

Chemopreventive effects of pequi oil (*Caryocar brasiliense* Camb.) on preneoplastic lesions in a mouse model of hepatocarcinogenesis

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Pequi (*Caryocar brasiliense* Camb.), a fruit from Brazil's central region, was evaluated for its chemopreventive effects on preneoplastic liver lesions induced by the carcinogen diethylnitrosamine (DEN) in mice. BALB/c mice, 14 days of age, received an intraperitoneal injection at 10 µg/g of DEN. The mice received either of two doses of pequi oil (100 or 400 mg/kg) daily from the age of 30 days and were killed at the age of 189 days. Stereological parameters, including the volume density (V_v) and the total volume (V_{tot}) of the lesions (preneoplastic and adenomas), were measured and the expression of cytokeratins CK8/18 was evaluated. The total volume of lesions and adenomas was reduced by 51% in the group treated with the carcinogen and 400 mg/kg of pequi oil administered daily by an oral gavage for 25 consecutive weeks. In addition, some mice in this group did not develop lesions. Among the remaining preneoplastic lesions in this group, the number of remodelled profiles increased by 2.4-fold in the 400-mg

pequi oil-treated mice relative to the 100-mg-treated mice. Our results show that pequi oil exerts a hepatoprotective effect against DEN-induced development of preneoplastic lesions and adenoma in mice and the potential for its use in the prevention of liver cancer. *European Journal of Cancer Prevention* 00:000–000 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Chemoprevention is an important strategy for potentially reducing the incidence and mortality caused by neoplasms if it can inhibit, suppress or reverse carcinogenesis (Sporn, 1976; Kelloff *et al.*, 1994; Surh, 2003; Steward and Brown, 2013). The search for chemopreventive agents has frequently focused on plant materials because they contain diverse, biologically active compounds with great potential for use in cancer prevention. These substances can be used as templates for the synthesis of medication for the treatment of diseases or they can also be used directly as a source of therapeutic compounds (Varanda, 2006; Souza-Moreira *et al.*, 2008; Gaascht *et al.*, 2013). There is evidence that some plants, or their isolated compounds, have chemopreventive properties in human carcinogenesis (Surh, 2003). Brazil harbours very diverse flora with great medicinal potential for the chemoprevention of neoplasms (Varanda, 2006). Previous studies from our group showed the anticarcinogenic effects of *Pfaffia paniculata* Kuntze, known as Brazilian ginseng (Da Silva *et al.*, 2005), and the antineoplastic and antitumour effects of *Paullinia cupana* Mart. Var. *sorbilis*, known as guarana (Fukumasu *et al.*, 2006). In this study, we evaluated *Caryocar brasiliense* Camb., another Brazilian plant known in the regional gastronomy as pequi, for potential

chemopreventive effects in cancer. Pequi pulp contains high concentrations of vitamin C (78.3 mg/100 g) and vitamin E (Almeida, 1998; Enes *et al.*, 2011). This fruit oil has been shown to contain several fatty acids, such as palmitic, oleic, palmitoleic, stearic, linoleic and linolenic acids (Facioli and Gonçalves, 1998; Lima *et al.*, 2007), along with a high concentration of carotenoids, such as β-carotene, lycopene (Oliveira *et al.*, 2006), ζ-carotene, criptoflavin, β-cryptoxanthin, antheraxanthin, zeaxanthin and mutatoxanthin (Ramos *et al.*, 2001; Oliveira *et al.*, 2006). This group of compounds is known for its antioxidant properties, which are related to the enhancement of the immune system and the reduction of risk from degenerative diseases, such as cancer (Britton, 1995; Rodriguez-Amaya, 1997; Ramos *et al.*, 2001). Previous studies with pequi oil-producing *C. brasiliense* reported antioxidant (Lima and Mancini-Filho, 2005; Miranda-Vilela *et al.*, 2011a), antigenotoxic and anticlastogenic effects (Khoury *et al.*, 2007; Miranda-Vilela *et al.*, 2008). When administered to athletes, pequi oil reduced DNA damage and tissue injury, decreased blood total cholesterol and LDL and showed anti-inflammatory activity (Miranda-Vilela *et al.*, 2009a, 2009b). To determine whether pequi oil affects the development of liver preneoplastic lesions (PNL), we used a well-established

young mouse animal model of hepatocarcinogenesis. PNL were induced in mice aged 12–15 days by diethylnitrosamine (DEN), which alkylates DNA (Heindryckx *et al.*, 2009) and causes mutations (Verna *et al.*, 1996). The high rate of proliferation of liver cells in this period of the mouse lifetime promotes the formation of PNL (Maronpot, 2009). The PNL markers used to evaluate the effect of the pequi oil were the foci of altered hepatocytes (FAH) and the expression of cyto-keratin CK8/18.

Materials and methods

This study was approved by the Research Ethics Committee for the School of Medicine of the University of São Paulo (protocol number 061/12) and by the Ethics Committee for Animal Research from the School of Veterinary Medicine and Animal Science of the University of São Paulo (protocol number 2402/2011).

Mice

Forty-nine 14-day-old BALB/c mice were maintained in the laboratory animal facility of the Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo. The mice remained in polycarbonate cages (five per cage), with food and water provided *ad libitum* (Nuvilab CR1). Room temperature was maintained at $22 \pm 2^\circ\text{C}$ and the relative humidity was 45–65% with a 12 h light/dark cycle and continuous circulation of ambient air.

Caryocar brasiliense (pequi) oil

Pequi oil was provided by Dr Cesar K. Grisolia from the University of Brasília. This oil was extracted from the pulp of fresh pequi through mechanical pressure and centrifugation to obtain extra-virgin oil. The oil was vacuum-filtered and stored in amber bottles under refrigeration. The pequi oil was administered by gavage at two different doses: 100 and 400 mg/kg. The 400 mg dose was based on previous studies (Miranda-Vilela *et al.*, 2009a, 2009b; Miranda-Vilela *et al.*, 2011a). Toxicological tests in mice were performed previously by Miranda-Vilela *et al.* (2008). The relative composition of the oil is shown in Table 1.

Experimental design of hepatocarcinogenesis

The original model of infant mice used here was described by Vesselinovitch and Mihailovich (1983). The DEN carcinogen (diluted in 0.9% saline solution; Sigma-Aldrich, St. Louis, Missouri, USA) was administered by an intraperitoneal injection in a single application at a concentration of $10 \mu\text{g/g}$ to BALB/c mice at 14 days of age. Mice in the control groups (C, $n=5$ and PO400, $n=5$) received only saline solution (0.9%). After weaning, the mice were allocated into five groups (Fig. 1). From the 30th to the 189th day of life, mice in the treatment groups PO400 (pequi oil 400 mg/kg, $n=5$), DEN + PO100 (DEN + pequi oil 100 mg/kg, $n=13$) and

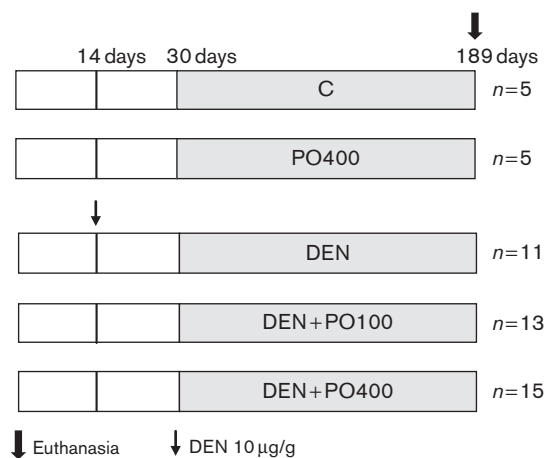
Table 1 Relative composition of (pulp oil from) pequi oil (*Caryocar brasiliense* Camb.)

Fatty acids		Vitamins (mg/100 g)	Carotenoids types (mg/100 g)
Saturated (%)	Unsaturated (%)		
Palmitic (41.78)	Oleic (54.28)	Vitamin E (0.607) ^a	Provitamin A (6.26–11.5)
Stearic (1.28)	Palmitoleic (0.67)		Lycopene (1.12–2.08)
Arachidic (0.12)	Linoleic (1.36)		
	Linolenic (0.51)		
Total (43.18)	Total (56.88)	Total (78.9)	Total (6.75–28.66)

Adapted with permission from Miranda-Vilela *et al.* (2011b). Adaptations are themselves works protected by copyright. So in order to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of the copyright in the translation or adaptation.

^aEnes *et al.* (2011).

Fig. 1



Hepatocarcinogenesis experimental design. C, control; DEN, diethylnitrosamine $10 \mu\text{g/g}$; PO400, treated only with pequi oil 400 mg/kg; DEN + PO100, diethylnitrosamine + 100 mg/kg of pequi oil; DEN + PO400, diethylnitrosamine + 400 mg/kg of pequi oil.

DEN + PO400 (DEN + pequi oil 400 mg/kg, $n=15$) received pequi oil once daily. Mice from the DEN group ($n=11$) and the control group (C, $n=5$) received only saline solution (0.9%) in this phase of the experiment. Body weight and feed intake were monitored weekly. The dose administered in the groups treated with pequi oil was adjusted after three weekly weighings. Animals were killed after 175 days after DEN application by a system of inhalation anaesthesia, where the drug (isoflurane; Cristália, Itapira, São Paulo, Brazil) was vapourized in 100% oxygen. The relative concentration of anaesthetic was increased gradually until respiratory and cardiac arrest was verified, then the livers were collected and weighed. Random fragments of liver lobes were collected and fixed in 4% formaldehyde for 24 h and subsequently embedded in Paraplast. Identification

and stereological evaluation of liver lesions were performed on 10 liver histologic sections and stained by haematoxylin and eosin. The basophilic FAH induced by DEN may be differentiated from the surrounding tissue because of its higher staining affinity for toluidine blue (Vesselinovitch *et al.*, 1985; Harada *et al.*, 1999). Therefore, additional liver sections were stained with 1% toluidine blue in 20% ethanol to better visualize small foci and remodelled PNL. For the characterization of the lesions, other histologic sections were subjected to an immunohistochemical test for CK8/18. All of the sections were 4.5 μm thick.

Stereological analysis of lesions

Stereological analyses were carried out using the (CAST) Visiopharm Stereology System software, version 3.6.5.0 (Visiopharm, Hoersholm, Denmark).

Liver volume (V_{liver})

The mass (or fresh weight) of the liver was converted from grams into volume (cm^3) by dividing the mass of the organ (M) by the specific density of the mouse liver ($d=1.10 \text{ g/cm}^3$). The following formula was used:

$$V_{\text{liver}} = \frac{M}{d}.$$

Volume density of the livers lesions

The volume density (V_v) was estimated using the Cavalieri principle (Gundersen *et al.*, 1999). V_v refers to the volume fraction occupied by the PNL and adenomas in the liver. To estimate V_v , the region of interest was overlapped onto a reference space (liver parenchyma). The total number of points over the region of interest (PNL and adenomas – P_{int}) and the total number of points on the reference space (liver parenchyma – P_{ref}) were counted. The following equation was used:

$$V_v = \frac{\sum_{\text{int}}^P}{\sum_{\text{ref}}^P}.$$

The V_v that results from this equation can range from 0 to 1, although the values can also be expressed as a percentage (Novaes *et al.*, 2012; Cupertino *et al.*, 2013; Santos *et al.*, 2013).

Total volume of the livers' lesions

The total volume of the PNL and adenomas were estimated by multiplying the V_v of the PNL by the liver volume (V_{liver}) (Howard and Reed, 2010; Marcos *et al.*, 2012). The following equation was used:

$$V_{\text{tot}} = V_v \times V_{\text{liver}}.$$

Expression of cytokeratin CK8/1

The primary guinea-pig polyclonal antibody CK8/18 (1 : 200 dilution; Lifespan Biosciences, Seattle, Washington, USA)

was used for immunological analysis. Tissue samples were fixed for 12 h in 4% paraformaldehyde. After dewaxing, antigen retrieval was performed with citrate buffer (pH 6.0) at 95°C for 10 min, followed by incubation with 10% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Nonspecific reactions were blocked with skimmed milk powder diluted to 5% in Tris HCl (pH 7.5) at 60°C for 1 h. The sections were then incubated with the primary antibody in a humid chamber overnight. On the second day, the sections were washed with Tris HCl buffer, followed by incubation for 1 h with a biotinylated secondary antibody supplied with the ABC Vectastain kit using the ABC method [Vectastain ABC Kit (guinea-pig IgG); Vector Laboratories, Burlingame, California, USA]. Visualization of the stained antigens was performed with 3,3'-diaminobenzidine tetrahydrochloride. The sections were dehydrated, mounted with Permount (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and photographed under an Olympus BX60 (Olympus, Tokyo, Japan) light microscope for the determination of CK8/18 immunopositivity and lesion characterization.

Statistical analyses

The density and total volume results of the lesions are expressed as the means, followed by the respective observed coefficients of variation (CV_{obs}), where CV_{obs} represents the ratio between the SD and average. Statistical analysis was carried out using Minitab 16 (Minitab Inc., State College, Pennsylvania, USA, 2010) statistical software taking into account the principles of systematic uniform random sampling (Gundersen *et al.*, 1999). Differences between groups were considered significant when P was less than or equal to 0.05. The data on the remodelling of PNL were analysed by one-way ANOVA, followed by the Tukey–Kramer test, which compared all of the groups together. InStat Software (GraphPad Software, Inc., La Jolla, California, USA) was used for these analyses.

Results

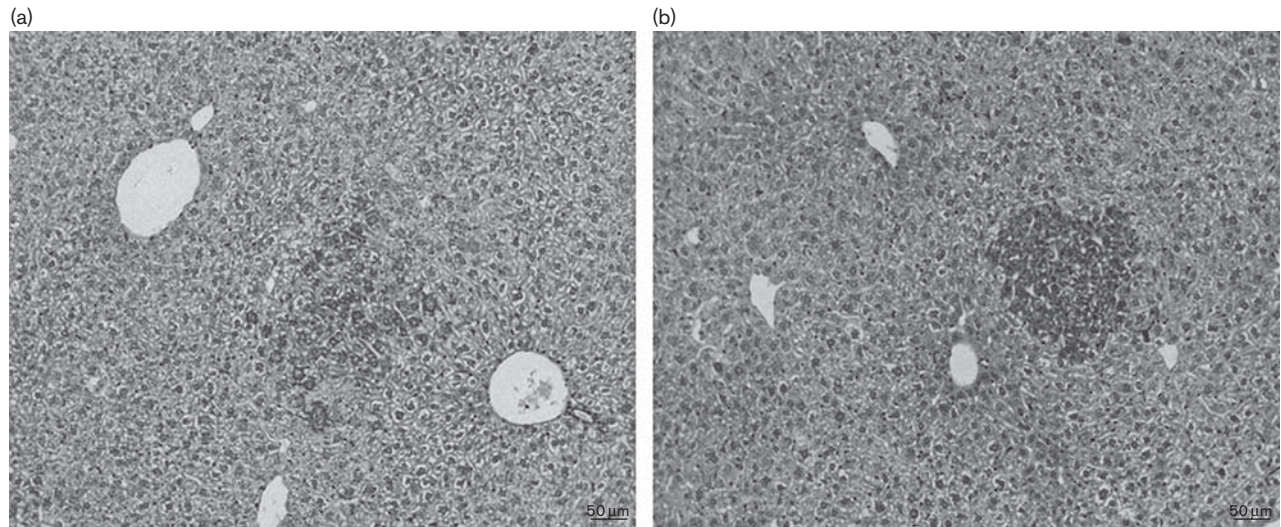
General observations

There was no death and no evident macroscopic signs of toxicity in mice during the experimental period. The final body weights and the total weight gain of mice in the different groups showed no differences. Compared with the control groups, the DEN+PO100 and DEN+PO400 groups showed an increase in liver weight and liver somatic index.

Histopathological examinations

PNL and adenomas were examined under a light microscope. The FAH of the basophilic, eosinophilic/acidophilic types and adenomas of the basophilic and clear cell types were observed. However, 98.33% of the section profiles of the observed PNL and adenomas were of the basophilic type. All of the mice in groups DEN and DEN+PO100 showed PNL. In the DEN+PO400

Fig. 2



Representative images of (a) remodelling and (b) persistent preneoplastic lesions in mice liver treated with DEN+pequi oil (DEN+PO400 and DEN+PO100, respectively, $\times 20$ objective). Toluidine blue staining.

group, 20% of the mice ($n = 3$) did not show any type of PNL or adenomas. The livers of the animals without lesions were further sectioned to confirm this observation. Remodelled preneoplastic lesions (rPNL) (Fig. 2) were characterized by morphology and toluidine blue affinity. Compared with persistent lesions, the rPNL lesions showed less uniformity with a mix of stained hepatocytes and clear hepatocytes. The rPNL were poorly delimited relative to the persistent PNL; the edges had a 'frayed' appearance because of the projection of the surrounding hepatocytes into the interior of the lesion (Mazzantini *et al.*, 2008). rPNL were also observed in all groups. However, in the DEN+PO400 group, more than 40% of the PNL profiles were of the remodelling type, representing a 2.4-fold of remodelling relative to that of the DEN group, with only 17% of rPNL (Table 2). Adenomas were diagnosed in the DEN, DEN+PO100 and DEN+PO400 groups. The last group (DEN+PO400) showed the lowest incidence of adenomas (20% or three mice) and the other groups had a higher incidence: 54.5% (six) in DEN and 46.1% (six) in

Table 2 Effect of pequi oil on the remodelling of PNL in the livers of DEN-treated mice

Group	Number of mice with PNL	Per cent of remodelling PNL relative to the total PNL
DEN	11/11	17.5 \pm 15.2 ^a
DEN+PO100	13/13	29.4 \pm 13.8 ^{a,b}
DEN+PO400	12/15	41.5 \pm 15.4 ^b

Data presented as mean \pm SD of the groups treated with DEN and DEN+pequi oil.

The means marked with the same letter do not differ statistically ($P = 0.05$).

DEN, diethylnitrosamine (10 mg/g); DEN+PO100 (DEN+100 mg/kg of pequi oil); DEN+PO400 (DEN+400 mg/kg of pequi oil); PNL, preneoplastic lesions.

DEN+PO100. Malignant neoplastic lesions were not observed in the experimental groups.

Stereological analysis of lesions

The total volume and V_v of lesions (PNL and adenomas) in the livers of mice treated with pequi oil at 400 mg/kg (DEN+PO400) were reduced by 51% compared with the DEN group. In addition, no significant reduction in the total volume or V_v was observed in the DEN+PO100 group. The liver volume remained uniform in all groups (Table 3).

Expression of cytokeratin CK8/18

Positivity for CK8/18 was observed in the bile ducts of all of the animals that were examined and was used as an internal control. Basophilic foci and cell adenomas were positive for CK8/18 (Fig. 3), although some foci were not

Table 3 Stereological parameters of the liver

Stereological parameters	Groups		
	DEN	DEN+PO100	DEN+PO400
Volumes			
Liver volume (cm ³) ^{NS}	1.48 \pm 0.21 ^a	1.38 \pm 0.14 ^a	1.52 \pm 0.10 ^a
Volume density of the livers lesions (V_v) [*]	0.044 \pm 0.025 ^a	0.032 \pm 0.010 ^{a,b}	0.021 \pm 0.009 ^b
Total volume of lesions (V_{tot}) (cm ³) ^{**}	0.067 \pm 0.041 ^a	0.044 \pm 0.013 ^{a,b}	0.032 \pm 0.015 ^b

Data presented as mean \pm SD. Animals treated with DEN 10 μ g/g; pequi oil 100 mg/kg and 400 mg/kg.

The means marked with the same letter do not differ statistically.

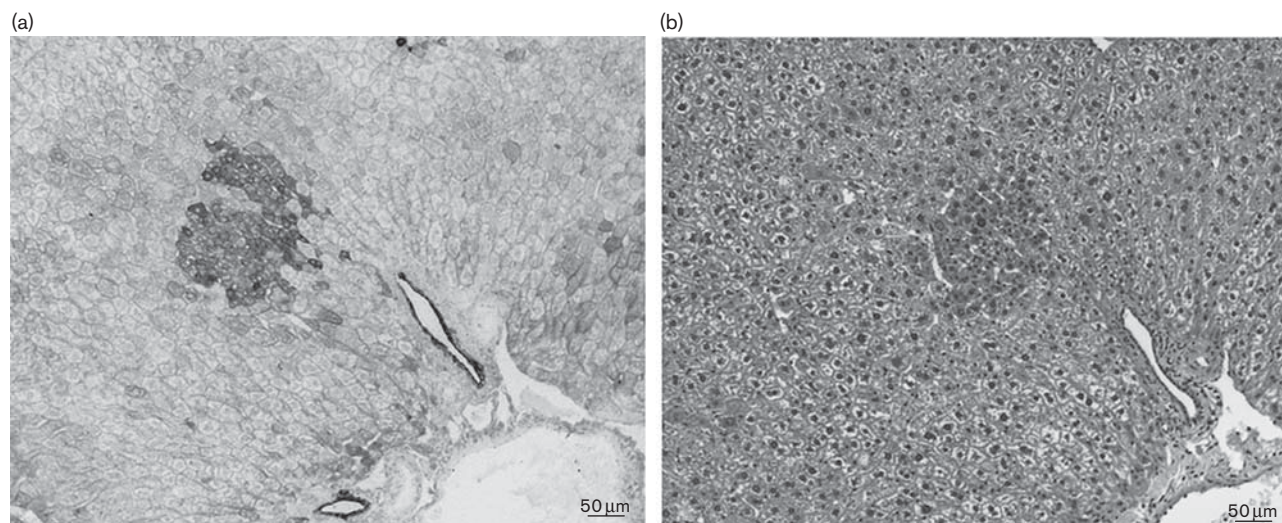
DEN+PO100 (DEN+100 mg/kg of pequi oil); DEN+PO400 (DEN+400 mg/kg of pequi oil); V_{tot} and V_v of preneoplastic lesions and adenomas.

^{NS} $P = 0.137$.

^{*} $P = 0.017$.

^{**} $P = 0.025$.

Fig. 3



Immunohistochemistry for CK8/18 (a) and haematoxylin and eosin (b) staining in serial sections in the liver of BALB/c mice treated with diethylnitrosamine + pequi oil (DEN + PO100, $\times 20$ objective). The basophilic focus observed in the centre of the image in (b) reacts positively for CK8/18 (a).

marked. A decrease in the number of FAH areas that were positive for CK8/18 in the DEN + PO400 group was observed.

Discussion

In this study, we evaluated the chemopreventive effect of pequi oil on chemically induced hepatocarcinogenesis in mice. The results indicate that pequi oil at a dose of 400 mg/kg showed hepatoprotective effects against DEN-induced carcinoma. The oil significantly reduced the total volume of PNL and adenomas in mice livers and reduced the number of persistent PNL, which are considered hepatocarcinoma precursors (Tatematsu *et al.*, 1983). The treatment also reduced the incidence of adenomas, suggesting that the pequi oil is effective in reducing the hepatocarcinogenesis promotion and progression steps. In addition, the number of mice that showed lesions preneoplastic or adenoma was reduced by 20%, which indicates that pequi oil potentially inhibited cellular proliferation after initiation by DEN. These results suggest a potential action of the pequi oil as a hepatocarcinogenesis blocking agent. DEN is a potent initiator that alkylates the DNA structure (Glauert *et al.*, 2010) and produces reactive oxygen species and substances that form DNA adducts. Both activities are responsible for DEN carcinogenic activity (Schuller, 2007; Glauert *et al.*, 2010). Large quantities of DNA adducts have been found in the livers of rats 20 days after administration of DEN (Dragan *et al.*, 1994; Verna *et al.*, 1996). DNA lesions may be reduced by pequi oil; Miranda-Vilela *et al.* (2009a) reported that upon administration of pequi oil to athletes after intense exercise, fewer DNA lesions were found. These results and our

observations suggest that pequi oil may act, at least partially, like other carcinogenesis-blocking substances. Like these other substances, pequi oil may function by inducing DNA repair (Surh, 2003; Steward and Brown, 2013) or contributing towards a better cellular environment through its antioxidant properties, which improve the efficiency of the cellular and DNA repair machinery. This result explains the lower (51%) total volume of PNL and adenomas found in the livers of mice that received pequi oil. Pequi oil has been used in popular medicine for the treatment of several diseases (Almeida and Silva, 1994; Mariano-Da-Silva *et al.*, 2009), including cancer control (Almeida *et al.*, 2000). Studies with *C. brasiliense* oil have shown the oil's inhibitory effects on oxidative cellular damage, which are related to its rich antioxidant content (Miranda-Vilela *et al.*, 2009a). These authors suggested that the effects of pequi oil on the mouse liver may be attributed to the antioxidant activity of the substances found in the oil, such as β -carotene, lycopene (Oliveira *et al.*, 2006) and vitamin C (Almeida, 1998). These compounds are inhibitors of oxidative stress, a process involving chemicals that can initiate or promote liver neoplasms (Glauert *et al.*, 2010; Santos *et al.*, 2014). Several studies have shown the chemopreventive effects of antioxidant compounds in both the initiation and the promotion phase of hepatocarcinogenesis (Gradelet *et al.*, 1998; Moreno *et al.*, 2002; Toledo *et al.*, 2003). The results are consistent with this evidence and suggest that pequi oil is a potential blocking agent and suppressor of hepatocarcinogenesis.

The ability of preneoplastic foci to be remodelled to apparently healthy liver tissue or to persist and develop

into a neoplasm is fundamental to the development of hepatocarcinogenesis (Bannasch *et al.*, 2001; Feo *et al.*, 2006; Scolastici *et al.*, 2014). Although there is relatively little information available on this process, some researchers have linked the persistence of PNL to redifferentiation and the inhibition of apoptosis (Feo *et al.*, 2006). The induction of preneoplastic remodelling is one of the mechanisms by which the pequi oil exerted its chemopreventive effects in this study. This effect is verified by the 2.4-fold increase in the rPNL in the group that received 400 mg/kg of pequi oil relative to the DEN group. This effect may be because of the antioxidant activity of the substances found in pequi oil, such as β -carotene, a carotenoid known to exert a protective effect on against hepatocarcinogenesis in rats through an increase in GST-positive remodelling lesions (Rizzi *et al.*, 1997; Fonseca *et al.*, 2005). Thus, the PNL volume reduction is associated with increased PNL remodelling by pequi oil, which strengthens the potential of this oil for use as a preventive agent against chemically induced liver cancer.

In the mouse liver, unlike the rat liver, GST expression is not a reliable marker of liver PNL (Kawai *et al.*, 2010). However, CK8 and CK18 are important markers of hepatocyte stress (Omary *et al.*, 2002; Kakehashi *et al.*, 2010; Kushida *et al.*, 2011). Although it was not evaluated quantitatively, we observed a tendency of a reduction in the area of CK8/18-positive PNL in DEN + PO400 animals. The increase in CK8/18 in basophilic foci has been associated with the induction of cell proliferation, the generation of hepatocyte stress and a resulting intracellular accumulation of these proteins (Kakehashi *et al.*, 2010; Kawai *et al.*, 2010). Therefore, the reduction in the CK8/18-positive FAH in the DEN + PO400 group may be related to the antiproliferative activity of substances present in *C. brasiliense* oil, suggesting that the antiproliferative mechanism of pequi oil is responsible for the reduction in the total volume of the PNL observed in our study.

In conclusion, *C. brasiliense* oil showed dose-dependent hepatoprotective activity. Administration of pequi oil reduced the development of liver PNL and adenoma and induced remodelling of the lesions in mice. The chemopreventive properties of the oil are likely related to the antioxidants contained in the oil. Therefore, pequi oil has potential for use in the prevention of liver cancer.

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Conflicts of interest

There are no conflicts of interest.

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