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Chemopreventive effects of a *Tamarindus indica* fruit extract against colon carcinogenesis depends on the dietary cholesterol levels in hamsters

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ABSTRACT

Tamarind has significant antioxidant potential. We showed that tamarind protects hypercholesterolemic hamsters from atherosclerosis. Hypercholesterolemia might increase the risk of colon cancer. We investigated whether tamarind extract modulates the risk of colon cancer in hypercholesterolemic hamsters. Hamsters (n = 64) were given tamarind and a hypercholesterolemic diet for 8 weeks. The groups were the control, tamarind treatment, hypercholesterolemic, and hypercholesterolemic treated with tamarind groups. Half of each group was exposed to the carcinogen dimethylhydrazine (DMH) at the 8th week. All hamsters were euthanatized at the 10th week. In carcinogen-exposed hypercholesterolemic hamsters, tamarind did not alter the cholesterol or triglyceride serum levels, but it reduced biomarkers of liver damage (alanine transaminase [ALT], and aspartate aminotransferase [AST]). Tamarind decreased DNA damage in hepatocytes, as demonstrated by analysis with an *anti*-γH2A.X antibody. In liver and serum samples, we found that this fruit extract reduced lipid peroxidation (thiobarbituric acid reactive substances [TBARS]) and increased endogenous antioxidant mechanisms (glutathione peroxidase [GPx] and superoxide dismutase [SOD]). However, tamarind did not alter either lipid peroxidation or antioxidant defenses in the colon, which contrasts with DMH exposure. Moreover, tamarind significantly increased the stool content of cholesterol. Although tamarind reduced the risk of colon cancer in hypercholesterolemic hamsters that were carcinogenically exposed to DMH by 63.8% (Metallothionein), it was still ~51% higher than for animals fed a regular diet. Staining colon samples with an *anti*-γH2A.X antibody confirmed these findings. We suggest that tamarind has chemoprotective activity against the development of colon carcinogenesis, although a hypercholesterolemic diet might impair this protection.

1. Introduction

Various healing properties have been reported about *Tamarindus indica* L. (*T. indica*; tamarind), such as digestive, carminative, laxative, expectorant, tonic, antioxidant, hypolipemic, anti-atherosclerotic, antioxidant, antihepatotoxic, anti-inflammatory, antimutagenic, and antidiabetic properties (Komutarin et al., 2004; Maiti et al., 2004; Martinello et al., 2006; Ramos et al., 2003; Rimbau et al., 1999). Our previous report showed that a *T. indica* fruit had approximately 34.02 ± 2.11 nM/ml of polyphenols and 35.51 ± 5.61 μg/ml of flavonoids (Martinello et al., 2006). Owen and colleagues profiled antioxidants in *T. indica* pericarp and seeds. They found that 73% of the phenolic antioxidants in pericarp were proanthcyanidins, while procyanidins were largely found in seeds (Sudjaroen et al., 2005). Recently, other research groups supported these findings that *T. indica* is rich in antioxidant compounds (Escalona-Arranz et al., 2016; Natukunda et al., 2016). Notably, we

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found that *T. indica* reduces the risk of atherosclerosis in hypercholesterolemic hamsters (Martinello et al., 2006).

However, the effects of natural compounds on colon carcinogenesis are controversial (Femia et al., 2003; Millen et al., 2007; Nalini et al., 2006; Shi et al., 2010). For instance, *T. indica* was previously found to promote pro-carcinogenic effects on colon tissue (Shivshankar and Shyamala Devi, 2004). Colon cancer is the third most common malignancy worldwide (Siegel et al., 2014). It affects over 1.2 million people in the USA, and prospective data suggest ~150 thousand patients will be newly diagnosed *per* year going forward (Siegel et al., 2012). Previous reports suggested that fatty acids and their metabolites promote free radical-induced DNA damage, which enhances the risk of colon cancer (Chithra and Leelamma, 2000; Cruse et al., 1979). Colon carcinogenesis develops through a multi-step sequence of changes (Fearon and Vogelstein, 1990), which means that colon tumors might arise from preneoplastic lesions (Bird, 1987; Fearon and Vogelstein, 1990; Maurin et al., 2007). Mutations and deletions in the colonic epithelial cellular DNA are the earliest changes that induce the development of colon cancer tumors (Maurin et al., 2007). In terms of whether phosphorylation on serine 139 of histone 2AX (γ-H2AX) signals the genomic machinery that a DNA damage event is taking place (Lobrich and Jeggo, 2007), new evidence demonstrated that this phosphorylated protein is the gold standard biomarker to study genomic damage (Orthwein et al., 2014). It seems that when colonocytes undergo massive and protracted DNA damage, histological modifications in colonic glands become detectable and have been named aberrant crypt foci (ACF) (Bird, 1987). Another biomarker, named metallothionein (MT), was shown to precisely determine the intensity of early carcinogenic events in the colon (Mori et al., 2012). Many preneoplastic lesions classically increase the risk of colon cancer because exposure to carcinogens promotes genomic instability and aberrant cell growth, leading to tissue transformation (Bird, 1995; Bird and Good, 2000; McLellan et al., 1991). High-expression of novel biomarkers, such as MT and γ-H2AX, demonstrates the risk of colon cancer resembles the development of ACF (Frajacomo et al., 2015).

Hamsters are the best animal models to study the effects of dietary cholesterol, which might illustrate the effects of hypercholesterolemia in humans (Lim et al., 2013; Martinello et al., 2006). Although a single report suggested that hamsters do not endure colon carcinogenesis after exposure to dimethylhydrazine (DMH) (Jeong and Kamino, 1993), there has been strong evidence indicating otherwise (Moore et al., 1987; Paulsen et al., 1996); for example, DMH in hamster drinking water was reported to induced angiosarcomas in the liver, lungs, muscle, heart, and pancreas (Toth, 1972). Paulsen et al. observed that DMH injections induce colon preneoplastic lesions in two different hamster strains (Paulsen et al., 1996). Additionally, Moore et al. demonstrated that hamsters exposed to DMH develop hepatic and hemangiocellular liver lesions, forestomach papillomas, and colon adenocarcinomas (Moore et al., 1987). Therefore, exposure of hypercholesterolemic hamsters to carcinogens seems a reasonable experimental model to evaluate the modulation of colon cancer risk with any hypocholesterolemic treatment.

Given that it seems odd an antioxidant-rich fruit, such as *T. indica* (Escalona-Arranz et al., 2016; Martinello et al., 2006; Natukunda et al., 2016; Sudjaroen et al., 2005), has been found to delay the development of renal cell carcinoma in rats (Vargas-Olvera et al., 2012) while promoting colon cancer (Shivshankar and Shyamala Devi, 2004), further investigation is required. Hence, we explored whether the antioxidant activity of *T. indica* could reduce the risk of colon cancer in hypercholesterolemic hamsters.

2. Methods

2.1. Preparation of tamarind fruit extracts

According to our previous report (Martinello et al., 2006), tamarind fruits (100 g) were macerated in 70% alcohol (300 ml; 4 °C; 72 h). Filtration and evaporation were used to remove the alcohol from the whole tamarind fruit extract. Before each treatment, dried tamarind extracts (5%) were diluted in water.

2.2. Experimental design

Sixty-four Golden Syrian male hamsters (±115 g; 30 days) were acclimatized for two weeks before starting the experiment (5 hamsters/plastic cage; 25 ± 2 °C; 12-12 h light-dark). The experiments followed the approved protocol by the Animal Care and Use Ethical Committee (n° 05.1.590.53.0). Hamsters were randomly divided into 4 groups (each group had 16 hamsters) at the beginning of the current experiments. These 4 groups were named standard diet (CT; Table 1), treatment with *T. indica* extract (TI), hypercholesterolemic diet (CL; full description of this diet is given below), and hypercholesterolemic diet plus tamarind treatment (TI-CL). After 56 days from the first day of tamarind treatment, half of the initial four groups (resulting in 8 groups with 8 hamsters each) were exposed to the carcinogen dimethylhydrazine (DMH; 30 mg/kg; single subcutaneous injection). At the end of the 10th experimental week, all 8 groups were euthanized. All hamsters had free access to food and liquids throughout the 10 weeks.

A hypercholesterolemic diet (CL) was prepared by adding 1% (w/w) cholesterol (92.5%; VETEC, Sigma-Aldrich, DE; V900415) to the standard chow (CT; Guabi Nutrilabor, Mogiana Food Industry, Brazil; Table 2). Briefly, CT pellets were milled before cholesterol was added and re-pelleting. After drying hypercholesterolemic pellets, each 300 g was vacuum sealed in dark containers and stored at 4 °C, which was repeated weekly.

2.3. Sacrifice

All hamsters were weighed at the beginning and end of the current experiment. Hamsters were sacrificed in a CO_2 chamber. Blood was collected by cardiac puncture and, centrifuged at 3,000*g* for 10 min. In individual autopsies, the liver and colon were carefully but quickly removed. Samples were homogenized according to our previous description (Martinello et al., 2006). Liver and colon samples were also fixed in 10% buffered formalin for 24 h.

2.4. Histopathological analysis

After paraffin-embedded colon samples had been sectioned at thickness of 4 μm, they were stained with H&E. According to our

Table 1
Composition of standard diet.

General compounds	%	Specific compounds	Amount
Carbohydrate	52	Niacin	80 mg
Protein	22	Calcium*	50 mg
Fat	4	Folic Acid	3 mg
Fiber	8	Biotin	0.1 mg
Calcium	1.2	Choline	1200 mg
Phosphorus	0.8		
Other Minerals	10		
Vitamin Mix	2		
Other compounds	0.01		

Standard diet (CT). Values are given for each 1000 g. *Calcium Pantothenate.

Table 2
Initial and final body weight values, and weight gain.

Period	Carcinogen	Treatment				Comparison	P <
		CT	TI	CL	TI-CL		
Initial	No-DMH	138.4 ± 16.7	133.9 ± 15.6	123.4 ± 20	122 ± 23.9	*vs CT	***0.001
(g)	DMH	130.1 ± 17.4	135.6 ± 16.6	129.3 ± 19.5	128.7 ± 20.1	§vs TI	*0.05
Final	No-DMH	160.7 ± 11.8	167.1 ± 8.7	163.2 ± 7.5	168.4 ± 11.8		§0.05
(g)	DMH	145.1 ± 18.4	143.3 ± 11.3	139.9 ± 9.8	147.1 ± 9.5		
Weight Gain	No-DMH	0.31 ± 0.07	0.47 ± 0.09*	0.56 ± 0.17***	0.66 ± 0.26§		
	DMH	0.21 ± 0.14	0.11 ± 0.07	0.15 ± 0.13	0.26 ± 0.15		

Standard diet (CT); hypercholesterolemic diet (CL); treatment with *Tamarind indica* extract (TI). Values are shown as the mean ± standard deviation. Two-Way ANOVA test (Bonferroni post hoc test) was used for all comparisons from both initial and final measurements. Weight gain = (initial-final/total days).

previous description (Kannen et al., 2012a), aberrant crypts with dysplastic features were enumerated under light microscopy at 400× magnification (Fig. 1A). The ratio of aberrant crypts *per* ~100 normal glands illustrated the risk of colon cancer.

Primary antibodies were applied to stain 4-μm paraffin-embedded sections according to the manufacturer's instructions. These antibodies were *anti*-MT [clone FL-61 (1:100); Santa Cruz Biotechnology, Santa Cruz, California, USA] and *anti*-γH2A.X [clone 3F2 (1:200); Abcam, Burlingame, California, USA]. Afterward, tissue sections were counterstained with Harris's hematoxylin. The ratio of positive MT crypts *per* total number of colonic glands validated the data from histopathologically determined to be aberrant crypts. The number of positive γH2A.X colonocytes *per* crypt demonstrated the intensity of DNA damage in colon samples. In the liver, this DNA damage intensity was determined to be the number of positive γH2A.X hepatocytes *per* microscopic field.

2.5. Biochemical analyses

Total cholesterol (mg/dL), triglyceride (mg/dL), high-density lipoprotein (HDL; mg/dL), low-density lipoprotein (LDL; mg/dL), alanine transaminase (ALT; U/L), and aspartate aminotransferase (AST; U/L) levels were measured in serum samples (triplicate) according to standard methods previously reported (Friedewald et al., 1972; Warnick et al., 1982) (Abbott VP Super System Autoanalyzer [Abbot, US]; commercial enzymatic kit [Labtest, Brazil]). A further description was previously reported (Martinello et al., 2006).

2.6. Fecal cholesterol content

Fecal cholesterol levels were analyzed in stool samples (1 g) according to a previous description (Ntanios and Jones, 1999). Briefly, a gas-liquid chromatography with a flame ionization detector (GLC; HP 5890 Series II, Hewlett Packard, US) and capillary column (SAC-5; Supelco, US) was used. Samples were first mixed to the internal standard compound 5α-cholestane (0.2 mg/g). Saponification was then performed with 0.5 M methanolic KOH (1 h; 100 °C). Then, distilled water and petroleum-ether were added to samples cooled at room temperature. Non-saponifiable materials at the upper solution layer were dried with liquid nitrogen and resuspended in hexane (300 μl). This mixture was injected into the GLC for analysis (2 μL). Identification peaks were confirmed with phytosterol (Sigma, US). Values are expressed as mg/dL.

2.7. Determination of a lipid peroxidation reaction and antioxidant enzyme levels in serum, colon, and liver samples

First, tissue samples were homogenized in ice-cold potassium phosphate buffer with a Potter-Elvehjem homogenizer. Centrifugation was then performed at 13,000 g (4min; 4 °C). For each of the following analyses, a full description was previously reported (Martinello et al., 2006).

2.7.1. Thiobarbituric acid reactive substances (TBARS)

TBARS activity (nM/g) represented the total lipid peroxidation in serum and tissue samples. Before the first incubation (6 h; 37 °C), a reaction buffer (KCl [125 mmol/L]; $C_8H_{18}N_2O_4S$/KOH [50 mmol/L]) was added to the samples. Then, the starting buffer was added (Fe $[NH_4]_2[SO_4]_2$ [50 μM/L]; $Na_3C_6H_5O_7$ [2 mM/L]). Centrifugation (1,900 g; 10min) was performed after lipid and protein precipitation (H_2SO_4 [12 N]; $H_3PW_{12}O_{40}$ [10%]). The pellet was resuspended in distilled water and $C_4H_4N_2O_2S$ (1%), which was kept at 95 °C for

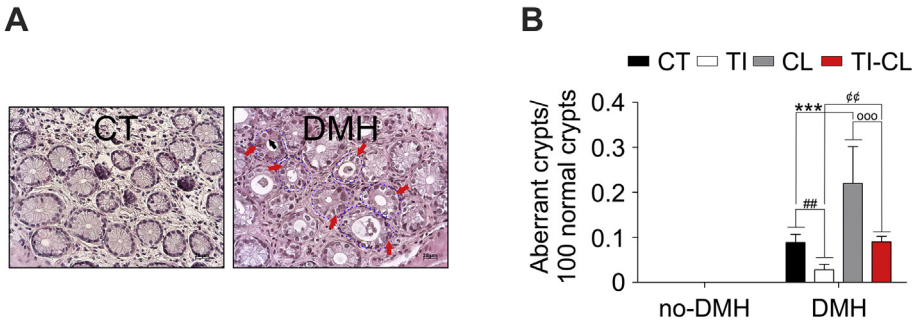

Fig. 1. Effects of tamarind treatment in colon carcinogenesis. (A) Representative images of normal (left-side picture) and aberrant crypts (right-side picture) in the colon of either carcinogen-unexposed (CT) or -exposed hamsters (dimethylhydrazine, DMH). Sectioned blue lines enclose aberrant crypts with dysplastic features. A black arrow shows a vacuolated Feulgen-positive body, while red arrows demonstrate the partial loss of cell polarity. Pictures were taken at 40× magnification (scale bars = 20 μm). (B) Relative values for colon preneoplastic lesions were determined to be the number of aberrant crypts *per* normal crypts (~100 standard glands). Standard diet (CT); hypercholesterolemic diet (CL); treatment with *Tamarind indica* extract (TI); and, TI plus CL (TI-CL). The two-way ANOVA test (Bonferroni's post hoc test) was applied for statistical analysis. Values are shown as the mean ± standard deviation ($^{\#\#}P < 0.01$, CT *vs* TI; $^{ooo}P < 0.001$, CL *vs* TI-CL group; $^{***}P < 0.001$, CT *vs* CL; and $^{¢¢}P < 0.01$, TI *vs* TI-CL group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1 h. Extraction with C_4H_9OH was performed in cooled samples at room temperature (Kannen et al., 2013). The reactions were finally analyzed (UV 515/553 nm wavelengths) in a Hitachi F4500 fluorimeter (Hitachi, US). Values were expressed as μM/g or mL.

2.7.2. Glutathione peroxidase (GPx)

A spectrophotometrical analysis (340 nm) was applied to determine the GPx activity. Samples were first mixed in the reaction buffer (KH_2PO_4 [0.1 M/L; pH 7.0]; $C_{21}H_{26}N_7Na_4O_{17}P_3 \cdot xH_2O$ [NADPH; 0.2 mM/L]; $[(HOOCCH_2)_2NCH_2CH_2]_2NCH_2COOH$ [1 mM/L]; GSH [1.5 mM/L]; and GR [7.3×10^{-6} U/mL]. The starting buffer (tBuOOH [0.12 mM/L]) was added afterwards (Flohe and Gunzler, 1984). Values were expressed as μM/min/g or mL.

2.7.3. Catalase (CAT)

Spectrophotometrical analysis determined the CAT activity in all samples. Samples were mixed with the reaction buffer (H_2O_2 [30 mM/L]; KH_2PO_4 [50 mM/L]; pH 7.0), which was then analyzed (Aebi, 1984). Values were expressed as mM/L of consumed H_2O_2 (cH_2O_2/min) *per* g or mL.

2.7.4. Superoxide dismutase (SOD)

SOD activity was measured by spectrophotometrical analysis (340 nm). Reaction buffer ($HN[CH_2CH_2OH]_2$ [100 mM/L]; NADPH [0.14 mM/L]; $[HO_2CCH_2]_2NCH_2CH_2N[CH_2CO_2H]_2/MnCl_2$ [2.27/1.14 mM/L; pH 7.0]) was first added to the samples. NADPH oxidation was started with $HSCH_2CH_2OH$ (1 mM/L) for analysis (Paoletti and Mocali, 1990). Values for the SOD activity (U/g or mL) were based on a standard curve (IC50 [NADPH oxidation inhibitions] = U [one SOD unit]).

2.8. Statistical analysis

Data were analyzed with the two-way ANOVA test (Bonferroni's post hoc test) using GraphPad Prism 5 (GraphPad Software, US). This statistical test enables different categorical independent endpoints to be analyzed on one dependent variable. Statistical significance was set at $p < 0.05$. All values are reported as the mean ± standard deviation.

3. Results

3.1. Cholesterol alters the chemoprotective effects of T. indica in the intestinal colon

Here, we investigated whether a tamarind fruit extract might modify the risk of colon cancer. We observed that hamsters endured well the current experiments (Tables 2 and 3). Although the final weight did not significantly change within groups exposed to DMH, carcinogen-unexposed hamsters treated with TI and CL gained more weight than control hamsters (Table 2). While the food intake remained unchanged, hamsters given TI drank fewer liquids than the controls (Table 3).

Histopathological analysis revealed tamarind reduced the development of dysplastic aberrant crypts (Fig. 1B). However, a careful statistical analysis showed that a hypercholesterolemic diet increased the risk of colon cancer in both tamarind-treated and -untreated groups that were carcinogenically exposed to DMH (Fig. 1B). We validated these results by staining colon samples with an *anti*-MT antibody, which is a biomarker linked to the development of preneoplastic lesions in this tissue (Frajacomo et al., 2015; Mori et al., 2012). It not only confirmed that tamarind has anti-carcinogenic effects but also demonstrated that a hypercholesterolemic diet impairs the benefits of this fruit (Fig. 2A). Phosphorylation on serine 139 of that histone 2AX (γ-H2AX) is broadly studied to determine the carcinogenic DNA double-strand breaks [DSB (Lobrich and Jeggo, 2007);]. This analysis supported that the chemoprotective effects of tamarind are impaired by increasing the dietary cholesterol intake (Fig. 2B; CT *vs* CL [$P < 0.001$]; TI *vs* TI-CL [$P < 0.001$]). Then, we biochemically analyzed the cholesterol levels in stool content and found this lipid compound was highly increased by tamarind in both DMH-exposed and unexposed hamsters (Fig. 2C). The dietary cholesterol content seems to impair some chemoprotective effects of tamarind fruit extract.

3.2. T. indica protects the liver from hypercholesterolemia- and carcinogen-induced lipid peroxidation

Given that small bowel uptake of cholesterol alters hepatic metabolism (Iqbal and Hussain, 2009), we biochemically analyzed several biomarkers to determine the further effects of tamarind extract in hamsters fed a hypercholesterolemic diet. According to Table 5, this dietary regimen increased the serum cholesterol levels in both carcinogenic exposed and unexposed groups. Moreover, carcinogenic exposure impaired the anticholesterolemic effects of tamarind. Fruit extract decreased the triglyceride levels in DMH-unexposed hamsters (Table 4). Although tamarind augmented the HDL concentrations in experimental groups that were not exposed to DMH, it did not protect hamsters against hypercholesterolemia and carcinogenic exposure (Table 4). Only carcinogen-unexposed hamsters showed reduced serum LDL levels with fruit extract treatment (Table 4). When biomarkers of liver damage were analyzed (ALT and AST), we found that potential damaging effects of DMH were counteracted by treating hypercholesterolemic hamsters with tamarind (Table 4).

To verify these events, we biochemically analyzed liver samples. Table 5 shows that hypercholesterolemic hamsters that were carcinogenically exposed to DMH had reduced hepatic lipid peroxidation when treated with tamarind. Although this fruit extract increased the hepatic levels of that scavenging enzyme GPx against lipid hydroperoxides, hypercholesterolemia reduced the intensity of these effects (Table 5). Tamarind extract only increased the hepatic content of CAT in normal hamsters (Table 5). In carcinogen-

Table 3
Daily food and water intakes.

Type	Carcinogen	Treatments				Comparison	P <
		CT	TI	CL	TI-CL		
Food	No-DMH	48 ± 4	43 ± 11	50 ± 1	49 ± 4	**vs* CT	***0.001
(g/day)	DMH	45 ± 6	47 ± 9	46 ± 4	47 ± 8	§*vs* TI	§§0.01
Liquids	No-DMH	138 ± 46	60 ± 19***	122 ± 51§§	58 ± 21$^{\circ\circ}$	$^{\circ}$*vs* CL	§§§0.001
(ml/day)	DMH	135 ± 36	55 ± 21***	130 ± 49§§§	60 ± 17$^{\circ\circ\circ}$		$^{\circ\circ}$0.01
							$^{\circ\circ\circ}$0.001

Standard diet (CT); hypercholesterolemic diet (CL); treatment with *Tamarind indica* extract (TI). Liquids mean either water or TI. Values are shown as the mean ± standard deviation. Two-Way ANOVA test (Bonferroni post hoc test) was applied for data analysis.

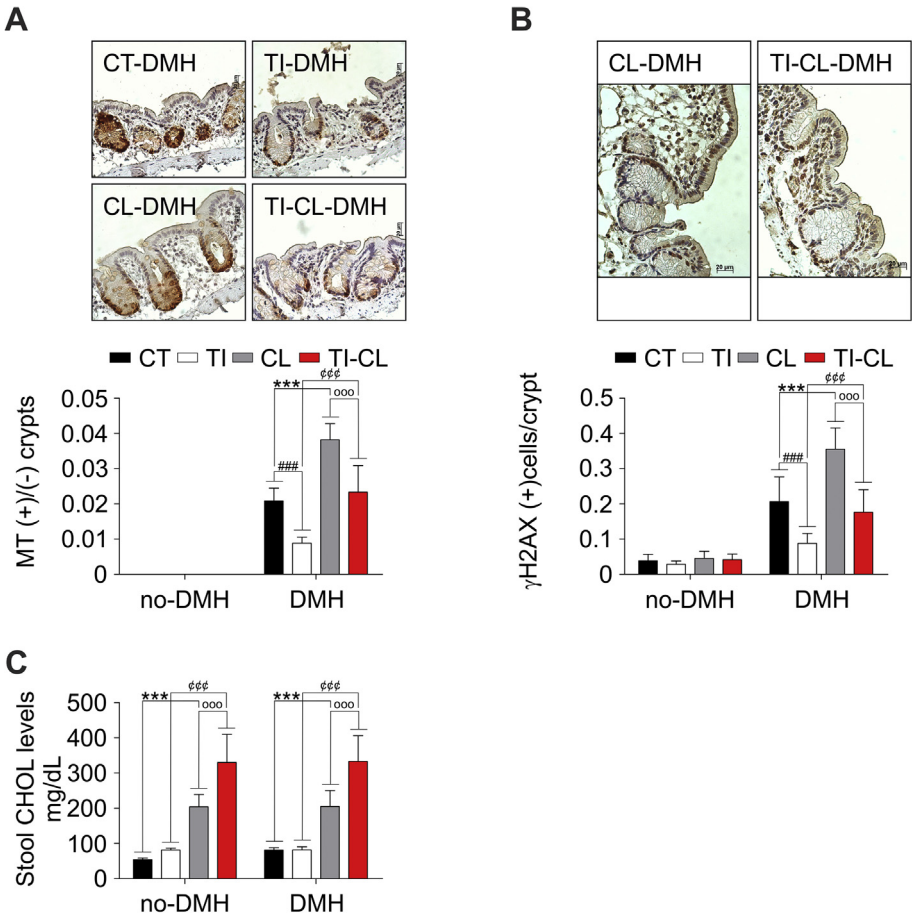

Fig. 2. Activity of tamarind treatment in colon carcinogenesis. (A) Representative pictures of metallothionein (MT) expression in colonic crypts for each group of carcinogen-exposed hamsters. Standard diet (CT); hypercholesterolemic diet (CL); treatment with *Tamarind indica* extract (TI); and, TI plus CL (TI-CL). Carcinogenic crypts are positively stained for MT (dark-brown). Images were taken at 40× magnification (scale bars = 20 μm). Relative values for the MT expression in colonic crypts from dimethylhydrazine (DMH) -exposed groups. (B) Representative images of phosphorylated histone 2AX (γH2AX) expression in colonic crypts for two groups of carcinogen-exposed hamsters (CL, and TI-CL). DNA damaged colonocytes are positively stained for γH2AX (dark-brown). Pictures were taken at 40× magnification (scale bars = 20 μm). Relative values for γH2AX expression in colonic crypts from DMH-exposed groups. (C) The cholesterol (CHOL) concentration in stool (mg/dL) was determined biochemically. The two-way ANOVA test (Bonferroni's post hoc test) was applied for statistical analysis. Values are shown as the mean ± standard deviation ($^{###}P < 0.001$, CT *vs* TI; $^{ooo}P < 0.001$, CL *vs* TI-CL group; $^{***}P < 0.001$, CT *vs* CL; and $^{¢¢¢}P < 0.001$, TI *vs* TI-CL group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unexposed and -exposed groups, cholesterol seems to impair the tamarind-related increase in hepatic SOD levels (Table 5).

Then, we histopathologically analyzed liver samples for DSB with an *anti*-γ-H2AX antibody (Fig. 3A). Although tamarind protects carcinogenically exposed hepatocytes from DNA damage in both dietary experimental conditions, cholesterol seem to interfere with its hepatic benefits (Fig. 3B). These findings might illustrate that tamarind protects hepatic functions from damage, while cholesterol might weaken its activity.

3.3. *T. indica* modulates serum lipid peroxidation, but not colonic lipid peroxidation

Table 7 reveals that the serum SOD levels were increased in control hamsters treated with tamarind. A hypercholesterolemic diet and carcinogenic exposure impaired this positive effect of tamarind (Table 6). Although tamarind increased the serum CAT levels in hamsters fed a regular diet and exposed to DMH, cholesterol blocked its protective activity (Table 6). Tamarind only improved enzymatic protection against lipid peroxidation in DMH-unexposed hamsters (Table 6), while cholesterol negatively impacted its protective effects (Table 6). Finally, we found that tamarind extract did not protect from the DMH-related impairment in antioxidant enzyme levels or promotion of lipid peroxidation in the intestinal colon (Table 7). Tamarind failed to improve the tissue antioxidant capacity of the intestinal colon.

4. Discussion

We treated hypercholesterolemic hamsters that were carcinogenically exposed with tamarind to reduce their lipid peroxidation and thus the risk of colon cancer. Our findings suggest that the benefits of tamarind in colon carcinogenesis might be related to its hepatic protection against lipid peroxidation. Of note, subcutaneous exposure to DMH promotes its slow release into the bloodstream, which is followed by hepatic lipid peroxidation throughout metabolic activation (Rajeshkumar and Kuttan, 2003). Specifically, hepatic CYP2E1 metabolizes DMH into azoxymethane (AOM), which later becomes methylazoxymethanol (MAM). Colonocytes then metabolize MAM into methyl diazonium ion, as well as methyl cation, that binds and damages DNA (Rosenberg et al., 2009). Another report showed that DMH promotes lipid peroxidation and malignant transformation in the colon (Dudeja and Brasitus, 1990). Indeed, dietary cholesterol promoted lipid peroxidation and colon adenomas, reducing the tissue antioxidant capacity in DMH-exposed rats (Tseng et al., 1996). On the other hand, tamarind has

Table 4
Serum fat acids and fecal cholesterol levels.

Analysis	Carcin	Treatments				Comp	P <
		CT	TI	CL	TI-CL		
CHOL mg/dL	No-DMH	153 ± 22.8	125 ± 13.4*	259 ± 34.2 ***	129.8 ± 11.9 ◦◦◦	*vs CT ◦vs CL	*0.05 ***0.001
	DMH	173.3 ± 8.6	158.9 ± 13	202.6 ± 28.6*	192.5 ± 24.8	§vs TI	◦◦0.01
TG mg/dL	No-DMH	380 ± 74.2	183 ± 29.5 ***	520 ± 74.8 ***	204 ± 25.9 ◦◦◦		◦◦◦0.001 §§§0.001
	DMH	286.3 ± 64.7	219.6 ± 48.2	476 ± 111.2 ***	337.1 ± 82.6 §§,◦◦◦		§§0.01
HDL mg/dL	No-DMH	48.2 ± 8.7	81.5 ± 3.1 ***	45.3 ± 4.4	73.2 ± 5.2 ◦◦◦		
	DMH	52.6 ± 7.4	67 ± 8.4 **	57.4 ± 6.9	65.5 ± 13.5		
LDL mg/dL	No-DMH	108.6 ± 25	52.3 ± 20.1***	214.6 ± 34.7***	56.6 ± 12.2◦◦◦		
	DMH	127 ± 26.3	94.9 ± 10.3	145.3 ± 33.6	118 ± 44.9		
ALT U/L	No-DMH	50.1 ± 12	36.7 ± 8	40 ± 4	39.8 ± 6.4		
	DMH	72.7 ± 25.8	69.8 ± 24	157.2 ± 36.6 ***	114.1 ± 14.9 §§§,◦◦◦		
AST U/L	No-DMH	44.4 ± 14.4	44.1 ± 17.6	41.8 ± 18.3	35.6 ± 9.3		
	DMH	91.6 ± 13.6	90.5 ± 5.9	170.9 ± 43.3 ***,§§§	127.8 ± 36.9 §§,◦◦		

Carcinogen (Carcin), Comparisons (Comp). Standard diet (CT), hypercholesterolemic diet (CL), treatment with *Tamarind indica* extract (TI). Cholesterol (CHOL), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine transaminase (ALT), and aspartate aminotransferase (AST). Values are shown as the mean ± standard deviation. Two-Way ANOVA test (Bonferroni post hoc test) was applied for data analysis.

Table 5
Hepatic oxidative stress.

Analysis	Carcin	Treatments				Comp	P <
		CT	TI	CL	TI-CL		
TBARS μM/g	No-DMH	601.7 ± 70.1	559.5 ± 100.1	634.6 ± 70.8	601.4 ± 68.7	*vs CT ◦vs CL	**0.01 ***0.001
	DMH	1084.7 ± 111.2	996.1 ± 107.9	1472.8 ± 151.2 ***	1309.6 ± 99.7 ◦◦,§§§	§vs TI	◦◦◦0.001
GPx μM/min/g	No-DMH	148.1 ± 4.3	218.6 ± 4.3 ***	146.8 ± 1.9	187.1 ± 3.2 ◦◦◦, §§§		◦◦0.01 ◦0.05
	DMH	140.8 ± 1.8	165.3 ± 4.5 **	136 ± 3.4	149.8 ± 0.8 ◦◦, §§		§§§0.001 §§0.01
CAT mM/L -cH_2O_2/min/g	No-DMH	1.2 ± 0.1	1.9 ± 0.1***	1.2 ± 0.1	1.3 ± 0.1		
	DMH	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1		
SOD U/g	No-DMH	327.3 ± 31.3	371.3 ± 20.3 **	259.2 ± 25.8 ***	278.4 ± 21.7 §§§		
	DMH	299.6 ± 32.2	325.3 ± 30.3	246.4 ± 22.4 ***	281.2 ± 18 ◦, §§		

Carcinogen (Carcin), Comparisons (Comp). Standard diet (CT), hypercholesterolemic diet (CL), treatment with *Tamarind indica* extract (TI). Cholesterol (CHOL), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine transaminase (ALT), and aspartate aminotransferase (AST). Values are shown as the mean ± standard deviation. Two-Way ANOVA test (Bonferroni post hoc test) was applied for data analysis.

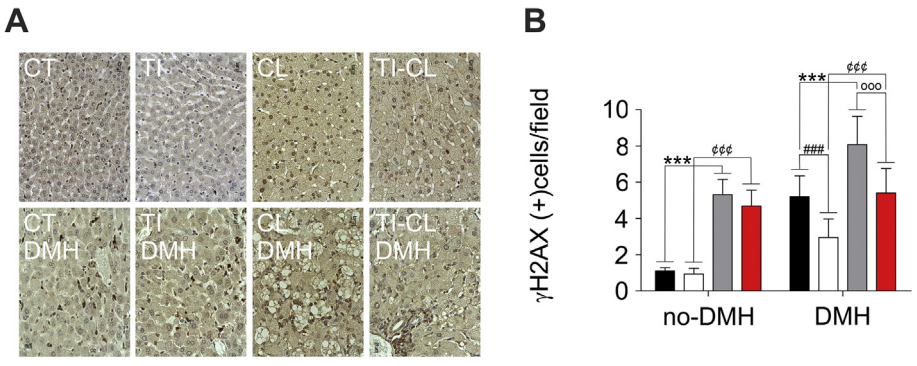

Fig. 3. Effects of tamarind treatment in hepatic biomarkers. Standard biochemical analyses determined the hepatic levels of thiobarbituric acid reactive substances (TBARS; μM/g; A), glutathione peroxidase (GPx; μM/min/g; B), catalase (CAT; mM/L of consumed H_2O_2 [cH_2O_2/min]/g; C), and superoxide dismutase (SOD; U/g; D). (E) Representative images of phosphorylated histone 2AX (γH2AX) expression in hepatocytes for all groups [standard diet (CT); hypercholesterolemic diet (CL); treatment with *Tamarind indica* extract (TI); TI plus CL (TI-CL); and, dimethylhydrazine (DMH)]. Images were taken at 40× magnification (scale bars = 20 μm). Relative values for γH2AX expression in liver samples. The two-way ANOVA test (Bonferroni's post hoc test) was applied for statistical analysis. Values are shown as the mean ± standard deviation ($^{###}P < 0.001$, CT *vs* TI; $^{ooo}P < 0.001$, CL *vs* TI-CL group; $^{***}P < 0.001$, CT *vs* CL; and $^{¢¢¢}P < 0.001$, TI *vs* TI-CL group).

Table 6
Serum oxidative stress.

Analysis	Carcin	Treatments				Comp	P <
		CT	TI	CL	TI-CL		
SOD U/mL	No-DMH	14.5 ± 1.8	18 ± 0.9 ***	6.3 ± 1 ***	6.6 ± 0.5 §§§	*vs CT ◦vs CL §vs TI	*0.05 **0.01 ***0.001 ◦◦◦0.001 ◦◦0.01 ◦0.05 §§§0.001 §§0.01
	DMH	10.4 ± 1.4	11.4 ± 1	10 ± 1	9.2 ± 0.7 §§§		
CAT mM/L -cH_2O_2/min/mL	No-DMH	0.023 ± 0.008	0.03 ± 0.005	0.017 ± 0.0046	0.02 ± 0.0035 §§		
	DMH	0.014 ± 0.007	0.024 ± 0.007 *	0.011 ± 0.004	0.014 ± 0.003 §§		
GPx µM/min/mL	No-DMH	9.36 ± 0.7	10.8 ± 0.2 **	8.8 ± 0.36	9.7 ± 0.46 ◦◦,§§		
	DMH	5.8 ± 0.34	6.1 ± 0.2	5.5 ± 0.5	5.9 ± 0.15		
TBARS µM/mL	No-DMH	5.2 ± 0.2	4.37 ± 0.3	7.2 ± 0.3 ***	5.8 ± 0.2 ◦◦◦,§§§		
	DMH	7 ± 0.09	6.6 ± 0.2	8.8 ± 0.4*	7.7 ± 0.2◦◦,§§		

Carcinogen (Carcin), Comparisons (Comp). Standard diet (CT), hypercholesterolemic diet (CL), treatment with *Tamarind indica* extract (TI). Cholesterol (CHOL), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine transaminase (ALT), and aspartate aminotransferase (AST). Values are shown as the mean ± standard deviation. Two-Way ANOVA test (Bonferroni post hoc test) was applied for data analysis.

Table 7
Colon oxidative stress.

Analysis	Carcin	Treatments				Comp	P <
		CT	TI	CL	TI-CL		
TBARS	No-DMH	221.1 ± 13.1	211.3 ± 10	230.2 ± 16	213.8 ± 11.2	None	None
nM/g	DMH	249 ± 11.6	242.2 ± 23.1	245.8 ± 20	244.6 ± 19		
GPx	No-DMH	151.4 ± 9.8	149.3 ± 8	147.4 ± 11	140.2 ± 10.3		
µM/min/g	DMH	129.2 ± 9.7	128 ± 11.2	127.8 ± 12	130 ± 13		
CAT	No-DMH	0.41 ± 0.05	0.4 ± 0.04	0.44 ± 0.03	0.42 ± 0.03		
mM/l -cH_2O_2/min/g	DMH	0.25 ± 0.02	0.2 ± 0.03	0.24 ± 0.03	0.23 ± 0.02		
SOD	No-DMH	150.2 ± 12.3	161 ± 12	142.8 ± 10.9	153.4 ± 14.6		
U/g	DMH	82.1 ± 6.9	90.2 ± 9	79.7 ± 7.1	77.1 ± 5.2		

Carcinogen (Carcin), Comparisons (Comp). Standard diet (CT), hypercholesterolemic diet (CL), treatment with *Tamarind indica* extract (TI). Cholesterol (CHOL), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine transaminase (ALT), and aspartate aminotransferase (AST). Values are shown as the mean ± standard deviation. Two-Way ANOVA test (Bonferroni post hoc test) was applied for data analysis.

significant free radical scavenging activity due to its large content of polyphenols and flavonoids, which provides its treated subject with a considerable level of antioxidants. Moreover, tamarind also seemed to augment the endogenous activity of antioxidant molecules such as GPx, SOD, and CAT (Martinello et al., 2006). Treating H_2O_2-challenged hepatic human cells with tamarind, Razali et al. confirmed that this fruit increased the endogenous antioxidant enzymes that we previously described (Martinello et al., 2006; Razali et al., 2015).

Although it seems exciting that tamarind reduced the risk of colon cancer, we should clearly state that cholesterol impaired the chemoprotective effects, as DNA-damaging events were not decreased compared to those in hamsters fed a regular diet. Hence, we should consider how dietary cholesterol acted pro-carcinogenically in the current experimental model. Adding 1% cholesterol to a regular diet increased, respectively, in 2.5-fold and 1.7-fold the stool content of this lipid compound and DNA-damaging process in colonic epithelial cells in DMH-exposed hamsters. While this carcinogen reduced the circulating cholesterol levels in hypercholesterolemic hamsters 1.7-fold, increasing the dietary cholesterol content augmented hepatic tissue damage 4-fold and 2.3-fold in lipid peroxidation. These observations suggest that dietary cholesterol might promote the development of colon carcinogenesis, enhancing lipid peroxidation in liver and colon tissues. Previously, we showed that dietary restriction alters hepatic metabolism, promoting lipid peroxidation and the risk of colon cancer (Kannen et al., 2013). Furthermore, rats fed a 14.6% lard-enriched diet had augmented abdominal and visceral adipose tissues and a higher risk of colon cancer (Kannen et al., 2012b). Another report from our group revealed that the surgical ablation of visceral adipose tissues reduced the development of colon carcinogenesis (Kannen et al., 2014).

Comparing groups treated with tamarind fed either a regular or hypercholesterolemic diet revealed that tamarind increased the release of cholesterol ~4-fold, while it doubled DNA-damaging events in carcinogenically hamsters. These findings demonstrate the benefits and limitations of tamarind treatment, which protects from the worsening activity of cholesterol without decreasing the risk of colon cancer to control rates. Although cholesterol impaired the benefits of tamarind treatment, tamarind has more benefits than a commercial drug named orlistat, which augments the stool content of cholesterol. We reported that orlistat increased the risk of colon cancer in rats given a high-fat diet (Garcia et al., 2006). In contrast, with tamarind, orlistat seems to lack some antioxidant capacity at the hepatic and systemic levels (Garcia et al., 2006; Morales et al., 2016). For instance, Gottteland and colleagues showed that the prebiotic oligofructose improved the intestinal flora activity, protecting patients from the damaging effects of orlistat, such as changes in inflammatory, oxidative, and metabolic events, at the systemic level (Morales et al., 2016).

The current findings also revealed that tamarind did not improve the antioxidant mechanism in the intestinal colon, which seems to clarify why this fruit was previously found to be unable to reduce MNU-induced preneoplastic lesions (Shivshankar and Shyamala Devi, 2004). Whether chemoprotective effects of tamarind are centered in the hepatic metabolism (Martinello et al.,

2006; Razali et al., 2015), this fruit could not counteract the direct carcinogenic effects of MNU (Shivshankar and Shyamala Devi, 2004) because it does not require any activation by the hepatic metabolism (Zarbl et al., 1985). A potential limitation of our study is that we were unable to evaluate the findings of Atawodi and Spiehelhalder in TI given to hamsters. These authors reported that out of 27 tropical plants, *T. indica* had the most primary amines and that harmful methylamine had the highest levels (Atawodi and Spiegelhalder, 1994). However, this previous study did not describe whether samples were collected in areas free of anthropogenic contaminants, which could be a bias of the reported data. A recent investigation showed that soil contamination increases the content of carcinogens in vegetables used as food by Nigerians (Inam et al., 2016). Here, tamarind fruits were collected from trees in the Brazilian countryside and were free of anthropogenic pollutants.

Taken together, we suggest that the tamarind extract has some chemoprotective effects against the development of colon carcinogenesis. However, we advocate for careful consideration because tamarind was unable to fully protect from the carcinogenic promoter activity of cholesterol in the colon. Further studies are warranted on this topic.

Conflict of interest

The authors declare that they have no conflicts of interest.

Competing interest

None.

Ethics approval

The ethical approved protocol #05.1.590.53.0 was a large project coordinated by Prof. Dr. Sergio Akira Uyemura. Some data (serum and liver samples; AST, ALT, glucose, total cholesterol, HDL, LDL, TG, TBARS, CAT, SOD, and GPx) from carcinogen-unexposed groups (STD groups [extract treated and untreated hamsters], and HCD groups [extract treated and untreated hamsters]) were already published (Martinello et al., 2006). These previous findings are used as control groups in this study. All experiments of carcinogen-unexposed and −exposed groups were performed together.

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