Increase of cellular recruitment, phagocytosis ability and nitric oxide production induced by hydroalcoholic extract from Chenopodium ambrosioides leaves


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Abstract

The leaves and the oil from the seeds of Chenopodium ambrosioides L. (Chenopodiaceae), a plant known in Brazil as ‘mastruz’, have been used by native people to treat parasitic diseases. Experimentally it was shown that Chenopodium ambrosioides inhibits the Ehrlich tumor growth, what could be due to an immunomodulator effect of this product. The aim of this study was to investigate the effect of hydroalcoholic crude extract (HCE) from leaves of Chenopodium ambrosioides on macrophage activity and on lymphoid organs cellularity. C3H/HePas mice received the HCE (5 mg/kg) by intraperitoneal via and were sacrificed 2 days later. HCE treatment did not alter the cell number in bone marrow, but it increased the cell number in peritoneal cavity, spleen and lymph node. The spreading and phagocytosis activity, the PMA-induced hydrogen peroxide (H2O2) release and the nitric oxide (NO) production were also increased when compared to control group. Similar results were obtained with concanavalin A (Con A), used as a positive control, with exception of the NO production that was only detected in HCE-derived macrophages. The in vitro treatment with HCE induced a dose-dependent NO production by resident macrophages, but did not enhance the NO production by HCE-derived macrophage, which however, was enhanced by Con A, suggesting that HCE and Con A induce NO production by different routes. In conclusion, HCE-treatment was able to increase the macrophages activity and also the cellular recruitment to secondary lymphoid organs, what could explain the previously related anti-tumor activity of Chenopodium ambrosioides.

Keywords: Chenopodium ambrosioides; Chenopodiaceae; Macrophage activity; Nitric oxide; Mastruz; Lymphoid organs

1. Introduction

Chenopodium ambrosioides L. (Chenopodiaceae) is an herbaceous shrub known in Brazil as ‘mastruz’ or ‘erva-de-Santa-Maria’ and as American wormseed, goosefoot, ‘epazote’ and ‘paico’ in other countries of America. Infusions and decoctions of the leaves, roots and inflorescences of Chenopodium ambrosioides have been used for centuries by native people as dietary condiments and as traditional medicine. According to literature, a decoction prepared with leaves and occasionally with flowers of Chenopodium ambrosioides and taken orally is used for dysmenorrhea and menses regulation and disorders. The leaves and flowers of Chenopodium ambrosioides are also used as a poultice to help ‘dissolve’ fibroids and to treat uterine hemorrhaging (Ososki et al., 2002).

Chenopodium ambrosioides has also been used to treat colds, as hemostatic, vermifuge and anthelmintic (Filipov, 1994). In Brazil, the leaves of Chenopodium ambrosioides have been
used to treat skin ulcerations caused by the *Leishmania* species (Franca et al., 1996; Moreira et al., 2002).

It was previously shown that the crude extract from *Chenopodium ambrosioides* is a strong stimulator of the murine lymphocytes (Rossi-Bergmann et al., 1997). Besides this *Chenopodium ambrosioides* is rich in flavonoids and terpenoids compounds that have diverse pharmacological properties including antioxidant and cancer chemopreventive effects (Di Carlo et al., 1999; Kiuchi et al., 2002; Liu, 2004). In fact, we have shown previously that the treatment of mice with *Chenopodium ambrosioides* reduced the growth of Ehrlich tumor (Nascimento et al., 2006a) however the immunological mechanism involved in this effect remains unclear.

One of the putative means to control the Ehrlich tumor growth is by increasing the macrophage activity (Kleeb et al., 1997; Pinello et al., 2006). Macrophages are central to cell-mediated and humoral immunity and they have an important role in the immune system as part of the host defense mechanism. These cells are present in different tissues as resident macrophages, which have moderate ability to secrete metabolites. Various agents, including interferon-γ (IFN-γ), lipopolysaccharide (LPS), or other microbial products, lectins and vegetal extracts are known to stimulate the macrophages (Fujihara et al., 2003; Ma et al., 2003; Nascimento et al., 2006b). The activated macrophages can inhibit the growth of a variety of tumor cells and microorganisms (Nascimento et al., 1998, 2002) due to the increase in the spreading and phagocytosis ability, and mainly, to the increase in the production of nitric oxide (NO), hydrogen peroxide (H$_2$O$_2$) and cytokines.

Despite the empiric and experimental evidences that *Chenopodium ambrosioides* extracts can improve the immunological responses, as far as we know, there are no evidences that *Chenopodium ambrosioides* can stimulate macrophage functions and cellular recruitment to lymphoid organs. Therefore, in this study we investigated the role of *Chenopodium ambrosioides* hydroalcoholic crude extract treatment on murine peritoneal macrophage activation by evaluating the peritoneal cellular influx, the spreading and phagocytosis activity, the H$_2$O$_2$ release and the NO production and also on lymphoid organs cellularity.

2. Material and methods

2.1. Mice

Male C3H/HePas mice (10/group), 8–12-week old, weighing 20–25 g have been maintained for many generations in the Animal Breeding Unit (Biotério Central da Universidade Federal do Maranhão, São Luís, MA, Brazil) under standard conditions. The animals were kept in well cross-ventilated room at 26 ± 2 °C, relative humidity 44–56%, light and dark cycles of 12 h. The animals had free access to sterilized food and acidified water. All procedures described were reviewed and approved by the Animal Ethics Committee in accordance with COBEA (Brazilian College of Animal Experimentation).

2.2. Plant material

Leaves of *Chenopodium ambrosioides* L. (Chenopodiaceae) were collected and identified at the Árico Seabra Herbarium of the Universidade Federal do Maranhão (São Luís, MA, Brazil) (voucher specimen no. 0998). The leaves were dried at 30 °C and powdered. Dry powdered leaves (200 g) were extracted with 1 L of ethanol (70%) and mixed each 8 h during 24 h. After this period the hydroalcoholic extract was filtered using a cotton funnel and the same procedure was repeated four times. After this process the hydroalcoholic crude extract (HCE) was concentrated under low pressure. The yield obtained was 10.4% (w/w). Finally, the extract was dried and the remainder was later lyophilized.

2.3. Treatment

To the in vivo experiments the lyophilized dry residue obtained was diluted in an isotonic phosphate buffered solution (PBS) at a concentration of 1 mg/mL. The animals were then weighed to adjust the dose of HCE to 5 mg/kg (mg of dried plant material/kg of body weight). The animals were treated by intraperitoneal (ip.) route with HCE and were killed 48 h later. The dose and time of treatment applied here were chosen based on the anti-tumor effect observed by Nascimento et al. (2006a,b) with the same dose and time of treatment. The negative control group was treated only with PBS and the positive control group was treated with concanavalin A (Con A) (Sigma, St. Louis, MO, USA) using a dose of 0.5 mg/kg of animal. This lectin is obtained from *Canavalia ensiformis*, which is an inducer of cellular migration to peritoneal cavity, a primer to macrophage activation in vivo and also a useful tool to induce cellular proliferation (Rodriguez et al., 1992; Nascimento et al., 2006b).

The in vitro assays were performed using resident macrophages or macrophages obtained from HCE-treated mice (HCE-derived macrophages). The resident macrophages were treated in vitro with 5, 50 and 500 μg of HCE/mL of medium by 24 and 48 h. The HCE-derived macrophages were cultured in the presence or not with HCE (5 μg/mL) or Con A (5 μg/mL) as a positive control of macrophage activation.

2.4. Peritoneal cell harvesting

Mice were killed by CO$_2$ asphyxia 48 h after the treatment with HCE or Con A or PBS (control) and the peritoneal cells were aseptically collected by washing the peritoneal cavity with 5 mL sterile ice-cold PBS devoid of calcium and magnesium ions. The resident macrophages were obtained from mice that had not received any treatment. For total cell determination, nine volumes of peritoneal cells were added to 1 vol. of 0.05% crystal violet dissolved in 30% acetic acid and the cells were counted using a bright-line hemocytometer (Sigma, St. Louis, MO, USA). Differential cell counts were determined by cytospin preparations stained with Instant-Prov (Newprov, Pinhais, Brazil).
2.5. Spleen, lymph node and bone marrow’s cells counting

After the collection of peritoneal fluid, the lymph node, spleen and femur were collected. The lymph node and spleen were processed in 1 or 5 mL of PBS respectively. The femur was washed with 1 mL of PBS to obtain the bone marrow’s cells. For total cell determination, nine volumes of peritoneal cells were added to 1 vol. of 0.05% crystal violet dissolved in 30% acetic acid and the cells were counted using a bright-line hemocytometer (Sigma, St. Louis, MO, USA).

2.6. Spreading assay

The spreading assay was performed according to Rabinovitch et al. (1977). The peritoneal cell suspensions containing 2 × 10^6 cells were centrifuged and suspended in 1 mL Dulbecco’s PBS containing 5 mM glucose. Fifty microlitres of cellular suspension were layered on glass coverslips and incubated for 1 h at 37 °C. The coverslips were gently rinsed in PBS; the glass adherent cells were fixed in 2.5% glutaraldehyde and examined with a phase contrast microscope at a magnification of 400×. Two hundred macrophages were counted and scored as round or spread. An index of macrophage spreading (SI) was then calculated as follows: SI = (number of spreading macrophages × 100)/200, i.e. SI = % of spreading macrophages.

2.7. Phagocytosis of zymosan particles

The phagocytosis assay was performed using the method described by Pinello et al. (2006) and modified for us. The peritoneal cell suspensions containing 2 × 10^6 cells were centrifuged and suspended in 1 mL of RPMI medium. The cells were dispersed over round glass coverslips (20 mm) in six-well flat-bottomed microtest plates (Costar, New York, NY, USA) and the cultures incubated at 37°C for 20 min. After incubation, the culture supernatants were aspirated and the non-adherent cells removed. Adherent monolayers were rinsed with PBS. Subsequently, 1 mL of RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 5% of heat-inactivated fetal bovine serum was added to the cultures. The cultures were maintained at 37°C for 1 h in the presence of Saccharomyces cerevisae zymosan (Sigma) (1 mg/mL). The cultures were then washed with cold PBS to remove the particles not internalized. The cells were then fixed with 0.5% glutaraldehyde (Sigma). An average of 200 macrophages was counted using phase contrast microscopy to determine the phagocytic percentage. An index of phagocytosis (PI) was then calculated as follows: PI = (number of macrophages with phagocytic activity × 100)/(200 adherent cells counted), i.e. PI = % of macrophages with at least two zymosan particle phagocytized.

2.8. Sequential analyses of macrophage functions using a single sample of macrophages

H$_2$O$_2$-release and NO-production was determined in a single macrophage sample using a method previously described by Nascimento et al. (2003). To evaluate H$_2$O$_2$-release, a horseradish peroxidase-dependent phenol red oxidation micro assay was used (Pick and Mizel, 1981). In this assay two million peritoneal cells were suspended in 1 mL freshly prepared phenol red solution that consisted of ice-cold Dulbecco’s PBS containing 5.5 mM dextrose, 0.56 mM phenol red (Sigma) and 8.5 U/mL horseradish peroxidase type II (Sigma). Hundred microlitres of the cell suspension were added to each well, and incubated in the presence or not of 10 ng phorbol myristate acetate (PMA) (Sigma), for 1 h at 37 °C in a humid atmosphere containing 5% CO$_2$ and 95% air. The plates were centrifuged once at 150 × g for 3 min and the supernatants were collected and transferred to another plate. The reaction was stopped with 10 μL 1N NaOH. The absorbance was measured at 620 nm with a micro plate reader (MR 5000, Dynatech Laboratories Inc., Gainesville, VA, USA). Conversion of absorbance to μM H$_2$O$_2$ was done by comparison to a standard curve obtained with known concentrations of H$_2$O$_2$ (5–40 μM) as previously described by Pick and Keisari (1980).

Thereafter the plates containing the cells were washed three times with PBS and the remaining adherent macrophages were cultured in 100 μL of complete RPMI 1640 medium supplemented with 10 mM HEPES, 11 mM sodium bicarbonate, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM l-glutamine, 23 mM l-asparagine, 1 mM folic acid, 0.1 mM pyruvic acid and 5% fetal calf serum (FCS) for 48 h at 37 °C in a humid atmosphere containing 5% CO$_2$ and 95% air. After the incubation, 50 μL of supernatants were collected and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthalene diamine dihydrochloride/2.5% H$_3$PO$_4$) for 10 min at room temperature, to quantify the accumulation of nitrite (Ding et al., 1988). The absorbance was determined at 550 nm. Conversion of absorbance to μM of NO was done by comparing to a standard curve obtained with known concentrations (5–60 μM) of sodium nitrite diluted in RPMI medium.

2.9. Statistical analysis

Results are expressed as the mean ± standard deviation from 10 animals per group. Statistical evaluation was done by ANOVA test. Differences were considered significant at p ≤ 0.05 and are represented by an asterisk. All experiments were repeated for at least two times.

3. Results

3.1. Peritoneal cellular influx by HCE ip. treatment

The HCE treatment induced a significant increase on the total peritoneal cell number when compared with control animals. This increase was similar to that obtained in Con A-treated group (Fig. 1A). The cellular influx induced by HCE and Con A was mainly constituted of macrophages that corresponded to 95 ± 2% and 97 ± 2% of total cells, respectively.

3.2. Macrophage activation induced by HCE ip. treatment

The HCE treatment significantly increased the ex vivo macrophage spreading and phagocytic ability when compared
Fig. 1. Effect of intraperitoneal treatment with hydroalcoholic crude extract (HCE) of Chenopodium ambrosioides or concanavalin A (Con A) on cellular influx to peritoneal cavity and on macrophage activation. C3H/HePas received 5 mg/kg of HCE or 0.5 mg/kg of Con A and were sacrificed 48 h later to collect the peritoneal cells. Total and differential counts were performed (A), the spreading and phagocytic ability (B), the spontaneous and PMA-induced hydrogen peroxide release (C) and the nitric oxide production (D) were evaluated. Data represent the mean ± S.D. (n = 10). *p < 0.05, significant as compared to the control group. #p < 0.05, significant as compared to the Con A group.

The addition of HCE and Con A to the cultures of resident macrophages induced a significant NO production (Table 1). The addition of HCE to the cultures of HCE-derived macrophage did not increase the NO production. However the addition of Con A to these cultures induced a synergistic increase in the NO production (Table 1). The NO production induced by the addition of HCE to resident macrophage cultures was dose- and time-dependent (Table 2).

3.3. Nitric oxide production by in vitro treatment with HCE

The addition of HCE and Con A to the cultures of resident macrophages induced a significant NO production (Table 1). The addition of HCE to the cultures of HCE-derived macrophage did not increase the NO production. However the addition of Con A to these cultures induced a synergistic increase in the NO production (Table 1). The NO production induced by the addition of HCE to resident macrophage cultures was dose- and time-dependent (Table 2).

3.4. Cellular recruitment to lymphoid organs induced by HCE ip. treatment

The HCE treatment did not alter the cell number of bone marrow (Fig. 2A), but it induced a significant increase on the

<table>
<thead>
<tr>
<th>Groups</th>
<th>Medium</th>
<th>Con A (5 μg/mL)</th>
<th>HCE (5 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>0.0 b</td>
<td>6.0 ± 1.1 c</td>
<td>10.5 ± 1.8 c</td>
</tr>
<tr>
<td>48 h a</td>
<td>7.1 ± 1.2 c</td>
<td>31.6 ± 3.2 d</td>
<td>22.7 ± 2.9 d</td>
</tr>
</tbody>
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a Culture supernatants were collected 48 h after the stimulation to quantify the nitrate (μM).
b In vitro stimulation.
c Data represent the mean ± S.D. (n = 3).
d In vivo stimulation.
p < 0.05, in comparison with unstimulated resident cells (medium).
** p < 0.05, in comparison with the resident macrophages cultures.
*** p < 0.05, in comparison with the unstimulated HCE-derived cells (medium), to HCE-stimulated cells and to resident macrophages.
cell number of spleen (Fig. 2B) and lymph node (Fig. 2C) when compared with control animals. This increase was similar to that obtained in Con A-treated group.

4. Discussion

In this work it was shown that a low dose of HCE from leaves of Chenopodium ambrosioides induced macrophage activation and increased the cell recruitment/proliferation in secondary lymphoid organs.

One of the activities improved by Chenopodium ambrosioides in macrophages was the spreading and phagocytic ability. The spreading ability of the macrophage is important to both the phagocytosis process and also to the macrophage migratory process to the inflammatory site (Tatefuji et al., 1996). Beside this, the phagocytosis and the secretion of microbicidal metabolites are the first line of interaction between macrophages and microorganisms or tumor cells.

It is known that phagocytosis and hydrogen peroxide release are related to almost all of the anti-microbial responses, since the burst oxidative is a sequential step after the phagocytosis. However, despite the increasing of the phagocytosis ability there was no spontaneous secretion of hydrogen peroxide. This result could be related to an anti-oxidant effect of Chenopodium ambrosioides since plant derived products such as flavonoids, terpenes and steroids that are constitutive of Chenopodium ambrosioides, have been shown to have anti-oxidant properties (DeFeudis et al., 2003; Takeoka and Dao, 2003).

Nevertheless the anti-oxidant property of Chenopodium ambrosioides seems not to be permanent, since the addition of PMA to the HCE-derived macrophages cultures, induced high levels of this metabolite (Fig. 1C). The PMA is a potent trigger of the burst oxidative since it activates the kinase protein directly. However the action of PMA is evident only when the phagocytic cells are primed in vivo. Then, our results indicate that the HCE-treatment acts as a primer to the macrophages, what could improve the subsequent microbicidal/tumoricidal activity of these cells.

Despite the treatment with HCE not having induced the spontaneous release of H$_2$O$_2$, it induced the spontaneous NO production. This production was significant when compared to the control and to the Con A-derived macrophages, which did not produce NO ex vivo (Fig. 1D). However, the mechanism of NO induction by HCE is unclear.

The expression of the inducible nitric oxide synthase (iNOS) and the consequent NO production usually requires one signal to prime and another to trigger macrophage activation. NO production by activated macrophages is a result of the induction and the genic expression of an enzyme, which is not present constitutively in these cells (MacMicking et al., 1997). Activated macrophages simultaneously release NO and superoxide anion and its dismutation product, H$_2$O$_2$. The release of these metabolites is an enzyme-controlled process that can be changed according to the performance of these enzymes. The inhibition of tyrosine kinases for example, completely abolished the H$_2$O$_2$ release by activated phagocytes, but increased NO production. On the other hand, inhibition of phosphotyrosine phosphatase, markedly decreases NO production and increases the release of O$_2$$. On the other hand, inhibition of protein kinase C diminishes the production of both H$_2$O$_2$ and NO (Carreras et al., 1997). So, it is possible that the HCE treatment can induce some alteration in the mechanism of activation of the tyrosine kinases in vivo, what could result in the abolishment of spontaneous H$_2$O$_2$ release and an improvement in the spontaneous NO production. This effect seems not to be extended to the protein kinase C since the PMA is enough to induce the ex vivo H$_2$O$_2$ release.

Besides the induction of NO production ex vivo, the addition in vitro of HCE to the resident macrophages cultures induced a dose- and a time-dependent NO production (Table 2). However, the addition of HCE to the cultures of HCE-derived macrophages did not alter the NO production (Table 1). Conversely, the addition of Con A to the cultures of the same macrophage population induced a synergistic increase in the NO production (Table 1).
This result suggests that Con A and HCE can induce the in vitro NO production by different routes.

Some plant-derived products have been demonstrated as NO-inducers or inhibitors by different mechanisms (Ignacio et al., 2001; Calixto et al., 2003; Nascimento et al., 2006b). Con A for example is a mannose-binding lectin which induces the NO production in vitro, but not in vivo, by a mechanism that is MHC-independent but co-stimulatory molecules- and cytokines-dependent.

The results obtained here show, at the first time, that Chenopodium ambrosioides treatment is a primer of macrophage activation. Besides this we have shown that the treatment with the HCE induced an increase in the cellular recruitment and/or proliferation in secondary lymphoid organs such as spleen and lymph node. This ability indicates that the HCE induces a positive immunomodulation in the organism what could explain the anti-tumor activity of this plant previously described (Effert et al., 2002; Nascimento et al., 2006a).

The activation of the macrophages by Chenopodium ambrosioides could also be the mechanism involved in the control of microorganisms’ growth, what could justify the popular use of this plant in the treatment of ulcers caused by the Leishmania species. Further studies are in progress in order to investigate these hypotheses and to characterize the bioactive compounds involved in the biological action of Chenopodium ambrosioides.

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References


Pfaffia paniculata (Brazilian ginseng) extract on macrophage activity. Life Sciences 78, 1287–1292.