



The ethanolic extract from *Erythrina mulungu* Benth. flowers attenuates allergic airway inflammation and hyperresponsiveness in a murine model of asthma

Jowanka Amorim^a, Marcos de Carvalho Borges^a, Alexandre Todorovic Fabro^a,
Silvia Helena Taleb Contini^b, Mayara Valdevite^b, Ana Maria Soares Pereira^b, Fabio Carmona^{a,*}

^a Ribeirão Preto Medical School, University of São Paulo, Av. Bandeirantes S/N, Monte Alegre, 14049-900 Ribeirão Preto, SP, Brazil

^b Department of Biotechnology in Medicinal Plants, Ribeirão Preto University, Av. Costábile Romano 2201, 14096-900 Ribeirão Preto, SP, Brazil



ARTICLE INFO

Chemical compounds studied in this article:

erysotrine (PubChem CID: 442219)

erysotrine-N-oxide (PubChem CID:

101851986)

hypaphorine (PubChem CID: 442106)

Keywords:

Allergic asthma

Herbal medicine

Inflammation

Fabaceae

Ovalbumin

Bronchial hyperresponsiveness

ABSTRACT

Ethnopharmacological relevance: *Erythrina mulungu* Benth. ("mulungu", Fabaceae) is a Brazilian native species with ethnopharmacological use for respiratory diseases. However, the effects of *E. mulungu* on the respiratory were never studied.

Aims of the study: To evaluate the effects of an ethanolic extract from flowers of *E. mulungu* in ovalbumin (OVA)-induced asthma in mice, and to study the mechanisms involved.

Materials and methods: OVA-sensitized mice were intraperitoneally (i.p.) treated with four doses (200, 400, 600, and 800 mg/kg) of the *E. mulungu* extract or dexamethasone (DEXA, 2 mg/kg) during seven consecutive days and simultaneously challenged with intranasal OVA. Bronchial hyperresponsiveness was evaluated *in vivo*, 24 h after the last OVA challenge. Bronchoalveolar lavage (BAL) was collected for counting the number of total and differential inflammatory cells. Blood was collected for measurement of anti-OVA IgE levels. Levels of cytokines interleukin (IL)–4, IL-5, IL-10, IL-13, and interferon (INF)- γ were measured in pulmonary homogenate by ELISA. The recruitment of inflammatory cells to the lung tissue was determined using hematoxylin and eosin staining (H&E). The extract's chromatographic profile was evaluated by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS).

Results: The treatment with *E. mulungu* extract significantly reduced bronchial hyperresponsiveness, significantly reduced the number of leukocytes, eosinophils, and lymphocytes in BAL, and significantly decreased the levels of IL-4 and IL-5, while increased levels of IL-13 and INF- γ . In addition, *E. mulungu* significantly decreased the cellular inflammatory infiltration in the lung tissue. Erysotrine, erysotrine-N-oxide, and hypaphorine were the major constituents identified in the extract.

Conclusion: Collectively, these results confirm the potential of *E. mulungu* for asthma treatment, through modulation of inflammatory response, supporting its ethnopharmacological use for respiratory diseases.

1. Introduction

Erythrina mulungu Benth. ("mulungu", Fabaceae) is a native Brazilian medicinal plant (Lorenzi and Matos, 2008) that has been traditionally used for anxiety, insomnia, seizures, menopause, gingivitis, hepatitis, respiratory diseases, and as a general anti-inflammatory (de Albuquerque et al., 2007; Lorenzi and Matos, 2008; Rodrigues and Carvalho, 2001; Vasconcelos et al., 2007, 2003). Many of these pharmacological properties were studied and well documented *in vitro* and *in vivo* (Lima et al., 2006; Vasconcelos et al., 2011, 2007). However, reports of the effects of *E. mulungu* on the respiratory system are scarce.

The objective of this study was to validate the ethnopharmacological use of *E. mulungu* for the treatment of respiratory conditions, by evaluating the effect of an ethanolic extract of *E. mulungu* flowers in bronchial hyperresponsiveness and markers of tissue inflammation in a murine model of ovalbumin (OVA)-induced asthma.

2. Material and methods

Preparation of the extract and phytochemical analysis were conducted at the Laboratory of Biotechnology in Medicinal Plants, University of Ribeirão Preto (UNAERP), while the experimental tests

* Corresponding author.

E-mail address: carmona@usp.br (F. Carmona).

<https://doi.org/10.1016/j.jep.2018.08.009>

Received 28 April 2018; Received in revised form 8 August 2018; Accepted 9 August 2018

Available online 10 August 2018

0378-8741/ © 2018 Published by Elsevier B.V.

were performed in the Laboratory of Experimental Lung Pathophysiology, Ribeirao Preto Medical School (FMRP), University of Sao Paulo (USP). The study was approved by local Ethics Committee on Animal Research (Protocol #072/2013).

2.1. Plant material

The flowers of *E. mulungu* were harvested at 10 a.m. at the rural area of Jardinopolis, Sao Paulo, Brazil (latitude 21°4'33" S, longitude 47°44'48" W), where it naturally grows. The specimens were identified by a specialist, Milena Ventrichi Martins, PhD. A voucher specimen was deposited in the Herbarium of Medicinal Plants at UNAERP (voucher #3168).

2.2. Preparation of the ethanolic extract of *E. mulungu*

The stem bark and the leaves are more frequently used as medicine, while flowers are less used (Flausino et al., 2007a, 2007b; Onusic et al., 2002, 2003; Ribeiro et al., 2006). We chose to use flowers because, in our clinical experience, they are more effective in the respiratory system, and also because harvesting roots or stem barks often leads to the death of the plant and can threaten the species. The flowers of *E. mulungu* were dried in a circulating-air oven at 45 °C for 48 h and sieved up to particle size of 40 mesh. The resulting powder is called powdered plant material. This powder (1.02 kg) was macerated with ethanol (5.6 L) for seven days, with daily agitation, filtered through paper filter, and then concentrated (rotary evaporator and freeze drier), resulting in 32.4 g of crude dry extract [drug-extract ratio (DER) of 31:1].

2.3. Extraction and isolation of alkaloids

The crude dry extract (32.4 g) was acidified with a 10% acetic acid solution and extracted with CHCl_3 . Acid solution was alkalized (pH 9–10) with NH_4OH and re-extracted with CHCl_3 , affording an alkaloid mixture fraction (10.7 g).

The alkaloid fraction (1.0 g) was purified by classical chromatography on silica gel 60 (15.0 g), eluting with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (8:2) to yield 90 fractions of 20 mL each. These fractions were analyzed by thin-layer chromatography on silica gel 60 with fluorescent indicator UV₂₅₄ (Macherey-Nagel®) plates eluted with $\text{CHCl}_3:\text{CH}_3\text{OH}$ (7:1). Spots were visualized after spraying with Dragendorff's reagent solution.

Fractions with similar TLC profiles were grouped, and fraction 4 (0.230 g) was purified by classical chromatography on silica gel 60 (3.0 g), eluting with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (9:1) to yield 20 fractions of 2 mL each. These fractions were analyzed by thin-layer chromatography on silica gel 60 with fluorescent indicator UV₂₅₄ (Macherey-Nagel®) plates eluted with $\text{CHCl}_3:\text{CH}_3\text{OH}$ (7:1). Spots were visualized after spraying with Dragendorff's reagent solution, and fractions with similar TLC profiles were grouped into eight fractions. Fractions 3, 4 and 5 were purified separately by preparative TLC on silica gel 60 with fluorescent indicator UV₂₅₄ (Macherey-Nagel®), eluted with $\text{C}_7\text{H}_8:\text{C}_3\text{H}_6\text{O}:\text{C}_2\text{H}_6\text{O}:\text{NH}_4\text{OH}$ (45:45:7:3). Spots were visualized after spraying with Dragendorff's reagent solution.

All these procedures resulted in purification of three alkaloids erysotrine (0.013 g), erysotrine-N-oxide (0.009 g) and hypaphorine (0.005 g), which were identified by NMR spectroscopy. ^1H - (500 MHz) and ^{13}C -NMR (125 MHz) spectra were obtained on a Bruker DPX 500 spectrometer.

2.4. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) analysis of ethanolic extract of the *E. mulungu*

The identities of the erysotrine, erysotrine-N-oxide, and hypaphorine, present in the extract, were confirmed by UPLC-MS analysis using authentic standards of compounds on a Waters (Milford, MA, USA) Acquity UPLC H-Class system equipped with a PDA detector and a

Waters Xevo TQ-S tandem quadrupole mass spectrometer with an electrospray source operating in the positive mode. The samples injection volume was 5 μL in a Zorbax Eclipse XDB-C18 column (150 $\times$ 4.6 mm i.d.; 3.5 μm particle size) from Agilent. The mobile phase used for gradient elution consisted of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) at a flow rate of 0.5 mL min⁻¹. The gradient elution program started with 15% B, raised B to 60% in the following 25 min, then raised B to 90% in the following 5 min, and after raised B to 95% in the following 2 min which remained at 95% B for 3 min, and returned to the initial condition (15% B) within the following 5 min. The source and operating parameters were optimized as follows: capilar voltage = 3.2 kV, source temperature = 150 °C, desolvation temperature (N_2) = 350 °C, desolvation gas flow = 600 L h⁻¹, and mass range from m/z 100–500 in the full-scan mode. For more details, see the [Supplementary Material](#) provided.

2.5. Experimental protocol

Male Balb/c mice (20–30 g), six to eight weeks old, were used in the experiments. The animals were kept in cages with controlled light/dark cycle (12 h each), temperature of 25 °C, and access to food and water *ad libitum*. Groups of 5–11 animals were used in all experiments. Briefly, animals were sensitized twice, on days 0 and 7, by intraperitoneal (i.p.) injection of 10 μg of ovalbumin (OVA) and 1 mg of aluminum hydroxide, in a total volume of 200 μL . On days 15, 17, 19, and 21, the animals were challenged in alternate days by nasal instillation of a solution containing 10 μg of OVA (total of 10 μL) while under mild sedation with isoflurane (100%) (Azevedo et al., 2018; Bates et al., 2009; Borges et al., 2013; Morel et al., 2016). *E. mulungu* or dexamethasone (DEXA) (Decadron®, Aché, São Paulo, Brazil), was administrated i.p., daily, for 7 consecutive days. Seven groups were studied, as described below:

- SAL/SAL – sensitized and challenged with normal saline (SAL), treated with SAL.
- OVA/SALTW – sensitized and challenged with OVA, treated with normal saline plus 2% Tween 80;
- OVA/EM200 – sensitized and challenged with OVA, treated with *E. mulungu* (200 mg/kg);
- OVA/EM400 – sensitized and challenged with OVA, treated with *E. mulungu* (400 mg/kg);
- OVA/EM600 – sensitized and challenged with OVA, treated with *E. mulungu* (600 mg/kg);
- OVA/EM800 – sensitized and challenged with OVA, treated with *E. mulungu* (800 mg/kg);
- OVA/DEXA – sensitized and challenged with OVA, treated with DEXA (2 mg/kg).

2.6. Lung mechanics

Bronchial hyperresponsiveness (BHR) assessment was made at increasing concentrations of methacholine (Mch), as previously described (Borges et al., 2013; Morel et al., 2016). Briefly, 24 h after the last challenge, animals were anesthetized with ketamine (10 mg/kg i.p.) and xylazine (80 mg/kg i.p.). A tracheal cannula was placed by tracheostomy, which was connected to a ventilator for small animals (flexiVent®, Scireq, Montreal, Canada). The respiratory rate was set at 150/minute and a positive end-expiratory pressure (PEEP) of 3 cm H_2O was used. Pancuronium bromide (1.2 mg/kg i.p.) was administered to induce complete muscle paralysis. Measurements of lung mechanics were performed under basal conditions and after exposure to increasing doses of methacholine (6.25, 12.5, and 25 $\mu\text{g}/\text{mL}$) with an ultrasonic nebulizer (Hudson RCI, Temecula, CA, USA). The parameters measured were selected from curves with coefficient of determination ≥ 0.9 . There were analyzed: the total respiratory system resistance (Rrs) and elastance (Ers), and tissue resistance (G) and elastance (H). After the experiment, the animals were disconnected from the ventilator.

2.7. Bronchoalveolar lavage (BAL) collection

BAL was collected immediately after disconnection from the ventilator, as described previously (Morel et al., 2017). Briefly, 1 mL of SAL was instilled in the tracheal cannula with subsequent aspiration with syringe. This was repeated twice, yielding 2 samples from each animal. The total number of cells was determined with Countess Automated Cell Counter (Life Technologies, Carlsbad, California, EUA). Differential cell counts, including eosinophils, lymphocytes, neutrophils, and macrophages, were determined by microscopic counting of 300 cells. For this, BAL samples were centrifuged (Cytospin™, Thermo Fisher Scientific, Waltham, USA). The cells were separated and stained using Diff-Quik Stain reagent (B4132-1A; Dade Behring Inc., Deerfield, IL) according to the manufacturer's instructions.

2.8. Measurement of serum IgE levels

Blood was collect from the right ventricle by direct puncture. Serum was obtained by centrifugation and stored at -80°C until assay. The measurement of OVA-specific IgE levels was performed using ELISA (OptEIA™, BD Biosciences, São Paulo, Brazil), according to the manufacturer's instructions and previously described (Prado et al., 2015).

2.9. Cytokines in lung homogenate

The right lung was harvested and stored in RNeasy™ (Ambion, Thermo Fisher Scientific) at -80°C until analysis. The lung homogenate was prepared with 50 mg of lung tissue and 2 mL of phosphate buffered saline (PBS) plus a complete protease inhibitor cocktail (Roche Diagnostics, Laval, PQ, Canada). After homogenized, the samples were centrifuged, and the supernatants were collected and stored at -80°C for subsequent quantification of cytokines. Th2 and Th1 cytokines [interleukin (IL)–4, IL-5, IL-13, IFN- γ , and IL-10] were quantified using ELISA kits (BD OptEIA™, BD Biosciences, São Paulo, Brazil), according to the manufacturer's instructions.

2.10. Histological assessment of lung tissue

The left lung was harvested and insufflated with 10% neutral buffered formalin (NBF) solution and then fixed in NBF for 3 days, embedded in paraffin, sectioned ($5\mu\text{m}$) and stained with hematoxylin & eosin for evaluation of inflammatory cell infiltration. Four to five airways per animal were photographed ($400\times$), and the photos were analyzed by two researchers, one of them blinded to treatment. An inflammation score for peribronchial and perivascular inflammation was used (Azevedo et al., 2018; Sur et al., 1999): 0, 1, 2, 3, and 4, corresponding to none, mild, moderate, marked, or severe inflammation, respectively. The sum of scores defined the total lung inflammation.

2.11. Statistical analysis

Results of different groups were compared with 1-way or 2-way repeated-measurements ANOVA, with Bonferroni correction for multiple comparisons. Significance level was set at 0.05. All statistical analyses were performed using Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Identification of major compounds within the ethanolic extract of the *E. mulungu*

The phytochemical analysis revealed the presence of the major constituents of the plant in the extract of *E. mulungu*: erysotrine, erysotrine-N-oxide, and hypaphorine (Fig. 1 and Fig. 2).

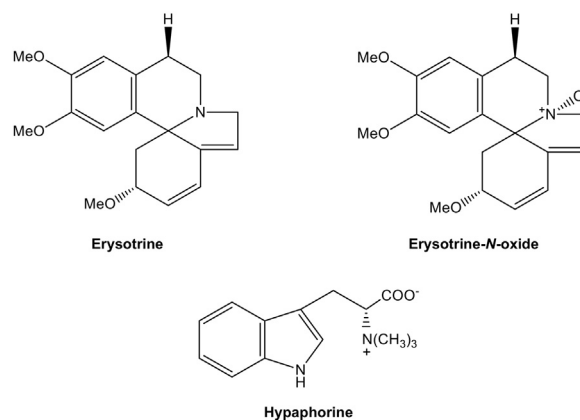


Fig. 1. Chemical structures of erysotrine, erysotrine-N-oxide, and hypaphorine.

3.2. Inflammatory cells in BAL

Induction of asthma resulted in higher counts of inflammatory cells (leukocytes, eosinophils, and lymphocytes) in BAL. Treatment with different doses of *E. mulungu* extract significantly decreased the total cell count (600mg/kg), eosinophil count (600 and 800mg/kg), and lymphocyte count (200, 400, and 600mg/kg), as did DEXA. The dose of 600mg/kg was consistently effective. The number of macrophages and neutrophils were not affected by any treatment. These results are shown in Fig. 3.

3.3. Lung mechanics

Results of BHR are shown in Fig. 4. Overall, induction of asthma resulted in worsening of all parameters measured (Rrs, Ers, G, and H). All treatments, including DEXA, resulted in significantly decreased parameters, as compared to OVA/SALTW, at Mch 25 and 50 mg/mL. Moreover, the effect of the extract, in all studied doses, was less marked than that of DEXA.

3.4. Concentration of cytokines in lung homogenate

OVA induced a significant elevation of IL-13 concentration, but not of other cytokines, in lung homogenate (Fig. 5). Treatment with *E. mulungu* extract at 600mg/kg, but not at other doses, induced a significant decrease on IL-4, IL-5, and IL-10 concentrations. DEXA also led to lower IL-4 concentrations. Interestingly, the extract of *E. mulungu* (400, 600, and 800mg/kg) induced further significant increases in IL-13 concentrations, in a dose-dependent manner, while DEXA elicited an opposite, significant effect. Moreover, *E. mulungu* extract (200 and 400mg/kg) led to significantly increased IFN- γ concentrations, while higher doses of *E. mulungu* or DEXA did no affect.

3.5. IgE serum levels

The allergic response induced by OVA increased the serum concentration of OVA-specific IgE. However, IgE levels were not affected by any of the treatments (Fig. 6).

3.6. Inflammatory cells in lung tissue

Peribronchial inflammation was more intense in OVA-induced asthmatic mice. Treatment with *E. mulungu* extract (400, 600, and 800 mg/kg) or DEXA significantly lessened the inflammatory infiltrates (Fig. 7).

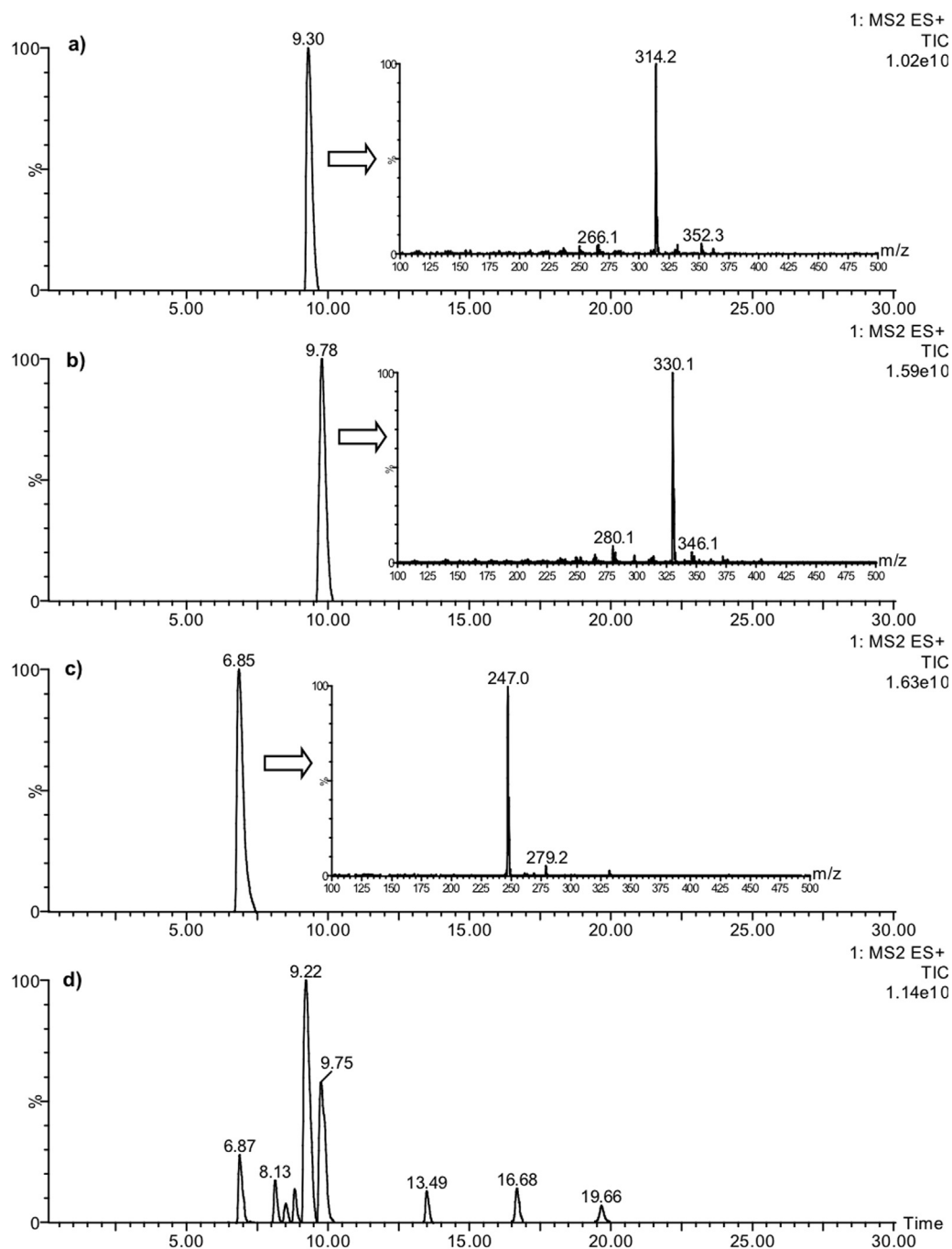


Fig. 2. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) of standards (a) erysotrine; (b) erysotrine-N-oxide; (c) hypaphorine; and (d) chromatograms of the crude ethanolic extract of *Erythrina mulungu* flowers.

4. Discussion

In our study, an ethanolic extract from flowers of *E. mulungu*, containing the alkaloids erysotrine, erysotrine-N-oxide, and hypaphorine, significantly improved lung mechanics in asthmatic mice, reduced the number of inflammatory cells in BAL and in lung tissue, lowered concentrations of IL-4, IL-5, and IL-10, and augmented levels of IL-13 and IFN- γ in lung homogenates. Taken together, our results suggest that flowers of *E. mulungu* have potential to treat asthma because of an anti-inflammatory effect. This is the first literature report of the use of flowers of *E. mulungu* to treat asthma, confirming its ethnopharmacological use for respiratory diseases.

The anti-inflammatory effects of *E. mulungu* have already been

shown in animal models (de Oliveira et al., 2011; Vasconcelos et al., 2011). These authors suggested that the anti-inflammatory effect of *E. mulungu* is due to the presence of anti-inflammatory compounds that modulate synthesis and release of several inflammatory mediators, such as prostaglandins, nitric oxide and cytokines like IL-1 and tumor necrosis factor (TNF)- α . Considering that eosinophils play an important role in asthma severity (Fahy, 2015) and exacerbations (Lee et al., 2009), we believe that, in our study, *E. mulungu*, decreased bronchial hyperresponsiveness because it reduced the migration of inflammatory cells, mostly eosinophils and lymphocytes, to the airways and lung parenchyma. We suggest that this reduced recruitment of eosinophils could be, at least in part, due to the inhibition of expression of IL-4 and IL-5, which stimulate maturation of eosinophils in bone marrow and

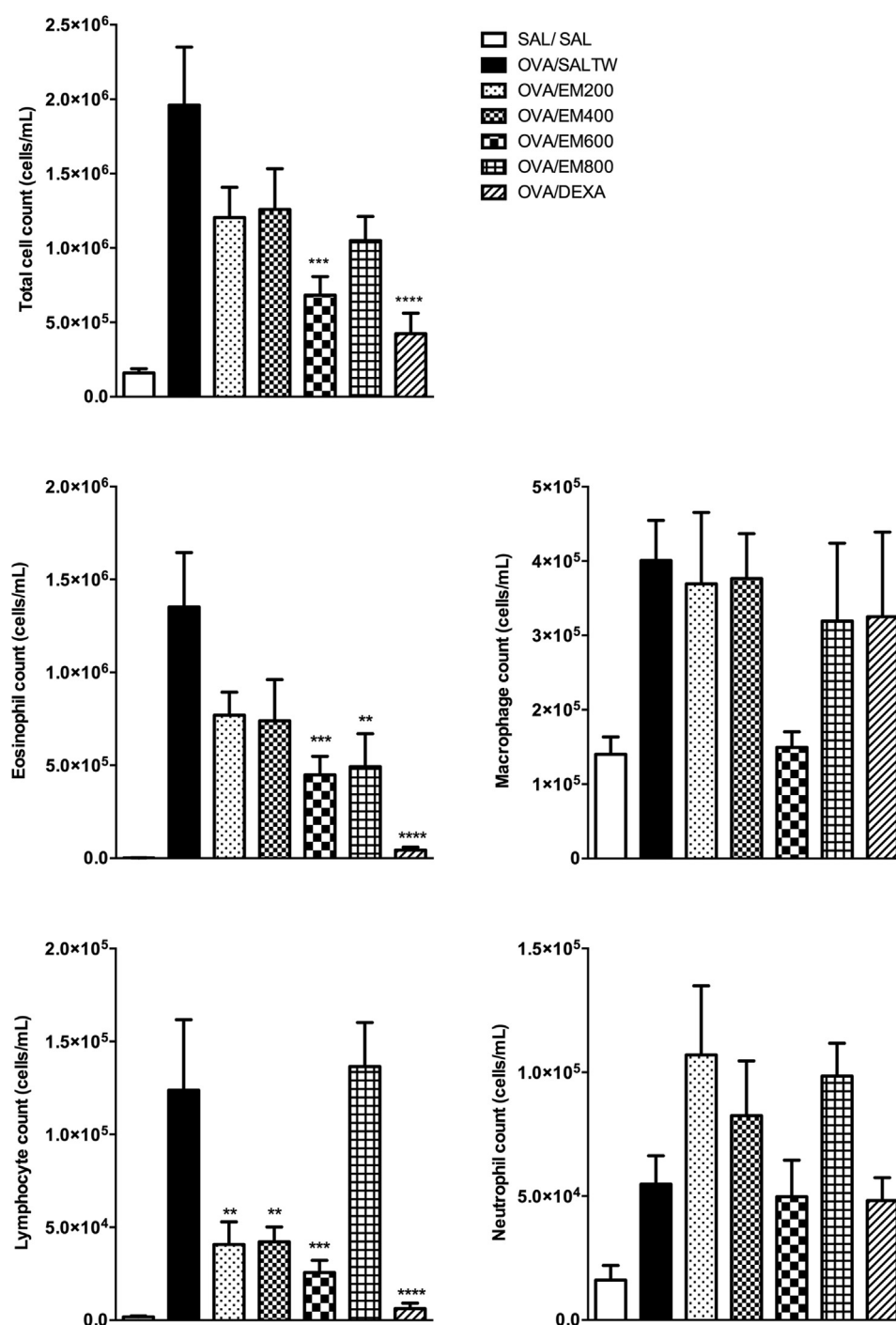


Fig. 3. Total and differential cell counts in bronchoalveolar lavage (BAL) in animals treated with different doses of the extract of *Erythrina mulungu*. Only significant treatment effects are marked. Legend: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; SAL, normal saline; OVA, ovalbumin; SALTW, normal saline plus 2% Tween 80; EM200, EM400, EM600, and EM800, *E. mulungu* extract, at 200, 400, 600, or 800mg/kg, respectively; DEXA, dexamethasone.

recruit these cells to the tissues (Abbas et al., 2012; Gour and Wills-Karp, 2015). The molecular mechanisms involved are yet to be clarified.

On the other hand, treatment with *E. mulungu* led to higher concentrations of IL-13 in the lungs. IL-13 is important in allergic responses, being involved in the migration of eosinophils, hyperresponsiveness, secretion of mucus, bronchoconstriction, and fibrosis. However, the underlying mechanisms for these effects are not well elucidated (Gour and Wills-Karp, 2015). The fact that *E. mulungu* led to higher IL-13 lung concentrations could explain why the treatments

were not effective in all doses or why we did not observe changes in IgE levels.

We also observed that treatment with *E. mulungu* resulted in higher concentrations of interferon (IFN)- γ in the lungs. IFN- γ is a typical cytokine from Th1 inflammatory responses (Iwasaki et al., 2016). Since inflammation in asthma is unbalanced towards the Th2 profile, any stimuli to Th1 may ameliorate asthma symptoms (Shen et al., 2008). This may have contributed to the effects we observed in lung mechanics and inflammation.

Overall, the extract of *E. mulungu* was less effective than DEXA,

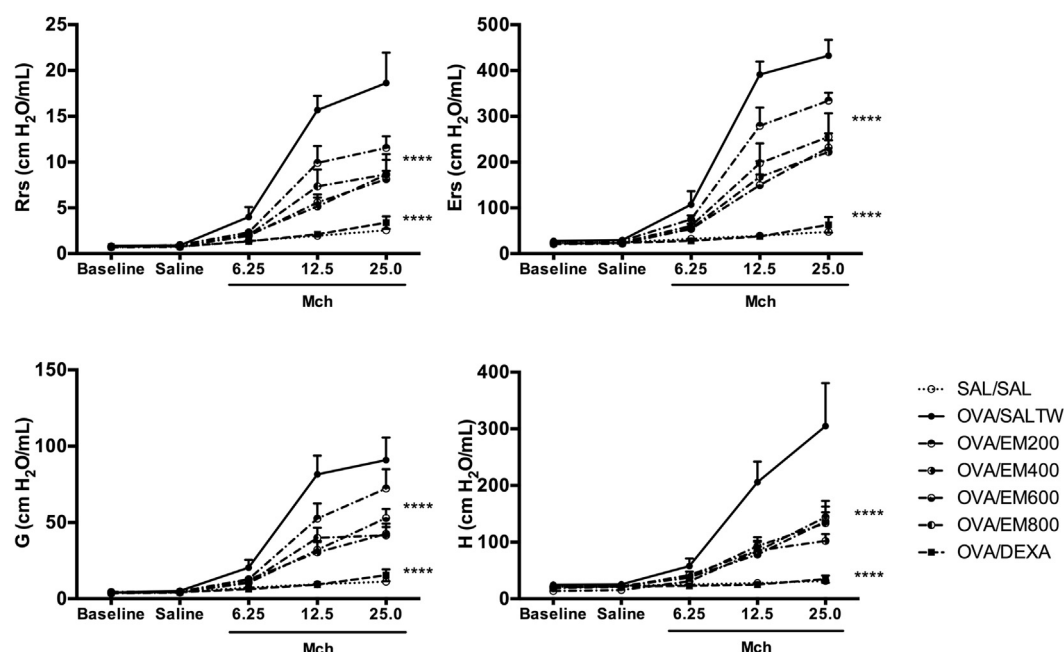


Fig. 4. *In vivo* measurements of lung mechanics under increasing concentrations of aerosolized metacholine (Mch) in animals treated with the extract of *Erythrina mulungu*. Legend: ****, $p < 0.0001$ for all treatments (EM and DEXA) versus OVA/SALTW at Mch 25 and 50mg/mL; Rrs, total respiratory system resistance; Ers, total respiratory system elastance; G, tissue resistance; H, tissue elastance; SAL, normal saline; OVA, ovalbumin; SALTW, normal saline plus 2% Tween 80; EM200, EM400, EM600, and EM800, *E. mulungu* extract, at 200, 400, 600, or 800mg/kg, respectively; DEXA, dexamethasone.

noticeably on eosinophil count in BAL, lung mechanics and peribronchial inflammation. However, besides in exacerbations, systemic corticosteroids are not chronically used in asthma treatment because they can elicit serious adverse events, such as osteoporosis, adrenal suppression, and metabolic syndrome. Therefore, *E. mulungu* presents as an alternative for chronic, intercritical asthma treatment.

The alkaloids (erysotrine, erysotrine-*N*-oxide, and hypaphorine) present in our extract are characteristic of the Fabaceae family. Indeed, more than a hundred different alkaloids have been reported in the genus *Erythrina*, including: (+)-11 α -hydroxyerythravine, (+)-erythravine, (+)- α -hydroxyerysotrine, erythrartine-*N*-oxide, and erythrastemin, among others (Amer et al., 1991; Flausino, et al., 2007a, 2007b; Sarragiotto et al., 1981).

Erysotrine was isolated from flowers of *E. mulungu* and other species of *Erythrina* before, and its anticonvulsant, anxiolytic, cytotoxic and antimicrobial actions were confirmed only in preclinical studies (Iranshahi et al., 2012; Mohammed et al., 2012; Santos Rosa et al., 2012; Wanjala et al., 2002). Erysotrine-*N*-oxide is even less studied. We did not find studies on anti-inflammatory effects of erysotrine or erysotrine-*N*-oxide.

Hypaphorine has been isolated from many species of *Erythrina* and other species of plants, and induces sleep in normal mice (Ozawa et al., 2008). In a recent bioinformatics study, a predictive model was used to screen natural products for potential anti-inflammatory activity from more than 3000 candidates. Hypaphorine was in the top ten scoring molecules for anti-inflammatory activity (Aswad et al., 2018). In fact, there are evidences that hypaphorine can decrease the production of inflammatory cytokines induced by lipopolysaccharide (LPS) in endothelial cells via inhibition of toll-like receptor 4 (TLR4) and activation of peroxisome proliferator-activated receptor (PPAR)- γ , dependent on AMP-activated protein kinase (AMPK) signaling pathway (Sun et al., 2017b, 2017c) or via inhibition of extracellular signal-regulated kinase (ERK) or/and nuclear factor kappa-B (NF- κ B) signaling pathways (Sun et al., 2017a).

The literature reports that *E. mulungu*, besides alkaloids, also contains different types of flavonoids, coumestanes, lignans, and terpenes. Many of these classes of compounds have anti-inflammatory activity

(Majinda et al., 2005). We speculate that these compounds, likely present in our extract, contributed, at least in part, to the observed anti-inflammatory activity, possibly synergistically.

E. mulungu is considered safe for internal use, but high doses can be dangerous. De Bona et al. have reported a LD₅₀ of 1370 mg/kg for the flowers (de Bona et al., 2012). In our study, a dose of 800 mg/kg was lethal for 45% of animals. Interestingly, the animals did not die immediately, but only after induction of anesthesia. We suspect that interaction or synergism between *E. mulungu* and the anesthetic agents led to respiratory depression and cardiac arrest. In fact, *E. mulungu* and erysotrine have well-documented effects on the central nervous system (Flausino et al., 2007a, 2007b; Onusic et al., 2002, 2003; Ribeiro et al., 2006). Therefore, caution should be taken when planning a clinical study, where concomitant use of any drug acting in the central nervous system being an exclusion criterion.

In summary, we hypothesized that the following events were sequentially elicited by *E. mulungu*: (1) modulation of production and release of cytokines (lower IL-4, IL-5 and IL-10, higher IL-13 and IFN- γ) by erythrinian alkaloids (mostly hypaphorine); (2) decreased migration of inflammatory cells to the lungs (less leukocytes, eosinophils, and lymphocytes in BAL and less leukocytes in lung tissue); and (3) better lung mechanics with decreased bronchial hyperresponsiveness (lower Rrs, Ers, G, and H).

Limitations of this study include: (a) a longer treatment course could have shown more expressive results on the release of IgE and cytokines; (b) our results may not apply to non-allergic types of asthma; and (c) the extract was administered i.p. and, therefore, we cannot assume that oral administration would elicit similar results.

5. Conclusion

In conclusion, an ethanolic extract from the flowers of *Erythrina mulungu*, containing, as major compounds, erysotrine, erysotrine-*N*-oxide, and hypaphorine, significantly improved lung mechanics in asthmatic mice, reduced the number of inflammatory cells in BAL and lung tissue, lowered concentrations of IL-4, IL-5, and IL-10, and augmented concentrations of IL-13 and IFN- γ in lung homogenates. Taken

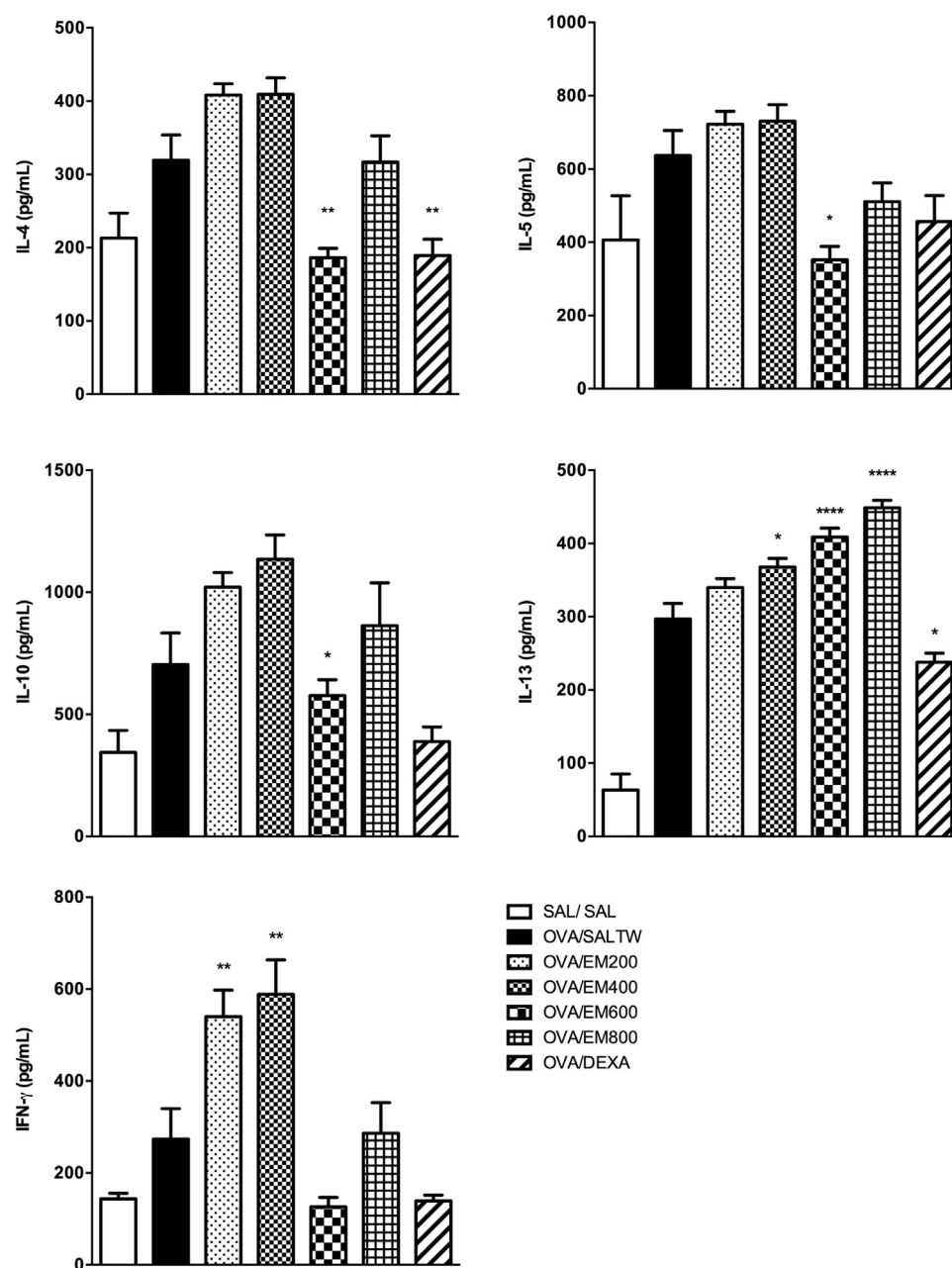


Fig. 5. Levels of cytokines in lung homogenate. Legend: *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; SAL, normal saline; OVA, ovalbumin; SALTW, normal saline plus 2% Tween 80; EM200, EM400, EM600, and EM800, *E. mulungu* extract, at 200, 400, 600, or 800mg/kg, respectively; DEXA, dexamethasone; IL, interleukin; IFN, interferon.

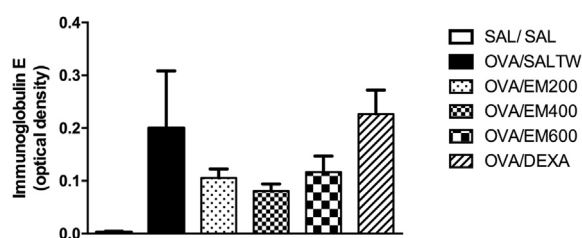


Fig. 6. Anti-ovalbumin IgE serum levels. Legend: SAL, normal saline; OVA, ovalbumin; SALTW, normal saline plus 2% Tween 80; EM200, EM400, and EM600, *E. mulungu* extract, at 200, 400, or 600mg/kg, respectively; DEXA, dexamethasone.

together, these results suggest that flowers of *E. mulungu* have potential to treat asthma because of an anti-inflammatory effect, supporting its ethnopharmacological use for respiratory diseases.

Acknowledgements

The study was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/PROSUP; Grant #00012/02–5), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Grant #473261/2013–8), and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Grant #2010/20600–4).

Conflict of interest

There is no conflict of interest.

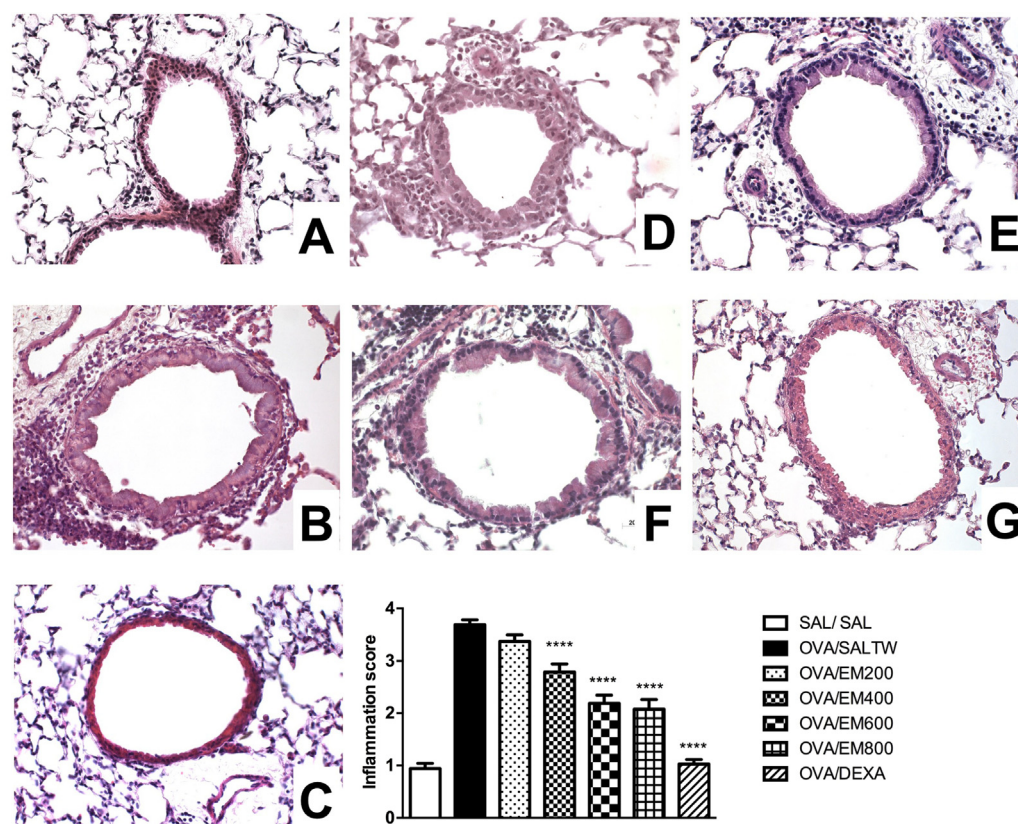


Fig. 7. Quantification of inflammatory cells in lung tissue. Panels contain selected airways from groups SAL/SAL (A), OVA/SALTW (B), OVA/DEXA (C), OVA/EM200 (D), OVA/EM400 (E), OVA/EM600 (F), and OVA/EM800 (G). Staining: H&E. Magnification: 400 ×. Legend: ****, $p < 0.0001$; SAL, normal saline; OVA, ovalbumin; SALTW, normal saline plus 2% Tween 80; EM200, EM400, EM600, and EM800, *E. mulungu* extract, at 200, 400, 600, or 800mg/kg, respectively; DEXA, dexamethasone.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jep.2018.08.009](https://doi.org/10.1016/j.jep.2018.08.009).

References

- Abbas, A.K., Lichtman, A.H., Pillai, S., 2012. *Imunologia Celular e Molecular* (7th ed.). Elsevier Ltda.
- de Albuquerque, U.P., de Medeiros, P.M., de Almeida, A.L.S., Monteiro, J.M., de Freitas Lins Neto, E.M., de Melo, J.G., dos Santos, J.P., 2007. Medicinal plants of the caatinga (semi-arid) vegetation of NE Brazil: a quantitative approach. *J. Ethnopharmacol.* 114, 325–354. <https://doi.org/10.1016/j.jep.2007.08.017>.
- Amer, M., Shamma, M., Freyer, A.J., 1991. The tetracyclic *Erythrina* alkaloids. *J. Nat. Prod.* 54, 329–363.
- Aswad, M., Rayan, M., Abu-Lafi, S., Falah, M., Raiyn, J., Abdallah, Z., Rayan, A., 2018. Nature is the best source of anti-inflammatory drugs: indexing natural products for their anti-inflammatory bioactivity. *Inflamm. Res.* 67, 67–75. <https://doi.org/10.1007/s00011-017-1096-5>.
- Azevedo, B.C., Morel, L.J.F., Carmona, F., Cunha, T.M., Contini, S.H.T., Delprete, P.G., Ramalho, F.S., Crevelin, E., Bertoni, B.W., França, S.C., Borges, M.C., Pereira, A.M.S., 2018. Aqueous extracts from *Uncaria tomentosa* (Willd. ex Schult.) DC. reduce bronchial hyperresponsiveness and inflammation in a murine model of asthma. *J. Ethnopharmacol.* 218. <https://doi.org/10.1016/j.jep.2018.02.013>.
- Bates, J.H.T., Rincon, M., Irvin, C.G., 2009. Animal models of asthma. *Am. J. Physiol. Cell. Mol. Physiol.* 297, L401–L410. <https://doi.org/10.1152/ajplung.00027.2009>.
- de Bona, A.P., Batistucci, M.C.P., Andrade, M.A., Riva, J.A.R., Perdigão, T.L., 2012. Estudo fitoquímico e análise mutagênica das folhas e inflorescências de *Erythrina mulungu* (Mart. ex Benth.) através do Teste de Micronúcleo em roedores. *Rev. Bras. Plantas Med.* 14, 344–351. <https://doi.org/10.1590/S1516-05722012000200014>.
- Borges, M.C., Marchica, C.L., Narayanan, V., Ludwig, M.S., 2013. Allergen challenge during halothane compared to isoflurane anesthesia induces a more potent peripheral lung response. *Respir. Physiol. Neurobiol.* 189, 144–152. <https://doi.org/10.1016/j.resp.2013.07.005>.
- Fahy, J.V., 2015. Type 2 inflammation in asthma—present in most, absent in many. *Nat. Rev. Immunol.* 15, 57–65. <https://doi.org/10.1038/nri3786>.
- Flausino, O., Santos, de Ávila, Verli, L., Pereira, H., Bolzani, A.M., Nunes-de-Souza, R.L., V.D.S., 2007. Anxiolytic effects of erythrinian alkaloids from *erythrina mulungu*. *J. Nat. Prod.* 70, 48–53. <https://doi.org/10.1021/np060254j>.
- Flausino, O.A., Pereira, A.M., da Silva Bolzani, V., Nunes-de-Souza, R.L., 2007. Effects of erythrinian alkaloids isolated from *Erythrina mulungu* (Papilionaceae) in mice submitted to animal models of anxiety. *Biol. Pharm. Bull.* 30, 375–378. <https://doi.org/10.1248/bpb.30.375>.
- Gour, N., Wills-Karp, M., 2015. IL-4 and IL-13 signaling in allergic airway disease. *Cytokine* 75, 68–78. <https://doi.org/10.1016/j.cyt.2015.05.014>.
- Iranshahi, M., Vu, H., Pham, N., Zencak, D., Forster, P., Quinn, R.J., 2012. Cytotoxic evaluation of alkaloids and isoflavonoids from the Australian tree *Erythrina vesperitio*. *Planta Med.* 78, 730–736. <https://doi.org/10.1055/s-0031-1298310>.
- Iwasaki, A., Foxman, E.F., Molony, R.D., 2016. Early local immune defences in the. *Nat. Publ. Gr.* <https://doi.org/10.1038/nri.2016.117>.
- Lee, M., Kim, S., Kwon, O.K., Oh, S.R., Lee, H.K., Ahn, K., 2009. Anti-inflammatory and anti-asthmatic effects of resveratrol, a polyphenolic stilbene, in a mouse model of allergic asthma. *Int. Immunopharmacol.* 9, 418–424. <https://doi.org/10.1016/j.intimp.2009.01.005>.
- Lima, M.R.F., Souza Luna, J., Santos, A.F., Andrade, M.C.C., Sant'Ana, A.E.G., Genet, J.P., Marquez, B., Neuville, L., Moreau, N., 2006. Anti-bacterial activity of some Brazilian medicinal plants. *J. Ethnopharmacol.* 105, 137–147. <https://doi.org/10.1016/j.jep.2005.10.026>.
- Lorenzi, H., Matos, F.J.A., 2008. *Plantas Medicinais no Brasil: Nativas e Exóticas*, 2a ed. Instituto Plantarum, Nova Odessa.
- Majinda, R.R.T., Wanjala, C.C.W., Juma, B.F., 2005. Bioactive non-alkaloidal constituents from the genus *Erythrina*. *Stud. Nat. Prod. Chem.* 32, 821–853. [https://doi.org/10.1016/S1572-5995\(05\)80070-5](https://doi.org/10.1016/S1572-5995(05)80070-5).
- Mohammed, M.M.D., Ibrahim, N.A., Awad, N.E., Matloub, A.A., Mohamed-Ali, A.G., Barakat, E.E., Mohamed, A.E., Colla, P.L., 2012. Anti-HIV-1 and cytotoxicity of the alkaloids of *Erythrina abyssinica* Lam. growing in Sudan. *Nat. Prod. Res.* 26, 1565–1575. <https://doi.org/10.1080/14786419.2011.573791>.
- Morel, L.J., de F., Azevedo, B.C., de Carmona, F., Contini, S.H.T., Teles, A.M., Ramalho, F.S., Bertoni, B.W., de Castro França, S., de Carvalho Borges, M., Pereira, A.M.S., 2016. A standardized methanol extract of *Eclipta prostrata* (L.) L. (Asteraceae) reduces bronchial hyperresponsiveness and production of Th2 cytokines in a murine model of asthma. *J. Ethnopharmacol.* <https://doi.org/10.1016/j.jep.2016.12.008>.
- de Oliveira, M.S.G., Aquino, A.B., da Silva, D.L., Aquino, P.G.V., Santos, M.S., Porfírio, A.P.R., Sant'Ana, A.E.G., Santos, B.V.O., Alexandre-Moreira, M.S., Araújo-Júnior, J.X., 2011. Antinociceptive and anti-inflammatory activity of hydroalcoholic extracts and fractions from *Erythrina mulungu*. *Braz. J. Pharmacogn.* 22, 157–161. <https://doi.org/10.1590/S0102-695X2011005000210>.
- Onusic, G.M., Nogueira, R.L., Pereira, A.M.S., Viana, M.B., 2002. Effect of acute treatment with a water-alcohol extract of *Erythrina mulungu* on anxiety-related responses in rats. *Braz. J. Med. Biol. Res.* 35, 473–477. <https://doi.org/10.1248/bpb.26.1538>.
- Onusic, G.M.O., Nogueira, R.L., Pereira, A.M.S., Flausino Júnior, O.A., Viana, M., de B., Flausino, Júnior, O.A., Viana, M., de B., Ogueira, L.N., Soares, M., Aparecido, O., Júnior, F.L., Iana, M.D.B.V., 2003. Effects of chronic treatment with a water-alcohol extract from *erythrina mulungu* on anxiety-related responses in Rats. *Biol. Pharm. Bull.* 26, 1538–1542. <https://doi.org/10.1248/bpb.26.1538>.
- Ozawa, M., Honda, K., Nakai, I., Kishida, A., Ohsaki, A., 2008. Hypaphorine, an indole alkaloid from *Erythrina velutina*, induced sleep on normal mice. *Bioorg. Med. Chem.*

- Lett. 18, 3992–3994. <https://doi.org/10.1016/j.bmcl.2008.06.002>.
- Prado, R.Q., Bertolini, T.B., Piñeros, A.R., Gembre, A.F., Ramos, S.G., Silva, C.L., Borges, M.C., Bonato, V.L.D., 2015. Attenuation of experimental asthma by mycobacterial protein combined with CpG requires a TLR9-dependent IFN- γ -CCR2 signalling circuit. *Clin. Exp. Allergy* 45, 1459–1471. <https://doi.org/10.1111/cea.12564>.
- Ribeiro, M.D., Onusic, G.M., Poltronieri, S.C., Viana, M.B., 2006. Effect of Erythrina velutina and Erythrina mulungu in rats submitted to animal models of anxiety and depression. *Braz. J. Med Biol. Res* 39, 263–270. <https://doi.org/10.1590/S0100-879x2006000200013>.
- Rodrigues, V.E.G., Carvalho, D.A. de, 2001. Plantas Medicinais no Domínio dos Cerrados. UFLA, Lavras.
- Santos Rosa, D., Faggion, S.A., Gavin, A.S., Anderson de Souza, M., Fachim, H.A., Ferreira dos Santos, W., Soares Pereira, A.M., Cunha, A.O.S., Belebony, R.O., 2012. Erysothrine, an alkaloid extracted from flowers of Erythrina mulungu Mart. ex Benth: evaluating its anticonvulsant and anxiolytic potential. *Epilepsy Behav.* 23, 205–212. <https://doi.org/10.1016/j.yebeh.2012.01.003>.
- Sarragiotto, M.H., Filho, H.L., Marsaioli, A.J., 1981. Erysotrine-N-oxide and erythartine-N-oxide, two novel alkaloids from Erythrina mulungu. *Can. J. Chem.* 59, 2771–2775.
- Shen, H.H., Wang, K., Li, W., Ying, Y.H., Gao, G.X., Li, X.B., Huang, H.Q., 2008. Astragalus Membranaceus prevents airway hyperreactivity in mice related to Th2 response inhibition. *J. Ethnopharmacol.* 116, 363–369. <https://doi.org/10.1016/j.jep.2007.12.002>.
- Sun, H., Cai, W., Wang, X., Liu, Y., Hou, B., Zhu, X., Qiu, L., 2017a. Vaccaria hypaphorine alleviates lipopolysaccharide-induced inflammation via inactivation of NF κ B and ERK pathways in Raw 264.7 cells. *BMC Complement. Altern. Med.* 17, 120. <https://doi.org/10.1186/s12906-017-1635-1>.
- Sun, H., Zhu, X., Cai, W., Qiu, L., 2017b. Hypaphorine Attenuates Lipopolysaccharide-Induced Endothelial Inflammation via Regulation of TLR4 and PPAR- γ Dependent on PI3K/Akt/mTOR signal pathway. *Int. J. Mol. Sci.* 18, 1227–1239. <https://doi.org/10.3390/ijms18040844>.
- Sun, H., Zhu, X., Lin, W., Zhou, Y., Cai, W., Qiu, L., 2017c. Interactions of TLR4 and PPAR γ , Dependent on AMPK Signalling Pathway Contribute to Anti-Inflammatory Effects of Vaccariae Hypaphorine in Endothelial Cells. *Cell. Physiol. Biochem.* 42, 1227–1239. <https://doi.org/10.1159/000478920>.
- Sur, S., Wild, J.S., Choudhury, B.K., Sur, N., Alam, R., Klinman, D.M., 1999. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* 162, 6284–6293.
- Vasconcelos, S.M.M., Rebouças Oliveira, G., Mohana de Carvalho, M., Rodrigues, A.C.P., Rocha Silveira, E., Maria França Fonteles, M., Florenço Sousa, F.C., Barros Viana, G.S., 2003. Antinociceptive activities of the hydroalcoholic extracts from Erythrina velutina and Erythrina mulungu in mice. *Biol. Pharm. Bull.* 26, 946–949. <https://doi.org/10.1590/S0102-695x2011005000134>.
- Vasconcelos, S.M.M., Lima, N.M., Sales, G.T.M., Cunha, G.M.A., Aguiar, L.M.V., Silveira, E.R., Rodrigues, A.C.P., Macedo, D.S., Fonteles, M.M.F., Sousa, F.C.F., Viana, G.S.B., 2007. Anticonvulsant activity of hydroalcoholic extracts from Erythrina velutina and Erythrina mulungu. *J. Ethnopharmacol.* 110, 271–274. <https://doi.org/10.1016/j.jep.2006.09.023>.
- Vasconcelos, S.M.M., Sales, G.T.M., Lima, N., Lobato, R., de, F.G., Macêdo, D.S., Barbosa-Filho, J.M., Leal, L.K.A.M., Fonteles, M.M.F., Sousa, F.C.F., Oliveira, J.L., Viana, G.S.B., 2011. Anti-inflammatory activities of the hydroalcoholic extracts from Erythrina velutina and E. mulungu in mice. *Rev. Bras. Farmacogn.* 21, 1155–1158. <https://doi.org/10.1590/S0102-695x2011005000134>.
- Wanjala, C.C., Juma, B.F., Bojase, G., Gashe, B.A., Majinda, R.R., 2002. Erythraline alkaloids and antimicrobial flavonoids from Erythrina latissima. *Planta Med.* 68, 640–642 (<https://doi.org/10.1055/s-2002-32891>).

Evaluation of ethanolic extract from the Brazilian medicinal plant *Erythrina mulungu* Benth. flowers in the treatment of asthma in an animal model

Jowanka Amorim^{1,*}, Marcos de Carvalho Borges¹, Alexandre Todorovic Fabbro¹, Silvia Helena Taleb Contini², Mayara Valdevite², Ana Maria Soares Pereira², Fabio Carmona¹.

SUPPLEMENTAL MATERIAL

The erythrinian alkaloids erysotrine and erysotrine-*N*-oxide were established on the basis of spectral data ¹H, ¹³C NMR, HMQC and HMBC (**Supplemental table 1**). Erysotrine (**1**) ¹H NMR spectrum showed signals at δ 6.79 (s) and 6.69 (s) assigned to aromatic hydrogens. Vinylic hydrogens produced signals at δ 6.57 (dd, 1H, *J*= 10.0 Hz), 5.99 (d, 1H, *J*= 3.0 Hz) and 5.73 (s), and it also was observed three singlets at δ 3.32 (s, 3H), 3.77 (s, 3H) and 3.80 (s, 3H) from methoxyl functional groups. The HMQC experiment shows connectivities between aromatic protons resonating at δ 6.79 (s) and 6.69 (s) with δ 109.2 and 111.4, corresponding to C-14 and C-17 carbons nucleus, respectively. Vinylic protons at δ 6.57 (H-1), 5.99 (H-2) and 5.73 (H-7) provided correlations between carbons at δ 124.9 (C-1), 132.0 (C-2) and 121.6 (C-7). HMBC spectrum shows long-range connectivities of the methoxyl protons at δ 3.32, 3.77 and 3.80 with the δ 75.6 (C-3), 147.2 (C-15) and 147.9 (C-16) carbons. All the correlations are summarized in Table X.

Erysotrine-*N*-oxide (**2**) ¹H NMR spectrum also showed the resonance of the aromatic, vinylic and methoxy protons (**Supplemental table 1**). The HMQC experiment shows connectivities between aromatic protons resonating at δ 6.60 (s) and 6.70 (s) with δ 108.7 (C-14) and 110.5 (C-17) carbons nucleus, and vinylic protons resonating at δ 6.64 (H-1), 6.11 (H-2) and 5.76 (H-7) with δ 125.6 (C-1), 133.5 (C-2) and 118.1 (C-7). The *N*-oxide structure was proposed based on changes in the shielding of the differences carbon atoms (C-4, C-5, C-6, C-7, C-8,

C10, C-11, C-12) neighboring N atom (Sarragiotto, Filho, & Marsaioli, 1981). Long-range connectivities observed in the HMBC spectrum provided strong evidence for the structure proposed to substance **2**.

According to **Supplemental table 2** ^1H NMR and HMQC spectra of indole alkaloid hypaphorine (**3**) showed aromatic hydrogens signals at δ 7.62 (d, 1H, $J=8.0$ Hz), 7.04 (d, 1H, $J=7.5$ Hz), 7.10 (d, 1H, 7.5Hz) and 7.33 (d 1H, 8.0Hz) correlating with carbons at δ 117.0 (C-4), 118.5 (C-5), 121.0 (C-6) and 111.0 (C-7), respectively. Aliphatic hydrogens produced signals at δ 3.90 (m, 1H) and 3.43 (m, 2H), and it also observed a signal at δ 3.30 (s, 9H) assigned to N-methyl groups. HMBC spectrum showed long-range correlations of quaternary carbons at δ 108.0, 127.0 and 137 with δ 7.18 (s), 7.62 (d, 1H, $J=8.0$ Hz) and 3.43 (m), indicating the presence of the indole skeleton.

REFERENCES

- Luan, G., Tie, F., Yuan, Z., Li, G., He, J., Wang, Z., & Wang, H. (2017). Hypaphorine, an Indole Alkaloid Isolated from *Caragana korshinskii* Kom., Inhibites 3T3-L1 Adipocyte Differentiation and Improves Insulin Sensitivity in Vitro. *Chemistry & Biodiversity*, 14(7). <https://doi.org/10.1002/cbdv.201700038>
- Sarragiotto, M. H., Filho, H. L., & Marsaioli, A. J. (1981). Erysotrine-N-oxide and erythartine-N-oxide, two novel alkaloids from *Erythrina mulungu*. *Canadian Journal of Chemistry*, 59(13), 2771–2775.

SUPPLEMENTAL TABLES

Supplemental table 1. Spectral data ^1H , ^{13}C NMR, HMQC and HMBC of erysotrine (**1**) and erysotrine-*N*-oxide (**2**).

C/H	1* ϕC	1 ϕH	1 ^3JCH	2* ϕC	2 ϕH	2 ^3JCH
C						
5	67.8		H-4, H-10, H-14	82.5		H-1, H-4, H-8, H-10
6	141.5		H-4	138.3		H-8
12	125.6		H-10, H-14	122.0		H-10, H-11, H-14
13	129.6		H-11, H-17	128.5		H-4, H-11, H-14, H-17
15	147.2		H-14, H-17	147.8		H-11
16	147.9		H-14, H-17	148.6		H-14, H-17
CH						
1	124.9	6.57 (<i>dd</i> , 10.0)		125.6	6.64 (<i>d</i> , 10.0)	
2	132.0	5.99 (<i>d</i> , 3.0)	H-4	133.5	6.11 (<i>d</i> , 10.0)	
3	75.6	4.04 (<i>s</i>)	H-4	75.4	4.10 (<i>m</i>)	H-4
7	121.6	5.73 (<i>s</i>)		118.1	5.76 (<i>s</i>)	H-8
14	109.2	6.79 (<i>s</i>)		108.7	6.60 (<i>s</i>)	
17	111.4	6.69 (<i>s</i>)	H-11	110.5	6.70 (<i>s</i>)	H-11
CH ₂						
4	40.2	1.83 (<i>t</i> , 10.5) 2.52 (<i>m</i>)		30.8	2.09 (<i>dd</i> , 11.6; 5.8) 2.80 (<i>m</i>)	
8	56.6	3.53 (<i>m</i>) 3.71 (<i>m</i>)	H-8, H-10	71.7	3.90 (<i>dd</i> , 15.3; 3.3) 4.00 (<i>m</i>)	H-10
10	43.7	2.93 (<i>m</i>) 3.47 (<i>m</i>)	H-11	59.6	3.70 (<i>m</i>)	H-11
11	23.8	2.61 (<i>m</i>) 2.88 (<i>m</i>)	H-17	27.2	3.11 (<i>m</i>)	H-17, H-10

OCH ₃						
3	55.8	3.32 (s)		55.7	3.29 (s)	
15	55.8	3.77 (s)		55.7	3.60 (s)	
16	55.8	3.80 (s)		55.7	3.73 s)	

Legend: Chemical shifts (δ) are in ppm from TMS and J values in Hz are presented in parentheses. All signals were assigned by HSQC and HMBC experiments (CD₃OD, 500MHZ). * Values in these columns were taken from the literature (Sarragiotto et al., 1981).

Supplemental table 2. Spectral data ¹H, ¹³C NMR, HMQC and HMBC of hypaphorine (**3**).

C/H	ϕ C*	ϕ H	³ JCH
C			
3	108.0		H-2, H-4, H-8, H-9
3a	127.0		H-2, H-5, H-7, H-8
7a	137.0		H-2, H-4, H-6
C=O	170.0		H-8, H-9
CH			
2	124.0	7.18 (s)	H-8
4	117.0	7.62 (<i>d</i> , 8.0)	H-6
5	118.5	7.04 (<i>d</i> , 7.5)	H-7
6	121.0	7.10 (<i>d</i> 7.5)	H-4
7	111.0	7.33 (<i>d</i> , 8.0)	H-5
9	79.0	3.90 (<i>m</i>)	N(CH ₃) ₃
CH₂			
8	24.0	3.43 (<i>m</i>)	H-9, N(CH ₃) ₃
N(CH₃)₃	52.0	3.30 (s)	H-8

Legend: Chemical shifts (δ) are in ppm from TMS and J values in Hz are presented in parentheses. All signals were assigned by HSQC and HMBC experiments (CD₃OD, 500MHZ). * Values in these columns were taken from the literature (Luan et al., 2017).