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

Juliane Pereira da Silva[1], Jamylle Nunes de Souza Ferro[1], Benisio Ferreira da Silva Filho[1], Luiz Antônio Ferreira da Silva[1], Tayhana Priscila Medeiros Souza[1], Heloisa de Carvalho Matos[1], Vinicius de Frias Carvalho[2], Renato Santos Rodarte[1] and Emiliano Barreto[1]*

[1]Institute of Biological Sciences and Health, Federal University of Alagoas, Maceio-AL, 57010-020, Brazil.
[2]Laboratory of Inflammation, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro-RJ, 21045-900, Brazil.

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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-

*Corresponding author. E-mail: emilianobarreto@icbs.ufal.br Tel: +55 82 3214 1704.

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).

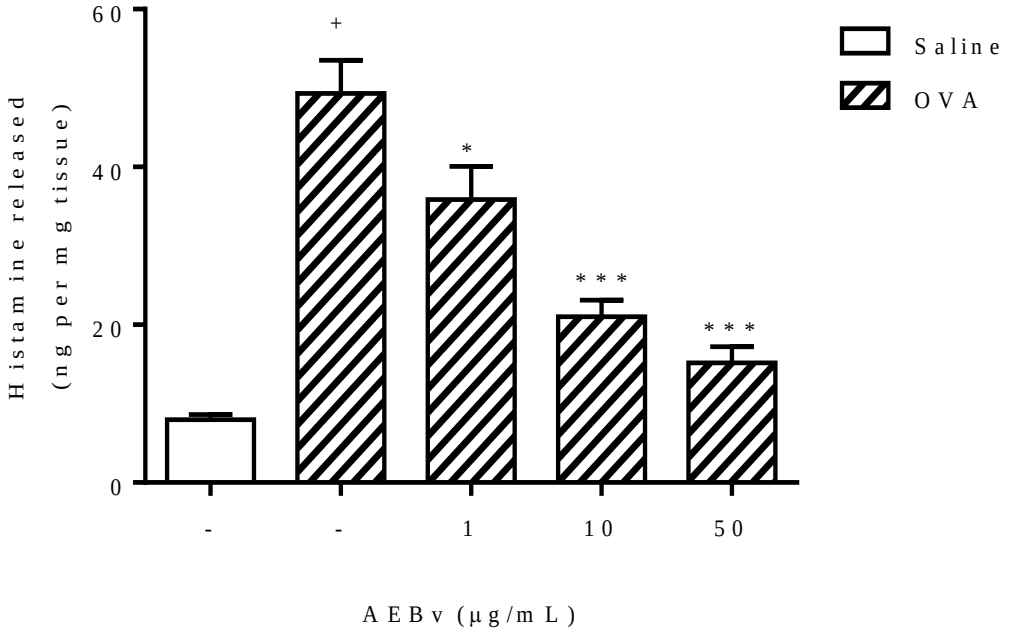
Phytochemical constituent	Results
Flavonoids	++
Condensed tannins	+++
Triterpenes	-
Saponins	++
Alkaloids	-
Steroids	+

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.

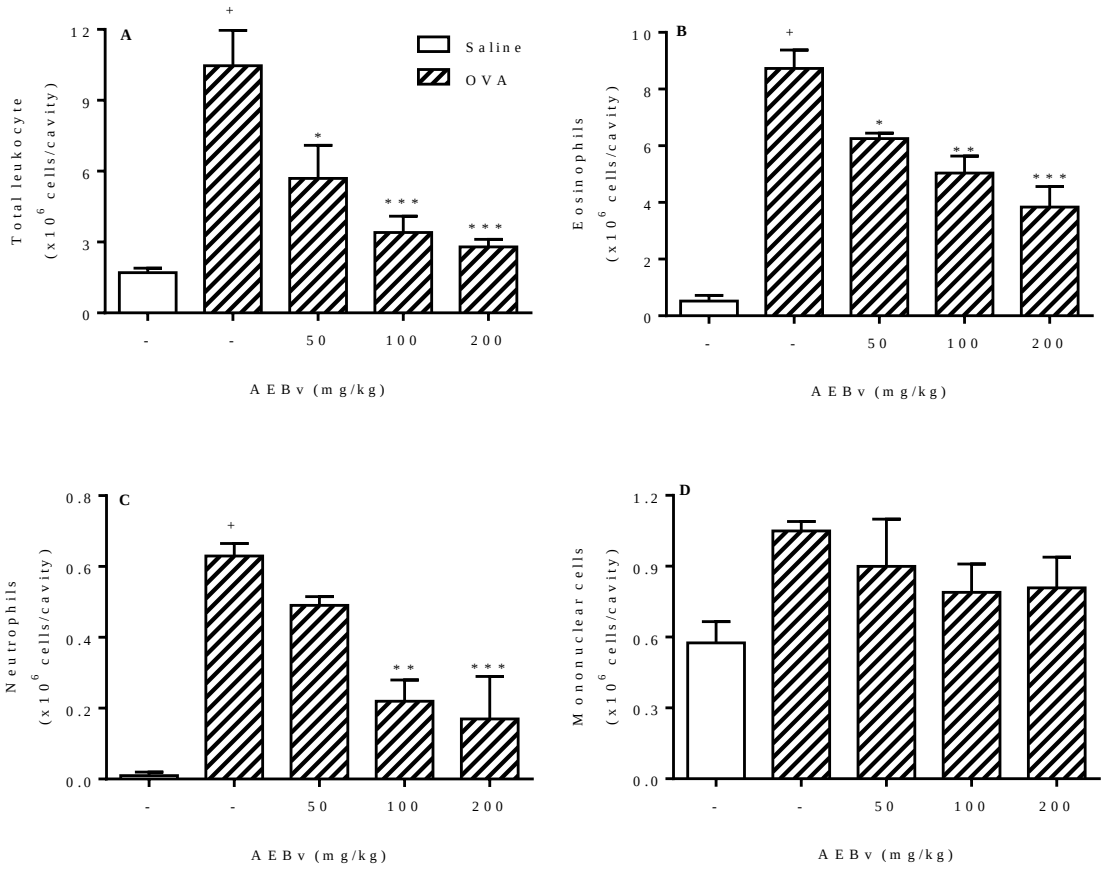


Figure 2. Inhibition of allergic pleurisy by oral pretreatment with the aqueous extract of *B. vigilioides* (AEBv). Pleurisy was induced in 14 day-sensitized mice by ovalbumin challenge (OVA: 12 µg/cavity). Total leukocyte (A), eosinophil (B), neutrophil (C) and mononuclear cell (D) counts were performed 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

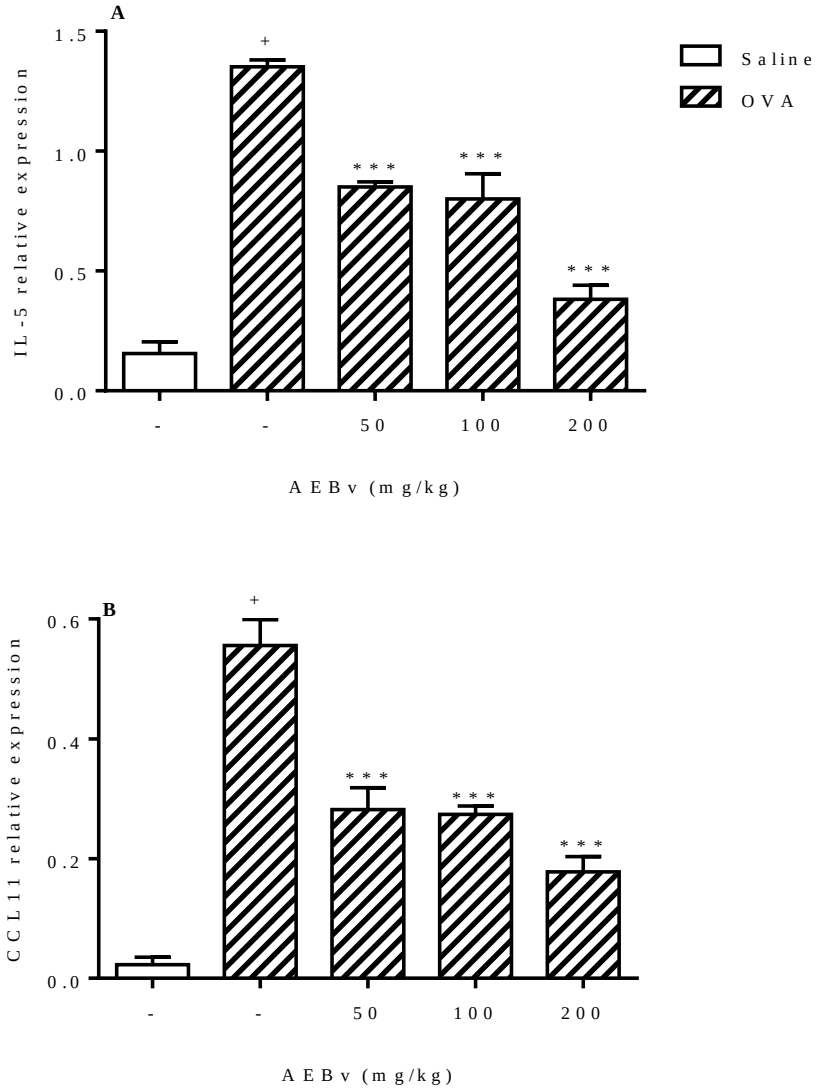


Figure 3. Effect of AEBv on the IL-5 (A) and CCL11 (B) mRNA expression in cells from pleural effluent after allergen-challenge. Total mRNA was extracted, and relative IL-5 and CCL11 mRNA amount were measured by real-time PCR. mRNA levels were expressed as fold changes over the basal after normalizing 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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