



Warifteine, a bisbenzylisoquinoline alkaloid, decreases immediate allergic and thermal hyperalgesic reactions in sensitized animals

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Abstract

Warifteine is a bisbenzylisoquinoline alkaloid isolated from the *Cissampelos sympodialis* Eichl (Menispermaceae). This plant is used in the folk medicine for the treatment of airway respiratory diseases. A murine model of immediate allergic reaction was used to evaluate warifteine treatment in the IgE production, leukocyte activation, thermal hyperalgesia, mast cell degranulation and scratching behavior. BALB/c mice treated with warifteine (0.4–10 mg/Kg) 1 h before OVA sensitization reduced OVA induced paw edema as well as the OVA-specific IgE serum titers as compared with non-treated and OVA-sensitized animals. Warifteine also reduced the mice death evoked by IgE-dependent anaphylactic shock reaction at 30 min after intravenous OVA challenge. To assess the effect of warifteine treatment on Tcell proliferative response, spleen cells from warifteine treated or non-treated and OVA-sensitized animals were evaluated. Spleen cells from warifteine treated animals (2.0 mg/kg) did not proliferate following OVA stimulation as compared with spleen cell cultures from non-treated animals. This response may be related with the increase of NO production as observed in peritoneal macrophage cultures treated with warifteine. Thermal hyperalgesia evoked by IgE or histamine/5-hydroxytryptamine challenge was inhibited on rats at dose of 4.0 mg/kg. Warifteine treatment (0.6 or 6.0 µg/ml) also decreased the IgE α DNP-BSA sensitized mast degranulation after DNP-BSA challenge measured by histamine release. In addition, compound 48/80-induced scratching behavior was also sensitive to warifteine treatment. These results demonstrate for the first time that warifteine treatment reduced the allergy-associated responses. © 2007 Elsevier B.V. All rights reserved.

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1. Introduction

Warifteine is a bisbenzylisoquinoline alkaloid isolated from the root bark and the leaves of *Cissampelos sympodialis* Eichl (Menispermaceae) [1,2]. This plant is used in the folk medicine for the treatment of rheumatism, cold, asthma, among other conditions [3]. The genus *Cissampelos* is rich in bisbenzylisoquinoline alkaloids and several studies have been shown that alkaloids have various activities such as induce apoptosis [4], inhibit macrophage NF κ B signals [5] and cellular proliferative response [6], suppress cellular immune responses [7] and showed a local anaesthetic activity [8] and anti-inflammatory effects [9].

In vitro studies demonstrated that warifteine induced muscle relaxation by inhibiting calcium channels and modifying the intracellular calcium stores sensitive to noradrenaline [1]. In addition, it was demonstrated the spasmolytic action of the alkaloid in guinea pig ileum contractions induced by histamine, carbachol or bradykinin [10].

Immunopharmacological investigations demonstrated that warifteine inhibited eosinophil recruitment, eotaxin and cisteinyl leucotriene productions into the pleura cavity and lungs of allergic mice [11]. These data highlights the role of warifteine as a potential anti-allergic molecule.

Immediate allergic responses are a health public problem mainly in industrialized countries. Allergens activate a subtype of T helper lymphocytes 2 (LTh2) which are responsible to mediate allergic reactions leading to B cell activation and immunoglobulin E (Ig) production [12]. Indeed, allergens are able to induce IgE production in genetically predisposed subjects [13]. IgE-dependent allergic reactions occur after cross-linking between allergen and high affinity IgE receptors in mast cell surface [14]. The mast cell exocytose delivers an array of inflammatory mediators on which histamine and leukotrienes have a potent effect on blood vessels and smooth muscle contraction [15,16]. The major sources of mast cells are the skin, gastrointestinal tract and lung, what may explain the clinical symptoms of immediate allergic reactions such as dermatitis, food allergy and asthma [17].

Additionally, pro-inflammatory mediators such as histamine and 5-hydroxytryptamine (5-HT) from mast cells lead to inflammation within minutes causing tissue damage, nerve injury and pain [18,19].

In this study we hypothesized that the oral warifteine treatment would modulate the allergic response in a murine model. Therefore we evaluated the effect of warifteine in IgE-dependent mechanisms, OVA-specific cell proliferation and NO production in peritoneal macrophages. Indeed, nociceptive response was observed as thermal hyperalgesia evoked by immunological and pharmacological challenges, histamine release from sensitized mast cells and the scratching behavior. These biological parameters are major symptoms observed in allergic diseases.

2. Materials and methods

2.1. Animals

Male or female BALB/c mice (25–30 g) and Wistar rats (250–300 g) were used throughout the study. The animals were supplied by the Animal Facility from the Laboratório de Tecnologia Farmacêutica

(Universidade Federal da Paraíba) and Oswaldo Cruz Foundation breeding unit. Experimental animals were maintained with water and food ad libitum in a room temperature ranging from 22 to 24 °C and a 12 h light/dark cycle. Groups of 5 to 10 animals were used in each test. All experiments were carried out with strict adherence to ethical guidelines [20] and approved by the Ethical Committee for Experimental Animal, Laboratório de Tecnologia Farmacêutica (CEPA Nº 0602/05) and by the Oswaldo Cruz Animal Welfare Committee.

2.2. Warifteine purification

Leaves from *C. sympodialis* Eichl (Menispermaceae) were obtained from the Botanical Garden of the Laboratório de Tecnologia Farmacêutica/ Universidade Federal da Paraíba (voucher specimen Agra 1456). The leaves were dried at 50 °C in an oven and pulverized. The powder was extracted with 70% ethanol in water at 70 °C for 5 days. The plant hydroalcoholic extract was submitted to procedures aimed to isolate the alkaloids, using column and thin layer chromatography (TLC). Briefly, the plant hydroalcoholic extract was dissolved in 3% HCl and extracted several times with CHCl₃. The aqueous fraction was basified with NH₄OH to pH 9 and again extracted with CHCl₃. The CHCl₃ extract was washed with H₂O, dried (MgSO₄) and the solvent evaporated to afford the total tertiary alkaloid fraction (TTA). The TTA was subjected to chromatography column over alumina, eluting with hexane containing increasing amounts of CHCl₃, CHCl₃ with increasing amounts of MeOH and finally with MeOH. The fraction eluted with CHCl₃–MeOH (49:1), after further purification by TLC (1.0 mm layer), yielded the isolation of the bisbenzylisoquinoline alkaloid warifteine (0.031%). The identification of the warifteine was performed by analyzing ¹H and ¹³C NMR spectral data compared with those published in the literature [1]. The warifteine solution was prepared using 1 mg of the crystal in 50 µl of HCl 1 N and 800 µl of distilled water. The pH was adjusted to 7.0 with NaOH 1 N. The volume was completed to 1000 µl and the dilutions were made in distilled water for the *in vivo* assay and in PBS for the *in vitro* assay.

2.3. Warifteine treatment and OVA sensitization

BALB/c mice were treated orally (p.o.) with warifteine (W–0.4, 2.0 or 10 mg/kg) 1 h before the sensitization protocol. On day 0 and 14th the treated mice (*n*=10) were actively sensitized with OVA (grade V Sigma Chemical, St. Louis, MO) by injection (i.p.) of 10 µg OVA adsorbed to 2,25 mg of alum adjuvant. The non-treated OVA-sensitized animals received only distilled water during the OVA sensitization. Non-sensitized group of animals received saline (i.p.) during the period of OVA sensitization. Three weeks after the first OVA injection, animals were exposed to aerosol of 1% (w/v) OVA in saline for 30 min [21].

2.4. Footpad swelling induced by OVA injection

Twenty-one days after initiation of OVA sensitization the warifteine treated, non-treated OVA-sensitized and non-sensitized groups were challenged with 400 µg of OVA in 20 µl of saline in the left footpad. The measurement of the edema was performed before (0 min) and after (30 and 60 min) OVA challenge into the left footpad whereas saline was injected into the right footpad as a control. The edema formation was measured with the aid of a gauge calliper (Mitutoyo).

2.5. Determination of OVA-specific IgE by the PCA test

Briefly, serum IgE titers were determined using the passive cutaneous anaphylaxis (PCA) reaction. Warifteine treated, non-treated OVA-sensitized and non-sensitized groups were bled and the

serum samples were diluted in PBS. Volumes of 50 μ l were inoculated intradermally on the shave backs of Wistar rats at marked sites. After 24 h the rats were challenged intravenously with OVA (2.0 mg) in 1% Evans Blue dye. Thirty minutes later the rats were euthanized and OVA-specific IgE titers were measured. The highest serum dilution giving an up 5 mm diameter flare or bluing reaction was taken as the PCA titer [22].

2.6. Anaphylactic shock reaction

For accomplishment of the anaphylactic shock, 3 days after the sensitization protocol, BALB/c mice were treated with warifteine at doses of 0.4, 2.0 or 10 mg/kg or water. One hour later the animals were challenged intravenously with OVA (500 μ g). The mortality was manually recorded with a chronometer at 30 and 60 min [23].

2.7. Cell preparation and proliferation assay

Spleen cell suspensions from each animal group were prepared and depleted of red blood cells by incubation in Boyle's solution (0.17 M Tris, 0.16 M ammonium chloride, BDH, UK). Cells were washed twice in RPMI 1640 medium by centrifugation at $200\times g$ for 10 min at 8 °C. The obtained pellet was resuspended in RPMI medium without phenol red and supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, 5 μ M β -mercaptoethanol and 10% fetal calf serum (FCS) (Gibco, Pisle, UK). Viable cell number was determined in a hemocytometer by the exclusion of the Trypan blue dye. Triplicate cultures were performed in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA) in a final volume of 200 μ l/well containing 5×10^5 cells. Ovalbumin (Grade V, Sigma) was added at 100 μ g/ml in the cell cultures to stimulate OVA-specific cells. Cultures were then incubated for three days at 37 °C, 5% CO₂. A volume of 100 μ l of the cell culture supernatants was removed, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide—Sigma Chem. Co., St. Louis, MO) was added (5 mg/ml) and the cells were further incubated for 4 h at 37 °C, 5% CO₂ [24]. After this period of time, SDS solution at 10% in HCl 0.01 N was added in each well and the plates were then incubated for 17 h at 37 °C, 5% CO₂ and read at 570 nm on a Microplate Reader (Bio-Rad model 550, Japan).

2.8. Peritoneal macrophage isolation and cell cultures

Mice were elicited by intraperitoneal injection of 1 ml of sodium thioglycolate (2.9%) and 5 days later the animals were euthanized by ether inhalation and the peritoneal macrophages rinsed out by injection of 10 ml of cold PBS plus 3% FCS. The cells were resuspended in RPMI 1640 medium supplemented with 10% FCS and added to 96-well tissue plates (4×10^5 cells/well). Cell cultures were incubated for 3 h at 37 °C in an atmosphere of 5% CO₂. Nonadherent cells were removed by washing with RPMI medium supplemented with FCS as above [25]. Macrophage cultures were stimulated with lipopolysaccharide (LPS, 10 μ g/ml) or warifteine (0.01–1.0 μ g/ml). The polymyxin B (0.5 μ g/ml) was added in the warifteine stimulated culture cells to inhibit any LPS contamination. A non-stimulated culture cells also received polymyxin B. All reagents were purchased from Sigma, St. Louis, MO, USA.

2.9. Nitrite measurement

NO synthesis was measured by a microplate assay that determines the levels of nitrite as previously described [26]. To measure nitrite, 100 μ L of the macrophage culture supernatants were removed and incubated in a microplate with equal volume of the Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride, 5% H₃PO₄) at room temperature for 10 min. The absorbance was read at 570 nm using a Microplate Reader (Bio-Rad

model 550, Japan). Nitrite levels were determined using a sodium nitrite solution as standard.

2.10. Algesimetric assay

Thermal hyperalgesic response was evaluated as previously described [27]. Briefly, male Wistar rats ($n=5$) were sensitized with murine IgE anti-dinitrophenylated-BSA (IgE α DNP-BSA 0.5 μ g/paw) into the left paw while the contra lateral paw was injected with vehicle isotonic saline. After 24 h groups of animals ($n=5$) were treated with warifteine (4.0 mg/kg) or water and 1 h later the animals were challenged with dinitrophenylated-bovine serum albumin (DNP-BSA). A control group of animals did not receive the IgE anti-DNP-BSA. The animals were individually placed on a hot plate (Eddy's Hot Plate) with the temperature adjusted to 51 °C and the withdrawal response latency of each hind paw determined at 15, 60 and 180 min after the allergen challenge. In another set of experiments, naïve rats were treated with warifteine (4.0 mg/kg) or water and 1 h later the animals received intraplantarly a combination of histamine (45 nmol/paw) plus 5-HT (2.6 nmol/paw). An equal volume of vehicle (isotonic saline) was injected into the contra lateral paws for comparison and the withdrawal response latency of each hind paw determined at 15, 60 and 180 min after pharmacological challenge. Isotonic saline was injected into both left and right hind paws (water-saline group). The heat source was maintained at constant intensity, which caused a stable withdrawal latency of approximately 8–10 s in sham-challenged paws. The animals were only tested in one series of measurements, and the typical responses were hind paw shaking and/or lifting. The latency to the response was manually recorded with a chronometer. The experiments were performed in a sound-attenuated and air-conditioned (20–22 °C) room. Hyperalgesia to heat was defined as a decrease in withdrawal latency and calculated as follows: Δ paw withdrawal latency (s) = right paw withdrawal latency – left paw withdrawal latency.

2.11. Peritoneal mast cells isolation and histamine release

Peritoneal mast cells of male Wistar rats were harvested and purified by percoll density gradient centrifugation. Mast cells (1×10^5 /well) were added to 24 well plate and then incubated with IgE anti-DNP (0.5 μ g/ml) for 12 h. Warifteine at 0.06–6.0 μ g/ml was added to each well and the plate was incubated for 1 h. After this period the plate was incubated with DNP-BSA (0.5 μ g/ml) for 1 h. After centrifugation at $170\times g$ for 10 min, the supernatant was collected and stored at –20 °C for histamine fluorimetric quantification, as previously reported [28].

2.12. Induction and evaluation of scratching behavior

An anti-itching property of warifteine was measured using an established model of scratching behavior in mice [29]. The animals had their rostral part of the skin clipped and were intradermally injected with compound 48/80 (10 μ g/20 μ l) or saline (negative controls). Immediately after stimulation, the mice were placed in the observation chamber and their behavior was evaluated for 60 min. Treatment with warifteine (0.4 and 2 mg/kg, sc) or vehicle was performed subcutaneously 1 h before provocation.

2.13. Statistical analyses

Data were analyzed by Student's *t*-test, Fisher's exact test, ANOVA followed by Dunnet using software GraphPad Prism (GraphPad, San Diego, CA). Values given are means $\pm$ S.E.M. A value of $P<0.05$ was considered significant.

3. Results

3.1. Effect of warifteine on the footpad swelling induced by OVA injection

BALB/c mice treated with warifteine (2.0 or 10 mg/kg) showed significant reduction in the paw edema formation at 30 min after the OVA challenge (7.7 ± 5.3 , 7.7 ± 2.0 mm, respectively) as compared with the non-treated OVA-sensitized group (13.6 ± 6.6 mm, $P < 0.05$) (Fig. 1). Furthermore the doses of 0.4, 2.0 or 10 mg/kg inhibited the paw edema formation at 60 min after the OVA challenge (7.3 ± 2.9 , 5.8 ± 3.4 , 6.2 ± 3.3 mm, respectively) as compared with the non-treated OVA-sensitized group (13.8 ± 5.5 mm, $P < 0.05$) (Fig. 1). The results demonstrated that warifteine treatment was able to inhibit the first phase of the immediate hypersensitivity in these animals. The non-sensitized mice did not present paw edema after the OVA challenge.

3.2. Effect of warifteine on OVA-specific-IgE production

In order to investigate the ability of warifteine to modify OVA-induced serum IgE titers the PCA assay was used. OVA sensitization increased the OVA-specific-IgE titer by 1:460 whereas oral administration of warifteine at doses of 0.4, 2.0 or 10 mg/kg during the OVA sensitization decreased the IgE titers by 1:166, 1:116 or 1:102, respectively (Table 1).

3.3. Protective effect of warifteine in anaphylactic shock reaction

Warifteine treated (2.0 or 10 mg/kg) and OVA-sensitized mice were protected (36.6% or 66.6% respectively, $P < 0.05$) by death at 30 min after intravenous OVA challenge in comparison with non-treated OVA-sensitized group. Non protective response was observed at 60 min after the OVA challenge (Table 1).

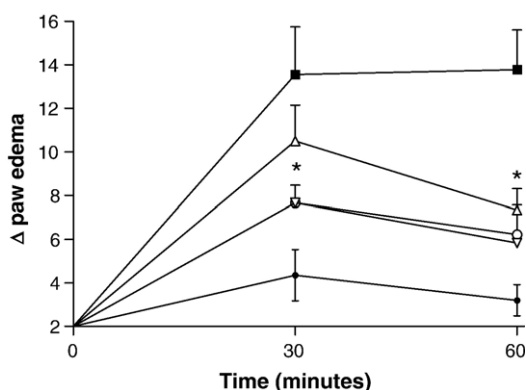


Figure 1 Effect of warifteine on footpad swelling in OVA-sensitized BALB/c mice. Animals ($n=6-9$) treated with warifteine at doses of 0.4 (Δ), 2 (∇) or 10 mg/kg ($\circ$) or water ($\blacksquare$) were analyzed to paw edema formation. Group of animals that did not receive any treatment was determined as control group ($\bullet$). After 30 and 60 min the intraplantar injection of OVA or saline into the left and right footpad, respectively, the edema was measured with the aid of a gauge caliper (Mitutoyo). Mean values $\pm$ S.E.M. * $P < 0.05$ as compared with water treated group (Student's t -test).

Table 1 Anaphylactic shock reaction and the IgE serum titers in warifteine treated and OVA-sensitized mice

Treatments ^a	% of protection –30 min. Alive/Total)	% of protection –60 min. Alive/Total)	OVA-specific IgE serum titer (PCA ^b)
OVA + water	0% (0/12)	0% (0/12)	1:460
OVA + W-0.4	08.3% (1/12)	8.3% (1/12)	1:166*
2.0	36.6% (4/11)*	27.3% (3/11)	1:116*
10	66.6% (7/11)*	27.3% (3/11)	1:102*

^aGroups of mice were treated with a single dose of warifteine (mg/kg) 1 h before OVA challenge (anaphylactic shock reaction) or before OVA sensitization (PCA). Control group received water instead of warifteine during the experimental protocols. After the treatment period, animals were challenged by intravenous OVA injection to evoke anaphylactic shock reaction. The number of living animals was counted after 30 and 60 min of the OVA challenge and the percentage of protection are indicated above. Sera of each animal group were collected to determine IgE titers by PCA test.

* $P < 0.05$ as compared with water treated group (Fisher's exact test).

* $P < 0.05$ as compared with water treated group (Student's t -test).

^bPassive Cutaneous Anaphylaxis Test.

3.4. Effect of warifteine on OVA-specific proliferative response

Spleen cells from warifteine treated (2.0 mg/kg) and OVA-sensitized mice did not proliferate in response to OVA (0.15 ± 0.01 OD, $P < 0.01$) as compared with cells from non-treated and sensitized mice (0.22 ± 0.01 OD). The dose used did not show increase the LDH activity in spleen cell cultures (data not shown). Indeed, either OVA or warifteine did not present changes in enzyme activation indicating no toxic effects. These data suggest that the warifteine treatment may be inhibiting the OVA-specific clones (Table 2).

3.5. Effect of warifteine on the nitric oxide production

The production of nitric oxide from peritoneal macrophages with different concentrations of warifteine (0.01, 0.1 or 1.0 μ g/ml)

Table 2 Effect of warifteine on the cell proliferative response

Animal Groups ^a	No stimuli (OD)	OVA stimuli (OD)
OVA + water	0.18 ± 0.01	$0.22 \pm 0.02^\dagger$
OVA + W 0.4	0.17 ± 0.01	0.19 ± 0.00
2.0	0.15 ± 0.02	0.15 ± 0.01

^aGroups of mice ($n=5-10$) were treated with warifteine at different doses (0.4 or 2.0 mg/kg) or water during the experimental protocol. Pooled spleen cells from each group were either un-stimulated or incubated *in vitro* with OVA (100 μ g/ml). The cell cultures were incubated for 3 days and the cell proliferation was measured by the MTT test. Values are mean of optical density $\pm$ SEM of triplicate cell cultures.

[†] $P < 0.05$, compared with non-stimulated culture cells (ANOVA followed by Dunnett).

OD = Optical density.

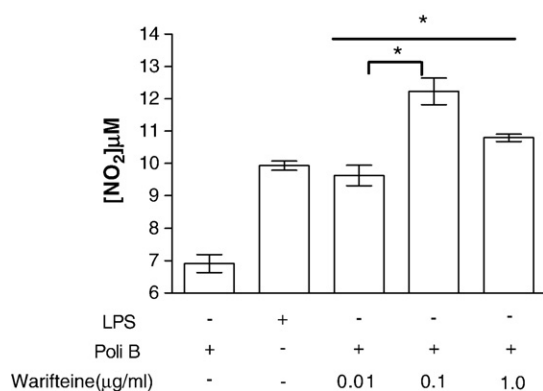


Figure 2 Effect of warifteine on nitrite production from peritoneal macrophages. Cell cultures were stimulated with LPS (10.0 μg/ml), warifteine (plus polymyxin B—Poli B—0.5 μg/ml) at different concentrations or non-stimulated (medium plus polymyxin B) and the nitrite levels were measured by Griess method. Mean values $\pm$ S.E.M analyzed by ANOVA (Dunnet). * $P < 0.05$ as compared with cell cultures in medium.

was increased (9.6 ± 0.3 , 12.2 ± 0.4 , 10.8 ± 0.1 μM respectively) as compared to the non-stimulated culture cells (6.9 ± 0.3 μM, $P < 0.05$). The best effect of warifteine was reached at 0.1 μg/ml similar to LPS effect (Fig. 2). The induction of NO by the warifteine treatment suggests that this alkaloid is one of the plant compounds responsible for its regulatory effect.

3.6. Effect of warifteine on thermal hyperalgesia

Warifteine at dose of 4.0 mg/kg 1 h before the DNP-BSA challenge in Wistar rats previously sensitized with IgEαDNP-BSA (0.5 μg/paw) decreased significantly the paw withdrawal latency at 15, 60 and 180 min (1.74 ± 1.0 , 1.0 ± 0.6 and 1.6 ± 0.5 s, respectively) as compared with the non-treated and IgEαDNP-BSA sensitized animals (6.4 ± 0.5 , 4.6 ± 0.7 , 4.6 ± 0.4 s, respectively, $P < 0.05$) (Fig. 3A). Similar effect of the warifteine was observed in naïve rats injected with histamine plus 5-HT, suggesting a protective role of this alkaloid on thermal hyperalgesia state (Fig. 3B). The group of animals that did not receive the passive sensitization or the mixtures of histamine plus 5-HT did not show hyperalgesic response.

3.7. Effect of warifteine on the histamine release

Warifteine significantly inhibited the DNP-BSA-induced histamine release from isolated rat peritoneal mast cells. Values dropped from 1303 ± 54.9 ng/ml in untreated cells to 1079 ± 17.7 ng/ml (0.6 μg/ml) and 971.5 ± 32.4 ng/ml (6.0 μg/ml) ($P < 0.05$) in warifteine treated cells. Warifteine at 0.06 μg/ml did not inhibit histamine release (Fig. 4).

3.8. Effect of warifteine on scratching behavior induced by compound 48/80

Fig. 5 shows the effect of warifteine on the compound 48/80-induced scratching behavior. The itching caused by the compound 48/80 was inhibited by warifteine treatment at doses of 2.0 and 0.4 mg/kg (98.5 ± 33.3 and 60.4 ± 21.5 , $P < 0.05$ respectively) as compared with non-treated animals (217.4 ± 10.6). Concentrations higher than 2.0 mg/kg induced a scratch-

ing behavior before challenge with compound 48/80 (data not shown).

4. Discussion and conclusion

Development of new drugs to treat allergic reactions has been aimed by an increasing number of laboratories around the world. In this study, we have evaluated the anti-allergic and anti-nociceptive effects of warifteine, an alkaloid isolated from *C. sympodialis*, which is described as a plant with a potential use for allergy treatment [30–32].

Our findings showed that the OVA-sensitized mice led to high levels of specific IgE, and reacted with a marked paw edema and anaphylactic shock reaction after OVA challenge. The warifteine-treatment reduced these responses. These results corroborate with previously data [32,33] that showed that the *C. sympodialis* extract also inhibited the IgE

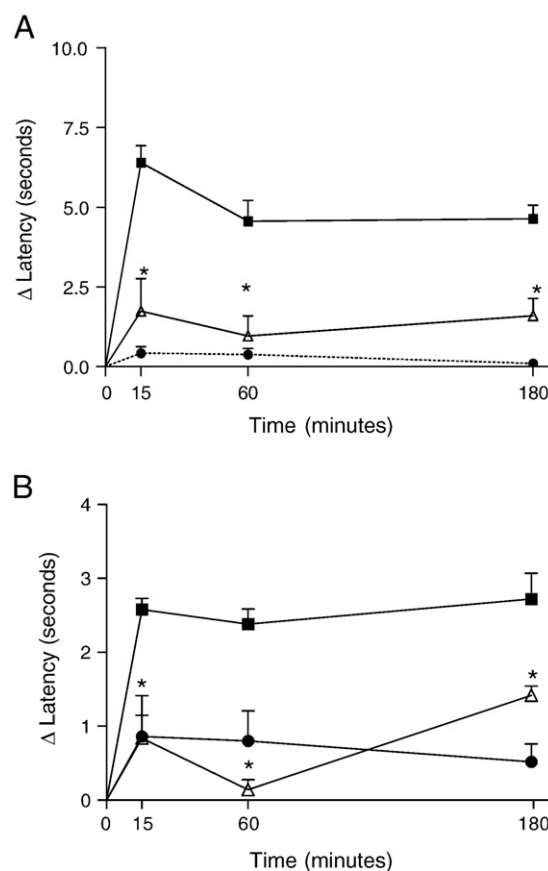


Figure 3 Effect of warifteine on thermal hyperalgesia in Wistar rats. IgEαDNP sensitized animals ($n = 5$) were treated with a single dose of warifteine 4 mg/kg (–Δ–) or vehicle (–■–) one hour before DNP-BSA challenge (A). In another set of experiments, animals were treated with warifteine (–Δ–) or vehicle (–■–) and challenged with a combination of histamine and 5-HT (B). Non sensitized animals subjected to a saline intraplantar provocation were used as a control group (–●–). Values are expressed as mean $\pm$ S.E.M from at least 5 animals. * $P < 0.05$ as compared to the positive control groups (sensitized and DNP-BSA-challenged group, panel A; Histamine + 5-HT challenged group, panel B).

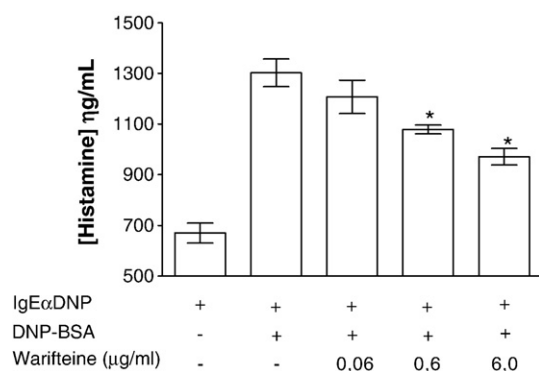


Figure 4 Effect of warifteine on allergen-evoked histamine release. Mast cells isolated from the rat peritoneal cavity were passively sensitized with IgEαDNP monoclonal antibody and treated with warifteine (0.06–6.0 μg/ml) or vehicle 1 h before DNP-BSA challenge. Values are the mean ± S.E.M from at least 4 experiments. * $P < 0.05$ as compared to the control (mast cells that were sensitized and challenged but remained untreated with warifteine).

production and anaphylactic shock reaction in OVA-sensitized BALB/c mice adding support to the interpretation that the alkaloid possesses anti-allergic properties.

Previous pharmacological studies have shown that warifteine relaxed smooth muscle cells by modifying the intracellular calcium stores sensitive to noradrenaline [1]. Calcium is crucial to B cell activation. The warifteine treatment might be reducing calcium stores in OVA-specific B cells and than regulating IgE production. On the other hand, warifteine may be modifying cyclic adenosine monophosphate (cAMP) levels by the inhibition of the phosphodiesterases in human neutrophils [34], which is resulting in the decrease of IgE production. This hypothesis seems also reasonable since the plant extract induced high levels of cAMP not only in anti-IgM stimulated B cell cultures [35] but also in guinea pig trachea strips [2]. The inhibition of the phosphodiesterase enzymes by warifteine may explain the up regulation of cAMP and the IgE inhibition.

The *in vivo* treatment with warifteine inhibited OVA-stimulated spleen cell proliferative responses. The bisbenzylisoquinoline alkaloid tetrandrine regulates macrophage activation resulting in failure of T cell activation and proliferation [6]. In search of a regulatory pathway it was measured the NO level in macrophages stimulated with warifteine. The results demonstrate that the alkaloid increased the level of NO, corroborating the idea that bisbenzylisoquinoline alkaloids can elevate NO levels and modulates the cell proliferation [36].

Of note, inhibitors of inducible NO synthase found in macrophages, clearly showed exacerbated the OVA allergen sensitization in experimental model of allergy [37]. Additionally, there is a causative relationship between synthesis of NO by macrophages and subsequent generation of IFN-γ [38] which is consistent with the findings of IFN-γ production following exposure of BALB/c spleen cells to *C. sympodialis* extract as reported [32]. Previous immunological studies with *C. sympodialis* extract showed induction of interleukin-10 and INF-γ in naive and OVA-sensitized mice [30–32]. These anti-inflammatory and anti-allergic cytokines are known for

their properties in regulate Th2 responses in allergic rhinitis and asthma.

Hyperalgesic response to allergen and vasoactive amines such as histamine and 5-HT was evaluated. Warifteine treatment decreased the paw withdrawal latency in both IgE sensitized and histamine/5HT-challenged rats. These results are in line with the interpretation that the anti-hyperalgesic effect exhibited by warifteine is at least in part accounted for by the blockade of vasoactive amine effect. Since itching is a major feature in allergic diseases, we have further studied the sensitiveness of this response to warifteine using a well established murine model of scratching behavior triggered by the standard mast cell activator compound 48/80 [29]. Our findings demonstrated that such a phenomenon is also clearly sensitive to treatment with warifteine.

Several studies state that mast cells are crucial triggers of allergic inflammatory processes, since once activated they rapidly secrete several pre-stored and neo-generated mediators [14]. We found here that warifteine was capable to down-regulate allergen-evoked histamine release from isolated peritoneal rat mast cells, suggesting that the protective anti-allergic effects raised by warifteine may result, at least in part, from its ability to stabilize mast cells. This finding is comparable to that exhibited by other alkaloids such as fangchinoline and tetrandrine that inhibited the phospholipase-mediated release of arachidonic acid from the cell membrane similar to the glucocorticoid effect [9]. Of note, in a previous study we showed that warifteine inhibited cisteinyl leukotriene production into the pleura cavity and lungs of allergic mice [11]. Since glucocorticoids are the most effective treatments for allergic dysfunctions available so far, the interesting possibility that warifteine may serve as a glucocorticoid sparing agent in allergic conditions is now under investigation.

The data described above highlights the anti-edema, anti-IgE and anti-hyperalgesic properties of warifteine. The anti-hyperalgesic and anti-edema effects of the warifteine suggest a decrease of the inflammatory process by interfering in the NO production and also in the pre-stored and neo-generated mediators. These findings suggest that warifteine may represent a new strategy to improve the therapeutic arsenal available for controlling immediate allergic reactions.

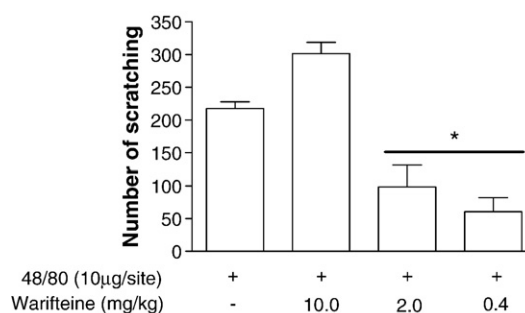


Figure 5 Effect of warifteine on the scratching behavior induced by compound 48/80. BALB/c mice ($n = 5-6$) were treated with warifteine (0.4–10 mg/kg) 1 h before compound 48/80 challenge. Scratching behavior was observed for 60 min after compound 48/80 injection. Values are expressed as mean ± S.E.M from at least 5 animals. * $P < 0.05$ as compared to the positive group (animals subjected to compound 48/80 stimulation only).

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