

Therapeutic potential of biodegradable microparticles containing *Punica granatum* L. (pomegranate) in murine model of asthma

Jéssica F. F. de Oliveira · Diego V. Garreto · Mayara C. P. da Silva ·
Thiare S. Fortes · Rejane B. de Oliveira · Flávia R. F. Nascimento ·
Fernando B. Da Costa · Marcos A. G. Grisotto · Roberto Nicolete

Received: 26 May 2013 / Accepted: 5 August 2013 / Published online: 25 August 2013
© Springer Basel 2013

Abstract

Objective and design Among the options for treatment of diseases affecting the respiratory system, especially asthma, drug delivering systems for intranasal application represent an important therapeutic approach at the site of inflammation. The present study aimed to evaluate the therapeutic effect of biodegradable microparticles formed by poly lactic-*co*-glycolic acid (PLGA) containing encapsulated pomegranate extract on a murine model of asthma. **Material** The extract was acquired from the leaves of *P. granatum* and characterized qualitatively by HPLC. A w/o/w emulsion solvent extraction–evaporation method was chosen

to prepare the microparticles containing pomegranate encapsulated extract (MP).

Treatment OVA-sensitized BALB/c mice were used as asthma model and treated with dexamethasone and *P. granatum* extract in solution form or encapsulated into microparticles.

Results MP were able to inhibit leukocytes' recruitment to bronchoalveolar fluid, especially, eosinophils, decreasing cytokines (IL-1 β and IL-5) and protein levels in the lungs. **Conclusions** This approach can be used as an alternative/supplementary therapy based on the biological effects of *P. granatum* for managing inflammatory processes, especially those with pulmonary complications.

Responsible Editor: Ji Zhang.

J. F. F. de Oliveira · M. A. G. Grisotto · R. Nicolete
Pró-Reitoria de Pós-Graduação, Pesquisa e Extensão,
Universidade CEUMA (UNICEUMA), Rua Josué Montello, 1,
São Luís, Maranhão 65075-120, Brazil

D. V. Garreto
Instituto Florence de Ensino, Rua Rio Branco, 216, São Luís,
Maranhão, Brazil

M. C. P. da Silva · T. S. Fortes · F. R. F. Nascimento
Department of Pathology, Laboratory of Immunophysiology,
Biological and Health Sciences Center, Federal University of
Maranhão (UFMA), São Luís, Maranhão 65085-580, Brazil

R. B. de Oliveira · F. B. Da Costa
Departamento de Ciências Farmacêuticas, Faculdade de Ciências
Farmacêuticas de Ribeirão Preto, Universidade de São Paulo,
Av. do Café s/n, Ribeirão Preto, São Paulo 14040-903, Brazil

R. Nicolete (✉)
Laboratório de Biotecnologia Aplicada à Saúde, Fundação
Oswaldo Cruz—Fiocruz Rondônia, Rua da Beira 7671, Porto
Velho, Rondônia 76812-245, Brazil
e-mail: rnicolete@gmail.com

Keywords *Punica granatum* L. · Inflammation ·
Asthma · Microparticles · Poly(lactic-*co*-glycolic acid)

Introduction

Asthma is chronic lung disease that involves many inflammatory cells and proteins. The complex inflammatory disorder that characterizes asthma is associated with eosinophilic inflammation, airway hyperresponsiveness, and hypersecretion of mucus by goblet cells [1, 2]. Inhaled corticosteroids are effective in treating chronic asthma, but the adverse side effects displayed by these substances severely limit their long-term use [3]. The extracts of numerous plants are reported as potent therapeutic materials in various diseases, including inflammatory processes such as asthma [4–8]. Experimental models to screen the pharmacologic activities of plant extracts well as isolated compounds (secondary metabolites) have been used with success in the course of a continued search for bioactive natural products derived from plants [8–10].

Punica granatum L., known as pomegranate, is a common fruit cultivated in tropical and subtropical regions of the world [11]. Pomegranate juice is a naturally rich source of polyphenols and other antioxidants, including punicalagin, ellagic acid, gallotannins, anthocyanins (cyanidin, delphinidin and pelargonidin glycosides) and also flavonoids (quercetin, kaempferol and luteolin glycosides) [12]. These components suggest a wide range of clinical applications for the treatment and prevention of several diseases where chronic inflammation is believed to play an essential etiologic role [13]. The extract has been used to treat diarrhea, dysentery, and dental plaque [14]. A study using the classical experimental model of ovalbumin-induced asthma performed with the plant (*Lafoensia pacari*), which belongs to the same family as the pomegranate showed that treatments made with the plant extract and ellagic acid (secondary metabolite) were able to interfere with the development and establishment of airway allergic inflammation, inhibiting leukocyte recruitment to the pulmonary parenchyma and also cytokines' production [9]. The described anti-inflammatory components of Pomegranate extract, i.e., punicalagin, punicalin, strictinin A and granatin B significantly reduced production of nitric oxide and PGE2 by inhibiting the expression of proinflammatory proteins [15, 16]. In this context, we can speculate that some metabolites from pomegranate, such as ellagic acid cited above, can contribute to the potential activity against allergic and inflammatory processes present in the asthma.

The use of polymeric microparticles as a pulmonary delivery vehicle for therapeutic drugs have been frequent due to their many advantages, including the controlled release of encapsulated drugs and easy administration in a minimally invasive manner [17–20]. When compared to conventional drug administration, the controlled release systems offer the great advantage of maintaining a sustained/constant drug concentration in the therapeutic range for a prolonged period [21]. The aim of this study was to evaluate the therapeutic potential of microencapsulated pomegranate extract using PLGA to confer a different approach to target and stimulate pulmonary cells during experimental asthmatic process.

Materials and methods

Materials

Poly(D,L-lactide-co-glycolide) (PLGA) with a co-monomer ratio of 50:50 (lactic/glycolic acid) and molecular weight of 78 kDa was obtained from Boehringer Ingelheim (Ingelheim, Germany). Poly(vinyl-alcohol) (Mowiol 40–88) was obtained from Aldrich Chemicals (Wankee, WI, USA). Methylene dichloride was purchased from Merck

(Dietikon, Switzerland). Chicken ovalbumin (OVA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA); dexamethasone was from Aché pharmaceutical laboratories S.A (Guarulhos, SP, Brazil); Griess' reagent was from Sigma Aldrich Brasil Ltda. (São Paulo, SP, Brazil).

Preparation of the Pomegranate extract

The fresh leaves of the plant were collected in Herbarium of the Universidade Federal do Maranhão, São Luis (Brazil). *Punica granatum* L. (Pomegranate) is deposited in the collection of the University Herbarium and cataloged under voucher 01002. The hydroalcoholic extract was obtained by maceration 20 g of plant leaves previously washed and crushed in a closed container containing 200 mL water–alcohol solution (70 % ethanol, v/v) at room temperature. Ethanol as solvent was chosen based on the results obtained by [22]. The solution was maintained for 8 days in amber glass under dark and with occasional agitation. Thereafter, we proceeded to filtration extractive liquid, which then was rotaevaporated at a temperature of 60–70 °C in thermostatic bath.

Analysis of the extract by high performance liquid chromatography (HPLC)

The analysis was conducted by high-performance liquid chromatography (HPLC) on reverse phase. The equipment used was a Shimadzu liquid chromatography (Japan) 10 SCL Avp coupled to UV-DAD detector Shimadzu SPD-M10Avp and an automatic gun. The gradient elution consisted of a binary mobile phase composed of H₂O (0.5 % AcOH) and MeCN in a linear gradient of 0–50 % of MeCN in 30 min and 50–100 % of MeCN in 30–35 min while maintaining at 100 % of MeCN for 5 min to clean the column. Two monolithic columns coupled (Onyx C18, 200 × 300 mm, Phenomenex) protected by a precolumn equivalent were used for the analyses. The sample was analyzed by dissolving 100 µL of the extract (100 mg/mL) in 500 µL of a solution of MeOH: H₂O (1:1 v/v), followed by subsequent filtration on PTFE filter (0.45 mm). The injected volume was 10 µL.

The presence of some classes of metabolites was inferred from their UV absorption spectrums, based on literature information about phytochemical profile of pomegranate [12, 13, 23] and also its general structures of the important phenolic compounds described [24].

Microparticles' preparation

The microparticles containing the extract of encapsulated pomegranate (MP) were prepared using the double emulsion/solvent evaporation method, as previously described [18] with some modifications. Briefly, 200 µL of the

extract (100 mg/mL) were added to dichloromethane solvent (5 mL) (JT Baker/Mallinckrodt, Phillipsburg, NJ, USA) containing 100 mg of PLGA polymer (Resomer RG 505, Germany) to produce a primary water-in-oil emulsion. Subsequently, this organic phase was stirred mechanically (mini-Q 235, Quimis, Brazil) at 1000 rpm for 3 min and mixed with 40 mL (3 % w/v) of an external aqueous phase (polyvinyl alcohol (PVA), Sigma Chemical Co.) to form a stable water-in-oil in-water emulsion. This was then placed under a magnetic stirring condition for 4 h to evaporate the solvent. Finally, microparticles formed were washed three times with doubly distilled water, centrifuged and then freeze-dried until further use. The control microparticles (MC) were prepared under the same conditions but without the addition of pomegranate extract (PGE).

Microparticles' characterization

The particles were reconstituted in distilled water for analysis at room temperature. Particle diameters were characterized using a particle size analyzer (LS 13 320 Laser Diffraction Particle Size Analyzer; Beckman Coulter, USA). Zeta potential analysis of the particles was performed using a Nano Zeta Sizer (Malvern instruments, England).

Animals

Female BALB/c mice weighing between 18 and 20 g (4–6 week old) were used for the induction of asthma. Mice were obtained from Suprilab to Biotery and Supplies Ltda. (Cachoeirinha-BA) and were maintained in the lab-

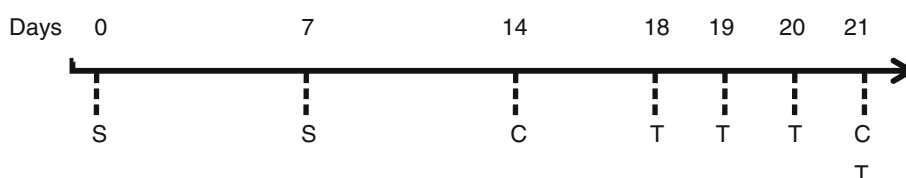
groups. To induce asthma, each mouse was sensitized with 75 µg ovalbumin and 2 mg aluminum hydroxide dissolved in 200 µL of saline on days 0 and 7, followed by two intranasal challenges (on post-sensitization days 14 and 21) with 50 µg of ovalbumin in 50 µL (25 µL per nostril) of saline delivered with the aid of a micropipette. This model is widely used and well characterized with similar features to those in human allergic asthma [25]. The control group received only saline without OVA and treatment.

Treatments

Groups of the asthma-induced mice were treated daily from days 18 to 21 [9] with microparticles containing pomegranate encapsulated extract (MP, 10 mg/mL, 25 µL per nostril) or with non-encapsulated pomegranate extract (PGE, 20 mg/Kg, 25 µL per nostril) by intranasal instillation. The concentration used for MP (10 mg/mL) was chosen based on published study of our group [18]. The dose of the Pomegranate extract was chosen based on published data regarding anti-inflammatory properties [24].

For comparison with classical therapy, mice in one group were treated with i.p. injection of dexamethasone (Dex, 0.4 mg/kg, 0.2 mL). In order to evaluate the efficiency of the encapsulated extract, another group was treated with control microparticles (MC, 10 mg/mL, 25 µL per nostril), without the extract. Animals were killed at 24 h after the second ovalbumin challenge. A sketch is provided below.

Sensitization (S), Challenge (C) and Treatments (T)



oratory of the Instituto Florence de Ensino, São Luís, Brazil, under standard laboratory conditions. All the protocols used in this study were approved by the Ethics Committee of Animal Experimentation of the Universidade Estadual do Maranhão, Brazil (Protocol CEEA/UEMA n° 022/2011).

Murine model of asthma

The mice were divided into six groups (six animals each), and the allergic lung inflammation was induced in five

Collection of bronchoalveolar lavage (BAL)

To collect BAL, a polyethylene cannula was introduced into the trachea and PBS was instilled in three aliquots (0.5 mL) in a total of 1.5 mL. The lavage was recovered and placed on ice. Total cell counts were immediately carried out using a Neubauer chamber. Differential cell counts were performed in light microscopy, according to standard morphologic criteria, by counting, in a blinded fashion, 100 cells in cytopsin preparations stained with Rosenfeld's stain. Following centrifugation (400×g, 5 min, 4 °C), supernatants of

the BAL were collected and stored at -70°C for subsequent measurement of cytokines and nitrites.

Cytokines' assessment

IL-1 β and IL-5 were determined by enzyme-linked immunosorbent assay (ELISA) using commercial assay kit (BD OptEIA, BD Biosciences, San Diego, CA) according to the manufacturer's protocol. The optical density (O.D) of samples was determined at 450 nm in a microplate reader and cytokine concentrations calculated from the standard curve.

Protein assessment

A quantification of total proteins in the BAL supernatants was performed based on the Biuret method using the standard Total Proteins Assay Kit from Labtest Diagnóstica S.A. (Lagoa Santa, MG, Brazil) according to the manufacturer's protocol. Albumin solution (4.0 g/dL) and sodium azide (14.6 mmol/L) were using as a standard. The absorbance of the test was determined in automated spectrophotometer at 545 nm.

Histological analysis

Lungs were fixed in 10 % neutral formalin, paraffinized, cut into sections (5 μm), and stained with hematoxylin and eosin for examining cell infiltration. Magnification 100 $\times$.

Statistical analysis

Data were expressed as a mean $\pm$ S.E.M. Multiple comparisons were performed by one-way ANOVA followed by Turkey post-test using a computer program GraphPad

Prism version 5.0. Values of $P < 0.05$ were considered statistically significant.

Results

HPLC profile of pomegranate extract (PGE)

The presence of some classes of metabolites in the extract was inferred through their absorption spectra in the UV, based on information in the literature about the phytochemical profile of Pomegranate [12, 13, 23]. The analysis of the extract of the leaves of *P. granatum* by HPLC showed peaks between 0 and 25 min, most of them well visualized at 325 nm. The lower retention times indicate a predominance of polar compounds, mainly of phenolic compounds (Fig. 1). UV absorption spectra of peaks 1, 2 and 3 suggest the presence of catechin (flavan-3-ols, band II at 270–280 nm). This class of substance has been described for the Pomegranate catechin, and epicatechin epicagalocatechin 3-gallate [13]. The peaks 11 and 12 suggest the presence of flavonols (I band at 250–270 nm band II at 350–380 nm). This class of substance was described for Pomegranate quercetin and kampferol [13]. The UV absorption spectrum of many peaks observed suggest the presence of hydrolysable tannins probably derived from gallic to ellagic acid. The peaks 4, 6 and 7 are probable gallic acid derivatives (gallic acid UV max = 269 nm, = derivatives II band around 260 nm to I band at 360–380 nm).

Size and zeta potential

Lyophilized particles were dispersed in distilled water and submitted to analysis of size distribution. For control microparticles (MC), the average diameter was 7.22 μm

Fig. 1 HPLC chromatograms of crude extract obtained from *P. granatum* leaves

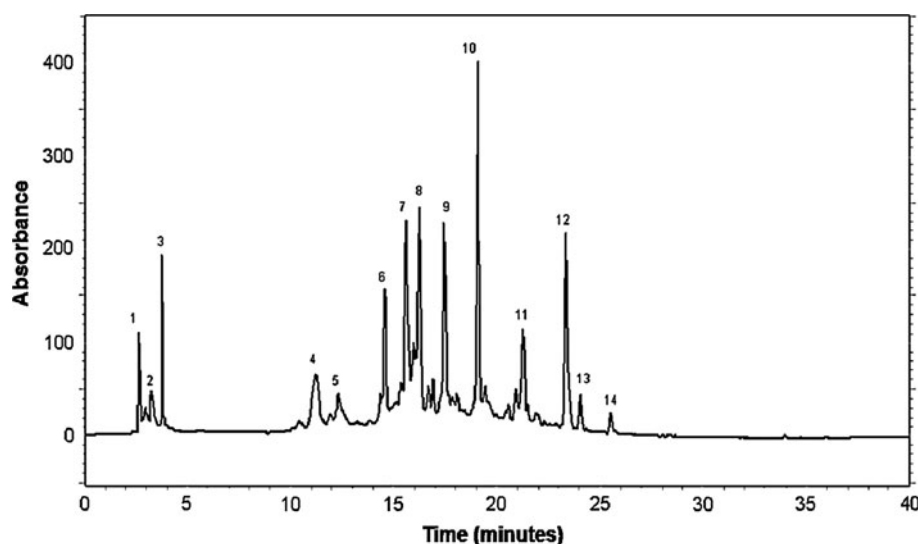
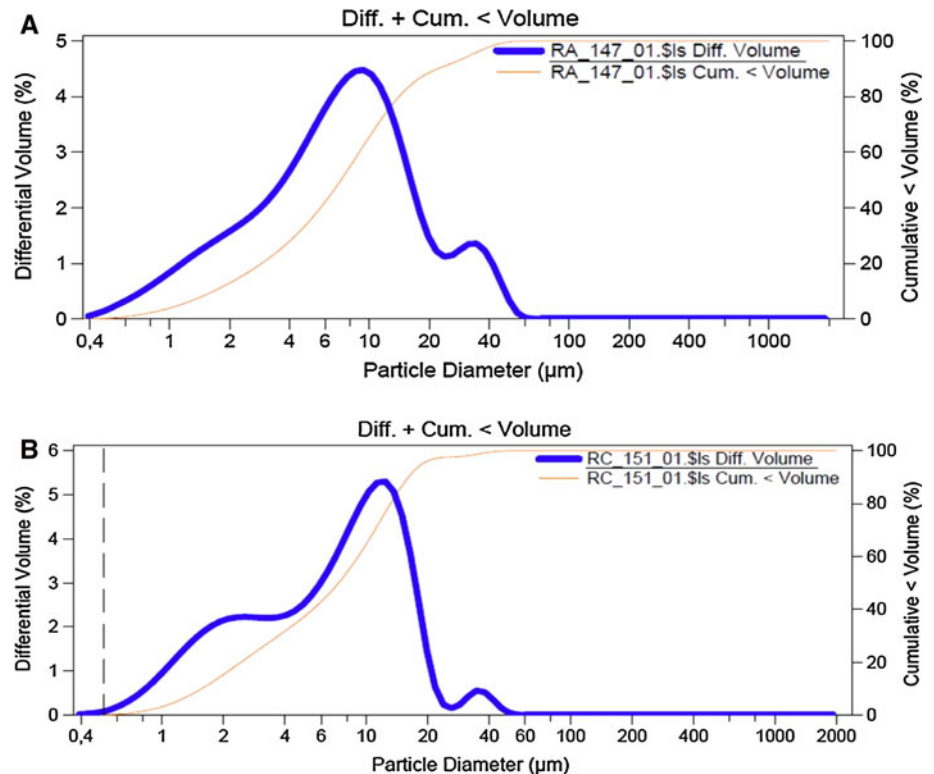


Fig. 2 Distribution of average diameters of **a** control microparticles and **b** microparticles containing pomegranate extract



(Fig. 2a) and for microparticles containing encapsulated extract (MP) was $7.31 \mu\text{m}$ (Fig. 2b). The average zeta potential was -0.70 and -1.37 mV , for MC and MP, respectively.

MP selectively reduce eosinophils' recruitment to BAL

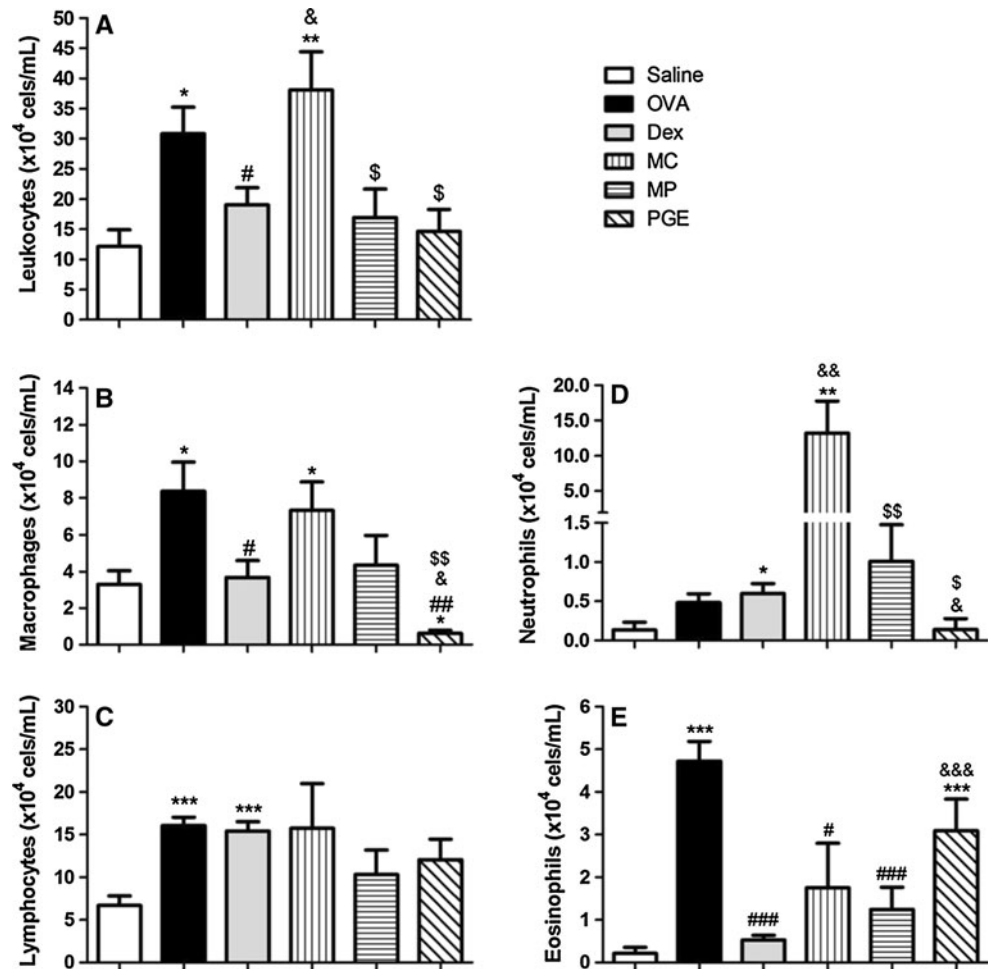
The therapeutic efficacy of the microparticles was assessed using murine model of asthma. Asthma was induced by sensitization and challenged with ovalbumin solution and the animals were divided into groups that received treatment with microparticles containing (MP) or not encapsulated Pomegranate extract (MC), dexamethasone (Dex) or non-encapsulated extract (PGE). The control group received only saline in sensitizations and challenges steps. The striking feature of asthma is the increase of inflammatory cells, such as macrophages, eosinophils, neutrophils and lymphocytes. Therefore, the total and differential cell counts were performed from the BAL of animals to evaluate the effects of the treatments (Fig. 3). The total number of inflammatory cells in mice with asthma (OVA) was $30.8 \times 10^4 \text{ cells/mL}$, which was substantially higher than that of control (no asthma-induced mice) (Fig. 3a). This number was significantly reduced compared to the treatment with microparticles containing pomegranate extract (MP, $16.9 \times 10^4 \text{ cells/mL}$), to those treated with dexamethasone (Dex, $19.0 \times 10^4 \text{ cells/mL}$) and to pomegranate non-encapsulated extract (PGE, $14.6 \times 10^4 \text{ cells/mL}$). The group that received control

microparticles (MC), unlike other treatment groups, showed a statistically significant increase in cell-recruited numbers. In relation to the cell types, the number of eosinophils (Fig. 3e), the “hall marker” cells involved in the inflammatory process of asthma, was substantially decreased in mice treated with MP ($1.24 \times 10^4 \text{ cells/mL}$) when compared to the asthma-induced mice (OVA, $4.7 \times 10^4 \text{ cells/mL}$). Animals that received MC and Dex treatments also showed low cell counts. Interestingly, mice treated with PGE did not show significant reduction compared to OVA group. The other cell types (macrophages, lymphocytes and neutrophils) did not present any unexpected recruitment that could be described comparing the groups treated.

MP reduce inflammatory/allergic cytokines' production in the BAL

Quantification of IL-1 β and IL-5 cytokines from the BAL of animals was determined by ELISA (Fig. 4). The results show that the asthma-induced group (OVA) had a significant increase in IL-1 β levels compared to the control group and this increase was significantly attenuated by all treatments (Fig. 4a). The groups treated with Dexamethasone (Dex) and microparticles containing the extract (MP) showed similar levels to the control group (saline), demonstrating relevant anti-inflammatory effect, especially comparing MP treatment to that with Dex, a classical therapeutic. The treatments with MC and PGE also showed

Fig. 3 Effect of the treatments with the preparations on murine model of asthma. Groups of BALB/c mice were treated from days 18 to 21 with dexamethasone (Dex), microparticles without encapsulated extract (MC), microparticles containing Pomegranate extract (MP) or non-encapsulated Pomegranate extract (PGE). BAL samples of mice were collected 24 h after the last OVA challenge and leukocytes' count was performed in a Neubauer chamber (a). Macrophages (b), lymphocytes (c), neutrophils (d) and eosinophils (e) counts were performed in cytopspin preparations stained with Rosenfeld's stain. Data are shown as mean $\pm$ SD. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared with negative control (saline); ## P < 0.01 and ### P < 0.001 compared with the Ova group; & P < 0.05 and && P < 0.001, compared with dexamethasone; \$\$ P < 0.01, compared to control microparticles group (MC)



significant decrease compared to the control group. Regarding IL-5 levels (Fig. 4b), treatment with MP showed a statistically significant reduction compared to OVA group. The other treatments also showed good reduction levels. The group treated with Pomegranate non-encapsulated extract (PGE) showed very low levels of IL-5, undetectable in the assay (data not shown).

MP reduce protein extravasation to the BAL

The onset of the inflammatory process is characterized by vascular events that happen right after the inflammatory stimulus, which causes vasodilation followed by increased vascular permeability and consequent leakage of molecules to the inflamed tissue. To assess whether the treatments used in asthma model showed effects on protein leakage into the lung tissue, a quantification of total protein was assessed in BAL of the animals (Fig. 5). Glycoprotein secretion (mucus) in the lungs was indirect measured as a part of total protein content. Significant reduction on protein levels was achieved for the

treatments with microparticles compared to OVA group, especially those containing Pomegranate extract. In contrast, PGE treatment did not show significant reduction on protein levels.

MP reduce airway inflammation in lung tissue

To investigate the effects of the pomegranate extract encapsulated or not on the pathological changes of the lung tissue in an asthmatic murine model, the lung tissues were stained with hematoxylin and eosin (HE) (Fig. 6). The asthma-induced mice (OVA) showed increased leukocytosis in the peribronchial tissues (Fig. 6b) (represented by white arrows), compared with control lung tissues (Fig. 6a). Animals that received Dex (Fig. 6c) also displayed lower leukocytes' infiltration in the lung tissue compared to OVA group. The Pomegranate encapsulated extract when delivered to pulmonary microenvironment, especially when compared to control microparticles, significantly inhibited pathological changes, showing a suppressive effect on airway inflammation (Fig. 6e).

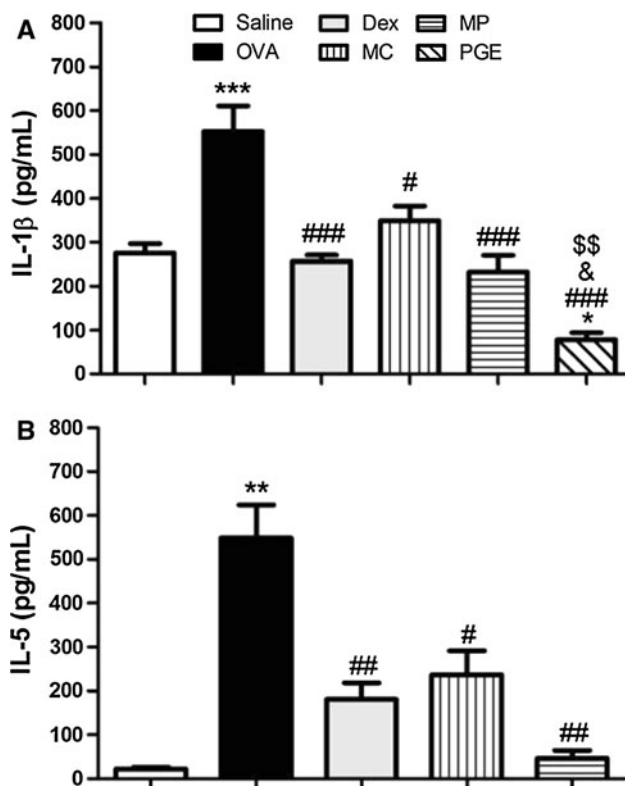


Fig. 4 Effects of the treatments with the preparations on the production of IL-1 β and IL-5 cytokines. Quantification levels were performed in the supernatant of BAL. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to negative control group (saline); # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to Ova group; & $P < 0.05$ compared to dexamethasone group. \$\$ $P < 0.01$, compared to control microparticles group (MC)

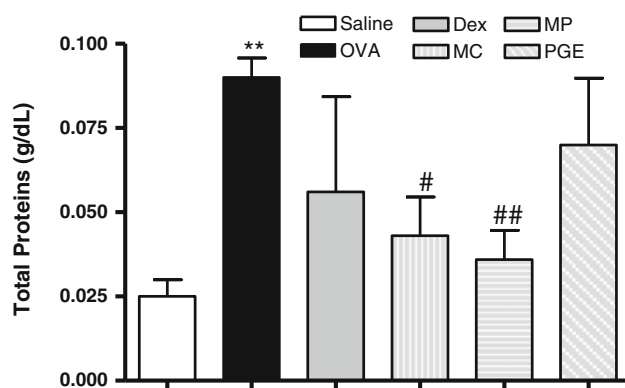


Fig. 5 Protein leakage in the BAL of animals 24 h after the last challenge with OVA. Samples were collected from the groups: saline, Ova, dexamethasone (Dex), control microparticles (MC), microparticles containing pomegranate extract (MP) and non-encapsulated pomegranate extract (PGE). Data are shown as mean $\pm$ SD. *** $P < 0.01$ compared with negative control (saline), # $P < 0.05$ and ## $P < 0.01$ compared with the Ova group

Moreover, the non-encapsulated extract also decreased histological alteration in the pulmonary parenchyma during asthmatic process (Fig. 6f).

Discussion

Biodegradable micro/nanoparticles have been frequently used as a vehicle for therapeutic drugs to the lung due to the various advantages offered by these systems, including the controlled release of encapsulated drugs and simple administration of minimally invasive manner [19]. In this study, we demonstrated that the biodegradable microparticles produced from PLGA polymer by the method of double emulsion and solvent evaporation without any bioactive or with *P. granatum* encapsulated extract had an average diameter within the range of sizes used for intranasal route of administration in animals as well as efficiency in the delivery of encapsulated compound to the lungs [26]. Porous PLGA microparticles with inhaled encapsulated corticosteroids and with similar diameter of the particles used in our study were able to reduce significantly the number of inflammatory cells in BAL of asthma-induced mice, as well as bronchial hyperresponsiveness and inflammatory cell infiltration observed in the lungs [19]. This corroborates our findings using biodegradable polymeric system to deliver pomegranate extract as promising approach in the treatment of asthma, especially based on the results comparing its efficiency with non-encapsulated extract.

The inflammatory cells migration into the lung tissue is the event that perpetuates in asthma, and characterizes the late stage and chronicity of the disease. We demonstrated that the microparticles containing *P. granatum* extract significantly decreased the number of cells in BAL compared to the group of asthmatic and non-treated animals (OVA). The same phenomenon was observed in groups treated with the non-encapsulated extract or Dexamethasone. This fact may be related to the expression of endothelial adhesion molecules. The trafficking of leukocytes between the bloodstream and the extravascular space is controlled by the vascular endothelium. A cascade of sequential leukocyte-endothelial cell adhesive interactions that involves a variety of cell adhesion molecules (CAMs) present on leukocytes and endothelial cells mediate the migration of leukocytes from the blood to sites of inflammation or injury extravascular [27]. In this context, a study with crude ethanolic extract prepared from freshly collected leaves of *P. granatum* assessed its ability to inhibit TNF- α -induced ICAM-1 (intercellular cell adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and E-selectin expression on human umbilical vein endothelial cells (HUVECs). The results showed that the extract significantly inhibited the expression of ICAM-1 (55 %), VCAM-1 (57 %) and E-selectin (51 %) on HUVECs. Also, it was screened the ability of ethanolic extract to inhibit the adhesion of neutrophils to the endothelium (56 %) [28]. In this regard and bringing those results to our study, we can suggest that the

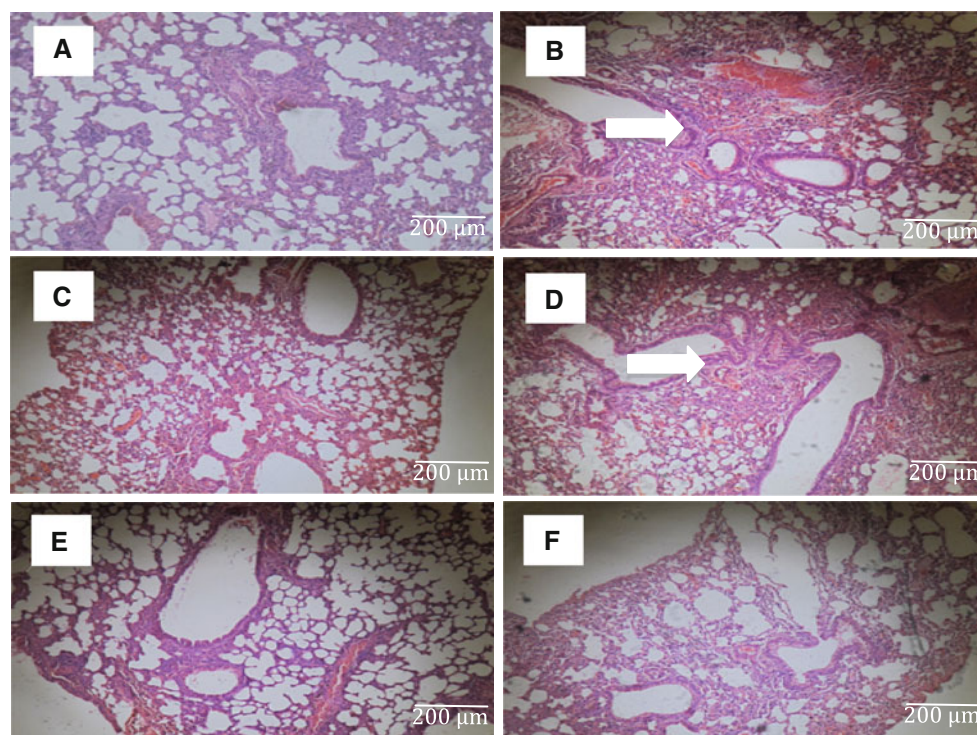


Fig. 6 Representative lung sections from the groups: saline (a), Ova (b), dexamethasone (c), control microparticles (d), microparticles containing Pomegranate extract (e) and non-encapsulated

Pomegranate extract (f). The cell infiltration in the lung tissues was confirmed by hematoxylin an eosin staining White arrows show leukocytes' infiltration. Magnification $\times 100$

decreasing numbers of total leukocytes present in the BAL (especially eosinophils) and also in the pulmonary parenchyma of animals observed in our study may be related to the inhibitory action of the extract released by the particles into the lungs after intranasal instillation. Studies showed that Pomegranate extracts are able to reduce inflammatory mediators in several experimental models, but few data are known about anti-allergic specific properties [24]. Interestingly, the group of animals that received treatment with microparticles without any encapsulated molecule showed an increase in cell numbers recruited to BAL, also reflected by cell infiltration in lung tissue. This finding might be due to the fact that microparticles per se constitute an inflammatory stimulus after the uptake process by phagocytic cells. This process leads to the translocation of the nuclear transcription factor NF- κ B to the cell nucleus and markedly stimulates the production of inflammatory cytokines, including IL-1 β [29]. This was evidenced here in our study by the significant increase in the number of neutrophils recruited to BAL from the group of animals that received control microparticles.

The analysis of the extract of *P. granatum* using HPLC showed phytochemical profile similarly to data described in the literature for this plant, which mainly describe the presence of hydrolysable tannins (punicalin, punicalagin, corilagin, casuarinin and pedunculagin) and flavonols

(quercetin and kampferol), catechins (catechin, epicatechin and 3-gallate epicagalocatechin) [13]. A variety of molecules present in this plant implies in many different biological activities, as well as a variety of mechanisms of action. In this context, the analysis of zeta potential was evaluated to verify whether the encapsulation of the ethanolic Pomegranate extract would modify the negative charge of the polymer used to produce the microparticles. The results showed that microparticles containing the extract present lower zeta potential value compared to control microparticles (-1.37 vs. -0.70 mV). This finding suggests that some molecules present in the ethanolic extract could provide ions to the polymer surface.

Regarding cytokines involved in the inflammatory process, IL-1 β is one that acts in the beginning of inflammatory process and performs multiple actions on endothelial cells, including the synthesis of adhesion molecules, induction of the release of various inflammatory mediators such as prostaglandins, prostacyclins and thromboxanes. The presence of this cytokine in asthma causes the persistence of the inflammatory and continuous cell recruitment to the lungs [30]. The results show that all groups of animals treated reduced the amount of IL-1 β . In relation to IL-5, this is the primary cytokine involved in the late phase of asthma, which is responsible for the proliferation, migration and accumulation of eosinophils from

bone marrow into the blood [31, 32]. Importantly, other cytokines not assessed in this study, including IL-4, IL-13, IL-8 [33], are also important during allergic process. In this context, we also assessed TNF- α production by leukocytes from BAL (data not shown) and although administration of microparticles and PGE had significantly decreased the levels of this cytokine compared with OVA group, no difference between the treatments was achieved. The treatment with microparticles containing Pomegranate extract showed a statistically significant reduction in IL-5 compared to asthmatic non-treated animals and also to those treated with dexamethasone. The levels detected were great lower than all other groups, especially compared to animals that received control microparticles. Interestingly, the group treated with non-encapsulated Pomegranate extract showed very low levels of IL-5, undetectable in the assay. These results are in agreement with the low cell counts, especially eosinophils, recovered from the BAL of animals that received instilled microencapsulated extract. This fact suggests that these microparticles, differently from PGE, were able to modulate the immune response involved in asthma, probably by regulating the Th1/Th2 axis, as well as protecting the extract of rapid metabolism, thus prolonging its local effect in the lung tissue. Especially regarding eosinophils counts, a significant reduction was observed for MP treatment compared with OVA group, but instead, macrophages and neutrophils counts showed that no significant reduction was achieved comparing the same groups, suggesting that MP can preferentially induce a Th1 response, modulating inflammatory cells pattern for decreasing allergic response (Th2).

The protein leakage into the lung tissue is an event, which appears in the beginning of setting of inflammation. The results achieved here show that MP were able to significantly decrease protein levels in the BAL compared to OVA group. In contrast, PGE treatment did not show significant reductions in the levels measured.

When compared to conventional drug administrations (solutions, suspensions, emulsions, etc.), where the drug concentration in the bloodstream or another site presents a peak and then declines, the controlled release systems offer the great advantage of maintaining a constant/sustained drug concentration in the therapeutic range for a prolonged period, using single or reduced doses. Also, without these intentions, these systems can confer a different pattern of cell activation for managing inflammatory/infectious processes. From a clinical perspective, therapeutic concentration control is important because it increases the therapeutic efficacy and also reduces the potential toxicity that could be achieved with crude extracts in solution forms.

Taken together, our results show that the treatment of asthma-induced mice with microencapsulated pomegranate

extract reduces the expression of the key markers involved in lung allergic inflammation. We can infer that the secondary metabolites present and preserved into microparticles play an important role (Th1 response) in inhibiting eosinophil recruitment, suggesting that this cell type may also be modulated by the extract released into the lungs.

In conclusion, in this study we demonstrated for the first time that the ethanolic extract from the leaves of *P. granatum* presents significant biological activity in a murine model of asthma and more important, this extract encapsulated in biodegradable microparticles has greater potential therapeutic effect than the extract in solution form. This approach can be alternatively/complementary delivered to the lungs, regarding the treatment of inflammatory/allergic diseases.

Acknowledgments This study was supported by Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Maranhão (FAPEMA) and Universidade Ceuma, São Luis, Maranhão. We would like to thank the technician Ana Cleia Pestana from the Universidade Ceuma for the assistance in the histological sections.

References

1. Barnes PJ. Pathophysiology of asthma. *Eur Respir Mon.* 2003;8:84–113.
2. Tattersfield AE, Knox AJ, Britton JR, Hall IP. Asthma. *Lancet.* 2002;360:1313–22.
3. Barnes PJ. New drugs for asthma. *Nat Rev Drug Discov.* 2004;3:831–44.
4. Yang EJ, Lee JS, Yun CY, Kim JH, Kim JS, Kim DH, Kim IS. Inhibitory effects of *Duchesnea chrysanth* extract on ovalbumin-induced lung inflammation in a mouse model of asthma. *J Ethnopharmacol.* 2008;118:102–7.
5. Yuk JE, Woo JS, Yun CY, Lee JS, Kim JH, Song GY, Yang EJ, Hur IK, Kim IS. Effects of lactose- β -sitosterol and β -sitosterol on ovalbumin-induced lung inflammation in actively sensitized mice. *Int Immunopharmacol.* 2007;7:1517–27.
6. Clardy J, Walsh C. Lessons from natural molecules. *Nature.* 2004;432:829–37.
7. Verpoorte R. Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. *Drug Discov Today.* 1999;3:232–8.
8. Koehn FE, Carter GT. The evolving role of natural products in drug discovery. *Nat Rev Drug Discov.* 2005;4:206–20.
9. Rogerio AP, Fontanari C, Borducchi E, Keller AC, Russo M, Soares EG, Albuquerque DA, Faccioli LH. Anti-inflammatory effects of *Lafoensia pacari* and ellagic acid in a murine model of asthma. *Eur J Pharmacol.* 2008;580:262–70.
10. Rogerio AP, SÁ-NUNES A, Faccioli LH. The activity of medicinal plants and secondary metabolites on eosinophilic inflammation. *Pharmacol Res.* 2010;62:298–307.
11. Jurenka JS. Therapeutic applications of pomegranate (*Punica granatum* L.): a review. *Altern Med Rev.* 2008;13:128–44.
12. Fischer UA, Carle R, Kammerer DR. Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD-ESI/MSn. *Food Chem.* 2011;127:807–21.

13. Lansky EP, Newman RA. *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *J Ethnopharmacol.* 2007;109:177–206.
14. Lansky E, Shubert S, Newman I. Pharmacological and therapeutic properties of pomegranate. *CIHEAM–Options Mediterraneennes.* 2004; 231–235.
15. Lee SI, Kim BS, Kim KS, Lee S, Shin KS, Lim JS. Immune-suppressive activity of punicalagin via inhibition of NFAT activation. *Biochem Biophys Res Commun.* 2008;371:799–803.
16. Romier B, Van De Walle J, During A, Larondelle Y, Schneider YJ. Modulation of signalling nuclear factor-kappaB activation pathway by polyphenols in human intestinal Caco-2 cells. *Br J Nutr.* 2008;100:542–51.
17. Nicolete R, Lima KM, Rodrigues Júnior JM, Baruffi MD, Medeiros AI, Bentley MVLB, Silva CL, Faccioli LH. In vitro and in vivo activities of leukotrienes B4-loaded biodegradable microspheres. *Prostaglandins Other Lipid Mediators.* 2007;83: 121–9.
18. Nicolete R, Rius C, Piqueras L, Jose PJ, Sorgi CA, Soares EG, Sanz MJ, Faccioli LH. Leukotriene B4-loaded microspheres: a new therapeutic strategy to modulate cell activation. *BMC Immunol.* 2008;9:1–11.
19. Oh YJ, Lee J, Seo JY, Rhim T, Kim S, Yoon HJ, Lee KY. Preparation of budesonide-loaded porous PLGA microparticles and their therapeutic efficacy in a murine asthma model. *J Controlled Release.* 2011;150:56–62.
20. Sung JC, Pulliam BL, Edwards DA. Nanoparticles for drug delivery to the lungs. *Trends Biotechnol.* 2007;25:563–70.
21. Pilcer G, Amighi K. Formulation strategy and use of excipients in pulmonary drug delivery. *Int J Pharm.* 2010;392:1–19.
22. Bekir J, Mars M, Souchard JP, Bouajila J. Assessment of anti-oxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of pomegranate (*Punica granatum*) leaves. *Food Chem Toxicol.* 2013;55:470–5.
23. Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant Activity of Pomegranate Juice and Its Relationship with Phenolic Composition and Processing. *J Agric Food Chem.* 2000;48:4581–9.
24. Ismail T, Sestili P, Akhtar S. Pomegranate peel and fruit extracts: a review of potential anti-inflammatory and anti-infective effects. *J Ethnopharmacol.* 2012;143:397–405.
25. Wager S, Lundblad LKA, Ekman M, Irvin CG, Bates JHT. The allergic mouse model of asthma: normal smooth muscle in an abnormal lung? *J Appl Physiol.* 2004;96:2019–27.
26. Nicolete R, Secatto A, Pereira PAT, Soares EG, Faccioli LH. Leukotriene B4-loaded microspheres as a new approach to enhance antimicrobial responses in *Histoplasma capsulatum*-infected mice. *Int J Antimicrob Agents.* 2009;4:365–9.
27. Cook-Mills JM, Deem TL. Active participation of endothelial cells in inflammation. *J Leukoc Biol.* 2005;77:487–95.
28. Balwani S, Nandi D, Jaisankar P, Ghosh B. 2-Methyl-pyran-4-one-3-O- β -D-glucopyranoside isolated from leaves of *Punica granatum* inhibits the TNF α -induced cell adhesion molecules expression by blocking nuclear transcription factor- κ B (NF- κ B). *Biochimie.* 2011;93:921–30.
29. Nicolete R, Santos DF, Faccioli LH. The uptake of PLGA micro or nanoparticles by macrophages provokes distinct in vitro inflammatory response. *Int Immunopharmacol.* 2011;11:1557–63.
30. Whelan R, Kim C, Chen M, Leiter J, Grunstein MM, Hakonarson H. Role and regulation of interleukin-1 molecules in pro-asthmatic sensitised airway smooth muscle. *Eur Respir J.* 2004;24: 559–67.
31. Faccioli LH, Mokwa VF, Silva CL, Rocha GM, Araujo JI, Nahori MA, Vargaftig BB. IL-5 drives eosinophils from bone marrow to blood and tissues in a guinea-pig model of visceral larva migrans syndrome. *Mediators Inflamm.* 1996;5:24–31.
32. Rogerio AP, Sá-Nunes A, Albuquerque DA, Anibal FF, Medeiros AI, Machado ER, Souza AO, Prado JC Jr, Faccioli LH. *Lafioensia pacari* extract inhibits IL-5 production in toxocariasis. *Parasite Immunol.* 2003;25:393–400.
33. Lampinen M, Carlson M, Hakansson LD, Venge P. Cytokine-regulated accumulation of eosinophils in inflammatory disease. *Allergy.* 2004;59:793–805.

Copyright of Inflammation Research is the property of Springer Science & Business Media B.V. and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.