Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.journals.elsevier.com/revista-brasileira-de-farmacognosia

Original article

Evaluation of anti-inflammatory, immunomodulatory, chemopreventive and wound healing potentials from *Schinus terebinthifolius* methanolic extract

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ARTICLE INFO

Article history:
Received 20 May 2014
Accepted 18 August 2014

Keywords:
Schinus terebinthifolius
Inflammation
Immunomodulation
Micronucleus
Comet assay

ABSTRACT

Inflammatory and genetic alterations are related to the development of chronic diseases such as cancer. *Schinus terebinthifolius* Raddi, Anacardiaceae, is used in folk medicine to treat inflammation, wounds and tumors. This study evaluated the anti-inflammatory, immunomodulatory, chemopreventive, and wound healing potentials of the methanolic extract from the leaves of *Schinus terebinthifolius*. The chemical composition of the extract was characterized using preliminary analytical LC methods. The results showed that the anti-inflammatory activity of the methanolic extract was similar to that of dexamethasone for edema reduction. Also, it inhibited the leukocyte migration into the air pouch and decreased plasma extravasation. In addition, the methanolic extract showed a healing action similar to that observed with collagenase. The methanolic extract is not genotoxic nor mutagenic, and in contrast it has chemopreventive activity, which elicits a high percentage of damage reduction by comet and micronucleus assay, preferably by bioantimutagenic action. The methanolic extract induced apoptosis and enhanced splenic phagocytosis in animals treated with cyclophosphamide. The methanolic extract contents, resolved by LC, include phenolic acid and flavonoids. Our results suggest a therapeutic potential for the methanolic extract.

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http://dx.doi.org/10.1016/j.bjp.2014.08.004

Introduction

Schinus terebinthifolius Raddi, Anacardiaceae, popularly known as "aroeira-vermelha", "aroeira-pimenteira", "Brazilian pepper" or "Christmas-berry" is an evergreen tree, native to Brazil, introduced in different parts of the world as South and Central America, Mediterranean Europe, Africa and the United States (Barbosa et al., 2007; El-Massry et al., 2009).

In popular medicine, stem bark and leaves of *S. terebinthifolius* are mainly used in the form of decoction or infusion (El-Massry et al., 2009) for the treatment of bacterial infections (Martinez et al., 1996; El-Massry et al., 2009), wound healing, anti-inflammatory and as an anti-ulcer agent (Martorelli et al., 2011; Santana et al., 2012). Moreover, *S. terebinthifolius* has antioxidant and anti-tumor activities (Queires et al., 2006, De Mesquita et al., 2009; El-Massry et al., 2009), due to the high content of phenolic compounds characteristic of the species, such as tannins, gallic acid and flavonoids (Queires et al., 2006; Ceruks et al., 2007; Santana et al., 2012).

It has been well documented in the literature that tissue damage determines the development of inflammation and wound healing process, by mechanisms that include the production of chemical mediators, the recruitment of specific cells and an increased rate of cell division. These inflammatory mediators, when present in excess, inhibit apoptosis (Barreto et al., 2011), leading to the loss of tissue homeostasis, which favors the onset of mutations that could lead to cancer development (Valko et al., 2007; Formagio et al., 2013b). Additionally, changes in the antioxidant defenses (counterbalance of free radical activities) of the organism relate also to the genetic/chromosome instability and the inflammation process, which show a strong relationship among the triad: inflammation x genetic instability x antioxidant action (Valko et al., 2007; Formagio et al., 2013a). Taking this into account, the present investigation evaluated the anti-inflammatory, immunomodulatory, chemopreventive and wound healing potentials of the *S. terebinthifolius* methanolic extract (MEST).

Material and methods

Plant material

S. terebenthifolius Raddi, Anacardiaceae, leaves were collected in June 2012 at the Botanical Garden of Federal University of Grande Dourados (UFGD). This area is located in the southern region of Mato Grosso do Sul, with an average altitude of 452 m; and coordinates 22°14'16" S latitude and 54°49'2" W longitude. The plant was identified by Maria do Carmo Vieira and a voucher specimen was deposited (# 4602) at the UFGD Herbarium.

Preparation of extract

S. terebenthifolius leaves were air-dried at 40°C for 4 days and the dried plant was cut and pulverized. The plant material was macerated for 7 days using methanol as solvent. The solvent was then eliminated using a rotary vacuum evaporator under reduced pressure and it was further lyophilized, representing a yield of 7.92% of the methanolic extract.

LC analysis of standards and samples

The extract obtained from the samples and standards were analyzed using an analytical LC (Varian 210) system with a ternary solvent delivery system equipped with an auto-sampler, a photodiode array detector (PDA) monitored at λ200-800 nm. The LC column was a C-18 (25 cm × 4.6 mm; particle size, 5 µm; Luna, Phenomenex, Torrance, CA, USA), with a small pre-column (2.5 cm × 3 mm) containing the same packing material, used to protect the analytical column. For each analysis, the flow rate and the injected volume were set as 1.0 $ml.min^{-1}$ and 20 µl, respectively. All chromatographic analyses were performed at 22°C. Elution was carried out using acetic acid (6%), and sodium acetate (2 mM) (solvent A) and acetonitrile (solvent B). The solvent gradient program was carried as follows: 0 min, 5% B; 45 min, 15%; 55 min, 50% B, 60 min; 65 min, 100% B and in 70 min returning to the initial.

Reagents

Spectroscopy grade acetonitrile was purchased from Merck (Darmstadt). Stock mixture of standards were constituted from individual solution, dissolved in acetonitrile and then used as external standard. Standards [caffeic acid (98%), *p*-coumaric acid (98%), luteolin (98%), quercetin (98%) and apigenin (95%)] were purchased from Sigma Aldrich.

Linearity

The standards content estimation in the samples was performed by external calibration. Aliquots of 20 µl of the dilutions were analyzed via LC, and each determination was carried out five times. For the standard, the corresponding chromatogram was obtained and a graph was constructed from the mean of the chromatogram areas plotted against the standards in concentration of the 1-50 $\mu g.ml^{-1}$. A linear least squares regression was performed for the peak areas as a function of concentrations to determine the correlation coefficients. The equation parameters (slope and intercept) of the standard curve were used to obtain the concentration values of the samples.

Experiment 1

Evaluation of the anti-inflammatory and wound healing properties of S. terebenthifolius

The experiments were conducted using male Swiss mice (25-35 g) or male Wistar rats (200-250 g) provided by UFGD. The animals were maintained under a 12 h light-dark cycle, under controlled humidity (60-80%), and temperature (22° ± 1°C). The animals were acclimatized to the experimentation room for at least 2 h before testing and were used only once throughout the experiments (n = 5/group). All experimental procedures were carried out in accordance with U.S. National Institute of Health, and were approved for research by the ethics committee on laboratory animal of UFGD (# 005/2010).

Wound creation, animal grouping and drug administration in rats

The backs of the rats were shaved, and 8 mm full thickness open excision wound was made by removing a patch of skin. Animals were anesthetized with ketamine and xylazine (intraperitoneal route) (Suguna et al., 1999). A total of 24 animals were divided into three groups: the control group that received only vehicle solution, MEST group and commercial colagenase group (that received Iruxol®, topical application). The control rats received 50 µl of unbuffered physiological saline, once daily, for a period of 11 days. The MEST rats received 50 µl of MEST (80 mg/ml, diluted with unbuffered physiological saline), applied topically, once daily, for a period of 11 days; while the commercial collagenase group received 50 µl of Iruxol. The wound closure is expressed in mm.

Croton oil-induced ear oedema in mice

Twenty-five adult male mice were used in this assay. MEST (0.1, 0.3 and 1 mg/ear), vehicle (acetone) or dexamethasone (0.2 mg/ear) were topically administered into the right ear of the mice 15 min prior to the application of croton oil. A total of 25 µl of 0.3% croton oil (dissolved in acetone) was topically applied to the inner surface of the right ear of each mouse. The left ear remained untreated as a negative control. Four and six hours after the administration of croton oil, the swelling induced by the phlogogen agent was measured using a digital micrometer in the right ear.

Air pouch model in mice

The air pouch model was performed as described by Garcia-Ramallo et al. (2002), with some modifications. Swiss mice were anesthetized with ketamine and xylazine and 4 ml of sterile air was injected subcutaneously on their backs on day 0. On day 3, a second injection of 2 ml of sterile air was performed into the preformed pouch. On day 6, animals were treated with vehicle (Physiologic Saline), DEX (1 mg/kg *s.c.*) as positive control drug or MEST (100 mg/kg, *p.o*) (per oral route); and one hour later the inflammatory response was induced by injection of a solution of carrageenan 0.1% (350 µl) directly into the pouch. Four hours after the inflammatory stimulus, the mice were euthanized and the pouches were washed with 1 ml of PBS/EDTA 10mM containing heparin. Exudates were collected and total cells were counted using a Neubauer chamber under light microscopy. Exudates were then centrifuged at 1000 *g* for 2 min at 4°C and the pellets suspended in 1 ml of 3 % albumin and added to previously-prepared slides. Cells were stained using the May-Gruenwald-Giemsa method, and analyzed under light microscopy; a total of 100 cells were counted. The supernatant obtained after exudate centrifugation was used to evaluate protein leakage to the inflammatory site. Protein levels were measured by Bradford´s reaction (Bradford, 1976).

Experiment 2

Evaluation of the toxigenic, chemopreventive, immunomodulatory and apoptotic properties of S. terebenthifolius

The experiments were conducted using male Swiss mice (20-30 g), provided by UFMS, divided into six experimental groups (n = 5). The animals were kept under a 12 h light-dark cycle, with controlled humidity (60-80%), and temperature (22° ± 1°C). The animals were acclimatized to the experimentation room for seven days. All experimental procedures were carried out in accordance with U.S. National Institute of Health, and were approved for research by the ethics committee on laboratory animals of UFMS (# 398/2012).

Cyclophosphamide (Fosfaseron®, Filaxis, Plot: D 06307), was used in these studies to induce DNA damage. It was diluted in 0.9% saline and administered intraperitoneally (*i.p.*), at a dose of 100 mg/kg (0.1 ml/10 g) of body weight (bw). The MEST was administered by gavage (*p.o.*), at a dose of 16.8 mg/kg (bw), and it was dissolved in 1% methanol.

The treatment protocols were: (1) Negative control: the animals received MEST (1% methanol) and cyclophosphamide (0.9% saline) vehicles at the first and second day of treatment. (2) Positive control: the animals received MEST and cyclophosphamide vehicles at the first day of treatment, and on the second day, they received the MEST vehicle and the cyclophosphamide. (3) MEST: the animals received MEST and cyclophosphamide vehicles on the first day, and on the second day, they received the cyclophosphamide vehicle and MEST. (4) Pre-treatment: the animals received MEST and cyclophosphamide vehicle at the first day and they received the MEST vehicle and cyclophosphamide on the second day. (5) Simultaneous treatment: the animals received the MEST and cyclophosphamide vehicles on the first day and on the second day they received both MEST and cyclophosphamide. (6) Post-treatment: on the first day animals received the MEST vehicle and the cyclophosphamide, and on the second day, they received MEST and the cyclophosphamide vehicle.

After treatment, the animals were weighed and euthanized by cervical dislocation. The spleen, kidneys, liver, heart and lungs were collected and weighed.

Toxigenic and chemopreventive evaluations: Comet assay and micronucleus in peripheral blood

To evaluate genotoxicity and antigenotoxicity, a comet assay was performed on peripheral blood according to method proposed by Singh et al. (1988) with modifications proposed by Oliveira et al. (2009). A sample of peripheral blood was collected by puncture of the tail vein 24 h (T1) after the last administration of the test compounds (cyclophosphamide or MEST). For each treatment, 100 cells were analyzed using a fluorescence microscope (Nikon), at 400 times of magnification, using an excitation bandpass of 420-490 nm, and barrier filter of 520 nm. The cells were classified as suggested by Kobayashi et al. (1995).

To evaluate mutagenicity and antimutagenicity, the micronucleus test was performed as described by Hayashi et al. (1990) with modifications proposed by Oliveira et al. (2009) in peripheral blood. Blood samples were collected at 24 h (T1), 48 h (T2) and 72 h (T3) after treatment by caudal vein puncture. A total of 2000 cells were analyzed per animal using a fluorescence microscope (Bioval® L-2000A), at 400 times of magnification, using an excitation filter of 490 nm and barrier filter of 420-520 nm.

Calculating the reduction of damage percentage

The chemopreventive activity of MEST was calculated by the percentage of DNA damage reduction (DR%).

$$DR\% = \left[\frac{\text{mean of postive control - mean of associated group}}{\text{mean of positive control - mean of neagtive control}}\right] \times 100$$

Immunomodulatory evaluation: splenic phagocytosis and differential count of blood cells

The method described by Hayashi et al. (1990) with modifications by Ishii et al. (2011) was used to evaluate splenic phagocytosis. The animal' spleens were macerated in 0.9% saline solution to obtain a homogeneous cell suspension. One hundred microliters of this material was deposited on a slide pre-stained with acridine orange (1 mg/ml) with a coated coverslip, and stored in a freezer until analysis. We analyzed 200 cells/animal using a fluorescence microscope (Bioval® G-2000A) at 400 times magnification using an excitation filter (490 nm) and barrier filter (420-520 nm), and classified them by the presence or absence of phagocytosis, according to the description of Hayashi et al. (1990).

In order to perform a differential cell count in peripheral blood, a drop of blood obtained by puncture of the tail vein 72 h (T3) after treatment was collected. Blood was smeared on a microscope slide and it was air dried and subjected to rapid staining using the Panoptic Instant-Prov Kit. One hundred cells were analyzed per animal and neutrophils, monocytes, eosinophils and basophils were distinguished (Ishii et al., 2011) using an optical microscope (Microscope Physis, EXP 100), at 1000 times magnification.

Assessment of apoptotic activity

The apoptosis assay was performed according to Mauro et al. (2011) with modifications. Fragments of spleen, liver and kidneys were macerated in 0.9% saline solution to obtain a homogeneous cell suspension. One hundred microliters of the solutions were used to do histological smear slides. The slides were air-dried and immersed afterwards in Carnoy's fixative solution for 5 min and then they were quickly immersed in plates with decreasing concentrations of ethanol dilutions (95-25%). The slides were washed in McIlvaine buffer for 5 min, stained with acridine orange (0.01%) for 3 min and washed in McIlvaine buffer. One thousand cells were analyzed per animal in a fluorescence microscope (Bioval® L-2000A), at 400 times of magnification, using excitation filter (490 nm) and barrier filter (420-520 nm). The cells were identified by DNA fragmentation patterns (Mauro et al., 2011).

Statistical analyses

All data are presented as mean ± SEM. Differences between groups were evaluated by analyses of variance (one-way ANOVA) followed by Student Newman-Keuls test or Tukey's test. Statistical differences were considered to be significant at $p < 0.05$. Graphs were drawn and statistical analysis was carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

LC analysis

The methanolic extract of *S. terebinthifolius* (MEST) exhibited potential activities as determined by functional assays, and LC data was developed to identify the constituents of their leaves from specimens collected in Mato Grosso do Sul, Brazil (Fig. 1). Identification of the compounds with the aid of a PDA detector scanning in the spectral range of 200-800 nm did not reveal interferences in retention time of the samples in LC by the developed elution method. Standards were easily identified and quantified based on their absorption spectra in the UV region and by retention time. Standards found in the extracts were unambiguously identified by co-injection experiments in which aliquots of the extracts and standards were mixed and diluted to a known volume, and analyzed through LC. The calibration curves were determined by linear regression using LC. The linearity for standards was assessed for seven concentration ranges. The average standard errors for the peak areas of replicated injections (n = 5) were less than 1.5%,

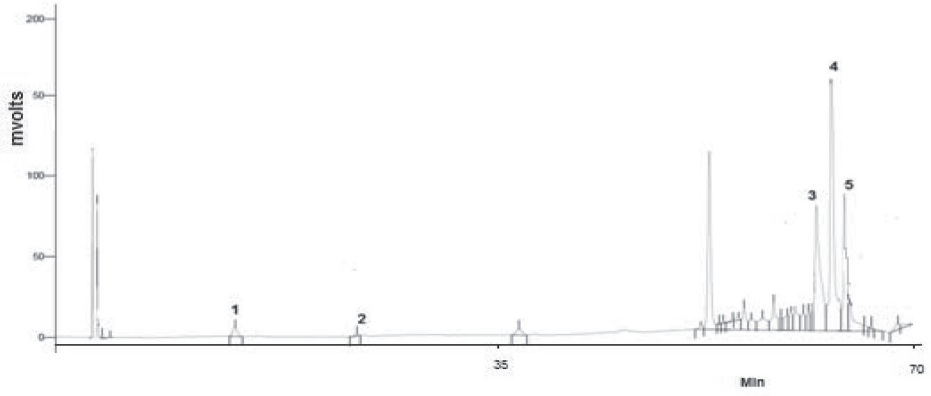

Figure 1 – LC-DAD chromatogram of methanolic extract of *Schinus terebenthifolius* leaves.

thus showing good repeatability of the calibration curve. The respective coefficients of determination (r^2) were 0.9994 for, caffeic acid and *p*-coumaric acid, and r^2 = 0.9996 for luteolin, quercetin and apigenin. The retention time of standards and contents in samples are shown in Table 1.

Experiment 1 - Evaluation of the anti-inflammatory and wound healing properties of S. terebenthifolius

Effects of MEST on wound healing

The topical application of the MEST dose (80 mg/ml) significantly decreased the diameter of the wound made, with a contraction of 2.33 ± 0.34% after eleven days, as illustrated in Table 2.

Table 1
Retention time of standards and contents in $\mu g \cdot g^{-1}$ of MEST in LC method.

Peak	Standards	Retention time (min)	Content in
1	caffeic acid	14.78	206
2	*p*-coumaric acid	25.82	167
3	quercetin	62.08	378
4	luteolin	64.62	527
5	apigenin	66.99	403

MEST, Methanolic extract of *S. terebinthifolius* leaves; LC, Liquid Chromatography.

Effects of MEST on croton oil-induced ear oedema

Topical application of croton oil to the ears of mice where oedema had been induced resulted in a significant increase in the weight of the treated right ear when compared with the untreated left ear. As shown in Fig. 2, the oral administration of MEST (0.1, 0.3 and 1 mg/ear) reduced the ear oedema. The maximal inhibitions reached at 1 mg/ear were 29 ± 2% and 47 ± 2%, after 4 and 6 h after croton oil, respectively. The positive control dexamethasone (0.2 mg/ear) also inhibited ear oedema at 30 ± 3% after 4 h, and 50 ± 2% after 6 h.

Effects of MEST on carrageenan-induced leukocyte migration and plasma leakage to air pouches

On the sixth day after induction of air pouch on the dorsal region of mice, a carrageenan injection was applied (350 µl at 0.1%), which after 4 h, promoted a plasma leakage and leukocyte migration, principally by polymorphonuclear leukocytes (Fig. 3). The results were expressed as leukocytes × 10^7, and plasma leakage as mg of proteins/100 µl. The oral treatment of the animals with MEST significantly inhibited leukocyte migration to air pouches in 69 ± 5% at 100 mg/kg. MEST significantly inhibited plasma leakage, and the inhibition percentage was 55 ± 8%. DEX used as positive control, reduced

Table 2
Effects of *Schinus terebinthifolius* methanolic extract on wound-healing.

Treatment	Mean area of closure of excision wound (mm)			
	Day 1	Day 3	Day 5	Day 11
Control	9.48 ± 1.04	7.99 ± 0.20	7.45 ± 0.18	3.23 ± 0.27
MEST	8.45 ± 0.22	7.07 ± 0.15	6.23 ± 0.32	2.33 ± 0.34*
Colagenase (ointment)	8.32 ± 0.34	7.80 ± 0.36	7.00 ± 0.32	2.15 ± 0.22*

MEST, Methanolic extract of *S. terebinthifolius* leaves; Mean ± S.E.M., Standard error of mean.
*Asterisk means statistical significance in comparison to the control group $p < 0.05$.
** $p < 0.01$ (Statistical Test, Analysis of Variance - one-way ANOVA/Student Newman-Keuls test).

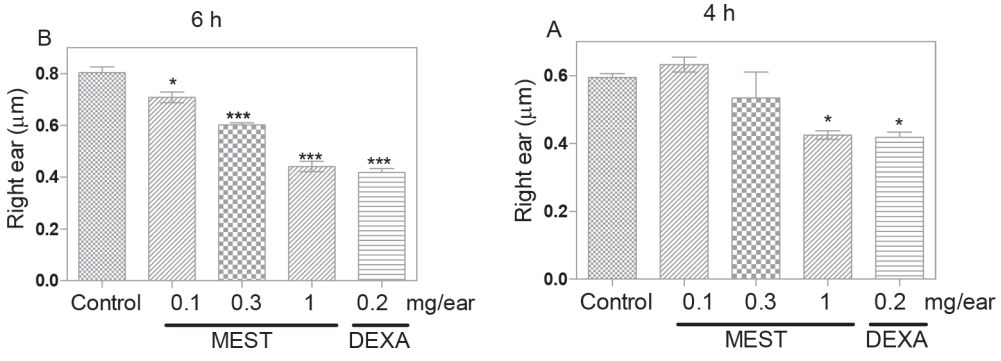

Figure 2 – Effects of MEST at doses of 0.1, 0.3 and 1 mg/ear administered topically during croton oil-induced ear oedema model in mice. Each column represents the mean (n = 5), and the vertical lines show the S.E.M.A. 4h after croton oil application; B. 6h after croton oil application. Asterisks denote the significance levels compared to the control values (croton oil): *$p < 0.05$ and ***$p < 0.001$.

about 67 ± 6% of leukocyte migration and 53 ± 7% the plasmatic extravasation.

Experiment 2 – Toxigenic, chemopreventive, immunomodulation and apoptotic evaluations

There was no body weight variation in all groups investigated, as showed in Table 3. A similar tendency for the relative weights of heart, lungs, liver and kidneys was observed. There was a reduction in the relative weight of the spleen in all groups that received cyclophosphamide.

Toxicogenic and chemopreventive evaluations: Comet assay and micronucleus in peripheral blood

The mean frequency of damaged cells, distribution among classes of damage and score related to genotoxicity and antigenotoxicity (comet assay) are presented in Table 4. There was absence of genotoxicity in the MEST group and decreased genotoxic damage was observed in all treatment protocols. The post-treatment group showed the highest DR (99.19%), with an average frequency of damaged cells (30.33 ± 12.45) similar to the Negative control group (30.00 ± 3.19). The DR% of the Pre-treatment and simultaneous groups were 69.95 and 10.01%, respectively.

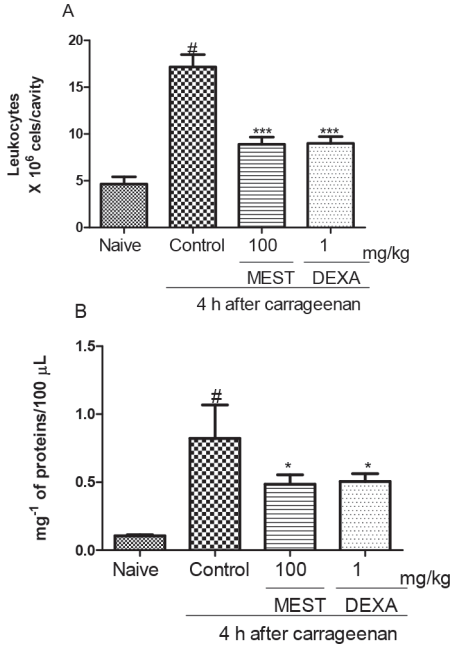

Figure 3 – Effect of MEST at dose 100 mg/kg on carrageenan-induced leukocyte migration and plasma leakage into the air pouch. Each column represents the mean (n = 5), and the vertical lines show the S.E.M. Cells were counted and plasma leakage was analyzed. A. Number of cells that migrates to the pouches 4h after carrageen an. Results are expressed as cell 107/cavity. B. Plasma leakage was measured by Bradford´s reaction. Results are expressed as protein/mg^{-1} of protein. $^{*}p < 0.05$; $^{***}p < 0.001$; compared to the vehicle group. Differences between groups were analyzed by analysis of variance (One-way ANOVA) followed by Newman-Keuls test.

The total frequency, frequency mean and percentage of damage reduction related to mutagenicity and antimutagenicity tests (micronucleus) are presented in Table 5. The micronucleus analysis indicated that MEST has no mutagenic activity and was effective in reducing the damage caused by cyclophosphamide in the groups analyzed, at different times, except for the pre-treatment group at T1. As it was observed, the chemopreventive activity displayed a time-dependent relationship, and there was an increased chemopreventive activity in the three analysis periods. The DR had a variation from 66.39% to 106.55% in T3.

Immunomodulatory evaluation: splenic phagocytosis and blood cells differential count

All data related to the splenic phagocytosis are illustrated in Table 6. The MEST group presented a higher ($p < 0.05$) incidence of splenic phagocytosis (138.25 ± 17.57) in comparison to the Negative control (111.8 ± 5.68). The pre-treatment (98.40 ± 5.19), simultaneous (88.40 ± 3.30) and post-treatment (82.00 ± 5.96) groups showed a trend of increased phagocytosis in comparison to the Positive control (68.75 ± 5.08).

The differential blood cell counts are presented in Table 7. Cell counts were similar between controls and treated groups, and the values are within the reference ranges, except for eosinophils, that present a slight increase in the negative control, MEST, pre-and post-treatment groups.

Assessment of apoptotic activity

All data related to apoptosis are illustrated in Table 8. The MEST when administered alone does not induce an increase in the frequency of apoptosis in liver, spleen and kidneys. Cyclophosphamide increased the frequency of apoptosis in the order of 3.11, 9.20 and 4.65 times in the liver, spleen and kidney, respectively. When cyclophosphamide was associated with MEST there was a decrease ($p < 0.05$) in the frequency of apoptosis in spleen, and an increase ($p < 0.05$) in the frequency of apoptosis in kidney. With the exception of post-treatment group, a tendency of increased apoptosis in liver was observed (Table 8).

Discussion

Medicinal plants are used to cure and/or prevent diseases, and they constitute a major source for the discovery of new therapeutic agents (Formagio et al., 2013a). *Schinus terebinthifolius* Raddi, Anacardiaceae, is a plant popularly used for the treatment of inflammation, infections, wounds, ulcers and tumors (Santana et al., 2012).

Previous studies using wound-healing models, demonstrated that the hydroethanolic extract from the leaves of *S. terebinthifolius* were effective for wounds on the tongue mucous of rats, and in the healing process of the alba linea (Nunes-Jr et al., 2006) surgical wounds (Coutinho et al., 2006) and cutaneous wound (Martorelli et al., 2011). The wound response were significantly ameliorated in all rodent models and the present study corroborated and extended these findings proving that the contraction of the wounds

Table 3
Initial, final and weight gain of the animals; absolute and relative weight of the organs of animals treated with *Schinus terebinthifolius* methanolic extract and /or cyclophosphamide.

Experimental Group	Negative Control	Positive Control	MEST	Pre-treatment	Simultaneous	Post-treatment
Animal's weight						
Inicial (g)	27.48 ± 1.57[a]	27.85 ± 0.72[a]	26.44 ± 0.43[a]	24.40 ± 1.04[a]	24.94 ± 1.09[a]	25.19 ± 0.44[a]
Final (g)	27.59 ± 1.27[b]	26.40 ± 0.41[a,b]	26.09 ± 0.40[a,b]	23.40 ± 0.85[a]	23.83 ± 0.95[a]	25.38 ± 0.38[a,b]
Liquid weight gain (g)	0.11 ± 0.71[a]	-1.45 ± 0.39[a]	-0.34 ± 0.14 [a]	-1.00 ± 0.45 [a]	-1.11 ± 0.21[a]	0.20 ± 0.11[a]
Absolute organ weight (g)						
Heart	0.157 ± 0.010[b,c]	0.165 ± 0.006[b,c]	0.191 ± 0.010 [c]	0.146 ± 0.003[b,c]	0.131 ± 0.010[a,b]	0.141 ± 0.012[b]
Lung	0.249 ± 0.020[a]	0.202 ± 0.007[a]	0.211 ± 0.008[a]	0.237 ± 0.037[a]	0.179 ± 0.009[a]	0.210 ± 0.007[a]
Liver	1.659 ± 0.072[c]	1.553 ± 0.043[b,c]	1.527 ± 0.051[b,c]	1.468 ± 0.081[b,c]	1.327 ± 0.084[a,b]	1.549 ± 0.050[b,c]
Kidney	0.384 ± 0.019[a]	0.411 ± 0.012[a]	0.360 ± 0.018[a]	0.349 ± 0.024[a]	0.333 ± 0.023[a]	0.364 ± 0.017[a]
Spleen	0.141 ± 0.007[c]	0.082 ± 0.003[b]	0.130 ± 0.006[c]	0.075 ± 0.003[a,b]	0.075 ± 0.004[a,b]	0.083 ± 0.004[b]
Relative organ weight (g)						
Heart	0.005 ± 0.000[a,b]	0.006 ± 0.000[a,b]	0.007 ± 0.000[b]	0.006 ± 0000[a,b]	0.005 ± 0.000[a]	0.005 ± 0.000[a]
Lung	0.009 ± 0.000[a]	0.007 ± 0.000[a]	0.008 ± 0.000[a]	0.010 ± 0.001[a]	0.007 ± 0.000[a]	0.008 ± 0.000[a]
Liver	0.060 ± 0.003[a]	0.059 ± 0.002[a]	0.058 ± 0.001[a]	0.062 ± 0.001[a]	0.055 ± 0.002[a]	0.061 ± 0.001[a]
Kidney	0.014 ± 0.000[a]	0.015 ± 0.000[a]	0.013 ± 0.000[a]	0.014 ± 0.000[a]	0.013 ± 0.000[a]	0.014 ± 0.000[a]
Spleen	0.005 ± 0.000[b]	0.003 ± 0.000[a]	0.005 ± 0.000[b]	0.003 ± 0.000[a]	0.003 ± 0.000[a]	0.003 ± 0.000[a]

MEST, Methanolic extract of *S. terebinthifolius* leaves; Mean ± S.E.M., Standard error of mean.
Different letters mean significant differences (Statistical Test, Analysis of Variance - ANOVA/Tukey; $p < 0.05$).

Table 4
Mean frequency of damaged cells ± S.E.M., distribution among classes of damage and score related to genotoxicity and antigenotoxicity tests by the comet assay of peripheral blood of Swiss male mice.

Experimental Group	Damaged cells	Classes of DNA damage				Score	DR%
		0	1	2	3		
Genotoxicity							
Negative Control	30.0 ± 3.1[a,b]	64.0 ± 6.5[c]	34.4 ± 5.8[a,b]	1.6 ± 1.0[a,b]	0.0 ± 0.0[a]	37.6 ± 7.2[a,b]	-
Positive Control	70.6 ± 5.0[c]	29.4 ± 5.0[a]	62.8 ± 4.7[c]	6.2 ± 1.7[c]	1.6 ± 0.9[a]	80.0 ± 6.2[c]	-
MEST	15.2 ± 3.4[a]	84.7 ± 3.5[c]	14.5 ± 3.6[a,b]	0.5 ± 0.5[a,b]	0.2 ± 0.2[a]	16.2 ± 3.6[a]	-
Antigenotoxicity							
Pre-treatment	42.2 ± 6.5[b,c]	57.8 ± 6.5[b,c]	39.4 ± 6.2[b,c]	2.4 ± 0.8[a-c]	0.4 ± 0.2[a]	43.4 ± 6.9[a,b]	69.9
Simultaneous	66.5 ± 4.1[c]	33.5 ± 4.0[a,b]	65.7 ± 4.3[c]	0.7 ± 0.5[a,b]	0.0 ± 0.0[a]	67.2 ± 3.9[b,c]	10.0
Post-treatment	30.3 ± 12.4[a,b]	69.7 ± 12.5[c]	30.3 ± 12.5[a,b]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	30.3 ± 12.4[a]	99.1

DR%, Percentage of Damage Reduction; MEST, Methanolic extract of *S. terebinthifolius* leaves; S.E.M., Standard error of mean.
Different letters mean significant differences (Statistical Test, Analysis of Variance- ANOVA/Tukey; $p < 0.05$).

Table 5
Total frequency, mean frequency ± S.E.M., percentage of damage reduction related to the tests for mutagenicity and antimutagenicity by micronucleus test of peripheral blood of Swiss male mice.

Experimental Group	MN frequency			Mean ± SEM			DR%		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
Mutagenicity									
Control	47	44	41	9.4 ± 0.9[a]	8.8 ± 0.7[a]	8.2 ± 1.0[a]	-	-	-
Positive Control	229	224	178	45.8 ± 2.8[c]	44.8 ± 2.6[d]	35.6 ± 2.3[d]	-	-	-
MEST	62	69	64	12.4 ± 0.9[a]	13.8 ± 2.8[a,b]	12.8 ± 0.9[a,b]	-	-	-
Antimutagenicity									
Pre-treatment	208	132	97	41.6 ± 5.0[c]	26.4 ± 1.6[c]	19.4 ± 1.5[c]	11.5	51.1	66.4
Simultaneous	143	103	79	28.6 ± 1.7[b]	20.6 ± 1.8[b,c]	15.8 ± 1.6[b,c]	47.2	67.2	81.1
Post-treatment	127	71	48	25.4 ± 3.0[b]	14.2 ± 1.5[a,b]	9.6 ± 0.6[a,b]	56.0	85.0	106.5

MN, Micronucleus; S.E.M., Standard error of mean; %DR, Percentage of damage reduction; T1, T2, T3, collection time.
Different letters mean significant differences (Statistical Test, Analysis of Variance - ANOVA/Tukey; $p < 0.05$).

Table 6
Mean frequency ± S.E.M. and percentage of cells with evidence of splenic phagocytosis in Swiss mice.

Experimental Group	Cells with evidence of phagocytosis	
	Mean ± S.E.M.	Percentage (%)
Negative Control	111.8 ± 5.7[b,c]	55.9
Positive Control	68.8 ± 5.0[a]	34.4
MEST	138.3 ± 17.6[c]	69.1
Pre-treatment	98.4 ± 5.2[a,b]	49.2
Simultaneous	88.4 ± 3.3[a,b]	44.2
Post-treatment	82.0 ± 5.9[a,b]	41.0

MN, Micronucleus; S.E.M., Standard error of mean; %DR, Percentage of damage reduction; T1, T2, T3, collection time.
Different letters mean significant differences (Statistical Test, Analysis of Variance - ANOVA/Tukey; $p < 0.05$).

of leukocyte migration and plasma leakage, observed in the air pouch model, are important parameters of inflammation. A recent work from our group showed that the essential oil obtained from *S. terebinthifolius* fruits also inhibited these two parameters in carrageenan-induced pleurisy in mice (Formagio et al., 2011). Maybe in whole parts of this plant the compounds responsible(s) for the anti-inflammatory activities are contained. These results contributed, at least in part, to explain the ability of *S. terebinthifolius* in folk medicine to combat the inflammatory and infectious diseases. Another important point of MEST activity is the demonstrated anti-inflammatory efficacy of topical application of MEST in the croton oil-induced oedema in mice. These results showed that MEST contain bioactive compound(s) that elicit topical anti-inflammatory effects in a dose-dependent manner.

Table 7
Mean frequency ± S.E.M. and percentage of cells with evidence of splenic phagocytosis in Swiss mice.

Cell types	Reference values	Experimental Group					
		Negative Control	Positive Control	MEST	Pre-treatment	Simultaneous	Post-treatment
Lymphocytes	55-95%	76 ± 5[a-c]	71 ± 4[a-c]	64 ± 3a	67 ± 4[a,b]	83 ± 2[c]	83 ± 2[c]
Neutrophils	10-40%	22 ± 4[a-c]	27 ± 4[a-c]	33 ± 3c	31 ± 4[c]	16 ± 2[a,b]	15 ± 2[a]
Monocytes	0.1-3.5%	0.4 ± 0.2[a]	1 ± 0.3[a]	0.2 ± 0.2a	0.4 ± 0.3[a]	0.2 ± 0.2[a]	0.4 ± 0.2[a]
Eosinophils	0-0.4%	1.4 ± 0.4[a,b]	0.4 ± 0.4[a,b]	1.8 ± 0.4b	0.8 ± 0.4[a,b]	0 ± 0[a]	0.8 ± 0.4[a,b]
Basophils	0-0.3%	0 ± 0[a]	0 ± 0[a]	0 ± 0 a	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]

MEST, Methanolic extract of *S. terebinthifolius* leaves; S.E.M., Standard error of mean.
Different letters mean significant differences (Statistical Test, Analysis of Variance - ANOVA/Tukey; $p < 0.05$).

Table 8
Evaluation of apoptosis in liver, spleen and kidney of mice.

Experimental Group	Liver	Spleen	Kidney
	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.
Negative Control	67.4 ± 13.7[a,b]	30.2 ± 4.9[a]	19.8 ± 1.7[a]
Positive Control	209.6 ± 21.5[d]	277.7 ± 14.3[d]	92.0 ± 10.7[b]
MEST	103.6 ± 7.5[b,c]	48.7 ± 5.9[a,b]	19.0 ±2.7[a]
Pre-treatment	232.2 ± 36.0[d]	103.6 ± 10.8[c]	159.8 ± 23.6[c]
Simultaneous	246.4 ± 12.4[d]	101.5 ± 10.7[c]	266.6 ± 21.1[d]
Post-treatment	181.2 ± 10.3[c,d]	72.8 ± 4.1[b,c]	97.4 ± 9.9[b,c]

MEST, Methanolic extract of *S. terebinthifolius* leaves; S.E.M., Standard error of mean.
Different letters meansignificant differences (Statistical Test, Analysis of Variance - ANOVA/Tukey; $p < 0.05$).

are induced with 80 mg/ml topical application of MEST. Our results showed that the extract obtained from *S. terebinthifolius* of Mato Grosso do Sul also induced wound healing. Moreover, the probable variation in chemical composition according to the part of plant (leaves) of *S. terebinthifolius*, culture and season did not influence this specific activity.

It is possible that MEST wound inhibition may be influenced by increase of inflammatory mediators (TNF-α, IL-1) and growth factors, as well as, inhibition of vascular permeability and leukocyte infiltration into the wound. To further confirm the role of MEST on leukocyte migration, experiments were carried out in an animal model of carrageenan-induced air pouch, which is a widely known model for massive leukocyte infiltration into artificially created pouches. MEST inhibition

Both wound-healing and croton oil-induced edema tests are models used for the development of new drugs. There is great differences between these two models regarding duration: in the ear oedema model it is possible to analyze the anti-inflammatory effects of a product in a couple of hours (Tonelli et al., 1965); while the the biological effects of a product in the healing model can be evaluated after 15 days (Nayak et al., 2013) or more. The topical application of croton produces oedema, cell infiltration and proliferation, production of prostanoids, leukotrienes, cytokines and other pro-inflammatory mediators (Patrick et al., 1987). The choice of lower doses for topical treatment of ear edema model was based on studies that showed that extracts from *S. terebinthifolius* in a systemic dose of 100 mg/kg exhibited effects

(Coutinho et al., 2006, Dos Santos et al., 2012). As expected, the present work showed that *S. terebinthifolius* extract inhibited ear edema at low doses of 0.1, 0.3, and 1 mg/ear. In contrast with low topical doses for ear edema, the dose for wound-healing model, 4 mg per wound per day, was chosen because in the wound healing model not only inflammation will be important but also activated keratinocytes, fibroblasts, and endothelial cells in the injury, all of which increase their expression of several different metalloproteinases (MMP) classes such as collagenases (MMP-1, MMP-8), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), the membrane type MMP (MT1-MMP) and several agents other agents (Eming et al., 2007). The topical treatment dose used of *S. terebinthifolius* extract induced healing. These results suggest that *S. terebinthifolius* extract elicits antiedematogenic and healing effects at different doses.

The phytochemical profile of *S. terebinthifolius* is characterized by high content of tannins, gallic acid and flavonoids (Queires et al., 2006; Ceruks et al., 2007; Santana et al., 2012). The chemical constituents of the methanolic extract of the leaves of *S. terebinthifolius* by LC reported the presence of important bioactive components. It has been described in the literature that these compounds have antioxidant, anti-inflammatory, healing, antimutagenic and antitumor properties, as well as, considered chemical inhibitors of carcinogenesis (Sghaier et al., 2011). The presence of phenolic compounds was also demonstrated in a previous study conducted by our research group, with MEST (Fedel-Miyasato et al., 2014).

Taking this into account, the present study evaluated the MEST toxicogenic and chemopreventive activities. The results have shown that in addition to not presenting genotoxic and mutagenic properties, MEST has chemopreventive activities, and cyclophosphamide-induced DNA damage reduction potential. The highest chemopreventive rate was observed in the post-treatment group, which presented a DR of 99.19% to genotoxic damage (T1) and 106.55% for mutagenic damage (T3), suggesting a preferential bio-antimutagenic activity mediated by repair enzymes (Kada and Shimoi, 1987; Antunes and Araújo, 2000; Oliveira et al., 2009), and the ability to reduce basal damage (Oliveira et al., 2002), a common event in chemoprevention studies (Oliveira et al., 2009). Our findings showed also, in lesser proportion, desmutagenic action observed in the pre-treatment group, which suggests the ability of MEST to inhibit compounds that cause DNA damage (Antunes and Araújo, 2000). Additionally, cyclophosphamide correlates to the process of immunosuppression (Ramadan et al., 2012). The MEST group had an increased phagocytosis, which suggests immunostimulatory activity. Moreover, the extract associated with cyclophosphamide also contributed to splenic phagocytosis. It is noteworthy that the spleen is a lymphoid organ related to hemocatheresis, storage and release of cells into the bloodstream (Ramadan et al., 2012). Therefore, the results of the phagocytic activity, when associated with antimutagenicity data suggest the phagocytosis of micronucleated cells by the spleen (Ishii et al., 2011). The present findings shows that, animals treated with cyclophosphamide exhibited an (I) increased frequency of micronuclei in peripheral blood; (II) decreased recruitment of macrophages by spleen, which determines the phagocytic activity reduction; and (III) a trend of increase of monocytes in circulation. Regarding the MEST-treated group, no increase in the micronuclei frequency was observed. However, there is evidence of increased phagocytosis, which could be correlated to the decreased monocyte number in peripheral blood, since they may have migrated to the spleen. Furthermore an association between cyclophosphamide and MEST (T3) was observed; we suggest that the percentage of damage reduction was due to (I) the antimutagenic activity which may have prevented (desmutagenesis) and/or repaired (bioantimutagenesis) DNA damage; and (II) the sequestration of micronucleated cells by the spleen. These results also indicate that the lower phagocytic activity took place in the post- treatment group. This confirms the increased bioantimutagenesis activity promoted by MEST. This mode of action was previously described in the *Allium cepa* model by our research group (Fedel-Miyasato et al., 2014).

Apoptosis was increased in the liver, which may be related to the metabolism of cyclophosphamide, indirect-acting agent, and the production of active metabolites that cause DNA damage leading to cell death (Ramadan et al., 2012). The highest rates of apoptosis were found for Pre- and Simultaneous treatment. This result is in accordance with the highest micronuclei frequency. Thus, it is suggested that MEST, besides being a chemopreventive agent, it is capable of stimulating the apoptosis of micronucleated cells. In addition, in the kidney was observed an increased apoptosis rate. It is known that this organ is responsible for blood filtration and removal of metabolic cyclophosphamide. Thus, it is suggested that the toxic product retention until the time of disposal could increase the rate of apoptosis in this organ.

The findings of this study indicate that MEST, in addition to treating inflammation and assisting the healing process, improves the integrity of the genetic material (chemopreventive activity), modulates splenic phagocytosis of mutated cells and increases the rate of apoptosis (Mauro et al., 2011). This latter fact is important for the development of new anti-inflammatory agents, since chemical-mediators such as cytokines, prostaglandins and leukotrienes, when produced in excess, may inhibit apoptosis (Barreto et al., 2011), which could favor cancerous development (Valko et al., 2007; Formagio et al., 2013a,b). It is noteworthy that the chemopreventive activities observed for MEST, are associated with the antioxidant activities of phenolic constituents of the species (Queires et al., 2006; Ceruks et al., 2007; Santana et al., 2012) that would be primarily responsible for the stability of the genetic material and for the resolution of inflammation.

In conclusion, our results showed that *S. terebinthifolius* presents significant wound-healing, anti-inflammatory and chemopreventive activities, which can be correlated with the prevention and/or treatment of degenerative diseases related to inflammation and mutagenic processes. This study also confirms that the extract of *S. terebinthifolius* can prevent and/or repair DNA damage by desmutagenic, and bioantimutagenesis processes. Further studies are needed to correlate the therapeutic properties

of the extract against the development of cancers originating from inflammatory processes, such as melanoma and colon cancer, and these are the next step of our research group.

Authors' contributions

LESFM, MOM, ALCL, ACDM carried out the laboratory work and the data analyses. ASNF, CALC, MCV obtained of the extract and plant herbarium confection. LESFM, CALK, SAA, RJO wrote the manuscript. CALK and RJO designed the study and supervised the laboratory work. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This project was supported by FUNDECT (Edital Chamada FUNDECT Nº 5/2011 – PPP – Processo Nº 23/200.702/2012 – Termo de Outorga Nº 0207/12; Edital Chamada FUNDECT/CNPq Nº 06/2011 – PRONEM).

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