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TRABALHO DE CONCLUSÃO DE CURSO

"*Canavalia ensiformis* urease effects on *Dysdercus peruvianus* hemocytes"

"Efeitos da urease de *Canavalia ensiformis* sobre hemócitos de *Dysdercus peruvianus*"

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Abstract

Jackbean (*Canavalia ensiformis*) ureases are entomotoxic to hemipteran insects, among others, an effect partially due to the release of internal peptides by insect's cathepsin like digestive enzymes. Here we studied the effects of the major jackbean urease isoform (JBU) on 5th instars *Dysdercus peruvianus'* isolated hemocytes. JBU (200 nM) was found to trigger *in vitro* microaggregation of the hemocytes, visualized under light microscopy and also after DAPI stain. Hemocytes were suspended in a calcium free saline before exposition to JBU, and the absence of external calcium did not change the aggregating effect of JBU. In the presence of JBU, there was a smaller number of melanized cells in the calcium free saline than in regular saline. The eicosanoid synthesis indirect inhibitor dexamethasone was used for testing a possible eicosanoid modulated aggregation. Dexamethasone (50 µM) was found to decrease the aggregated response caused by JBU. The results suggest that JBU is activating immune reactions in *D. peruvianus* isolated hemocytes, probably through eicosanoid metabolites, as it was seen with other mammal cellular models.

Resumo

Ureases do “feijão-de-porco” (*Canavalia ensiformis*) são entomotóxicas para insetos hemípteros, entre outros, sendo o efeito parcialmente dependente da liberação de peptídeos internos por enzimas digestivas, do tipo catepsinas, de insetos. Neste trabalho estudamos os efeitos da isoforma majoritária de urease do “feijão-de-porco” (JBU) sobre hemócitos isolados de ninfas de quinto instar de *Dysdercus peruvianus*. Foi observado que JBU (200 nM) é capaz de in vitro induzir microagregação dos hemócitos, visualizados por microscopia óptica e também com marcação da sonda fluorescente DAPI. Hemócitos suspensos em uma solução salina livre de cálcio antes da exposição a JBU foram testados, observando-se que a ausência de cálcio externo não altera o efeito da JBU. Na presença de JBU, observou-se um menor número de células melanizadas presentes quando em solução salina livre de cálcio do que na salina comum. O inibidor indireto da síntese de eicosanóides dexametasona, foi utilizado para testar uma possível modulação da agregação de hemócitos por eicosanóides. Foi observado que dexametasona (50 μM) é capaz de diminuir a agregação causada por JBU. Os resultados sugerem que JBU está ativando reações do sistema imunitário em hemócitos isolados de *D. peruvianus*, possivelmente através de metabólitos de eicosanóides, da mesma forma como já foi observado em outros modelos celulares.

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1. Introduction and Objectives

1.1. Ureases

The legume *Canavalia ensiformis*, popularly known as 'Jackbean', is highly resistant to insects and its seeds are rich sources of proteins such as ureases (Sumner 1926; Carlini and Polacco, 2008). Ureases are nickel dependent metalloenzymes that catalyze urea hydrolysis into two molecules of ammonia and one molecule of carbon dioxide (Dixon *et al*., 1975). These proteins can be found in plants, fungi and bacteria but are not synthesized by animals (Mobley and Hausinger, 1989). Previous studies have shown that *C. ensiformis* ureases are lethal when fed to insects and show persistence of their insecticidal property after treatment of an irreversible urease inhibitor proving that a protein domain distinct from the active site is involved in entomotoxic activity (Carlini *et al.,* 1997; Follmer *et al*., 2004). This toxicity is partially dependent on urease hydrolysis inside the insect's midgut by cathepsin B and D digestive enzymes which cleave the molecule in specific sites for the release of peptides with toxic activity (Piovesan *et al*., 2008; Defferrari *et al*., 2011). After *in vitro* hydrolysis of an urease isoform from Jackbean, called canatoxin (cntx), by the coleopteran *Callosobruchus maculatus'* digestive enzymes, a 10 kD peptide was isolated, which was highly toxic when administered orally to insects of different orders. Based on the sequence of this peptide, a recombinant peptide was designed and called Jaburetox-2Ec which is also toxic to all insects tested so far (Ferreira-DaSilva *et al*., 2000).

The physiological role of urease in plants has also been investigated suggesting it may have a protective function against pathogens and phytophagous insects. Ureases were detected in various plants by testing immunoreactivity with polyclonal anti-canatoxin antibodies, suggesting a process of evolutionary conservation of antigenic determinants (Carlini *et al.*, 1991). In addition, reports that the content of urease increases progressively during seed maturation reinforces the idea that these proteins play an important role in plant defense (Barcellos *et al*., 1993). Studies have demonstrated fungistatic and fungicidal activity of filamentous fungi, including pathogenic, by different ureases, like cntx (Oliveira *et al*., 1999) and the major Jackbean urease (JBU), soybean urease (*Glycine max*) and the bacterial *Helicobater pylori* urease (Becker-Ritt *et al*., 2007). Also, tests have been done to demonstrate the effects of the intact JBU on the hemipteran model *Rhodnius prolixus*, where it was observed that JBU potentiates serotonin-stimulated contractions of the anterior midgut and that one ciclooxygenase

inhibitor is capable of blocking this effect. In the same study it was demonstrated that cyclooxygenase products (prostaglandins) content increases inside the midgut after JBU treatment, suggesting eicosanoids might be playing a role as second messengers on this effect (Stanisçuaski *et al*., 2010). Ureases are able to activate different cell models through eicosanoids pathways, such as *H. pylori* urease that induces platelet activation via the lipoxygenase and subsequent release of hydroperoxides (Wassermann *et al*., 2010) and as cntx that activates the production of eicosanoids and pathways of lipoxygenase, inducing exocytosis and changes in levels and flows of intracellular Ca^{2+} in different mammalian cells (Carlini *et al*., 1985; Barja-Fidalgo *et al.*, 1991a and 1991b; Ghazaleh *et al.,* 1992 and 1997).

1.2. Eicosonoids and immune reactions in insects

Eicosanoids are synthesized from fatty acids, mainly from arachidonic acid (AA) (20: 4n–6) released upon cell stimulation from membrane phospholipids, via activation of a phospholipase A2 (PLA2) (Fig. 1). AA then follows three possible oxygenation pathways, cyclooxygenase (COX - yielding prostaglandins), cytochrome P450-expoxygenase and lipoxygenase (LOX). Most of the work done with insects focuses on the products of COX, specially the prostaglandins (PGs). Two forms of COX are known by studies with mammals but it is not known how many forms of COX genes are expressed in insects. This is because the genes responsible for encoding the COX enzymes and COX-like are still unknown. PGs and other eicosanoids serve as central mediators within insect immunity (Stanley, 2000; Stanely *et al.*, 2009), and were detected in reproductive tissues of crickets (Loher *et al*., 1981), in the gut of caterpillars (Buyukguzel *et al*., 2002) and in tissues of the immune system, like hemocytes (Gadelhak *et al*., 1995) and fat body (Stanley-Samuelson and Ogg, 1994; Tunaz *et al*., 2001). Cellular reactions are coordinated by eicosanoids and are involved in several defense functions, such as phagocytosis, microaggregation, nodulation, cell-spreading, encapsulation and hemocyte migration. Insect immunity has at least 3 types of cellular reactions to invasions: phagocytosis, nodulation and encapsulation. Microaggregation is a part of the nodulation process and clears bacterial infections from distribution in the hemocoel. Nodulation begins with the entrapment of bacterial cells that form microaggregates that grow by joining with additional cells to form nodules. The nodulation process is finished when layers of phagocytes are attached to mature nodules. The melanization process leaves observable nodules connected to the inner sides of the body or wall of various organs (Stanely *et al*., 2009).

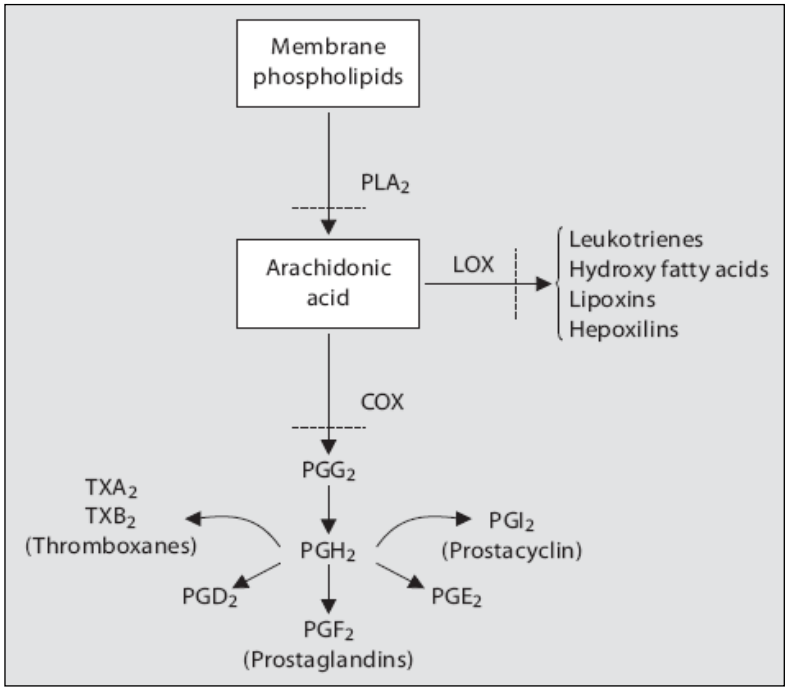

Figure 1. Eicosanoids biosynthesis scheme (Stanley *et al*., 2009).

PLA_2 - phospholipase A2

LOX- lipoxygenase

COX-cyclooxygenase

1.3. *Dysdercus peruvianus* and hemocyte classification

Insect hemocyte's classification can vary among species, but the following have been identified and are present in most species studied: (1) plasmatocytes, (2) granular hemocytes or granulocytes, (3) oenocytes, (4) prohemocytes, (5) spherule cells or spherulocytes (Lavine and Strand, 2002; Price and Ratcliffe, 2004; Costa *et al*., 2005; Ribeiro and Brehelin, 2006; Giglio *et al.*, 2008; Strand, 2008). Plasmatocytes have been found to be the most copious hemocytes and have many pseudopods resembling polymorphic amoebocytes. Granulocytes show scattered cytoplasmic granules and are usually in likeness too plasmatocytes. Oenocytes are oval and elongated in shape with a smaller nucleus than the others. Prohemocytes are small, round cells that are normally found undergoing mitotic division. Spherulocytes, or adipohemocytes, are large hemocytes that have the appearance of circulating fat bodies with small nuclei (Barracco *et al.*, 1987).

Here, we were able to study the effects of vitality and quantity of hemocytes after JBU treatment based on these previous descriptions of hemocytes. Our model of study was the phytophagous hemipteran *Dysdercus peruvianus*, also known as the "cotton stainer bug". *D. peruvianus* feeds on cotton seeds, harming them and also staining the cotton fibers, which minimizes cotton production, besides being a vector for phytopathogenic microorganisms (Gallo *et al*., 1988). Nymphs have been found to be susceptible to urease isoforms, while adults were not (Stanisçuaski *et al*., 2005; Piovesan *et al*., 2008). Therefore, we used 5th instars as our subjects of study.

Discoveries for pharmaceutical products that selectively inhibit specific enzyme routes have been a focus in research because of the reactions seen in humans. In mammals, the glucocorticoid dexamethasone acts by increasing the expression of annexin A1, a protein that binds to and inhibits phospholipase A2 (PLA2) eicosanoid production by blocking the activity of PLA2 (Herbert *et al*., 2007). Annexin A1 is active in various aspects of cell biology (Lim and Pervaiz, 2007). These inhibitors, like Dex, are generally used in such aspects as the improvement in pain or inflammation. Dex's effects have been found to be reversed within insects once treated with arachidonic acid (AA). Therefore, it can be inferred that the actions of Dex in insects are linked to the biosynthesis of eicosanoids (Stanley *et al*., 2000). From the previous evidence of effects caused by urease that produced eicosonoid reactions in both mammalian models and *Rhodnius prolixus*, our objective is to study the activity of *Canavalia ensiformis* urease on the hemocytes of *Dysdercus peruvianus*. Aforementioned, the immune

system of insects has been found to be centrally mediated by eicosanoids, where cellular reactions are the first response. This is an interesting target for the understanding of the mechanism of action of JBU on insects. This potential finding could also open doors to new pharmaceutical methods in the treatments of infections that deflect the mammalian immune system.

2. Materials and Methods

2.1. Reagents

Canavalia ensiformis urease (JBU), dexamethasone and Trypan blue were obtained from Sigma Chemical Company (Saint Luis, USA). DAPI (4,6–Diamidine-2-phenylindole dihydrochloride) was obtained from Roche Diagnostics GmbH (Mennheim, Germany).

2.2. Insects

A colony of *D. peruvianus*, established and operated in our laboratory for eleven years, is maintained as described (Stanisçuaski *et al*., 2005). The insects develop from eggs through five nymphal stages, in about 20–25 days. The insects were fed with cotton seeds (*Gossipium hirsutum*) and had free access to water. 5^{th} instars were used in all experiments.

2.3. Hemolymph collection and hemocytes isolation

D. peruvianus hemolymph samples were collected carefully from a cut leg with a 20-µl micropipette, as described by Machado *et al*. (2006), with some modifications. The samples consisted of a total of 8 bugs and approximately 4 µl of hemolymph per bug. The collections were immediately added to ice cold anticoagulant solution (62 mM sodium chloride, 10 mM ethylene diamine tetraacetic acid, 26 mM citric acid, 100 mM glucose, pH 4.6) at a proportion of 1:5 (anticoagulant: hemolymph). Hemolymph was then centrifuged at 4°C, 4,400 *g,* for 2 min and the pelleted hemocytes were washed two times and then re-suspended in insect saline, prepared as described by Meredith *et al*.(1984) with modifications: 20 mM NaCl, 24 mM KCl, 2 mM $CaCl_2$, 4 mM $NaHCO_3$, 2 mM $MgCl_2$, 6.7 mM glucose, pH 6.9, and 20 mM NaCl, 24 mM KCl, 4 mM $NaHCO_3$, 2 mM $MgCl_2$, 6.7 mM glucose, pH 6.9 for the calcium free saline. The volume of saline for hemocytes re-suspension was the same as the initial hemolymph volume.

2.4. Microaggregation assays

Hemocytes *in vitro* microaggregation was assayed following the method of Miller and Stanley (2001) as adapted by Garcia *et al.* (2004). After re-suspension in saline, cells were treated with JBU (200 nM) and incubated for 1 hour at room temperature. In order to analyze the involvement of eicosanoids, cells were incubated at room temperature for 30 minutes with dexamethasone in different concentrations prior to addition of JBU. Control groups were divided in three groups: (1) cells were re-suspended in saline and incubated for 1 hour at room temperature, (2) cells were re-suspended in saline and treated exclusively with dexamethasone (0.05 mM) for 1.5 hour, room temperature, and (3) cells were re-suspended in saline and incubated in presence of ethanol for 1.5 hour – this serving as a control for the dexamethasone experiments, since the reagent was diluted in ethanol. After the cells were submitted to treatments, DAPI (0.03 µM) was used to fluorescently stain the viable cells, and was incubated for 30 minutes at room temperature. Alternatively, Trypan blue (1:10) was added to DAPI untreated cells prior to microcopy analysis in order to differentiate damaged from intact hemocytes (where unstained cells are viable and blue cells are in lysis process). The samples were analyzed in a Neubauer chamber under optical and/or epifluorescence microscopy. The microscope was an Axioskop 40 from Zeiss (Germany), and maginification used was 20x/ 0.5 for all pictures.

3. Results

3.1. Hemocytes microaggregation induced by JBU

In vitro hemocyte microaggregate formation may be influenced by various factors, including the handling involved when setting up the preparations (Garcia *et al*., 2004). Here we observed that after one hour incubation at room temperature, JBU (200 nM) induced hemocyte microaggregation (Fig. 2B and Table 1). Control hemocytes, which were collected and kept in the same conditions as JBU treatments, were incubated in saline alone and did not have the formation of microaggrates (Fig. 2A), indicating that the handling and the protocol were adequate. Besides microaggregates formation, it was possible to detect the presence of melanized cells and larger microaggregates, suggesting that JBU is not only inducing cellular reactions but also leading to a humoral response (Fig. 3A)

JBU-induced microaggregation was tested in the presence of a PLA2 indirect inhibitor, glucocorticoid dexamethasone, which apparently inhibited aggregation (Fig. 4), suggesting that the effect might be mediated by eicosanoids. The hemocytes were not affected and did not aggregate when tested only with dexamethasone or ethanol, in the absence of JBU (data not shown). The importance of calcium on JBU activity was seen in a previous study, where extracellular calcium was found to be necessary to JBU's effect on *Rhodnius prolixus* diuresis inhibition (Stanisçuaski *et al*., 2009). We also tested JBU effect on hemocytes in calcium free saline, which did not change JBU aggregative activity completely, but reduced the melanized cells and aggregates (Fig. 3B and Table 1).

Table 1. The table represents the counted hemocytes on a Neubauer chamber under optical microscopy with 20x/0.5 magnification. Hemocytes were incubated in regular saline (control), JBU (200 nM) in regular saline or JBU (200 nM) in calcium free saline.

Treatment	**Number of cells per mL of hemolymph**	**Number of microaggregates per mL of hemolymph**
Regular saline (control)	67 $x10^{-4}$	0
Regular saline + JBU	57 $x10^{-4}$	19 $x10^{-4}$
Calcium free saline + JBU	57 $x10^{-4}$	15 $x10^{-4}$

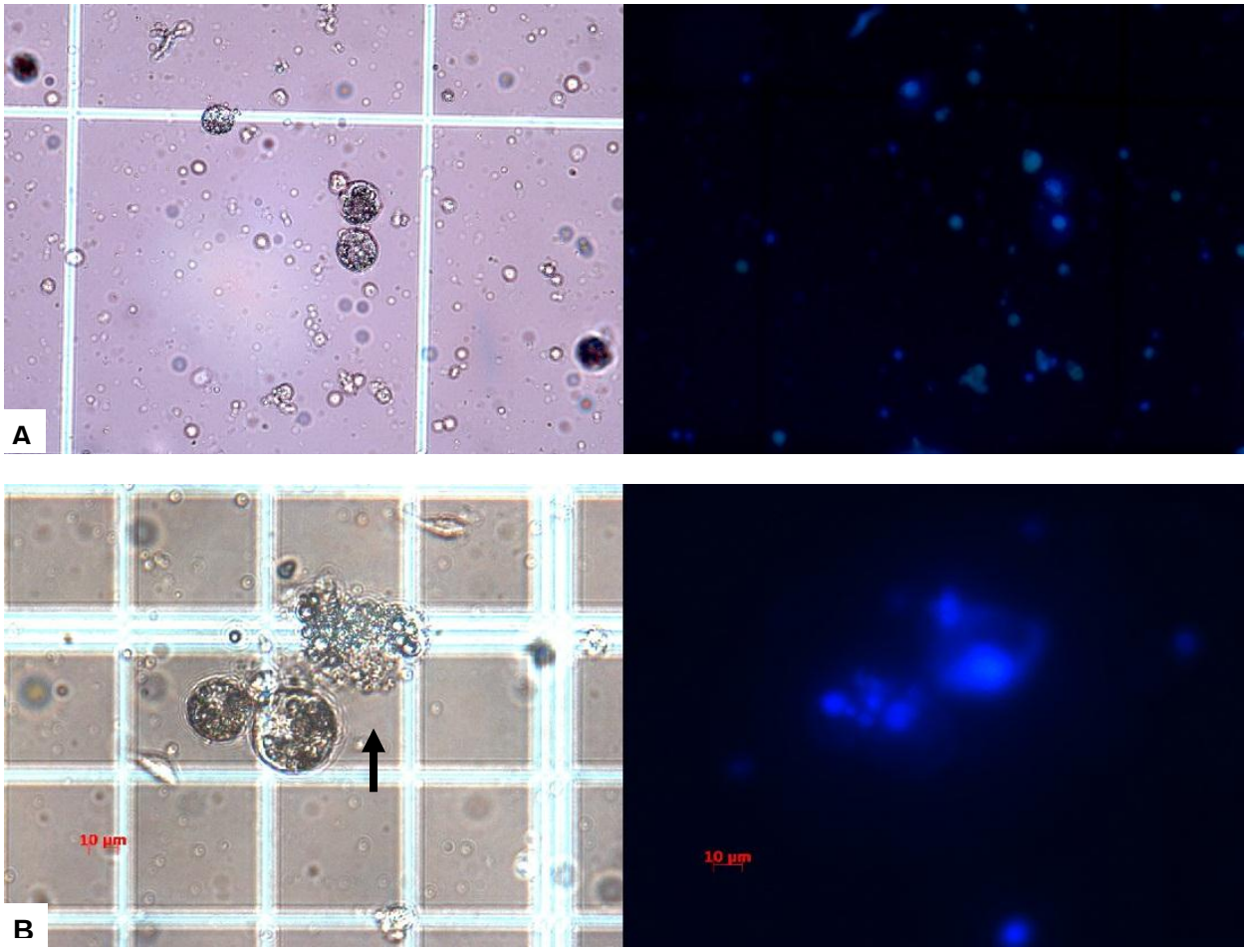

Figure 2. *Dysdercus peruvianus*' hemocytes microaggregation induced by JBU.

Hemolymph was collected from 5th instars in anticoagulant solution and centrifuged for 2 min at 4,400 *g*. Hemocytes were suspended in saline and treated with JBU (200 nM) for 1 hour at room temperature or kept in saline for 1h at room temperature. Both treatments were subsequently incubated with DAPI for 30 min. (A) Control hemocytes, (B) JBU treated hemocytes. Left side – optical microcopy, right side – epifluorescence microscopy. 20x/0.5 magnification. The arrow indicates a microaggregate.

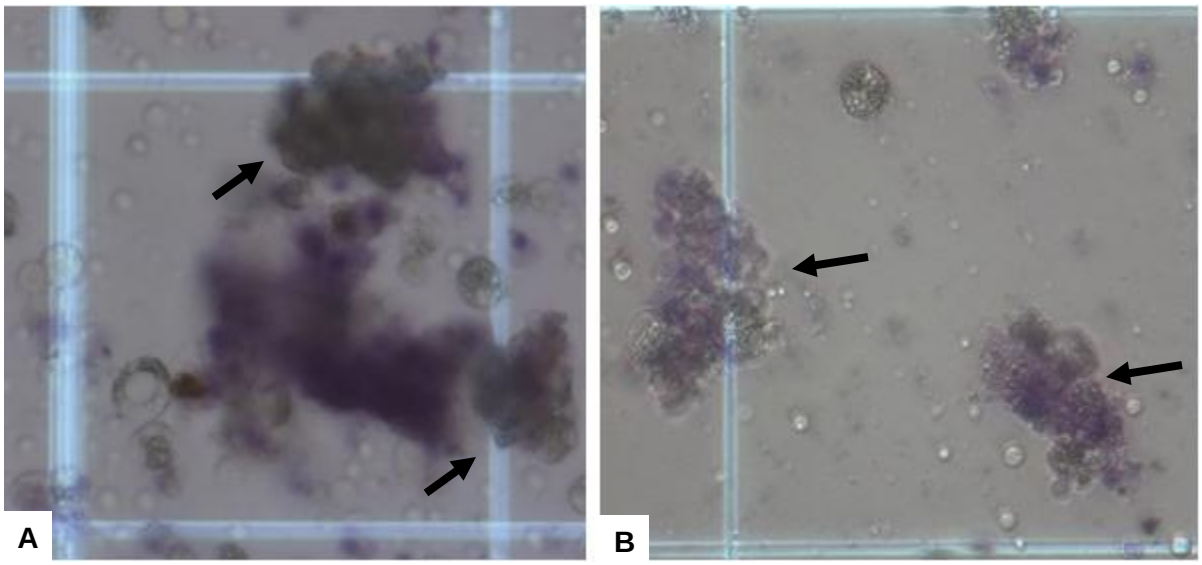

Figure 3. *Dysdercus peruvianus*' hemocytes microaggregation induced by JBU in the absence of Calcium.

Hemolymph was collected from 5th instars in anticoagulant solution and centrifuged for 2 min at 4,400 *g*. Hemocytes were suspended in saline and treated with JBU (200 nM) for 1 hour at room temperature. Trypan blue was added to hemocytes right before microscopy analysis. (A) regular saline, (B) calcium free saline. Regular saline or calcium free saline were used from the first wash to the incubation, respectively. Arrows indicate microaggregates. 20x/0.5 magnification.

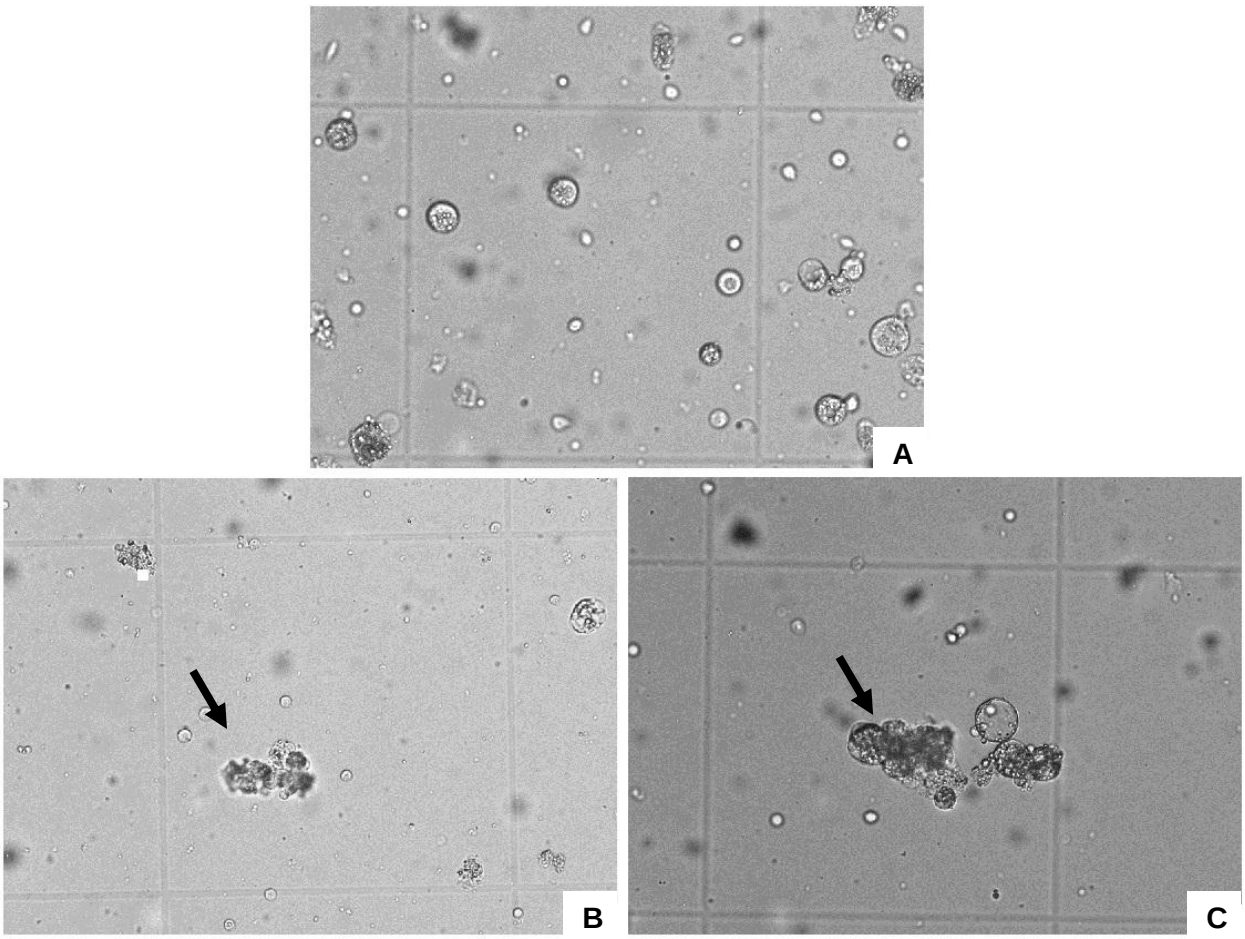

Figure 4. Dexamethasone inhibition of microaggregation induced by JBU in *D. peruvianus*' hemocytes.

Hemolymph was collected from 5th instars in anticoagulant solution and centrifuged for 2 min at 4,400 *g*. Hemocytes were suspended in saline and treated with dexamethasone in different concentrations for 30 min at room temperature. Subsequently, hemocytes were incubated with JBU (200 nM) for 1 hour at room temperature. (A) 0.05 mM dexamethasone, (B) 0.01 mM dexamethasone, (C) 0.005 mM dexamethasone. The arrows indicate the microaggregates. 20x/0.5 magnification.

4. Discussion and Perspectives

The results presented in this work show that hemocytes respond to *Canavalia ensiformis* (jackbean) urease through microaggregation of *D. peruvianus* hemocytes.

Canavalia ensiformis seeds are the natural source of the glucose/mannose specific lectin concanavalin A, well known for its hemagglutinating activity (Carlini & Guimarães, 1991). In this study we used highly purified crystalline jackbean urease (JBU) to exclude the presence of contaminant concanavalin A. On the other hand, jackbean urease itself was shown to behave as a univalent lectin (thus not able to produce agglutination of cells), binding to sialic acid containing glyconjugates (Follmer et al., 2001). Since insects do not synthetize sialic acid (Friedman, 1985), a possible lectin-like effect of JBU producing the microaggregation of hemocytes was discarded.

In this study, *Canavalia ensiformis* urease was tested for potential actions on immune signaling in the hemipteran model *Dysdercus peruvianus*. Previously mentioned, cellular reactions are coordinated by eicosanoids and are involved in several defense functions in insects. The results presented in this work show that *D. peruvianus* hemocytes respond *in vitro* to urease through microaggregation. Also in this study, we showed that the glucocorticoid dexamethasone, a PLA2 indirect inhibitor, might have an influence in this reaction. Although dexamethasone has been used as an inhibitor before, nothing has been published about its specificity on eicosanoid pathways within insects. Eicosanoids are known to mediate cellular reaction in insects inoculated with fungi and bacteria (Dean *et al*., 2002; Jurenka *et al*., 1999; Lord *et al*., 2002; Mandato *et al*., 1997; Stanley, 2000; Stanley-Samuelson *et al*., 1991 and 1997) and previous studies showed dexamethasone counteracting the suppression of the immune system by infection within animal models (Garcia *et al*., 2004). Taking into consideration that eicosonoids are molecules that signal inflammatory responses in mammals and signal immune responses in insects, we could deduce that urease is prompting an inflammatory response in *D. peruvianus*. When the experiment was developed with calcium free saline, JBU aggregative activity did not change completely, but reduced the melanized cells and number of aggregates.

In conclusion, JBU was found to directly activate *D. peruvianus* hemocytes in nanomolar doses by inducing an aggregated response. Furthermore, dexamethasone was found to decrease this aggregated response of *D. peruvianus* hemocytes, suggesting eicosanoid metabolites are involved in the reaction. Additional experiments need to be done to elucidate

these initial results. Studies with feeding assays and injections of JBU in the hemolymph of *D. peruvianus,* for *in vivo* assays; the use of different eicosanoid synthesis inhibitors, such as indomethacin and nordihydroguairetic acid; the analysis of hemocytes differentiation; and the establishment of hemocyte cultures, in order to develop different protocols. With this study we were able to show that JBU is acting in hemocyte cellular signaling, opening doors to a novel chapter of this great research topic, the multifaceted insecticidal activity of plant ureases.

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