberg DA, Smith EA, et al. Vitamin C antagonizes the cytotoxic effects of antineoplastic drugs. Cancer Res 2008;68:8031–8.
[59] Berthiaume JM, Oliveira PJ, Fariss MW, Wallace KB. Dietary vitamin E decreases doxorubicin-induced oxidative stress without preventing mitochondrial dysfunction. Cardiovasc Toxicol 2005;5:257–67.
[60] Carrillo C, Cavia MdM, Alonso-Torre SR. Antitumor effect of oleic acid; mechanisms of action: a review. Nutr Hosp 2012;27:1860–5.
[61] Crowe FL, Allen NE, Appleby PN, Overvad K, Aardestrup IV, Johnsen NF, et al. Fatty acid composition of plasma phospholipids and risk of prostate cancer in a case-control analysis nested within the European Prospective Investigation into Cancer and Nutrition. Am J Clin Nutr 2008;88:1353–63.
[62] Bartsch H, Nair J, Owen RW. Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. Carcinogenesis 1999;20:2209–18.