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Full Length Research Paper

Aqueous extract of *Bowdichia virgilioides* stem bark inhibition of allergic inflammation in mice

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This study evaluated the anti-allergenic properties of the aqueous extract of the stem bark from *Bowdichia virgilioides* (AEBv). Oral administration of AEBv inhibited the plasma protein leakage at 30 min post allergenic challenge in Swiss mice actively sensitized with ovalbumin (OVA). AEBv inhibited OVA-induced histamine release *in vitro* in tissues obtained from sensitized mice. AEBv-treated mice exhibited a lower influx of neutrophils and eosinophils in allergen-induced pleurisy 24 h post OVA-challenge. This treatment also reduced tumor necrosis factor (TNF)-α content in the pleural effluent. Furthermore, AEBv treatment drastically inhibited the high levels of interleukin (IL)-5 and CC chemokine eotaxin (CCL11) mRNA expression in pleural leukocytes after OVA-challenge. Preliminary toxic effects were assessed, and a qualitative phytochemical profile was performed. The extract contained condensate tannins, flavonoids, saponins, and steroids but not triterpenes and alkaloids. Oral treatment with AEBv did not induce signs of systemic toxicity or genotoxic effects. These results demonstrated that AEBv is a potent inhibitor of contributors to the allergic inflammatory response, supporting its use in folk medicine to treat allergic conditions.

Key words: *Bowdichia virgilioides,* allergy, inflammation, natural product.

INTRODUCTION

In allergic inflammation, exposure to allergens through an IgE-dependent mechanism induces mast cell release from different mediators, including histamine and several proinflammatory cytokines, which contribute not only to immediate hypersensitivity but also to later reactions (Barnes, 2011). Eosinophils are known to be important effector cells in allergic reactions, and they are one of the most abundant leukocytes at the inflammatory site (Wang et al., 2007). Eosinophil accumulation depends on the release of cytokines and chemokines, such as interleukin (IL)-5 and CC chemokine eotaxin (CCL11), in response to allergen challenge (Ochkur et al., 2007). Once tissue is exposed to an allergen, activated eosinophils release several mediators, including leukotrienes, platelet-

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activating factor (PAF), and tumor necrosis factor (TNF)-α, which contribute to extensive tissue damage (Luana-Gomes et al., 2011). Therefore, reducing these allergic hallmarks, including protein extravasation, and the release of pro-inflammatory mediators and eosinophil infiltration are key to relieving allergic inflammatory symptoms.

Bowdichia virgilioides Kunth, a member of the family Fabaceae, is a tree that grows in South American countries (Deharo et al., 2001). In Brazil, the bark is used in folk medicine to treat different symptoms of inflammatory diseases (Brandao et al., 1992). Previous studies have reported that the extracts from *B. virgilioides* possess anti-inflammatory (Thomazzi et al., 2010), antinociceptive (Silva et al., 2010), and antioxidant (Dos Santos et al., 2014). However, the anti-allergic inflammatory effect of *B. virgilioides* has not yet been studied. Thus, in the present study, we investigated the effect of the aqueous extract of the stem bark from *B. virgilioides* (AEBv) on allergic inflammation in mice actively sensitized with ovalbumin (OVA).

MATERIALS AND METHODS

Plant material

Stem bark of *B. virgilioides* Kunth were collected at the *Arboretum* of Federal University of Alagoas and taxonomically identified by Prof. Rosângela P. Lyra Lemos. A voucher specimen (No. MAC29914) was deposited at the Herbarium MAC of the Institute for the Environment, Maceió, AL, Brazil.

Preparation of extract

The preparation of aqueous extract was carried out according to the traditional method. After collection, the stem bark was dried at ambient temperature and triturated. The aqueous extract of *B. virgilioides* (AEBv) was prepared by infusing 50 g of powdered plant material for 20 min using 200 mL of boiling water. The extract was filtered and lyophilized. The yield of the infusion was 17.2% (w/w). At the time of use, extract was reconstituted in 0.9% NaCl (saline) at the required concentrations.

Phytochemical screening

Chemical tests were carried out on the AEBv using standard procedures to identify the constituents using the methods described by Matos (2009).

Animals

Male Swiss mice weighing 18-22 g were obtained from breeding colonies of the Federal University of Alagoas. Animals were housed at the Institute of Biological and Health Science animal housing facility at 22 ± 2°C with a 12-h/12-h light/dark cycle and free access to food and water. Experiments were performed during the light phase of the cycle. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian Society of Laboratory Animal Science. All experimental protocols were approved by the institutional Ethics Committee (License nº 12614/11-65).

Allergic pleurisy

Allergic pleurisy was performed as described by Martins et al. (1993) with modifications. Mice were actively sensitized by dorsal subcutaneous injection of a mixture containing 50 mg OVA and 5 mg aluminum hydroxide in a final mixture volume of 0.2 mL. Fourteen days later, sensitized animals received an intrapleural (i.pl.) injection of OVA (12 µg/cavity) that was dissolved in sterile 0.9% NaCl (saline solution) immediately before use. All i.pl. injections were performed under inhalation anesthesia (2% isoflurane) and in a final volume of 0.1 mL. At distinct post-challenge time points, the mice were killed under CO_2 atmosphere and the pleural cavity was rinsed with 1 mL of PBS containing EDTA (10 mM), pH 7.4. At 24 h post-challenge, the pleural effluent was collected to analyze the cellularity and the amount of TNF-α. Sensitized mice that were injected (i.pl.) with saline were used as negative control.

To measure plasma protein leakage, the pleural effluent was collected 30 min after allergenic challenge and centrifuged at 1500 ×g for 10 min. Then, the protein content of the supernatant was quantified in a spectrophotometer (650 nm) by means of the Follin–Lowry technique. To measure pleural leukocytes, the pleural effluent was collected 24 h after stimulation with OVA, and the total leukocyte counts were determined in a Newbauer chamber with exudates diluted in Turk solution (1:20). Cytospin preparations of exudates were stained with May-Grunwald-Giemsa for the differential count, which was performed under an oil immersion objective.

Quantification of TNF-α in the pleural effluent

The amount of TNF-α produced in the pleural cavity was assessed 24 h post OVA challenge. The pleural lavage recovered was centrifuged at 770 ×g for 10 min. TNF-α was quantified in the supernatant free of cells by ELISA, following the manufacturer's protocols (BD-Bioscence Pharmingen, San Diego, CA).

Quantification of histamine secreted from tissue stimulated with antigen in vitro

The anaphylactic histamine release from mice subcutaneous tissue fragments *in vitro* was determined using a method described by Carvalho et al. (2008) with modification. Briefly, dorsal skin tissue (hypoderm layer) was removed from actively sensitized mice, washed with Tyrode solution, and placed in 24-well plates containing Hank's balanced salt solution containing Ca^{++} and Mg^{++} (HBSS). Tissues were treated with AEBv (1, 10, and 50 µg/mL) 1 h after challenge with OVA (0.4 mg/mL). Thirty minutes after stimulation, the plates were centrifuged at 150 ×g for 10 min, and the samples were collected and added to perchloric acid (0.8 N). After centrifugation at 170 ×g for 10 min, the supernatant was recovered to quantify histamine content, as described by Shore et al. (1959). The results were expressed as the amount of histamine released (ng) per amount of tissue (mg).

Real-Time RT-PCR assay

Total RNA was isolated from leukocytes collected in pleural effluent using the RNeasy kit with the addition of RNase-free DNase, according to the manufacturer's instructions (QIAGEN, Valencia, CA). cDNA was synthesized from purified RNA with random primers using MultiScribe reverse transcriptase, random hexamers, and

reverse transcriptase reagents (Applied Biosystems, Branchburg, NJ). cDNA was amplified using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Inc., Hercules, CA), as suggested by the manufacturer (1 µL cDNA, 10 µL of 2x SYBR Green supermix, and 200 nM of each specific primer). The mouse β-2-microglobulin (B2M), IL-5, and CCL11 primer pairs used were synthesized by Invitrogen and described as follows: B2M: forward 5´-GCTATCCAGAAAACCCCTCA-3´, reverse 5´-GCGGGTGGAACTGTGTTAC-3´; IL-5: forward 5´-TCATAAAAATCACCAGCTATGC-3´, reverse 5´-TTGGAATAGCATTTCCACAGT-3´; CCL11: forward 5´-GCTCACCCAGGCTCCATC-3´, reverse 5´-TGTTGTTGGTGATTCTTTTGTAGC-3´. Real-time quantitative PCR thermal cycling conditions were 95°C for 3 s, followed by 95°C for 15 s, and the T_m for 45 s for 45 cycles. Data were analyzed according to the $2^{-\Delta(\Delta CT)}$ comparative C_t Method (User Bulletin #2, ABI PRISM Sequence Detection System; Applied Biosystems) and were normalized to β-2-microglobulin expression in each sample.

Preliminary toxicity study

The preliminary toxicity of AEBv was investigated by oral administration at the higher dose (200 mg/kg body weight) for 7 consecutive days. Animal behavior (socialization, mobility, and piloerection), death, cellularity of bone marrow, and blood leukocyte counts were assessed 24 h after the last administration. The control group received vehicle (0.9% saline solution).

To assess total cell counts from blood, sample blood from the caudal vein was collected and diluted 1:10 with 1% acetic acid to lyse the red blood cells. Blood smearing following staining with May-Grunwald-Giemsa stain was used to observe leukocyte subtype counts. Bone marrow cellularity was obtained after the femurs were harvested and its medullar channels were washed with PBS plus EDTA (10 mM). The single suspension obtained by mechanical dissociation was then centrifuged at 770 ×g for 10 min and re-suspended in 1 mL PBS. Total and differential cell counts were performed similar to the blood samples.

Possible genotoxic effects of orally administered AEBv were analyzed by the comet assay according to Tice et al. (2000). At 24 h after the last treatment with AEBv, total cells from blood and bone marrow were collected to perform the comet assay. Mice treated with cyclophosphamide (50 mg/kg) were used as positive controls.

A 10 µL aliquot of blood or bone marrow was mixed in 70 µL of 0.5% low melting point agarose. The mixture was rapidly spread onto microscope slides pre-coated with a 300 µL layer of normal melting agarose (1%). After solidification, slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10 % DMSO) for 1 h at 4°C and washed with 1X PBS. Slides were incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min at 4°C prior to electrophoresis. Electrophoresis was performed at 4°C for 20 min at 300 mA and 25 V (0.90 V/cm). Slides were then neutralized (0.4 M Tris, pH 7.5), stained with SYBR Green solution in 1X PBS (1:200), and inspected using a fluorescence microscope at 400× magnification.

The extent and distribution on DNA damage was examined in 100 cells per slides that were selected randomly with a blind analysis. Cells were scored visually using a damage index (DI) according to tail size and grouped into four classes ranging from undamaged (0) to maximal damage (3), so that each animal had a single DNA damage score. Cells with DI class 0 did not show a tail; cells with damage class 2 exhibited a tail length of 1 times the head diameter; in damage class 3, the tail was longer than 2 times the head diameter. Comets with no head and images with nearly all DNA the in the tail were excluded. The total score for 100 comets was obtained by multiplying the number of cells in each class by the damage class, ranging from 0 to 300.

Statistical analysis

Data are reported as the mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism software, version 5.0 (San Diego, CA, USA). Comparisons between the experimental groups were performed using a one-way ANOVA followed by a Tukey post hoc analysis test, and the P values less than or equal to 0.05 were considered statistically significant.

RESULTS

Phytochemical prospection of AEBv

Preliminary phytochemical screening of AEBv showed the presence of condensed tannins at higher concentrations and flavonoids and saponins at moderate concentrations. Low levels of steroids were observed, while no triterpenes or alkaloids were observed. These results are presented in Table 1.

Effect of AEBv on plasma leakage triggered by allergen

Initially, to assess the effect of AEBv on allergic reaction, we quantified the plasma protein leakage caused by mast cell degranulation after allergen-induced anaphylactic reaction in sensitized animals. The challenge with allergen (OVA, 12 µg/cavity) in pleural space triggered significant protein extravasation 30 min after injection, as shown in Table 2. Oral pretreatment with AEBv (50, 100, and 200 mg/kg) suppressed protein extravasation with an inhibition of about 40, 53 and 79%, respectively. As expected, pretreatment with dexamethasone, a reference drug, was able to suppress plasma leakage (to 20.3 ± 2.7 µg/cavity).

Effect of AEBv on histamine release evoked by allergen from tissue in vitro

In this set of experiments, we investigated the effect of AEBv on mast cell degranulation by means of histamine release from tissue fragments *in vitro*. As shown in Figure 1, stimulation with allergen (OVA, 0.4 mg/mL) induced a drastic increase in histamine release. Pretreatment of sensitized tissue with AEBv (1, 10, and 50 mg/mL) *in vitro* significantly inhibited the release of histamine caused by antigen.

Effect of AEBv on allergic pleurisy

As illustrated in Figure 2, the intrapleural injection of allergen (OVA, 12 µg/cavity) into actively sensitized mice led to an intense pleural inflammatory response, which was characterized by a massive accumulation of inflammatory cells (that is, total cells; Figure 2A), eosinophils (Figure 2B), and neutrophils (Figure 2C) in

Table 1. Phytochemical evaluation of aqueous extract of barks from *Bowdichia virgilioides* Kunth (Fabacea).

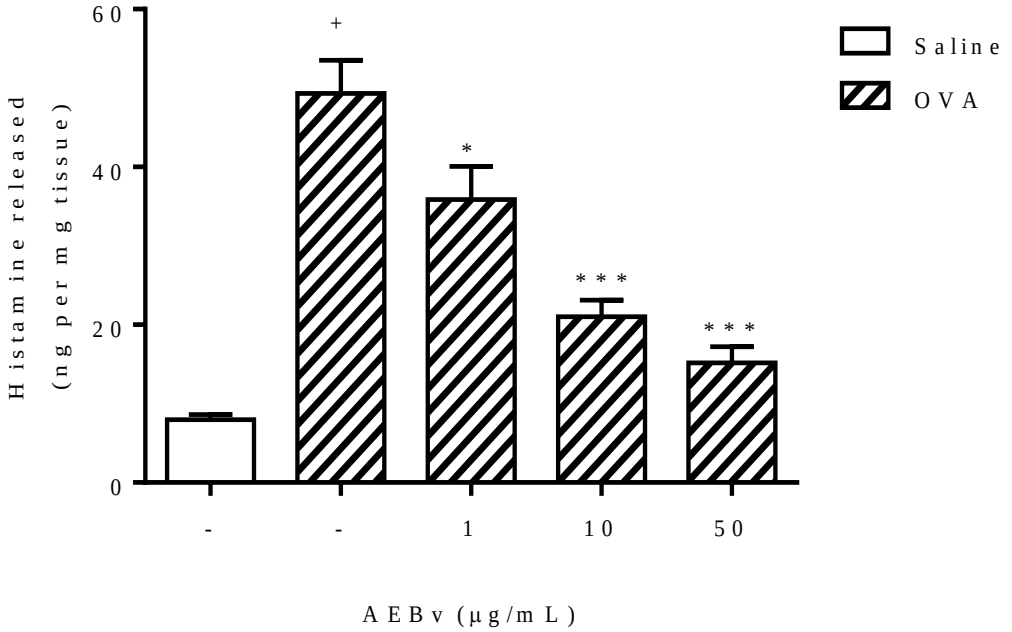
Phytochemical constituent	Results
Flavonoids	++
Condensed tannins	+++
Triterpenes	-
Saponins	++
Alkaloids	-
Steroids	+

Key: Not detected (-); Low presence (+); Moderate presence (++); Strong presence (+++).

Table 2. Effect of AEBv on protein extravasation induced by allergen in sensitized mice.

Oral pré-treatment	Stimulus	Total protein (µg/cavity)
Saline	Saline	1.43 ± 0.9
Saline	OVA	31.2 ± 2.5 $^{+}$
AEBv (50 mg/kg)	OVA	18.6 ± 1.9 ***
AEBv (100 mg/kg)	OVA	14.6 ± 2.8 ***
AEBv (200 mg/kg)	OVA	6.3 ± 1.5 ***

Treatments were performed 1 h min before allergen challenge. The groups were challenged intrapleurally with ovalbumin (OVA, 12 µg/cavity) or saline solution (vehicle) and analysed 30 min post-challenge. Each value represents the mean ± S.E.M. from at five animals. $^{+}$P < 0.001 compared to saline-challenged animals and pre-treated with saline; **P < 0.01 and ***P < 0.001 compared to OVA-challenged animals and pre-treated with saline.



Figue 1. Effect of AEBv on tissue histamine release induced by allergen *in vitro*. Bars represents the means ± S.E.M. from six fragments. $^{+}$P<0.001 compared to saline-challenged fragments. *P<0.05 and ***P<0.001 compared to OVA-challenged fragments.

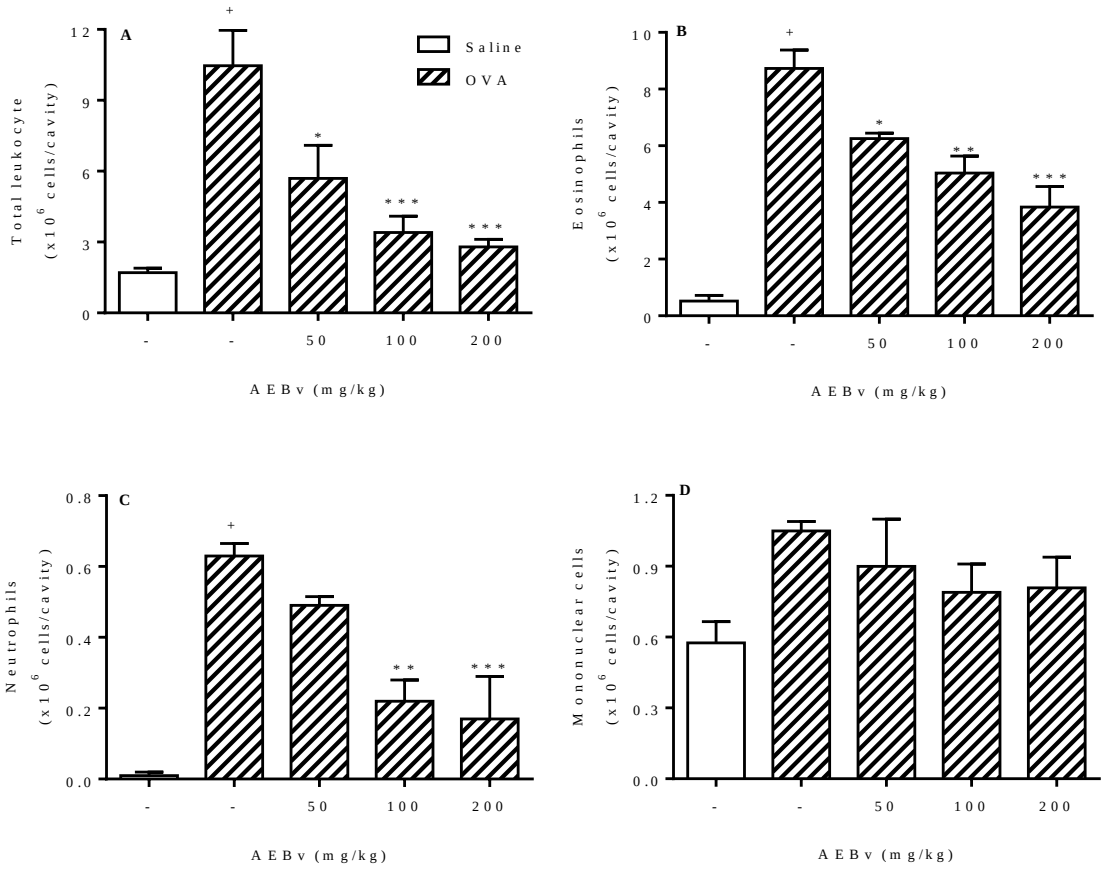


Figure 2. Inhibition of allergic pleurisy by oral pretreatment with the aqueous extract of *B. vigilioides* (AEBv). Pleurisy was induced in 14 day-sensitized mice by ovalbumin challenge (OVA: 12 µg/cavity). Total leukocyte (A), eosinophil (B), neutrophil (C) and mononuclear cell (D) counts were performed 24 h after challenge. Bars represent the mean ± SEM for at least 5 animals. $^{+}P < 0.001$ compared to saline-challenged animals; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to OVA-challenged animals.

pleural effluent, while mononuclear (Figure 2D) cell count remained slightly increased.

Pretreatment with AEBv 1 h before challenge dose-dependently suppressed pleural accumulation of total leukocytes (Figure 2A), including eosinophils (Figure 2B) and neutrophils (Figure 2C), 24 h after OVA administration. Nevertheless, AEBv failed to alter the mononuclear cell profile 24 h post-challenge (Figure 2D). As expected, treatment with dexamethasone (1 mg/kg) also significantly reduced cell accumulation in the pleural cavity 24 h post-challenge. Values of cell accumulation to steroid-treated groups were 10.4 ± 1.5 to 6.1 ± 1.4 x10^6 total leukocytes per cavity, 6.7 ± 0.5 to 2.9 ± 0.9 x10^6 eosinophils per cavity, 1.6 ± 0.3 to 0.5 ± 0.03 x10^6 neutrophils per cavity, and 3.0 ± 0.2 to 1.7 ± 0.1 x10^6 mononuclear cells per cavity.

Since TNF-α has a relevant role in leukocyte motility in allergic inflammation, we evaluated how pretreatment with AEBv interfered with levels of this cytokine in the pleural effluent 24 h post-challenge. Antigen challenge led to an increase in TNF-α levels, from 0.8 ± 0.1 µg/cavity in saline-injected mice to 3.4 ± 0.1 µg/cavity in OVA-challenged animals. When animals were pretreated with 50, 100, and 200 mg/kg AEBv 1 h before allergenic challenge, TNF-α levels decreased to 2.9 ± 0.2 µg/cavity, 1.9 ± 0.07 µg/cavity, and 1.5 ± 0.09 µg/cavity, respectively. As expected, the TNF-α level of the dexamethasone-treated group was 1.1 ± 0.01 µg/cavity.

In an allergic response, eosinophils are known to be key effector cells for inflammation triggered by an allergen. In line with this rational, in this study, we noted that eosinophil count in the pleural effluent was significantly higher than that of other cell types at 24 h post-challenge. In OVA-challenged mice, the percentage of eosinophils reached about 80% of the total leukocyte count at the inflammatory site (Figure 2).

Effect of AEBv on the relative expression of IL-5 and CCL11 mRNA induced by allergen challenge

Considering the eosinophil influx into the pleural cavity

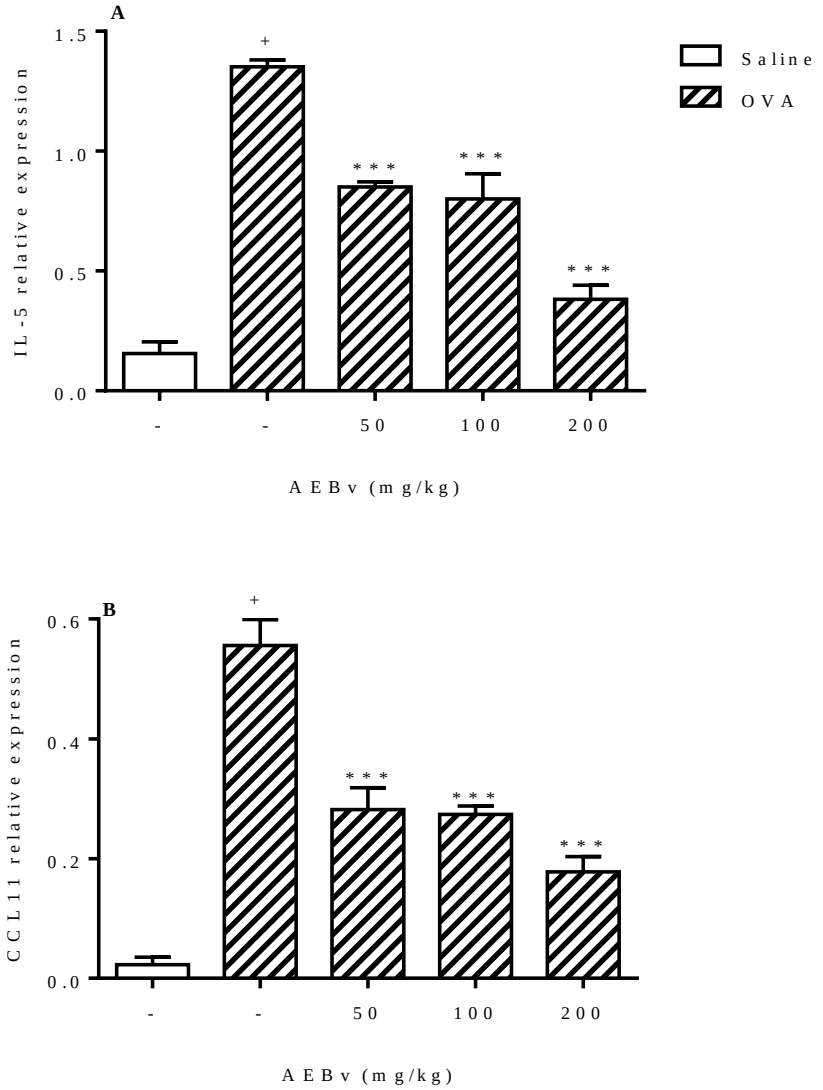


Figure 3. Effect of AEBv on the IL-5 (A) and CCL11 (B) mRNA expression in cells from pleural effluent after allergen-challenge. Total mRNA was extracted, and relative IL-5 and CCL11 mRNA amount were measured by real-time PCR. mRNA levels were expressed as fold changes over the basal after normalizing to beta-2-microglobulin. Bars represent the mean ± SEM of relative fluorescence intensity. $^{+}$P<0.001 compared with saline-challenge animals. ***P<0.001 compared with antigen-challenged sensitized group.

after OVA challenge, IL-5 and CCL11 mRNA expressions were examined in inflammatory cells harvested 24 h after antigen challenge to verify whether AEBv would be able to mediate its effects on eosinophilia pleural through the inhibition of IL-5 and CCL11.

As illustrated in Figure 3, at 24 h after OVA challenge, IL-5 and CCL11 mRNA expression levels increased in cells of the pleural effluent compared to those in saline-challenged mice (Figure 3A and B). Pretreatment with AEBv, in all doses tested, significantly reduced IL-5 and CCL11 mRNA expression levels in cells that were recovered from the pleural space after OVA-challenge (Figure 3A and 3B). As expected, dexamethasone (1 mg/kg) administered 1 h before antigenic challenge reduced the mRNA expression of IL-5 and CCL11 by 72 and 66%, respectively, compared to OVA-stimulated animals.

Preliminary toxicity study

To estimate the potential toxicity of ABEv, single oral administrations of AEBv for 7 consecutive days at 200 mg/kg were performed. No signs or symptoms of toxicity, such as reduction in locomotion, altered breathing, piloerection, body tremor, or diarrhea, were observed in

Table 3. Effect of treatment with AEBv on cell counts in blood and bone marrow.

Treatment	Blood cells ($x10^5$/mL)	Bone marrow ($x10^6$/mL)
	Total cells	**Total cells**
Saline	12.2 ± 1.0	12.4 ± 4.5
AEBv	11.7 ± 0.6	12.8 ± 3.4
	Mononuclear cells	**Mononuclear cells**
Saline	10.1 ± 1.2	8.9 ± 2.1
AEBv	9.8 ± 2.1	8.1 ± 1.1
	Neutrophils	**Neutrophils**
Saline	2.1 ± 0.4	3.6 ± 0.7
AEBv	1.9 ± 0.2	3.7 ± 0.9
	Eosinophils	**Eosinophils**
Saline	0.01 ± 0.01	0.1 ± 0.1
AEBv	0.01 ± 0.01	0.1 ± 0.1

Data show the leukocyte counts (mean ± S.E.M of 5 animals) after oral treatmet with AEBv (200 mg/kg) or saline solution.

Table 4. Absence of genotoxic effects in cells from blood and bone marrow after treatment with AEBv in mice.

Treatment	Damage Index (Arbritari Unity)	
	Blood cells	**Bone marrow**
Saline	4.5 ± 2.5	6.5 ± 1.9
AEBv	8.0 ± 3.5	7.6 ± 2.0
Cyclophosphamide	116.0 ± 6.0	106.6 ± 7.8

Data show the damage index in cells from blood and bone marrow in range from 0 (completely undamaged. 100 cells × 0) to 300 (with maximum damage 100 × 3).

treated animals 24 h after the last administration. Moreover, ABEv treatment also did not modify the cell count profile in the blood or bone marrow (Table 3).

In order to evaluate the potential genotoxic effects induced AEBv treatment, cells from blood and bone marrow were used to perform the comet assay. Table 4 shows that only cyclophosphamide (used as positive control) was genotoxic in peripheral blood and bone marrow, as indicated by the increase in the DI values compared to the saline group. AEBv did not have a genotoxic effect.

DISCUSSION

In the current study, we showed that AEBv was able to inhibit OVA-induced inflammation in a murine model. OVA-induced pleurisy has been recognized as an experimental model characterized by anaphylactic plasma leakage and leukocyte influx (e.g. eosinophils) in tissue after antigen challenge. In current work, the oral administration of AEBv 1 h prior to OVA challenge resulted in a significant suppression in the extravasation of plasmatic protein at 30 min post-stimulation. In addition, treatment with AEBv inhibited the release of histamine triggered by OVA in tissues obtained from sensitized animals. Moreover, AEBv treatment attenuated leukocyte influx, particularly eosinophils, and TNF-α levels in pleural effluent 24 h post OVA challenge. In allergen-induced diseases, elevations in eosinophillotactic attractants, including IL-5 and CCL11, are important for the recruitment of eosinophils to inflamed tissue. We also demonstrated that AEBv suppressed IL-5 and CCL11 mRNA expression. Thus, the mechanism for the reduced influx of eosinophils to the pleural cavity after OVA challenge has, at least in

part, been identified. In addition, AEBv did not produce any toxic effects or death.

Previous studies report that seeds and bark from *B. virgilioides* are traditionally used to treat diabetes and inflammatory conditions (Macedo and Ferreira, 2004). Confirming the pharmacological potential of the extracts from this plant, we and other authors have previously demonstrated that extracts from its bark and leaves are able to alleviate painful and inflammatory conditions in experimental models (Barros et al., 2010; Silva et al., 2010; Thomazzi et al., 2010). Indeed, these pharmacological effects are related to natural phytochemical constituents present in the extracts.

Here, AEBv was prepared in accordance with its use in folk medicine. Preliminary phytochemical studies of this extract reveal abundance of tannins, while flavonoids, saponins, and steroids were found at low concentrations. Interestingly, terpenoids and alkaloids were not observed. Consistent with our findings, previous chemical investigation of *B. virgilioides* has revealed the presence of phenolic compounds (Arriaga et al., 2000; Dos Santos et al., 2014). Nowadays, phenolic compounds are well known because of their anti-inflammatory properties (Chan et al., 2013; Lu et al., 2014).

Tannins belong to a group of phenolic compounds that are often encountered in food and medicinal plants (Yoshida et al., 1987). Previous studies have reported the effect of condensed tannins on compound 48/80-induced mast cell degranulation (Tokura et al., 2005) and on histamine release from RBL cells (Kanda et al., 1998). Apart from these, tannins were also able to suppress the release of β-hexosaminidase from RBL-2H3 cells after trigger by IgE (Yamada et al., 2012). Considering the abundance of tannins in AEBv, it is possible that the anti-allergenic activity exerted by this extract might to be associated with this class of metabolites.

Our results reveal that oral pretreatment with AEBv causes a marked reduction in the anaphylactic allergen-evoked plasma leakage in mice following active sensitization. This phenomenon could be associated with functional changes in the behavior of microvessels and/or decreased responsiveness of endothelial cells to vasoactive agents. Alternatively, the less intense edema formation provides evidence that AEBv might exert a suppressive effect on mast cell population. In line with this proposition, we also noted that allergen-provoked histamine release from isolated tissue fragments *in vitro* was inhibited after AEBv treatment, thus reinforcing the possibility that AEBv acts on the mast cell population.

Plasma protein accumulation as well as leukocyte influx into the inflammatory site may play a role in the pathogenesis of allergies. Treatment with AEBv inhibited leukocyte influx (mostly eosinophils) 24 h after OVA challenge, similar to what was noted in the dexamethasone treatment group. AEBv treatment also inhibited the increase in TNF-α levels in pleural effluent. This inhibitory effect on TNF-α production appears to be of great relevance, since this cytokine has long been recognized as a key mediator in the pathogenesis of allergic reactions (Brightling et al., 2008). Previous reports have demonstrated that TNF-α is able to induce the migration and activation of leukocytes (Babu et al., 2011) and stimulate the synthesis of CCL11 in different cell types (Matsukura et al., 1999). Furthermore, blocking the TNF-α utilization of the soluble TNF-α receptor (sTNF-αR) results in decreased IL-5 levels in inflammatory exudate in allergic animals challenged with antigen (Nam et al., 2009). Thus, the inhibition induced by AEBv on TNF-α levels at the inflammatory site appears to be important for attenuating signals that act as amplifiers of the allergic response.

According to previously published data, OVA challenge induced a marked increase in total leukocyte numbers in the pleural cavity within 24 h, with a significant accumulation of eosinophils (Martins et al., 1993). Based on that data, we examined leukocyte influx into the pleura cavity, and our results show that AEBv treatment attenuated polymorphonuclear accumulation, mainly eosinophils influx, in the allergic pleurisy without a significant change in mononuclear cell recruitment.

Considering the recognized effect of pro-inflammatory mediators, such as IL-5 and CCL11, on the recruitment of eosinophils on inflammatory tissue after allergen challenge (Weller et al., 2005), we decided to evaluate whether oral treatment with AEBv could influence the generation of these mediators in cells from inflammatory infiltrate after allergen challenge. Our results show that IL-5 and CCL11 mRNA expression were increased by leukocytes 24 h after OVA challenge, suggesting that inflammatory cells might contribute to changes in IL-5 and CCL11 levels at the inflammatory site. Our results show that AEBv inhibited the increase in CCL11 and IL-5 levels at the inflammatory site, suggesting, at least in part, reduced eosinophil influx into the pleural cavity after OVA stimulation. Consistent with our results, previous studies report that extracts of plants that are rich in phenolic compounds, such as tannins, appear to be capable of inhibiting parameters of allergic inflammation in experimental models *in vivo* and *in vitro* (Kimura et al., 1987; Zhou et al., 2011). Moreover, previous studies report that phenolic compounds inhibit the production of IL-5 and eotaxin-3 after immune stimulation (Hurst et al., 2010; Mao et al., 2002). Therefore, our results allow us suggest that AEBv might inhibit allergic eosinophilia via the inhibition of eosinophilotactic mediators involved in the allergic response.

Obtaining information on the toxicity of plants is very important before further exploring its development as a new herbal medicine (Saad et al., 2005). In addition, the toxic effects, which are often unknown, are problematic aspects that limit the use of medicinal plant extracts. Here, we also verified that AEBv was well-tolerated after oral administration, since mice did not show signs of systemic toxicity. In view of the beneficial effects of AEBv

and the absence of any data on its genetic toxicity, we extended our analysis to evaluate the potential genotoxic effects of AEBv. Moreover, our results revealed that oral treatment with AEBv did not induce DNA damage based on a genotoxicity test using the comet assay. This set of results shows that AEBv did not interfere with genomic stability. Interestingly, some authors showed that tannins from plants were able to inhibit DNA damage in *in vivo* and *in vitro* studies (Dauer et al., 2003; Fukumasu et al., 2006). Moreover, it has been reported that flavonoids, another class of secondary metabolites found in AEBv, have the ability to protect DNA from damage (Jothy et al., 2013). Taken together, our results suggest that AEBv has low toxicity.

Conclusion

The findings here indicate that AEBv has anti-allergenic activity, which was observed by the inhibition of distinct parameters triggered by an allergen in sensitized animals. Furthermore, it should be noted that this effect was not accompanied by toxic side effects. These results suggest that aqueous extract of the stem bark from *B. virgilioides* may be useful as a potential therapeutic agent for allergic inflammatory response.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Sensitization with babassu mesocarp induces activation of murine splenocytes against tumor cells

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***Attallea speciosa* Mart. (babassu) fruit contains a mesocarp that is rich in carbohydrates with immunomodulatory effects. The induction of the tolerogenic response is a tumor escape mechanism, and immunomodulator adjuvants have been studied to reestablish host immunogenicity. This study evaluates the adjuvant potential of babassu mesocarp carbohydrates in a tumor model. The babassu mesocarp extraction (BME) yield was 75.54%, and the total sugar concentration was 29.79 mg ml^{-1} containing monosaccharides, reducing sugars, polysaccharides and 0.506 mg ml^{-1} total protein. Chromatography analysis identified glucose, sucrose and fructose. Sensitization increased the spleen weight in the tumor group compared with the control, and a comparatively lower frequency of T helper and higher frequency of B-lymphocytes was also observed. The tumor+BME group had more cytotoxic T lymphocytes compared with the control. After co-culture with cancer cells, the tumor splenocytes showed lower proliferation, lower frequency of T helper cells and higher concentrations of interleukin (IL)-2, IL-6 and IL-10. However, the tumor+BME splenocytes presented results similar to the control, suggesting a reduction in the regulatory response of the tumor group. These results demonstrated that BME sensitization with cancer cells modulated an immune response in Balb/c animals, indicating an immunogenic effect.**

Key words: Arecaceae, *Attallea speciosa* Mart., adjuvant, carbohydrate, antitumor.

INTRODUCTION

The palm tree *Attallea speciosa* Mart., known as babassu, is a common species in Northeastern Brazil. The mesocarp of babassu fruit predominantly consists of carbohydrates (Silva and Parente, 2001) and is popularly used as a health food. Previous studies have demonstrated that the babassu mesocarp has been

traditionally used to treat inflammatory diseases (Agra et al., 2007), and the native communities have reported the use of babassu mesocarp to treat gastritis, leukorrhea and rounds (Souza et al., 2011). The biological effects of babassu mesocarp have been reported, including anti-inflammatory effects (Silva and Parente, 2001; Baldez, 2006; Nascimento et al., 2010; Silva et al., 2015). However, previous studies have shown that aqueous babassu mesocarp extract induces both *in vitro* and *in vivo* nitric oxide (NO) and tumor necrosis factor α (TNF-α) production in peritoneal macrophages (Nascimento et al., 2006). In addition, immunomodulatory (Guerra et al., 2011; Pessoa et al., 2014) and antitumoral effects (Rennó et al., 2008; de Souza et al., 2011; de Sousa et al., 2013) have been reported.

Herbal immunoadjuvants are substances that stimulate the immune response (Khyati, 2012), and carbohydrates are promising adjuvants because of their low cytotoxicity, good biocompatibility, and strong immune enhancement (Li and Wang, 2015).

Immune responses against tumors involve both innate and adaptive immunity (Lehrnbechter et al., 2008; Loose and Van de Wiele, 2009; Achyut and Arbab, 2016). Innate immunity includes natural killer (NK) cells and macrophages, while adaptive immunity involves cytotoxic T lymphocyte (CTL) cells, which are responsible for a more specific response against tumor antigens and the establishment of an immunologic memory (Klebanoff et al., 2006; Coulie et al., 2014; Dimeloe et al., 2016). Therefore, drugs with broad-spectrum activities, combining antitumoral and immunomodulatory effects with the capacity to mobilize the host immune system towards immunogenicity, are of great interest.

Considering its immunomodulatory activity and composition that is rich in carbohydrates, we hypothesized that the carbohydrates extracted from babassu mesocarp could act as adjuvants in sensitization against tumor cells. This study shows the *ex vivo* phenotypic, functional and cytokine production characteristics of splenocytes obtained from Balb/c mice sensitization using the extract of babassu mesocarp and tumor cells.

MATERIALS AND METHODS

Extract preparation and carbohydrate analysis

To prepare the babassu mesocarp extract (BME), the powder of babassu mesocarp supplied by the Cooperative of Coconuts breakers of Maranhão, located in Esperantinópolis-MA (Latitude: 04° 52' 00" S, Longitude: 44 42' 30" W) was used. The powder showed similarity regarding all of the botanical and phytochemical aspects compared with the mesocarp flour prepared in the laboratory (Nascimento et al., 2006). The botanical identifications were obtained from the Herbario Ático Seabra, State of Maranhão, Brazil (authenticated voucher specimen number 1135).

The babassu mesocarp powder was macerated in water at a concentration of 20 mg/ml for 24 h (Fortes et al., 2009), and subsequently, an aqueous babassu mesocarp extract was obtained (BME). The quantitative carbohydrate analysis was performed using a phenol-sulfuric acid method, with glucose as a standard (Dubois et al., 1956). The extract sample was deionized with a cationic-exchange column (Dowex 50W X 8 - 100) and anionic-exchange column (Dowex 1 X 8 -100). Next, the sample was filtered through 0.45-µm membrane filters. Samples with equivalent glucose concentrations (400 ug ml^{-1}) were analyzed by anion exchange chromatography coupled with pulsed amperometric detection (HPAEC/PAD) using a DIONEX ICS3000 chromatograph and CarboPac PA-1 (2×250 mm). The carbohydrate separation was performed using a multi-step gradient obtained after mixing eluent A (water) and eluent B (250 mM sodium hydroxide): 0 to 15 min, 100 mM; 15.1 to 20 min, 200 mM; and 20.1 to 25.5 min, 100 mM. The applied PAD potentials for E1 (0 to 0.4 s), E2 (0.41 to 0.42 s), E3 (0.43 s) and E4 (0.44 to 1.00 s) were 0.1, 2.0, 0.6 and 0.1, respectively, and the flow rate through the column was 0.25 ml^{-1} min. The peaks were identified through comparison with authentic standards of Mio-inositol, Glucose, Fructose, Sacarose, Raffinose and Estaquiose, with 99% purity (Sigma) (Figure 1). The protein concentration was determined using the Bradford test (Bradford, 1976).

Animals

Balb/c male mice (25 to 30 g, 30 days old) were obtained from the Biotery at the University of São Paulo. The study was approved by the Ethics Committee on the Use of Animals of UFMA (Application number: 8608/2011-00).

MCF-7 cancer cells

The MCF-7 breast cancer cell line was obtained from the Cell Bank of Rio de Janeiro. The cells were cultured in flasks containing Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator at 37°C under 5% CO_2. When confluent, the cells were detached by addition of 3 ml of 0.025% trypsin-ethylene diamine tetraacetic acid and incubated at 37°C in a humidified incubator under 5% CO_2 for 5 min. Subsequently, the cell suspension was transferred into a 15 ml polyethylene tube containing 12 ml of RPMI medium supplemented with 10% FBS, centrifuged at 800 *g* for 10 min at 18°C, and washed again. The cells were adjusted to a concentration of $1×10^5$ cells and resuspended in 100 µl of enzyme assay buffer (EAB; at a concentration of 10 mg/kg) or saline solution (0.9%) 2 h prior to the sensitization assay.

Sensitization protocol

The animals were randomly divided into 4 groups (n=6/group): the

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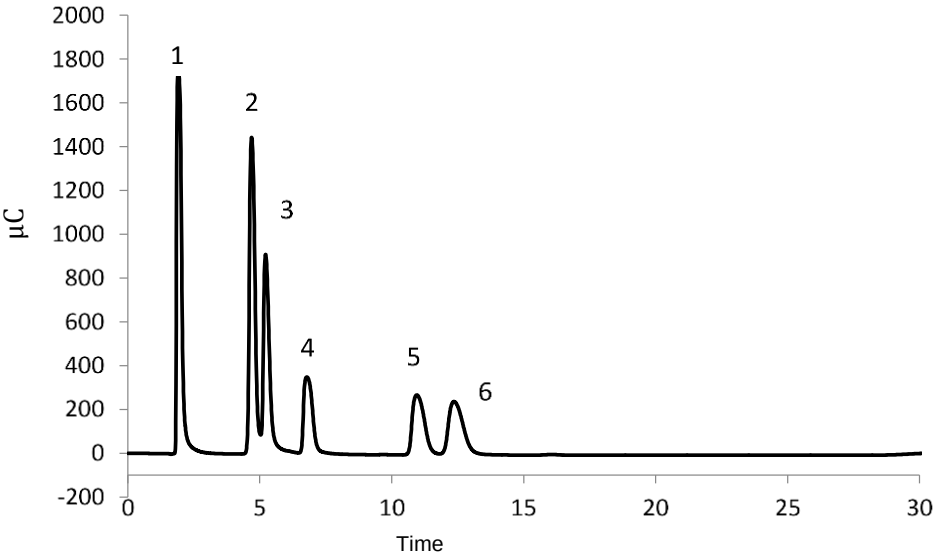

Figure 1. Separation of neutral and amino monosaccharides derived from glycoproteins. The peaks were identified by comparison with authentic standards of (1) Mio-inositol (2) Glucose (3) Fructose (4) Sacarose (5) Rafinose and (6) Estaquiose

control group in which the animals were sensitized with 100 µl sterile saline solution; the BME group in which the animals were inoculated with 100 µl BME (66,6 mg/kg); the tumor group in which the animals were inoculated with 100 µl MCF-7 cancer cell suspension at a concentration of 1×10^6 ml^{-1} in saline solution; and the tumor+BME group in which the animals were sensitized with a 100 µl MCF-7 cancer cell suspension at a concentration of 1×10^6 ml^{-1} in BME (66.6 mg/kg). Sensitization was subcutaneously performed 3 times on the dorsum, at an interval of 5 days. On the 15th day, the animals were euthanized through intraperitoneal access with an excess of anesthetics, including 2% xylazine hydrochloride (20 mg/kg), and 5% ketamine hydrochloride (25 mg/kg). Blood, bone marrow cells, and splenocytes were collected.

Blood collection and leukocyte count

The blood was collected in a micro hematocrit capillary tube by puncturing the orbital plexus. The blood was collected after adding HEMSTAB EDTA anticoagulant and leukocyte (white blood cells, WBC) count was determined using the hematology automatic analyzer.

Collection and counting of bone marrow cells

To collect bone marrow cells, the femur was removed and perfused with 1 ml phosphate buffered saline (PBS) solution. To count the cells, 90 µl of the suspension was mixed with 10 µl of 0.1% crystal violet solution. Subsequently, the cells were counted under an optical (light) microscope in a Neubauer chamber.

Cell collection and splenocyte count

The spleen was surgically removed, weighed, and homogenized in 3 ml PBS. The suspension was stored on ice until counting. For quantification, 90 µl of cell suspension was mixed with 10 µl of crystal violet, and the cells were counted under an optical (light) microscope in a Neubauer chamber. The splenocytes were resuspended in RPMI medium supplemented with 10% FBS at a concentration of 1×10^6 ml^{-1} and stored in a Petri dish for 2 h in a CO_2 incubator. After incubation, non-adherent cells were removed for phenotype characterization and co-culture experiments.

Phenotype characterization of splenocytes

The phenotypes of adherent and non-adherent cells, isolated from the spleen, were characterized with commercial monoclonal antibodies (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. Two panels of antibodies were used, one for adherent cells, including anti-CD14 (FITC), anti-IA/IE (PE), and anti-Ly6G (PE-Cy5), and the other for non-adherent cells, anti-CD3 (FITC), anti-CD19 (PE-Cy5), anti-CD4 (PE), and anti-CD8 (PE-Cy5). After the acquisition of 10,000 events in a FACSCalibur flow cytometer, the obtained data were analyzed using FlowJo software.

Establishment of a co-culture with MCF-7 cancer cells

For co-culture experiments, MCF-7 tumor cells (1×10^4/well) were seeded in 96-well microtiter plates (Corning Costar) in RPMI medium supplemented with 10% FBS. After 24 h, non-adherent splenocytes were seeded at a concentration of 3×10^5 cells/well in RPMI medium supplemented with 10% FBS. After three days, the splenocytes were used for proliferation assays and phenotype characterization, and the supernatant was used to determine cytokine production.

Lymphoproliferation assay

To evaluate cell proliferation, the splenocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE) using the CellTrace

CFSE cell proliferation kit according to the manufacturer's instructions and subsequently added to co-culture plates containing MCF-7 cancer cells. After three days, 10,000 events were acquired using a FACSCalibur flow cytometer. The proliferation capacity was determined as the decay of the average fluorescence intensity of the population corresponding to splenocytes, according to the Side and Forward Scatter (SSC and FSC, respectively) obtained using FlowJo software. The proliferation index was calculated as the ratio between the mean fluorescence intensity (MFI) of CFSE obtained in control splenocytes and the MFI of CFSE obtained in the other splenocytes.

Phenotype characterization of splenocytes after co-culture

The phenotype of the non-adherent splenocytes in co-culture was characterized using the commercial monoclonal antibodies (BD Biosciences, San Jose, CA) anti-CD3 (FITC), anti-CD4 (PE), and anti-CD8 (PE-Cy5) according to the manufacturer's instructions. After the acquisition of 10,000 events using a FACSCalibur flow cytometer, the obtained data were analyzed using FlowJo software.

Cytokine quantification by flow cytometry

The quantification of IL-2, IL-6, and IL-10 cytokines in the supernatant of the co-culture was performed using the Cytometric Beads Array (CBA) commercial kit according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism version 5.0 software with Student's *t*-test and Newman-Keuls post hoc ANOVA test. Statistical significance was determined at a value of $p<0.05$. The data are expressed as the average±standard deviation of 6 animals per group.

RESULTS

The babassu mesocarp aqueous extract was rich in carbohydrates

The babassu mesocarp aqueous extract showed a yield of 75.54%. The aqueous extract showed a total sugar concentration of 29.79 mg/ml, with positive results for the presence of monosaccharides, reducing sugars, aldoses and ketoses, confirming the presence of polysaccharides. HPAEC analysis identified glucose, sucrose and fructose (Figure 2). The protein concentration in the aqueous extract was 0.506 mg/ml.

Sensitization with tumor cells altered the balance between T and B lymphocytes of the spleen

After the sensitization of Balb/c mice, the numbers of cells in the blood, spleen, and bone marrow were determined. The data showed that sensitization did not alter the number of circulating leukocytes and bone marrow cells in the different groups. However, the animals in the Tumor group showed an increase in spleen weight compared with the control group (Table 1).

Phenotyping of the cellular population of the spleen revealed that the frequency of B cells was higher in the tumor group compared with the other groups (Figure 3A). In contrast, the frequency of T cells was lower in the tumor group compared with the other groups (Figure 3B). The decreased frequency of T cells in the tumor group was followed by a reduction in the frequency of CD4+ T cells (Figure 3C). However, the frequency of CD8+ T cells in the tumor group was similar to that in the other groups (Figure 3D). No changes were observed in the frequency of CD14+, IA/IE+, and Ly6G+ splenocytes in any of the groups analyzed (data not shown).

Lymphoproliferative activity of splenocytes co-cultured with MCF-7 cells

To evaluate whether sensitization interferes with the proliferative capacity of splenocytes, non-adherent cells, and primarily represented by lymphocytes, were co-cultured with MCF-7 cancer cells. The data showed that splenocytes obtained from the BME group presented 3 times higher proliferative activity compared with the control group. No significant difference was observed in the tumor+BME group compared with the control group; however, significantly higher cell proliferation was observed compared with the tumor group (Figures 4A and B).

Phenotype characterization of splenocytes after co-culture with MCF-7 cancer cells

Splenocytes and MCF-7 tumor cells were co-cultured and the phenotypic analysis of T cells was performed. The results showed that the percentage of CD4+ T lymphocytes was lower in the tumor group compared with the other groups (Figure 5A). The results obtained in the tumor+BME group were similar to those obtained in the control group. However, no changes in cytotoxic T cells were observed (Figure 5B).

Cytokine production and release in the co-culture supernatant of splenocytes and MCF-7 cancer cells

The supernatant of the co-culture was removed to determine cytokine production. The data revealed that the concentrations of IL-6 and IL-10 were lower in the BME group compared with the control group. However, the tumor group showed significantly higher levels of IL-2, IL-6 and IL-10 than the control and BME groups. The splenocytes obtained from animals in the tumor+BME

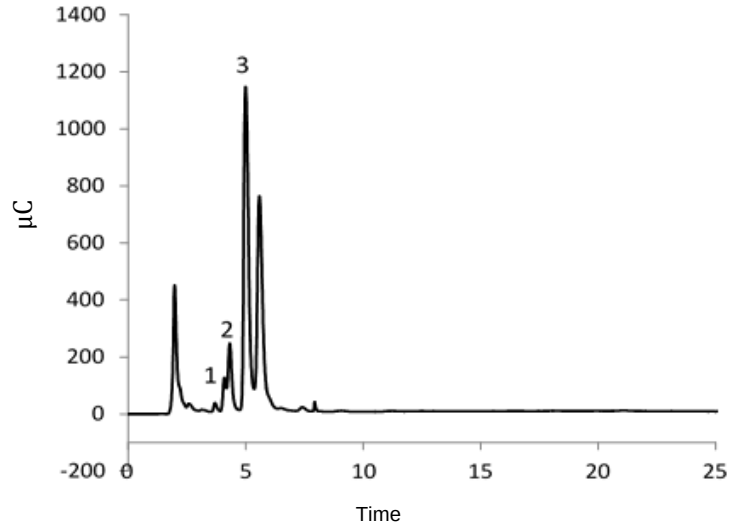

Figure 2. HPAEC chromatography identified of carbonhydrate the aqueous extract obtained from babassu mesocarp. HPAEC analysis identified type [(1) glucose, (2) fructose, and (3) sucrose in aqueous extract babassu]. The extract sample was deionizated in cationic-exchange column (Dowex 50W X 8-100)and anionic-exchange column (Dowex 1X 8-100). After, the sample was filtered through 0.45 µM membrane filtres. Samples with glucose equivalent (400 µg/ml) was analysed by anion exchange chromatography coupled with pulsed amperometric detection (HPAEC/PAD) using DIONEX ICS3000 chromatogram and carboPac PA-1 (2×250 mm). The carbonhydrate separation ws performed by using a multi-step gradient by mixture eluent A (water) and eluent B (250 mM sodium hydroxide): 0-15 min, 100mM; 15.1-20 min, 200 mM; and 20.1-25.5, 100 mM. the applied PAD potential for E1 (0-0.4 s) E2 (0.41-0.42 s), E3 (0.43 s) and E4 (0.44-1.00 s) were 0.1, 2.0. 0.6 and 0.1, respectively, and the flow rate through the column was 0.25 ml/min. The peak were identified by comparism with authentic standards of Mio-inositol, Glucose, Fructose, Sacrose, Rafnose, and Estaquiose with 99% purity Sigma.

Table 1. Immunological parameters.

Parameter	Groups (n=6/group)			
	CTL	BME	Tumor	Tumor+BME
WBC/µL (10^3)	7.8±1.7	6.2±1.4	6±1.7	5.8±1.1
Bone marrow cells (10^5)	58.1±7	52.6±5	61.4±8	65.3±7
Spleen weight (mg)	90±13	90±16	140±19*	110±13
Splenocytes (10^5)	1.4 ±304	1.4±216	1.7±436	1.2 ±208

The results are expressed as the average ± standard deviation of the total leukocyte count in the blood (WBC), number of bone marrow cells and weight and number of splenocytes obtained from groups sensitized with saline solution (Control group), babassu mesocarp extract (BME) alone (BME group), MCF-7 cancer cells resuspended in saline solution (Tumor group), and MCF-7 cancer cells resuspended in BME (Tumor+BME group). The results were analyzed using the Newman-Keuls post hoc ANOVA test, and * indicates $p < 0.05$ compared with the Control group (n=6/group).

group did not show a significant difference in cytokine production compared with the other groups, but there was a decrease in the IL-6 and IL-10 concentrations compared with the tumor group (Table 2).

DISCUSSION

In the present study, Balb/c mice were subcutaneously sensitized with MCF-7 cancer cells, which are ideal for

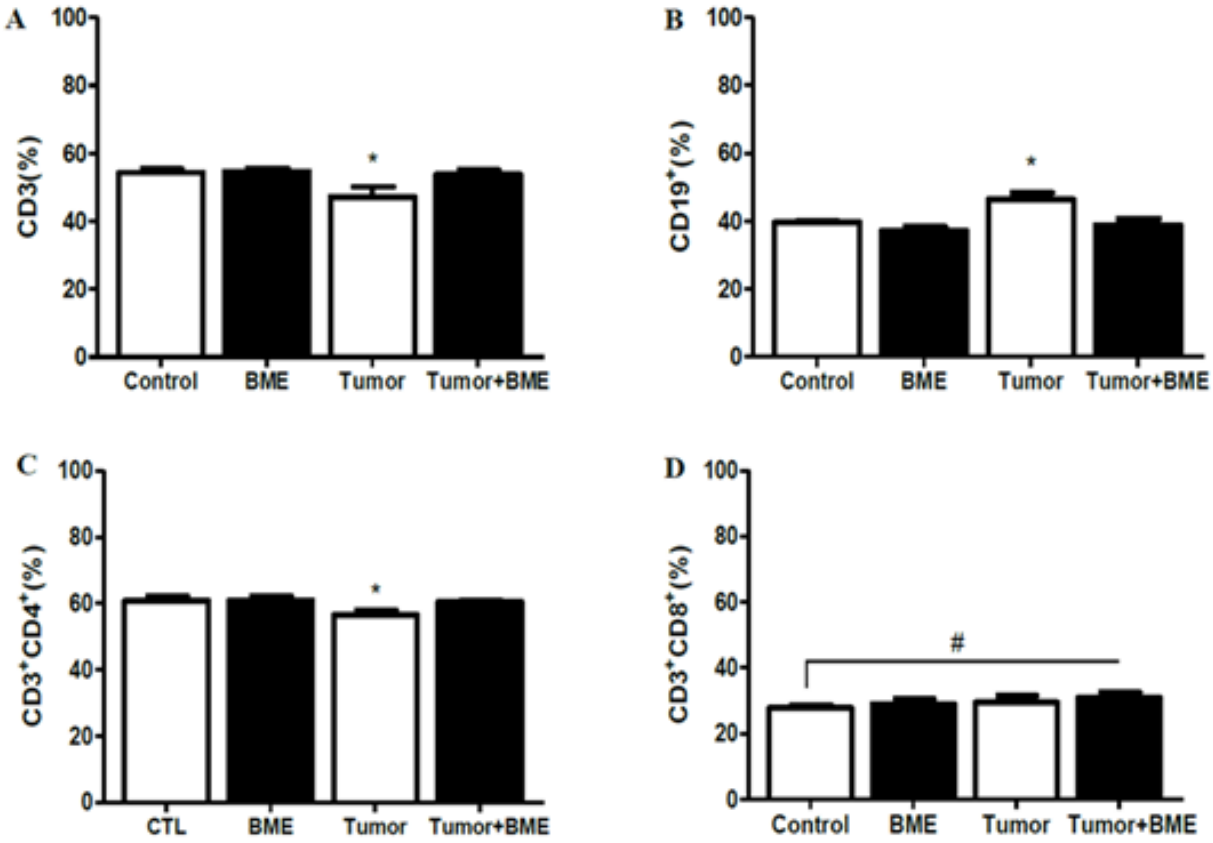

Figure 3. Phenotyping of splenocyte cultures obtained after sensitization. Frequency of T cells (A), B cells (B), and subpopulations of T helper (C) and cytotoxic T cells (D), respectively, were analyzed in the spleen of animals after subcutaneous sensitization with saline solution (Control group), extract rich in carbohydrates obtained from Babassu mesocarp extract (BME) alone (BME group), suspension of MCF7 cancer cells in saline solution (Tumor group), or suspension of MCF7 cancer cells in BME (Tumor+BME group). Values are expressed as average ± standard deviation and analyzed by the Newman-Keuls post-hoc ANOVA test with *$p<0.05$ compared with all groups and $^{\#}p<0.05$ compared with a single group.

antigen recognition by resident immune cells and subsequent migration and presentation to secondary lymphoid organs. In the context of immunization with tumor cells, the immune system might experience two types of naive responses: the low capacity of the host to trigger local inflammation that might compromise tumor antigen presentation, and poor immunogenicity in response to the tumor (Edelman and Jefford, 1968). Balb/c mice induced an adequate response after immunization (Crowther and Wasgstaff, 1983; Ochsenbein et al., 2001; Ochesenbein, 2002). Although these cells do not proliferate in mice, upon sensitization, human MCF7 cancer breast cancer cells induce the proliferation of splenocytes and after fusion, form hybridomas that produce specific monoclonal antibodies (Ochsenbein et al., 2001; Ochsenbein, 2002; Schunck and Macallum, 2005).

In the present study, the results showed no significant differences in the leukocyte number and cellularity of the lymphoid organs. However, increased spleen weight and a lower frequency of T helper cells were observed in animals sensitized with MCF-7 cells. These data contradict the expectation, as the inoculation of human cells in mice should lead to a xenograft reaction involving cell rejection and T lymphocyte stimulation (Mandal-Ghosh et al., 2007; Menard et al., 1983). However, studies with athymic mice showed that the overexpression of transforming growth factor beta (TGF-β) induces immunosuppression in the MCF-7 cancer cell line (Sun et al., 1992; Arteaga et al., 1993; Sachs, 1995; Schunk and Macallum, 2005; Koch et al., 2013). Although, the thymus of the animals was maintained in the present study, we suggest the existence of potential mechanisms that might be involved in the steps occurring between xenograft rejection and tumor cell escape, presumably affecting the T helper cell response.

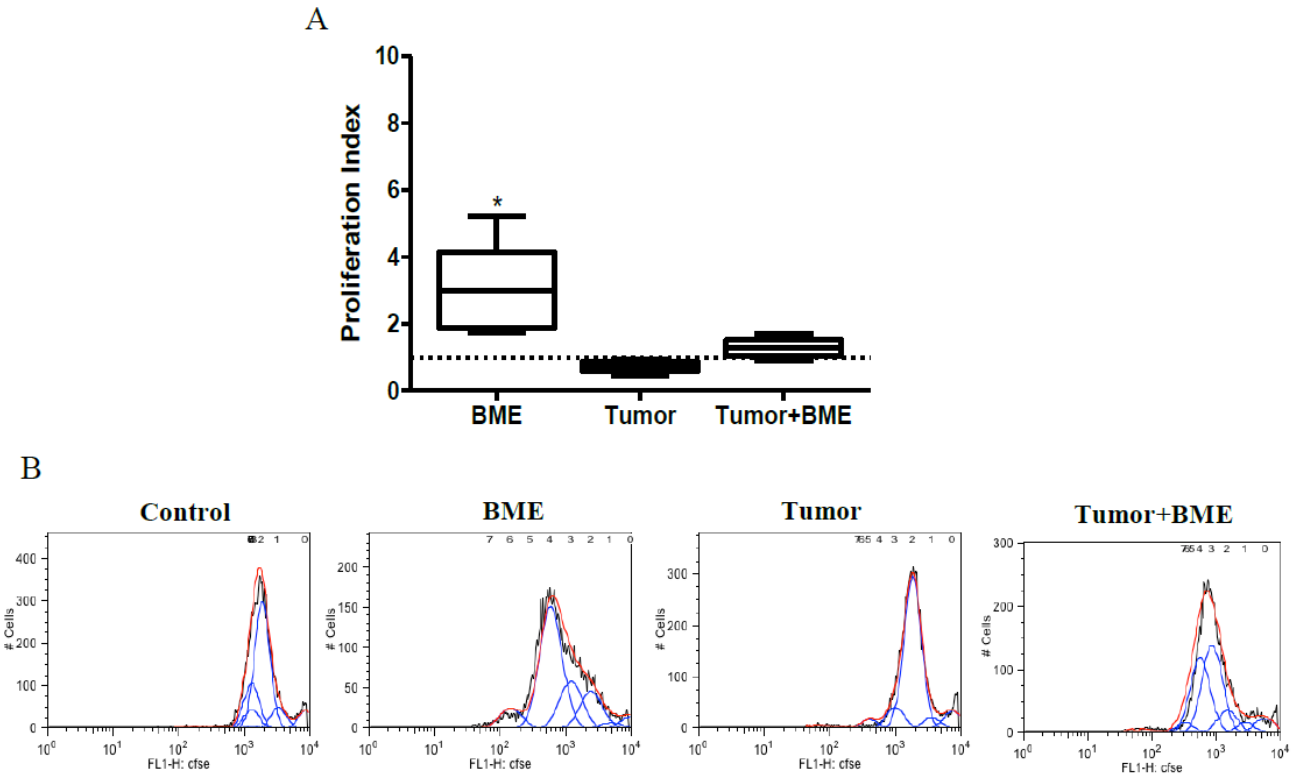

Figure 4. Proliferation of splenocytes after sensitization and maintainance in culture with MCF7 cancer cells. The proliferation rate was calculated from the ratio between the mean fluorescence intensity (MFI) of carboxyfluorescein succinimidyl ester (CFSE) in the control group sensitized with saline solution and the other groups by determining the number of times that the group sensitized with Babassu mesocarp extract (BME) alone (BME group), MCF7 cancer cells (Tumor group), or MCF7 cancer cells and BME (Tumor+BME group) promotes cell division compared with the control (dashed line) (A). Histograms for the different groups illustrate CFSE fluorescence intensity; lines inside the peak indicate the shift of CFSE MFI to the left, which is proportional to increased cell proliferation (B). Values are expressed as average ± standard deviation and analyzed by the Newman-Keuls post-hoc ANOVA test with * indicating $p<0.05$ compared with all groups.

However, the frequency of CD3+CD4+ cells in the group sensitized with MCF-7 cancer cells and BME was similar to that in the control group. Although the difference was not significant, sensitization with babassu mesocarp, with or without MCF-7 cancer cells, increased the frequency of IA/IE$^+$ cells, demonstrating the occurrence of improved antigen presentation, the modulation of the resident cells' pro-inflammation response (Azevedo et al., 2003), or the generation of complexes with tumor proteins that increase immunogenicity (Richmond and Su, 2008; Ju et al., 2001). Nascimento et al. (2006) showed that in animals intraperitoneally treated with babassu mesocarp, the migration of cells into the peritoneum increased, primarily through the activation of macrophages that release hydrogen peroxide (H_2O_2) and nitric oxide (NO). This finding indicates that babassu mesocarp promotes an M1 type response. M1 macrophages are activated through classical pathways and play an important role in eliminating tumor cells via the production of high levels of pro-inflammatory cytokines that lead to a Th1 response (Koch et al., 2013; Schunk and Macallun, 2005; Richmond and Su, 2008; Sachs, 1995; Arteaga et al., 1993; Sun et al., 1992). Using isogenic C57Bl/6 and Balb/c mice, Fortes et al. (2009) showed antitumor activity and improved survival of animals sensitized with solid and ascitic Ehrlich tumors. In the present study, in addition to the recovery of T helper cells, sensitization with BME and tumor cells increased the frequency of cytotoxic T cells and reduced the frequency of B cells compared with animals sensitized with the tumor alone. These data reinforce the idea that the carbohydrates of babassu mesocarp might exhibit immunomodulatory activity through the induction of a cytotoxic response.

In the proliferation assay, the group sensitized with MCF-7 cancer cells did not present any differences compared with the control group, and the population of T helper cells remained lower after co-culture. These data reinforce the hypothesis that the presence of a tumor

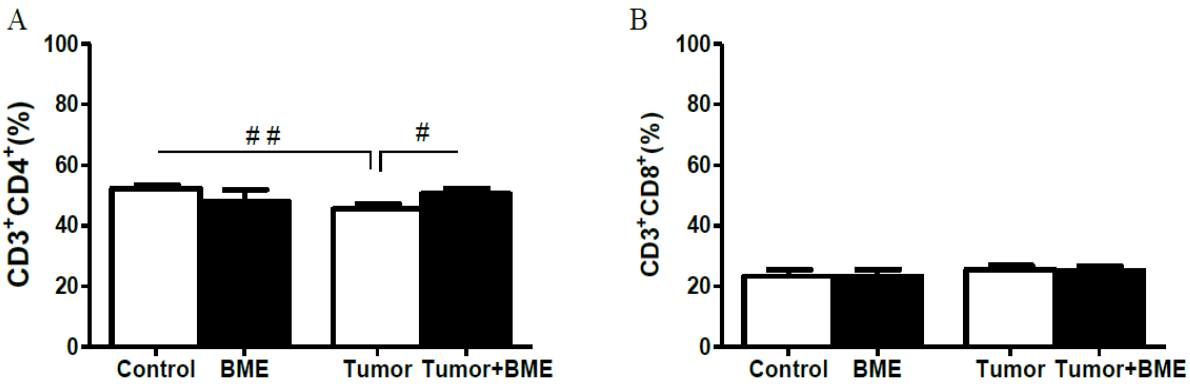

Figure 5. Phenotyping of splenocytes obtained after sensitization and co-cultured with MCF-7 cells. Frequency of T helper cells (A) and cytotoxic T cells (B) were analyzed 3 d after co-culturing MCF-7 tumor cells with non-adherent splenocytes obtained from animals sensitized subcutaneously with saline solution (Control group), Babassu mesocarp extract (BME) rich in carbohydrates (BME group), suspension of MCF-7 tumor cells in saline solution (Tumor group), or with the suspension of MCF-7 tumor cells in BME (Tumor+BME group). Values are expressed as average ± standard deviation and analyzed by Newman- Keuls post-hoc ANOVA test with $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with a single group.

Table 2. Cytokine concentrations in the supernatant of splenocyte and MCF-7 tumor cell co-cultures.

Parameter	Groups			
	Control	BME	Tumor	Tumor+BME
IL-2 (pg/ml)	0.5±0.2	0.05±0.1	14.5±5.7*	3.5±3.7
IL-6 (pg/ml)	122.9±3.0	32.0±14.3*	363.1±172.8*	194.4±128.9
IL-10 (pg/ml)	89.1±7.6	14.8±12.2*	115±54.8*	78.2±38.6

The results are expressed as the average of the duplicates ± standard deviation of interleukins (IL) 2, 6, and 10 concentrations in the supernatant of splenocyte and MCF-7 cancer cell co-cultures obtained from groups sensitized with saline solution (Control group), BME alone (BME group), MCF-7 cancer cells resuspended in saline solution (Tumor group), and MCF-7 cancer cells resuspended in BME (Tumor+BME group). The results were analyzed using the Newman-Keuls post hoc ANOVA test, and *p < 0.05 compared with the Control group (n=6/group).

might activate immunosuppressive mechanisms that could affect effector lymphocyte ability. However, the proliferation rates of the splenocytes obtained from the BME and tumor+BME groups were higher, suggesting that BME treatment induced the immune response against tumor-associated immunosuppression.

The data show that increased levels of IL-2, IL-6, and IL-10 were observed in the supernatant of the splenocyte co-culture obtained from animals of the tumor group, thus indicating a regulatory response. The splenocytes obtained from the animals of the tumor+BME group showed a decrease in all cytokines, similar to the control group. The data reinforce a Th1 adjuvant role for the carbohydrates extracted from babassu mesocarp, as the entire response induced in Balb/c mice stimulated with MCF-7 cancer cells was reversed when the tumor stimulus was associated with BME.

The mesocarp of the babassu fruit is rich in carbohydrates and in particular, a mucopolysaccharide, MP1, which was shown to induce phagocytic activity (Silva and Parente, 2001). Other studies demonstrated that treatment with mesocarp exerts immunoregulatory effects. Nascimento et al. (2006) showed that intraperitoneal injections of BME increased cellular migration to the peritoneal cavity, promoted the activation of peritoneal macrophages both *in vitro* and *in vivo*, and increased the expression of major histocompatibility complex (MHC) class II and the recruitment of immune cells (Nascimento et al., 2006). Fortes et al. (2009) showed that BME exerted an immunomodulatory effect, resulting in increased survival of the animals and a decreased size of the solid Ehrlich tumors when this tumor cell line was pretreated with BME. Guerra et al. (2011) demonstrated that immunization with promastigote forms of *Leishmania amazonensis* and the addition of BME induced a Th1-dependent immune response in

Balb/c mice, thus showing promising effects compared with other adjuvants. Despite their poor immunogenicity, the ability of carbohydrates to exert an immunomodulatory effect through the formation of complexes with tumor proteins potentiates immunospecific responses. This is the case for the oligosaccharides in Lewis Y-containing glycoproteins, which are used in mice to form complexes that induce the production of antibodies that exert cytotoxic effects against MCF-7 cells through the activation of macrophages (Kudryashov et al., 1998; Silva and Parente, 2001; Mantovani et al., 2004) by the binding of these molecules to membrane receptors (Um et al., 2002; Moretão et al., 2003; Ma et al., 2010) or the increase in the host immune response against tumors, which enhances leukocyte action and cytokine production (Mosser, 2003). The results showed that BME induces cell proliferation, increases T helper cells and reduces IL-6 and IL-10 levels, thereby reversing tumor-associated immunosuppression.

Therefore, this study showed that BME modulates the frequency of T helper cells and B-lymphocytes in the spleen of Balb/c mice after sensitization with human MCF-7 cancer cells. Moreover, *in vitro* experiments demonstrated that BME improved the lymphoproliferative activity and modulation of cytokines in the presence of MCF-7 cancer cells. These data reinforce the immunomodulatory effect of the carbohydrates extracted from babassu mesocarp, thus demonstrating an adjuvant role in the development of antitumor vaccines.

Conflict of Interests

The authors have not declared any conflicts of interests.

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Full Length Research Paper

Curcuma comosa ameliorates cisplatin-induced nephrotoxicity: COX-2 expression and ultrastructure changes

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To determine the protective effects of ethanol extracts of *Curcuma comosa* on kidney injury by cisplatin, mice were randomly assigned into 4 groups: Control, cisplatin control (12.5 mg/kg body weight (BW), i.p.), *C. comosa*+cisplatin (pretreatment with *C. comosa* at dose 200 mg/kg BW orally for 4 consecutive days before cisplatin injection), and *C. comosa* control groups. After five days, the renal tissues were collected to evaluate histopathological changes and inflammatory markers. This study elucidates the postulate of using *C. comosa* to counteract effect of cisplatin in terms of renal toxicity. The outcome shows that incidence of nephrotoxicity in cisplatin given along with *C.comosa*, decreased clinically and statistically, comparing with cisplatin given alone. It shows less of renal tubular damages and COX-2 expression. Also, the microscopic alteration showed a decreased number of swollen cells, necrotic and apoptotic cells. These aforementioned results proved the benefit use of *C. comosa* in aspect of renal protection. *C. comosa* can ameliorate cisplatin-induced kidney injury through the suppression of the inflammatory cytokine (COX-2) and its anti-oxidant properties. Therefore, it is a promising alternative regimen for the prevention of nephrotoxicity during cisplatin therapy.

Key words: Randomized controlled trial, *Curcuma comosa*, kidney injury prevention, histopathology, COX-2 expression.

INTRODUCTION

cis-Dichlorodiammine platinum (II) or Cisplatin is one of the most effective alkylating agent widespread use in chemotherapy regimen for solid organ tumor including head, neck, lung, testis, ovary and breast. As a platinum-based anticancer agent, cisplatin acts through inhibition of DNA inter-strand and intra-strand cross-linking process. This mechanism leads to decreased cell proliferation; and eventually cell death (Chvalova et al., 2007). Despites the use of cancer eradication, cisplatin also causes many harmful side effects such as, ototoxicity, gastro-toxicity, myelosuppression and nephrotoxicity, with the incidence up to 20% in those

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receiving high-dosed cisplatin (Hill and Speer, 1982; Cooley et al., 1994). The pathogenesis of cisplatin-induced kidney injury is caused by direct cell injury from the uptake of free-form cisplatin in tubular cells. This uptake can be observed at inner medullae and outer cortices as well. As a result, this substance caused tubular dysfunction and lead to kidney injury (Kuhlmann et al., 1997).

Damage to kidney could be manifested in many ways such as reduction of glomerular filtration rate, reduction of renal blood flow, polyuria, hypomagnesemia, hypokalemia and hypocalcemia (Winston and Safirstein, 1985; Hutchison et al., 1988; Schilsky and Anderson, 1979). Many renal protective protocols have been postulated in order to lessen the occurring of cisplatin-induced nephrotoxicity, for example normal saline solutions, mannitol and furosemide infusion, and sodium thiosulphate (Cvitkovic et al., 1977; Pera et al., 1979; Heidemann et al., 1985; Hirosawa et al., 1989). According to ESRP SIG or European Society of Clinical Pharmacy Special Interest Group cancer care recommendation, only normal saline infusion was recommended to be used for the prevention of the adverse effect (Launay-Vacher et al., 2008). Even though aggressive hydration, especially with normal saline solutions as the suggestion is routinely performed, the number of nephrotoxicity is still not decreasing (Hayes et al., 1977; Einhorn and Donohue, 1977). Thus, the protective manner for cisplatin-induced nephrotoxicity is still needed for a new method.

Curcuma comosa Roxb. (*Curcuma* species, Zingiberaceae family) is commonly a traditional herbal medicine under the name Wan-Chak-Mod-Luk in Thailand. Thai traditional practitioners use its rhizome as an anti-inflammatory agent for the treatment of postpartum uterine bleeding. It enhanced involution and reduced inflammation of uterus after vaginal delivery (Piyachaturawat et al., 1995). Interestingly, *C*. *comosa* and its diarylheptanoid component were reported to have anti-oxidant and anti-inflammatory properties (Jantaranotai et al., 2006; Suksamrarn et al., 2008). Its anti-inflammatory effect is proved by the evidence of reduction in release of pro-inflammatory cytokines, tumor necrosis factor α (TNF-α) and interleukin-1β from monocytoid U937 cell line in phorbol-12-myristate-13-acetate (PMA)-stimulated PBMC. The two *C*. *comosa* diarylhepatanoids were mentioned to reduce the expression of TNF-α and suppress expression of IκB kinase and activation of nuclear factor kappa B (Sodsai et al., 2007). Moreover, the ethanol extract of *C*. *comosa* exhibited effective protection against cisplatin-induced nephrotoxicity through its antioxidant activity by exhibiting radical scavenging activities, such as lipid peroxidation, glutathione (GSH) content, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities (Jariyawat et al., 2009). Thus, this study was intended to investigate the ameliorative effects of ethanol extract of *C*. *comosa* on cisplatin-induced nephrotoxicity in mice for the possibility of usage as a pretreatment regimen in the future.

MATERIALS AND METHODS

Ethanol extract of *C. comosa* and chemicals

The rhizomes of *C*. *comosa* were sliced, dried at 50 to 60°C, pulverized and extracted in a Soxhlet extraction apparatus with hexane first and then with ethanol. The *C*. *comosa* ethanol extract was dissolved in 10% dimethyl sulfoxide (DMSO) and suspended in olive oil later. The two main substances extracted are 7-(4-hydroxyphenyl)-1-phenyl-(1E)-1-hepten-3-ol and (3S)-7-(3, 4-dihydroxyphenyl)-1-phenyl-(1E)-1-hepten-3-ol. All chemicals and solvents used throughout this investigation were of analytical grade. Cisplatin was purchased from Pharmacia (Perth) Pty limited (WA, Australia) and dissolved in saline to give a 1 mg/ml solution.

Animal and experimental design

Male ICR mice (8 weeks old, 25 to 30 g) were obtained from the National Animal Center of Thailand, Mahidol University, Salaya Campus, Nakornpathom, Thailand. They were given a standard laboratory diet and water *ad libitum,* maintained in room with controlled temperature (25 ± 2°C), humidity 65% and a 12-h dark/light cycle, and allowed to acclimatize for 1 week before use. All experimental mice were performed in accordance with the guidelines of National Laboratory Animal Center, Mahidol University, Bangkok, Thailand.

Forty mice were randomly assigned into 4 groups of 10 animals each: solvent control group, Cisplatin group (12.5 mg/kg body weight (BW), intra-peritoneal or i.p.), *C*. *comosa*+cisplatin group (pretreatment with *C*. *comosa* extract at dose 200 mg/kg BW orally for 4 consecutive days before cisplatin injection), and *C*. *comosa* group. Five days after kidney removal in all mice, unfixed cryostat sections (6 µm thick) were prepared for cyclooxygenase-2 or COX-2 staining. The renal tissues were then collected and fixed in 4% neutral buffered paraformaldehyde for evaluation under light microscopy (LM) and 2.5% glutaraldehyde for evaluation under transmission electron microscopy (EM). The project was submitted and approved by the Siriraj Animal Care and Use Committee (SI-ACUC), Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Histopathological preparation

Light microscopy

The mouse kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, cross-sectioned (4-µm thickness), and stained with hematoxylin-eosin. The histopathological analyses were performed blindly under a light microscope (LM). The renal sections graded by semi-quantitative scale to evaluate the degree of tubular changes. The study of changes was limited to the area of proximal tubules, S1, S2 and S3. These parameters were evaluated under a 5-point scale: 0 = normal; 1 = minimal proximal tubular cells swelling; 2 = moderate proximal tubular cells swelling with cast in tubular lumen; 3= maximal proximal tubular cells swelling with cast in lumen, early necrosis and apoptosis of cells; 4 = necrosis, apoptosis and sloughing of proximal tubular cells. The mean score for each group was calculated. Statistical significance was assessed by the two-sided Student's *t* test for independent samples, and was indicated if

the *P*-value was 0.05 or less.

Transmission electron microscopy (TEM)

Upon animal sacrificing, renal samples were dissected into 1-mm cube tissue, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 1 to 2 h, rinsed with the buffer, post-fixed in 1% OsO_4 for 1 h, then rinsing again with the buffer and post-fix in 2% Uranyl acetate aqueous solution for 30 min. Then the specimens were rinsed in distilled water, dehydrated in a graded series of alcohols, treated with propylene oxide two times for 20 min each, immersed in the mixture of propylene oxide and araldite plastic (2:1) for 60 min and then immersed again in the mixture of propylene oxide and araldite plastic (1:2) for overnight, lastly embedded in araldite plastic, and overnight incubated. Ultrathin sections then were cut and mounted on the copper grids, washed in distilled water, viewed and photographed under transmission electron microscope.

Immuno-histochemistry cyclooxygenase staining

Fresh renal specimens were cut with cryostat at 4 µm thicknesses, air-dried and fixed in 0.1% formalin for 5 min. The sections were fixed in 95% ethanol for 20 min; and incubated with 3% H_2O_2 in ethanol for 5 min in order to inactivate endogenous peroxides. Non-specific antibody binding sites were locked by using 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) pH 7.4. The tissue sections were incubated in 1:200 rabbit polyclonal antibody to human cyclooxygenase-2 (COX-2) as primary solution in a humidified chamber at 4°C overnight, washed with PBS and then incubated for 1 h with peroxidase labeled polymer as secondary solution (1:1 Envision+/ HRP anti-rabbit antibody; Dako Laboratories), washed again with PBS and re-incubated in a solution of 0.1 M 3,3'-diaminobenzidine (DAB) in 0.05 M TBS with 0.5 ml 3% H_2O_2 DAB solution (Dako Laboratories) for 5 min. The slides were then counterstained with hematoxylin. The expression of COX-2 was stained in brownish color. The analysis of immuno-histochemical staining method was assessed by light microscopy and analyzed with four sections from each tissue block. The COX-2 staining was examined by one double blinded observer who was blinded to the origin of the sections. The expression of COX-2 was evaluated according to the intensity in semi-quantitative grading; 0=negative staining, 1= minimal, 2= mild, 3= moderated, 4= strong-positive staining. Images were captured at 400× magnification and the entire outer stripes of medulla were measured. Total immuno-stained (brown) cells were averaged and expressed as the mean of intensity of stained area per field on 20 microscopic fields, each responding to an area of 0.042 mm^2, mean score for each group was calculated.

Statistical analysis

Data are reported as the mean ± standard error of mean (SEM). Statistical different between groups were analyzed using one-way analysis of variance (ANOVA) and followed by Bonferroni test. Student's unpaired *t*-test was used for the evaluation of scores of renal damage between two groups. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Histopathological changes of renal tissues

The renal tissues of mice in control group (Figure 1A and E) and those given *C*. *comosa* (Figure 1 and H) showed no structural change in renal cortex and medulla. The renal tissues of mice induced by cisplatin revealed damages mainly at proximal tubular with widespread lesions to the outer stripe of outer medulla (S3 segment) and medullary ray of cortex. The affected proximal tubules displayed dilated lumens and large amount of necrotic epithelial cells, characterized by loss of brush border. Along with the aforementioned necrotic process, apoptosis was significantly noted as evidenced by diminished cell size with small amount of eosinophilic cytoplasm, condensed nuclear chromatin and karyorrhexis (Figure 1B and F). Comparing to cisplatin-given alone group, the renal tissues of those with *C*. *comosa*-pretreatment, showed positive improvement in the histopathology predominantly at proximal tubules in outer stripe of outer medulla (S3 segment) and medullary ray in cortex. Necrosis and apoptosis are noted less. The morphological change of this group showed only minimal cell swelling and few proximal tubular casts. The regeneration of the proximal tubular cells and lining epithelial cells, especially the S3 segment are also marked. The proximal tubular cells in this group are low cuboid-shaped cell with basophilic granular cytoplasm and large vesicular nuclei with numerous mitotic figures (Figure 1C and G). The descriptive and semi-quantitative grading of the structural change in each group is summarized in Table 1. The result reveals that higher score is significantly found in those with cisplatin-given alone comparing to those control group, *C*. *comosa*-given alone group, respectively (2.90 ± 0.10 vs. 0 vs. 0). On the other hand, score in *C*. *comosa* pretreatment with cisplatin treated group manifests markedly low in comparison to the group with cisplatin-given without *C*. *comosa* pretreatment (1.40 ± 0.16 vs. 2.90 ± 0.10).

Ultra-structural changes of renal tissues

Transmission electron microscopy (TEM) was used as a method to evaluate intracellular structural change in proximal tubular cells of kidney tissues from each group in this study. The tubular cells of cisplatin-given without *C*. *comosa*-pretreatment group showed typical apoptotic nucleus and early features of apoptosis, including cell shrinkage, nuclear chromatin clumping with accumulation at nuclear rim. Also, mitochondria were marked to be decreased in numbers; and differed in shape into wavy appearance with irregular or ruptured membrane. The lysosomes in this group were found to be in extremely electron-dense formations. The endoplasmic reticulums were also broken down into small vesicles. Most of the proximal cells showed stunt and expanded microvilli, and discontinuous plasma membrane (Figure 2B). Comparing to those cisplatin alone group, the severity of morphological damage of proximal tubules in the *C*. *comosa* pretreatment group was found to be less with evidence of decreased number of apoptotic cell (Figure 2C).

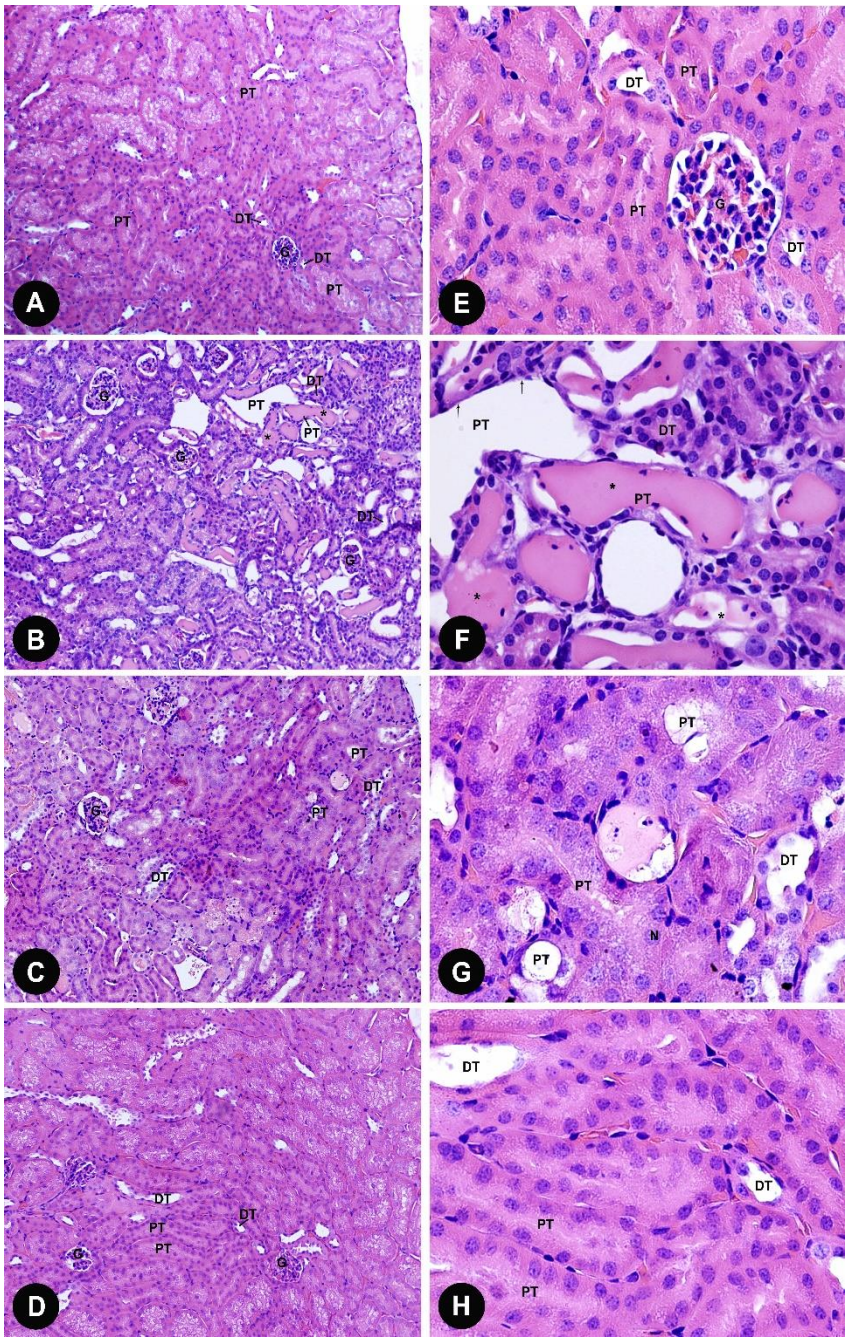

Figure 1. Light micrographs stained with H&E and the summary of semi-quantitative lesions after cisplatin administration, the extensive histological damages including tubular dilatation, cast formation (*), necrosis, apoptosis, loss of brush border and sloughing of proximal tubular cells (arrows) were observed in the cisplatin group (B, F). However, these changes were less pronounced in *C. comosa* pre-treated group (C, G). Normal morphology of the proximal tubules was observed in control (A, E) and *C. comosa* control (D, H). The cisplatin-treated group revealed a significantly higher score versus controls. However, the pretreatment of *C. comosa* significantly lowered the score level compared with cisplatin treatment.

Cyclooxygenase-2 expression

COX-2 is an inducible enzyme which is usually undetectable in most normal tissues, unless during inflammation process. Five days after administration of cisplatin, COX-2 -positive cells were found in dark brown

Table 1. Descriptive and semi-quantitative grading of the structural change in each group.

Animal groups	n	Histopathological classification					Average score
		0	1	2	3	4	
Control	10	10	0	0	0	0	0.00 ± 0.00
Cisplatin	10	0	1	2	7	0	2.90 ± 0.10[a]
Cisplatin + *C. comosa*	10	3	6	1	0	0	1.40 ± 0.16[a,b]
C. comosa	10	10	0	0	0	0	0.00 ± 0.00

The level of tubular damages was scored at 5 levels; 0 = normal kidney; 1 = minimal proximal tubular cells swelling; 2 = moderate proximal tubular cells swelling with cast in tubular lumen; 3= maximal proximal tubular cells swelling with cast in lumen, early necrosis and apoptosis of cells; 4 = necrosis, apoptosis and sloughing of proximal tubular cells. Data are mean ± SE, n = number of mice. [a]*P*<0.05 versus control gr., [b]*P*< 0.05 versus cisplatin gr. (DT, distal tubule; G, glomerulus, PT, proximal tubule; A-D, x100; E-H, x400).

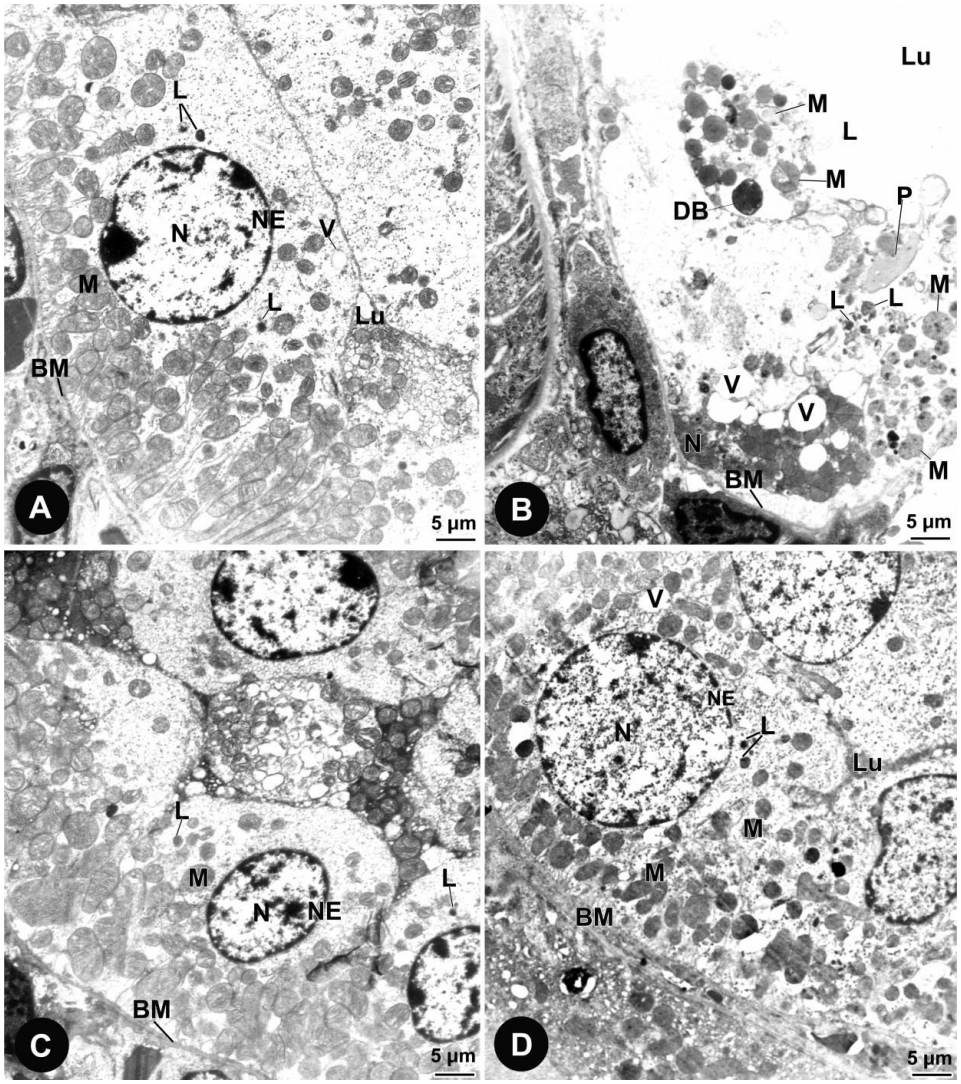

Figure 2. Transmission electron micrographs (TEMs) comparing part of proximal tubular cells of kidneys from various groups, apoptosis as evidenced by diminished cell sizes with condensed cytoplasm and chromatin, and loss of their brush border. The condensed chromosome accumulating at the nuclear rim and relatively intact cell organelles were observed in the cisplatin group (B). However, the tubular cell damage was reduced by pre-treatment with *C. comosa* (C) and normal tubular cells were observed in intact control (A) and *C. comosa* control (D) (DB, dense body; BM, basement membrane; L, lysosome; Lu, lumen; M, mitochondria; N, nucleus; NE, nuclear envelope; x3600).

color as peroxidase stain on the tissue sections. Accumulations of COX-2 expressed mainly at nuclear membrane, circumferential perinuclear cytoplasm, and diffuse cytoplasm (Figure 2B). The COX-2 expression was observed predominantly in the proximal tubules of outer stripes of outer medullas (S3 segment) and medullary rays. However, some parts in medullary rays were damaged beyond the inflammatory process into necrosis and apoptosis; whereas they expressed less staining. Comparing to control group, the COX-2 expression in cisplatin given alone group was significantly increased with mean score of 2.67 ± 0.37. The area involved mainly at the S3 segment of proximal tubules in the outer stripes of outer medullas (Figure 3). These changes were found to be in correlation with lesions found in histopathological and ultrastructural views.

With induction of *C*. *comosa* before cisplatin, the intense of COX-2 staining is profoundly decreased in the proximal tubules of the outer stripes of outer medullas and cortical medullary rays. The COX-2 expression is manifested in the same pattern as those cisplatin-given groups, but only in relatively small and scattered groups of cells. On immunohistochemistry detection, those with *C*. *comosa* pretreatment at dosage of 200 mg/kg showed marked decreased of COX-2 staining as compared to those of cisplatin alone group at the mean score of 1.32 ± 0.39 versus 2.67 ± 0.37 (Figure 3).

Thus, this study demonstrates the evidences of less inflammation process both in histopathology and immunohistochemistry aspects in pretreatment with *C*. *comosa,* in order to prevent nephrotoxicity in ciplatin given subjects.

DISCUSSION

Cisplatin has been chosen as a key drug to combat against various types of malignancies for decades. However, one third of the total numbers of patients are still suffering from the renal injury despites various pretreatment protocols (Prasaja et al., 2015). The prove of better outcome from *C. comosa* in this study will facilitate its novel use in standard practice.

In this study, we look into inflammatory cytokine COX-2 level and ultra-structural changes for evaluation of kidney injury. The outcome displays improvement of renal tissue in term of decreased pathologic changes, less tubular necrosis and apoptosis and reduction of expression levels of COX-2 evidently at proximal tubules in the outer stripes of outer medullas and cortical medullary rays in group with *C. comosa* pretreatment before cisplatin induction. Thus, *C. comosa* is proved in this study that it can be considered as a protective substance for cisplatin-induced nephrotoxicity. The mechanism of injury is caused by unbound free-form of cisplatin that filtered freely through glomeruli and uptake in tubular cells by various organic transporters. The concentration of the substance is highest at proximal tubular, followed by inner medullae and outer cortices orderly (Kuhlmann et al., 1998). Once cisplatin is collected inside the cell, it causes damages by inducing reactive oxygen species which trigger program cell death or apoptosis through both intrinsic and extrinsic pathways (Ozben, 2007; Martindale and Holbrook, 2002). Besides, the oxidative stress injury directly to DNA. COX-2 expression is used to monitor inflammation markers in this study because of its ability to maintain in low level in baseline and elevated with cisplatin induction. Also, pathological changes are found to be correlated with area of high COX-2 expression (Jia et al., 2010). According to Jariyawat et al. (2009), the effect of free radical scavenger activities (EC_{50}) of diarylheptanoids extracted from ethanol extracts of *C.comosa*, 7-(4-hydroxyphenyl)-1-phenyl-(1E)-1-hepten-3-ol and (3S)-7-(3,4-dihydroxyphenyl)-1-phenyl-(1E)-1-hepten-3-ol are 134.32 ± 7.14 and 6.14 ± 0.05 µg/ml, respectively. The ethanol extracts of *C. comosa* in this experiment has marked ability to counteract inflammation with EC_{50} 21.32 ± 0.40 µg/ml, vitamin C only has EC_{50} of 4.58 ± 0.18 µg/ml. The damaged area showing under LM, TEM correlates with the straining of COX-2. Thus, it is clear that the substance has direct effect of anti-oxidation process on kidney protection, however, the exact mechanism is sophisticated to be explained by one phenomenon.

In conclusion, this study exhibits convincing evidences in inflammation and morphological alterations, indicating benefit of *C. comosa* extract usage for renal protection through its effect of anti-inflammation and regeneration of tubular cells. Therefore, this extract could be a potential solution in the combination chemotherapy regimen with cisplatin to prevent from its kidney complication.

Conflict of Interests

The authors have not declared any conflict of interests.

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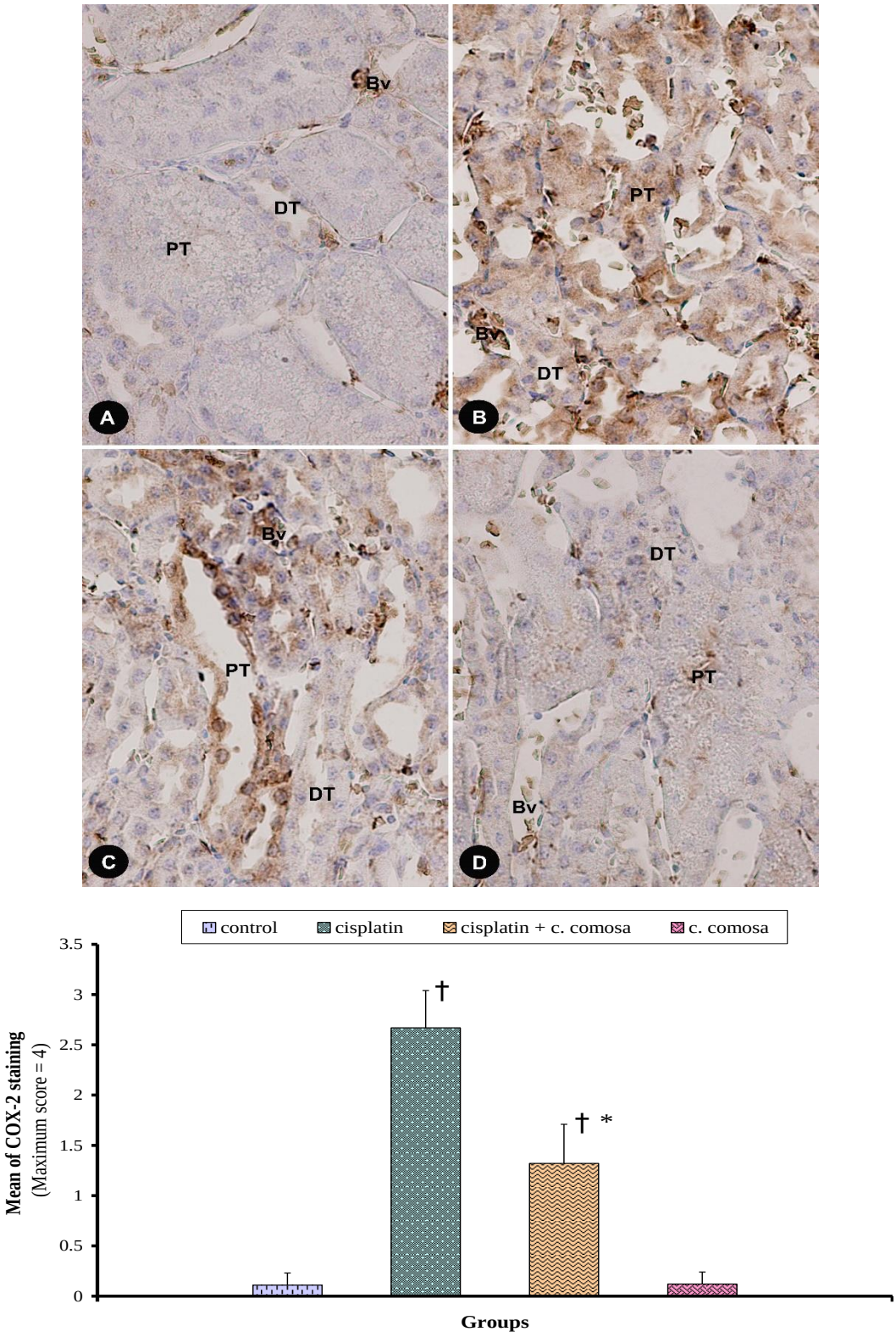

Figure 3. Higher magnification of the outer stripe of outer medulla of kidney, in cisplatin kidney (B), the intense of COX-2 expression were noted at proximal tubular cells (PT) as compared to control (A) and *C. comosa* groups (D). However, pretreatment with *C. comosa* (C) **s**howed markedly decreased stained at proximal tubular cells**.** The semi-quantitative evaluation of COX-2 immuno-staining (E), the extent of COX-2 expression was graded on area field 0.042 mm^2 in 4×20 fields per slide of outer stripe of outer medulla. Data represent mean ± SEM from all experimental groups ($^{\dagger}P$<0.05 vs. control; *P< 0.05 vs. cisplatin; DT, distal tubule; Bv, blood vessel; ×400).

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