INTRODUCTION

Helminthic infections are potent inducers of Th2-type responses in both humans and experimental models. Th2-type responses are characterized by eosinophilia, high titers of circulating IgE, enhanced Th2 cytokine profile, and reduced Th1 cytokine profile (Silveira et al., 2002; Lawrene 2003). *Toxocara canis* is an intestinal parasite of dogs, and it is the etiologic agent of Visceral Larva Migrans Syndrome (VLMS) in humans (Beaver, et al,1952). Humans are infected by ingesting embryonated eggs which develop into larvae in the gastrointestinal tract and then penetrate through its wall invading multiple organs (ex. lung, liver, kidney, brain, and eye) (Beaver, et al1956; Hoffmeister et al., 2007).

The presence of antigen-secreting (TES) eggs in the tissues initiates Th2-type immunological response which is marked by both eosinophilia and high levels of IgE. *Harpagophytum procumbens* (Hp) has been used as treatment for a variety of inflammatory illnesses. The Decrease on the levels of IL-5, IL-4 and IgE was observed after the administration of Hp, suggesting strongly that Hps has therapeutic benefits regarding the eosinophilic inflammation triggered by *Toxocara canis*.

Humans are infected with *Toxocara canis* by accidental ingestion of embryonated eggs (larvae) from the environment or contaminated food and water. The mechanisms of molecular and immunological development of the symptomatic syndromes of VLMS are largely unclear. Moreover, the presence of antigen-secreting (TES) eggs in tissue initiates a Th2-type immunological response that is marked by eosinophilia and high levels of IgE. *Harpagophytum procumbens* (Hp) has been used as treatment for a variety of inflammatory illnesses. The present study examined the role of Hps in the recruitment of eosinophils as well as the immune response in a murine model of VLMS. Our results demonstrated that Hps led to persistent lower eosinophilia during *Toxocara canis* infection. The Decrease on the levels of IL-5, IL-4 and IgE was observed after the administration of Hp, suggesting strongly that Hps has therapeutic benefits regarding the eosinophilic inflammation triggered by *Toxocara canis*.

Natural products have long been used in medicine as alternative treatment for various diseases, including inflammatory processes of various origins. *Harpagophytum procumbens* (Hp) has been used as treatment for a variety of illnesses, including, indigestion, diabetes mellitus, hypertension, fevers, skin cancer, infectious diseases (including tuberculosis), allergies, osteoarthritis, fibrosis and rheumatism, and it is particularly effective in small joint diseases (Van Wyk and Gericke, 2000). *In vitro* studies have evaluated the ability of Hps to inhibit the lipid mediators generated by arachidonic acid metabolism (prostaglandin and eicosanoid) (Grant et al., 2007).

Other studies demonstrated that Hps extracts were able to suppress the biosynthesis of cysteinyl-leukotrienes (Cys-LT; LTC₄, LTD₄, and LTE₄) and TXB₂. (Tippler et al 1997; Loew, 2001). However, no reports on its parasite related anti-
inflammatory capability have been yet described. The anti-inflammatory effect and the potential for modulation and recruitment of eosinophils that cause systemic eosinophilia of an ethanolic extract of *H. Procumbens* will be evaluated during VLMS, our experimental model.

**Materials and Methods**

**Animals**

Female Balb/c mice, weighting between 15 and 18 grams, 4 weeks old, from the animal facilities from the Faculty of Pharmaceutical Sciences in Ribeirão Preto (FCFRP-USP), pathogen free (Specific Pathogen Free - SPF) were used. This project was approved and conducted in accordance with the guidelines established by the Federal University of São Carlos (UFSCar) Animal Care Committee (Protocol n° 046/2009). Animals were divided into 3 groups: control, infected and untreated (Tc) and infected and treated with 100 mg/Kg/animal of *H. Procumbens* ethanolic extract of (Tc + Hp). Infected animals received 500 animal eggs of *T. canis* by gavage. Either kept treated or untreated with *H. procumbens*.

**Experimental infection**

Eggs were obtained from female parasites recovered from young dogs by administration of mineral oil with piperazine hydrochloride. Female *T. canis* were recovered and dissected to extract their wombs, which were dissolved in Petri dishes containing 2% formalin. Material was filtrated on gauze to obtain eggs and stored in a formalin solution in a Petri dish, left at room temperature to reach the infective stage (L3). Animals were infected with eggs containing larvae (L3) of *T. canis* (gavage/500 eggs/0.5 ml saline/animal) (Sugane and Oshima, 1982). Animals were euthanized on day 18 post parasite infection.

**Preparation of *H. Procumbens* ethanolic extract**

Crude ethanol extract of *Harpagophytum procumbens* (Hp) was prepared at Natural Products Laboratory at the Chemistry Department of the Federal University of São Carlos (DQ-UFSCar), with the guidance of Prof. Dr. Cezar Paulo Vieira. Hp secondary roots maceration was performed using ethanol and water. After that, aliquots of the extract were kept refrigerated at -20 °C until use.

**Treatment**

Animal treatment was administrated using the mean weight of the animals on the group. Aliquots of the extract were kept refrigerated at -20 °C, daily treatment was prepared with the diluted extract. Treatment with *H. Procumbens* extract at a 100 mg/Kg/animal dose began after infection and was maintained throughout 18 days. The administration was performed orally by gavage daily. After this period the animals were euthanized and the cells obtained from the peritoneal cavity lavage (LCP), bronchoalveolar fluid (BAL) and whole blood were analysed.

**Collection of lavage of the peritoneal cavity (LPC), bronchoalveolar fluid(BAL) and Plasma**

Blood, LPC and BAL smears were prepared and stained with dye-Panotico (Laborclin). On each slide 100 cells were counted using light microscopy with a final magnification of x1000. The total number of cells /mm³ in the different compartments was determined using Turk solution to lyse the erythrocytes (Stibbe et al. 1985) at 1:20 dilution and subsequent counting in a Neubauer chamber, and stained with dye-Panotico (Laborclin). After blood coagulation, plasma was collected and stored at -20°C.

**Cytokines analysis in the plasma**

ELISA were performed according to the manufacturer instructions (BD) using purified mAb as capture Abs and biotinylated mAb as developing Abs, followed by incubation with streptavidin-alkaline phosphatase substrate. Plates were read in a 96-well spectrophotometer (Microquant-Sellex, Inc 450nm) and data was analysed using software by comparison against a standard curve generated using recombinant cytokine at known concentration.

**Analysis of anti- *T. canis* IgE – ELISA**

Plasma samples were collected on day 18 post parasite infection and stored at -20°C until its use for IgE analysis. For detection of this immunoglobulin, IgE Kit (BD) was used - plates with 96 wells coated with 200ng of total protein antigen
of Trichuris canis (diluted in carbonate buffer and applied 100 l/well) incubated at 4°C overnight.

Graph 3 Top panels evaluation of IL-5 (A) and IL-4 (B) in the plasma. Bottom panels, IgE levels of anti-T. canis present in serum on day 18 post parasite infection. Data represent mean±SEM (n=7 animals) of two independent experiments. *** P<0.001 represents significant difference between the results obtained from the Tc+Hp groups compared with the control group and ** P<0.01 represents significant difference between the results obtained from the Tc group compared with Tc+Hp group using the test two-way ANOVA.

Blocking was then performed using 1% BSA in PBS for 60 min at 37°C. 50 ul of each diluted sample (plasma dilution 1:2) in carbonate buffer was added to each well. Subsequently, the plates were washed and 100 mL of biotin-conjugated secondary Ab (4 μl Ac + 10 ml of carbonate buffer) was added. After 1h, the plates were washed and then added 100 ul of enzyme streptavidin (1:200 dilution). The plates were washed and 100 ml of a substrate 1:1 mixture of H2O2-tetramethylbenzidine (TMB) (BD-OpTEIA) was added. The reaction was stopped by the addition of 50 μL/well of H2SO4. Absorbance was read at a wavelength of 450 nm in an ELISA – READER (Microquant – Sellex Inc.).

Statistical analysis

The results were expressed as mean±SEM. The results obtained in different experiments were analysed using ANOVA. Statistical analysis was performed using the PRISM-TWO (ANOVA) (San Diego, California, USA). The level of significance was 5%.

RESULTS

Eosinophils accumulation after T. canis infection and treatment with H. procumbens

Graph 1 Eosinophils accumulation after T. canis infection in blood, LPC and LBA

During the course of T. canis infection, an intense inflammatory reaction characterized by an increase in the total number of leukocytes and eosinophils in the blood, LPC and BAL was observed in the infected groups when related to the control group (Figure 1). Our data also demonstrated that H. procumbens administration was able to decrease total numbers of eosinophils on Tc+Hp groups when compared to the infected untreated group both for blood, LPC and BAL compartments. (Figure 1D, 1E and 1F).

Cytokines analysis in the plasma

Graph 2 Plasma levels of IL-5 and IL-4 cytokines

Analysis of the serum obtained 18 days after infection revealed a significant increase in plasma IL-5 in infected animals (Tc) when compared to the control group. Additional analysis revealed that IL-5 concentration significantly decreased in the H. Procumbenstreated group(Tc+Hp) when compared to the infected untreated animals (Figure 2).

Analysis of anti-T. canis IgE – ELISA

Graph 3 Antibody production IgE

The infected (Tc) group showed a significant decrease in the levels of IgE when compared to the group control in the 18th day post infection. Levels of serum IgE showed a significant decrease in treated (Tc+Hp) mice with H. procumbens compared to the group only infected (Tc) the 18th day post infection (Figure 3).

DICUSSION

Parasitic infections represent a serious problem for human health (Menezes et al, 2008), mainly caused by helminthic infections, which are characterized by long-term infections and can lead to a specific state of morbidity or even death (Maizels and Yasanbakhsh, 2003). Immune responses during nematode infections include mainly a Th2-response pattern which is characterized by the production of cytokines such as IL-4, IL-5, IL-13 and also regulatory cytokines such as IL-10, resulting in the differentiation of B lymphocytes into serum cells secreting high levels of antibodies (Lawrene, 2003; Maizels and Yasanbakhsh, 2003; Pinelliet al, 2007).

There are a wide variety of disorders associated with eosinophilia (Mendes et al, 2000). However, in our country this increase is related mainly with intestinal helminthic infections, asthma and various allergic processes (Poseet al, 2007). Eosinophila has been implicated by several authors as one of the most outstanding characteristic of the VLMS in naturally infected human or experimental models (Beaver, et al, 1952; Sugane and Oshima, 1994; Meeusen and Balic, 2000). VLMS eosinophilia peaks on day 18 post parasite infection and has been correlated with the production of IL-5 by Th2 CD4+ lymphocytes in the lungs as well as with elevated production of IgE (Faccioli et al, 1996; Anibalet al, 2007). There is no proven effective treatment for VLMS, although anthelmintic drugs such as diethylcarbamazine, ivermectin, thiabendazole, mebendazole and albendazole have been used, these being sometimes associated with an antihistamine (Cunha, 2005).

Plant-derived compounds are sources of anti-inflammatory agents. Harpagophytm procumbens has been used as an analgesic, as a medication for fevers and allergies and by San bushmen in Africa to stimulate gastric enzymes and digestion (Chrubasik et al, 2000), and it has been used traditionally for centuries by indigenous people for treating a range of conditions (Wegener and Luleke, 2003). Other studies demonstrated the anti-inflammatory effect of Harpagophytm procumbens in various disorders such as rheumatic diseases, diabetes, atherosclerosis, malaria, indigestion, and fever among others (Lanhaeret al, 1992; Andersenet al, 2004; Clarksenet al, 2006). Here, we have investigated the effect of H. procumbens administration in mice infected with T. canis.

Similar to previous data (Faccioli et al, 1996, Anibalet al, 2007, D. Carlos et al, 2011) our findings demonstrated an increased numbers of eosinophils in blood (1D), LPC (1E) and BAL (1F) in infected animals when compared to the control group. Eosinophilia have long been recognized as a
characteristic feature of helminthic infections (Ponte et al., 2007).

The larvae of *T. canis* have an allergenic fraction responsible for the stimulation of eosinophils, which explains the eosinophilia (Pellouxat et al., 2004). In our results the *H. procumbens* treatment was able to inhibit eosinophilia in the blood, LCP and BAL. Given these data our next step was to investigate whether HP treatment was able to reduce interleukin-5 (IL-5) that has been correlated with an increased eosinophilia. From our results, we can say that infected mice showed a significant increase in the plasma IL-5 concentrations when compared with the control group (Graph 2A). Thereby, eosinophilia peaks are correlated with production of IL-5 in this experimental infection. However, the role of IL-5 on eosinophilia has been demonstrated in several parasitic and allergic models. Experiments using anti-IL-5 or mice lacking IL-5 gene show that eosinophilia is IL-5-dependent (Nakajima et al., 1996; Hamelmann et al., 2000).

*H. procumbens* treatment was able to significantly reduce the concentrations of IL-5 in animals receiving treatment when compared with those infected and untreated (Graph 2A). Thus we suggest that the reduction in eosinophilia in the compartments LBA, LCP and blood, observed after HP treatment could be explained because IL-5, a cytokine produced by CD4 + T cells, plays an important role in the inflammatory response observed during the Th2 immune response. In addition to eosinophilic inflammation and a shift in the cytokine pattern toward a Th2 profile, one of the clinical signs seen during VLMS is an increment in the production of antibodies specific for *T. canis* antigens. IL-4 is a cytokine essential for development of a Th2 pattern response (Bacharier and Geha, 2000).

We found, however, that treatment with *H. procumbens* generally reduce IL-4 in serum, on day 18 post *T. Canis* infection using Balb/c mice treated with *H. procumbens* (Graph 2B) when compared to infected untreated mice. These findings are consistent with other studies, which report the prevalence of these cytokines in this model and that specify a pattern of Th2-type response as characteristic in VLMS (Buijs et al., 1997; Altcchehet al. 2003).

Among others, *Toxocara canis* is one of the most commonly parasites associated with increased levels of IgE, a proposed mechanism to explain the increase in total IgE levels is the secretion by parasites of factors that stimulate the production of IL-4 leads to an increased levels of IgE (Sorensen et al., 2006). By analysing the pattern of specific antibodies in serum from animals infected with *T. canis*, an increase of IgE antibody was found when compared with infected animals treated with *H. procumbens*. IgE levels in the infected mice were higher than those in the control group (Graph 3). Ours findings also clearly suggests that the administration of *H. procumbens* inhibit production of IgE on day 18 post parasite infection. IgE immunoglobulin plays a central role in the pathogenesis of immediate hypersensitivity reactions due to its ability to bind specifically to receptors with high (FcεRI) and low activity (FcεRII-CD23) present on various cells such as mast cells, basophils, B lymphocytes and T and activated eosinophils with the objective of expanding cellular and humoral immune responses in allergic reactions (Delespesse et al. 1992; Turner and Kinet, 1999; Mutizani et al., 2012). The increase in total IgE levels might be explained by the fact that factors that stimulate IL-4 also increases the level of IgE production (Pinelli and Aranzamendi, 2012). This finding is in agreement with others that report *Toxocara canis* as one of the most commonly parasites associated with IgE increased levels (Mendonça et al., 2012). Thus, our results corroborates previous studies, which report that IL-4 acts in the regulation of the immune response by acting on the induction of IgE (Mutizani et al., 2012; Zavadiak and Rosário, 2005), because in our study concentrations of IL-4 (2B) are reduced in the same period in which it was possible to observe the reduction in serum levels of IgE in animals that were treated with *H. procumbens* (Graph 3).

In conclusion, our findings suggested an early blockage on IL-5, IL-4 and specific IgE production apparently in the effective on subsequent blockage of eosinophilic inflammation. So, given the above, we suggest that the extract of *H. procumbens* presented an anti-inflammatory effect in the model of VLMS. However, further investigations will be required to contribute to the understanding of the mechanisms by which the extract of *H. procumbens* can interfere with the migration of eosinophils, production of cytokines and antibodies in this model, since this event is characterized by factors of order complex and multifactorial.

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Referências


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