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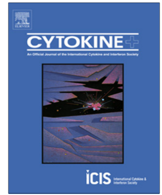

Homeopathic medicine Cantharis modulates uropathogenic *E. coli* (UPEC)-induced cystitis in susceptible mice

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A B S T R A C T

Objective: This is a random blinded placebo controlled murine experimental model to study the effects of Cantharis 6 CH, a homeopathic medicine, on *E coli*-induced cystitis.
Methods: 24 adult susceptible female BALB/c mice were inoculated with *E coli* – UPEC O4:K-:H5 by a transurethral catheter. Cantharis 6cH or vehicle (placebo) was offered to mice by free access into the drinking water (1:100), during 24 h after infection. Spleen, bladder and kidneys were processed for quantitative histopathology after immunohistochemistry, using anti-CD3, CD79, MIF, NK and VEGF antibodies; the cytokines present in the bladder washing fluid were measured using a LUMINEX-Magpix KIT. Mann-Whitney and Fisher exact test were used as statistical analysis.
Results: Cantharis 6 CH increased IL12p40, IFN-γ and decreased IL10 concentrations in the bladder fluid ($p \leqslant 0.05$); in the bladder mucosa, it increased the ratio between B and T lymphocytes (31%) and between B lymphocytes and MIF+ macrophages (57%, $p \leqslant 0.05$). In the pelvis, instead, it decreased the B/T cells ratio (41%, $p \leqslant 0.05$) and increased the M1/M2 macrophage ratio (42%, $p \leqslant 0.05$). No differences were seen in the kidney and spleen analysis.
Conclusion: The inverted balance of inflammatory cells and cytokines in bladder and pelvis mucosa shows specific local immune modulation induced by Cantharis 6cH.

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1. Introduction

Escherichia coli is one of the agents commonly involved in digestive and extra-intestinal infections, such as those in the urinary tract infection (UTI), being this pathotype called UPEC (uropathogenic of *E. coli*) [1]. The infection happens when uropathogens ascend and colonize the lower or upper parts of the urinary tract, originating cystitis and/or pyelonephritis [2]. That is due to the unbalance of the host-parasite relation which involves two simultaneous factors: the rupture of the hosts' organism's defense mechanisms and the presence of sufficient number of virulent microorganisms capable of adhering, multiplying and persisting in a portion of the urinary tract [3]. Since uropathogens present great resistance to available antibiotics, exploring alternative strategies for managing UTI is a theme of interest [4–7].

Two adhesins participate in uropathogenic processes: type 1 fringes and pili, both involved in bacteria colonization in the urinary tract [8–10]. The interaction between *E. coli* and the hosts' tissue, however, also depends on the immune condition. It is known that B-lymphocytes produce immunoglobulins capable of fixing complement and inducing bacterial lysis; the pro-inflammatory macrophages (M1) are capable of secreting cytokines, that amplify the interaction between lymphocytes and phagocytes, enhancing the presentation of antigens and specificity of the immune response [11]. A recent study developed *in vitro* showed that the interaction between B-lymphocytes and macrophages can ease the expression of pro-inflammatory cytokines by the later [12].

The development of medications capable of optimizing those interactions and, thus, decreasing the vulnerability of hosts bearers of UPECs to ascendant and systemic infection, would be very valuable for controlling UTI in susceptible populations. Homeopathy is

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appointed as a potentially useful tool in such cases. Recent results show that different homeopathic medications are capable of modulating the interaction macrophage-parasite *in vivo* and *in vitro*, modifying the dynamics of cellular migration between lymphoid organs and the infection site, as well as the phagocytic activity [13–17]. This dynamic was also reported to Cantharis in an experimental UTI [18].

Other studies have demonstrated the action of homeopathic medicines in inflammatory cells. In [19], Falkowski-Temporini and co-workers showed the increase in megakaryocytes and Kupffer cells, as well the predominance of Th1 response (increased TNF-a, IL-10, TNF-a/IL-4, TNF-a/IL-17, and decreased IL-6, IL-6/IL-4) were seen in mice infected with *Trypanosoma cruzi* and treated with *Lycopodium clavatum*. Besides the reported studies performed by our group [20,21], other studies about the properties of homeopathic products on the regulation of inflammatory process has been recently published [17,22–26].

The choice of the homeopathic medicine *Cantharis vesicatoria* occurred because of its characteristic action on the urinary tract, mainly in situations where the constant urging to urinate is seen, as well the presence of burning pain and frequent painful urination [27]. Despite of the fact that the traditional use of *Cantharis vesicatoria* is very stablished in homeopathic medicine, few scientific studies about its mechanisms are seen in the literature. This is the main contribution of this work, show some aspects of the mechanisms involved in its therapeutic effect, regarding to the regulation of local inflammatory process. The 6 CH dilution (or potency) was chosen because this is routinely used in the clinical practice, in acute cases, as reported by Fontes et al. [18] and Tarevnic e Pinto [28].

The aim of this study was to verify, in a murine experimental model, if homeopathic medicine Cantharis can interfere in the physiopathological aspects of *E. coil*- induced cystitis.

2. Methods

2.1. Animals and ethics

The animals used in this study were in accordance with Brazilian standards and ethic procedures regarding the use of laboratory animals. The project was approved by Paulista University ethics committee (CEUA-UNIP) under protocol n° 062/11 CEP/ICS/UNIP, in February 9th, 2012. The study used Balb/c female adult mice (N = 32) kept in controlled conditions of temperature (22–26 °C) and humidity (50–65%), in micro-isolators (Techniplast®) located in the SPF (Specific Pathogen Free) vivarium of UNIP's Research Center. The animals were fed *ad libitum* with water and food and were kept with light/dark periods of 12/12 h (light period at 7:00 AM). The use of animals in this study was inexorable, since the balance of cell migration among different levels of urinary tract has to be observed in a systemic approach.

2.2. Murine model of ascendant urinary infection

This protocol was defined after a series of previous pilot studies. The *Escherichia coli* strain JJ079, prototype of urosepsis (UPEC O4: K-:H5, genotype *pap*+, *sfa*+, *fimH*+, *hly*+, *cnf*1+, *fyuA*+, *traT*+, *malX* +), was cultivated overnight in LB medium (DIFCO®), centrifuged for 30 min at 15,000 RPM, 25 °C and suspended in sterile PBS to obtain the concentration of 7.5×10^{11} UFC/mL (5,0 Mac Farland scale). The pathogenicity of bacteria was checked before all the experimental procedures, by the hemagglutination test. The bacterial suspension was inoculated in each animal through a transurethral sterile catheter under sterile conditions. The mice were deprived of water for 4 h to warrant the emptiness of the bladder before inoculation. Two hours before, all animals were treated intraperitoneally with 5 mg/kg of disodium phosphate dexamethasone (0.1 ml/10g of body weight) in order to increase their susceptibility to bacterial infection. For the bacterial inoculation, the sedation was performed with association of two parts of xylazine 2% and one part of ketamine chlorhydrate 10%. This mix was diluted once again in 4 parts of sterile physiologic solution, to be administered intraperitoneally (0.4 ml/10g body weight). After verifying sedation, the urethral inoculation was carried out with 50 μL of UPEC JJ079 suspension (7.5×10^{11} UFC/mL), kindly inserted into the bladder with a 22G probe. After 24 h, the animals Were euthanized with an association between xilazine (50 mg/kg) and ketamine (125 mg/kg), injected intraperitoneally.

2.3. Preparation of Cantharis 6CH and 20% hydro-alcoholic solution (vehicle)

The homeopathic medication Cantharis 6cH is obtained from a beetle called *Cantharis vesicatoria*, and the mother tincture is obtained from the maceration of such insect. Herein, the medication preparation was prepared in an ANVISA (Brazilian Agency of Health) accredited commercial pharmacy and the method used to prepare this medication followed the Brazilian Homeopathic Pharmacopeia, 3rd edition, 2011 [29]. Thus, serial 1:100 dilutions of the matrix (*Cantharis versicatoria*) was made in 20% alcohol followed by automatic 100 vertical automatic mechanical agitations in a proper device (Autic®). The samples of the medications are stored in sterile amber flasks and kept in room temperature. This procedure was repeated 6 times in order to reach the final flask (6CH or 6th centesimal dilution following Hahnemann's method) to be used in the experimentation. For the preparation of the control, 20% hydro-alcoholic solution, the same dilution procedures were performed using only alcohol (vehicle). After the dilution of 1: 100 into the drinking water, there was no further agitation and the liquid just remained available to the animals.

2.4. Experimental design

The experimental animals were divided in 2 groups, with N = 12 per group, which were:

- ***Placebo group***: treated with the vehicle;
- ***Experimental group:*** treated with Cantharis 6 CH.

An additional ***Control Group,*** N = 8, composed by no inoculated nor treated animals, was also added for standardization of the histological features of the colony. All treatments started immediately after bacteria inoculation up to 24 h. Medicines were added 1:100 into the drinking water and offered to mice as free access. The pH of each drinking bottle was measured and no alteration was observed after the drugs adding (pH = 7.5 to 7.7). The free access in the water is a kind of homeopathic administration strategy used experimentally in previous studies and it is particularly useful in population veterinary medicine, as shown in *E coli* infection control in pig farms [13,14,16,30].

2.5. Necropsy and material harvesting

A unique blood sample was harvested with calibrated loop and seeded directly in a MacConkey agar (DIFCO®) plate, for UFCs counting. The bladder was divided in two equal parts. The first part was fixed in 8% paraformaldehyde for posterior histological procedures. The second fragment was grinded in a drop of sterile PBS and the washing fluid was frozen for posterior cytokines dosage. The histology was performed according to conventional paraffin-embedded inclusion and hematoxylin-eosin (HE) staining method.

Additionally, some sections were submitted to immunohistochemistry. The same procedure was done with both kidneys and spleen, from which a central fragment was harvested and fixed by the same method described above.

The qualitative analysis of the inflammatory spots in bladder and kidney revealed by the HE slides guided the choosing of materials sent to immunohistochemistry and cytokines evaluation. Eight slides were selected per group for this purpose, being priority the choice of positive samples, with the presence of clear inflammatory spots within the epithelial surface. The spleen fragment was used to evaluate the proportion between red and white pulp tissues, in order to know the lymphoid reactivity.

2.6. Immunohistochemistry and histometry

The entire surface of the spleen cuts were registered in an Eclipse E 200 photomicroscope (NIKON®) added to a Coolpix® camera and a LCD monitor, using a 4 fold objective. Photographs were analyzed in the Metamorph® authomatic system, in order to measure the number of pixels related to the delimited area corresponding to white and red pulps. Then, the ratio between these areas was, then, calculated.

The immunohistochemistry was used to identify different leucocytes populations present in the inflammatory infiltrate of bladder and kidneys, including the pelvis. The following markers were used: anti-CD3 (T-lymphocytes), CD79 (B-lymphocytes), MIF (M1 macrophages), NK (natural killer cells) and VEGF (M2 macrophages) (Fig. 1). After paraffin removal from slides, they were submitted to antigen unmasking process using 1% sodium dodecyl sulphate (SDS, SIGMA-ALDRICH®) solution for 5 min, with a triple washing procedure in PBS. The endogenous peroxidase blockage was done by immersion of cuts into 20% H_2O_2 in methanol, for 5 min. The nonspecific adsorption sites were blocked with regular equine serum 2.5% (VECTOR®), for 30 min. The cuts were incubated with monoclonal antibodies in different dilutions, overnight and at 4 °C, in humid chamber. The dilutions and clone details are resumed in Table 1. Following that, the slides were incubated with secondary antibody linked to polymer and peroxidase (*Kit Impress Universal* VECTOR®) for 30 min, in room temperature. The positive cells were highlighted after staining with DAB (VECTOR®). Negative controls of all reactions were made using only the diluent of the antibodies (DAKO®) instead of the primary antibody. The number of positive cells for each marker was registered, spot by spot; even those with restricted amount of cells were carefully examined.

2.7. Cytokines dosage

The bladder washing fluids were defrosted and aliquoted in a 96-well plate to be processed in a Luminex MAGPIX® (EMD Millipore®) apparatus, using a MILLIPLEX MAG PIX for 13 cytokines kit, according to the manufacturers' instructions. IL1a, IL1b, TNF alfa, IL 6, IL 10, IL12 p40, IL 12 p70, MIP-1b, INF gamma, MCP-1, RANTES/CCL 5, GM-CSF and VEGF-A were measured, in duplicate samples. Results were expressed in pg/mL. Samples with quantification below the detection limit for the analysis were registered as "zero" and samples above the quantification limit of the standard curb were registered with value equal to the highest value of the curb.

2.8. Statistical analysis

The data were statistically analyzed Mann-Whitney test for comparison between means, according to the sample homoscedas-

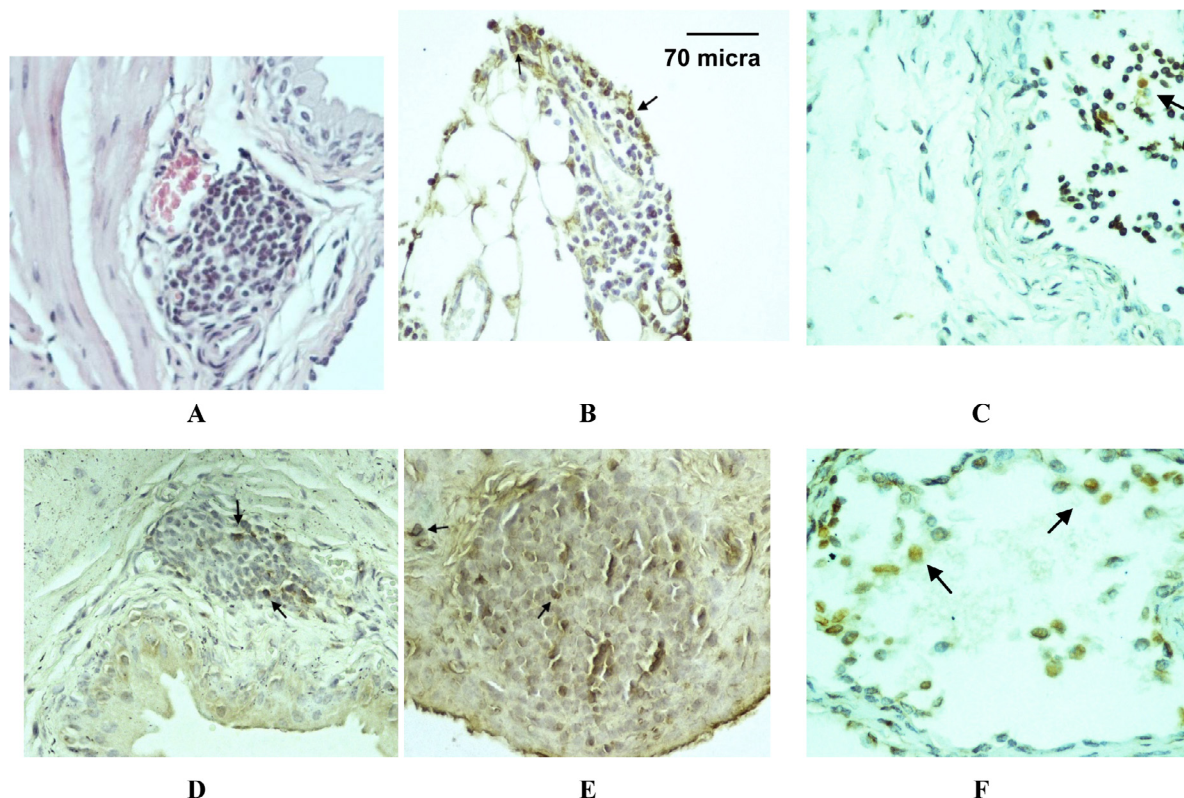


Fig. 1. A. Inflammatory infiltrate (cystitis) in bladder tissue stained by HE; B. CD3+ cells (T-lymphocytes) in bladder mucosa; C. CD79+ cells (B-lymphocytes) in the pelvis; D. MIF+ macrophages in bladder mucosa; E. GM1+ cells (Natural killer cells) in the pelvis; F. VEGF+ (M2) macrophages in the bladder mucosa. Immunohistochemistry revealed by DAB and counterstained by Harris hematoxylin. Images captured using 40 X.

Table 1
Markers used for immunohistochemistry.

Marker	Purpose	Molecular target	Donor species	Clone	Dilution	Brand
CD3	T-lymphocytes	Trans membrane protein	Rat	Clone KT3	1: 40	Serotec®
CD79	B-lymphocytes	Trans membrane protein	Mouse	Monoclonal	1:300	Serotec®
MIF	Marker of activated macrophages (M1)	Migration inhibitory factor	Rabbit	Polyclonal	1:200	Santa Cruz®
Anti-asialo GM 1 (NK)	Natural Killer Cell	Membrane ganglioside	Rabbit	Polyclonal	1: 300	Wako Pure Chemical Industries Ltd
VEGF	Marker of macrophages (M2)	Growth factor	Rat	VG-1 monoclonal	1:200	Abcam®

ticity, as verified previously by the Bartlett Test (software GraphPad Prisma, version 5.0). Fisher exact test (software GraphPad Instat 3.0) was used to compare proportions between groups in relation to the cystitis positive and negative mice and the two cell subtypes. In all cases, the significance level was fixed at $p \leqslant 0.05$.

3. Results

3.1. Bacterial Growth and cystitis gross pathology

There was no bacterial growth from the blood samples, but the animals presented acute cystitis with erythema, being the incidence equal to 100% in the vehicle group (12/12 animals) and 50% in Cantharis 6cH group (06/12 animals)

The presence or absence of erythematous mucosal in the bladder was registered by 2 independent observers, to avoid biases due to interpretation. The Fisher exact test revealed that $p = 0.0137$, being the relative risk equal to 0.3333 and the 95% confidence interval: 0.1734 to 0.6407, using the approximation of Katz.

3.2. Immunohistochemistry and histometry

According to Fisher exact test analysis, the treatment with Cantharis 6 CH increased the rate of B-lymphocytes when compared to the populations of T-lymphocytes and MIF+ macrophages in the bladders' mucosa ($p \leqslant 0.05$). In the renal pelvis, as an opposite, the animals treated with Cantharis 6cH presented significant decrease of B-lymphocytes when compared to T-lymphocytes and MIF+ macrophages ($p \leqslant 0.05$), as well as decrease in the rate of VEGF+ macrophages when compared to MIF+ macrophages ($p \leqslant 0.001$) (Tables 2 and 3, Fig. 2). The ratio of white pulp and red pulp of the spleen in the different groups showed no statistical significance (data not shown).

3.3. Citytokines dosage

The group treated with Cantharis 6 CH showed significant increase in IL12 ($p = 0.041$; $U = 22.50$) and IFN-γ ($p = 0.015$; $U = 17.50$) concentrations, when compared with the vehicle group; moreover, a decrease of IL10 ($p = 0.019$; $U = 18.50$) concentration was statistically significant too (Table 4).

4. Discussion

Urinary tract infection (UTI) is a public health issue, caused by *Escherichia coli* [31,32] in 75% of cases [33]. Chronic infections of the urinary tract are recurrent problems, due to the populations' aging and the increase in resistant organisms to antibiotics [4,32,34,35], for this reason, there is an effort in discovering new options of disease control. The knowledge of the pathogen-host interaction [33] and the immunomodulatory properties of cytokines [36,37], mainly those produced in UTIs [19], can help in this sense. The immune inflammatory response is regulated by the body, to maintain homeostasis, in this respect, the development of new immunotherapies it's important [38].

The activation of the inflammatory process can happen through IL-1, IL2, IL6, IL8, IL-12, IL-17, TNF-α and INF-γ, which can aid in the elimination of pathogens and resolution of the inflammatory process, as well as lead to the activation of macrophages, Natural Killer cells (NK), proliferation of B and T-lymphocytes and immunoglobulins secretion. The IL-4, IL-10, IL-13, TGF-β, considered anti-inflammatory cytokines, act as balance factors [37]. It is known that B-lymphocytes are responsible for Gram negative bacterial decrease in the context of an infection, including those caused by *Escherichia coli* [32]. In macrophages stimulated by LPS (lipopolysaccharide present in *E. coli*) *in vitro*, the IFN-γ and IL-6 production can be modulated in the presence of B-lymphocytes [12].

Our results show interferences in the balance between MIF+ and VEGF+ macrophages and between B and T lymphocytes in the pelvis mucosa after treatment with Cantharis 6cH. Similarly, changes in the balance between B and T cells in the inflammatory cells infiltrate of the bladder mucosa were also seen. Taking the data together, different populations are seen along the different segments of the urinary tract, in such a way that the major concentration of B lymphocytes is found in the bladder of mice treated

Table 2
Sum of T-lymphocytes, NK cells, MIF+ macrophages, B-lymphocytes and VEGF+ macrophages by group (N = 8 spots). Bladder, renal pelvis and kidney.

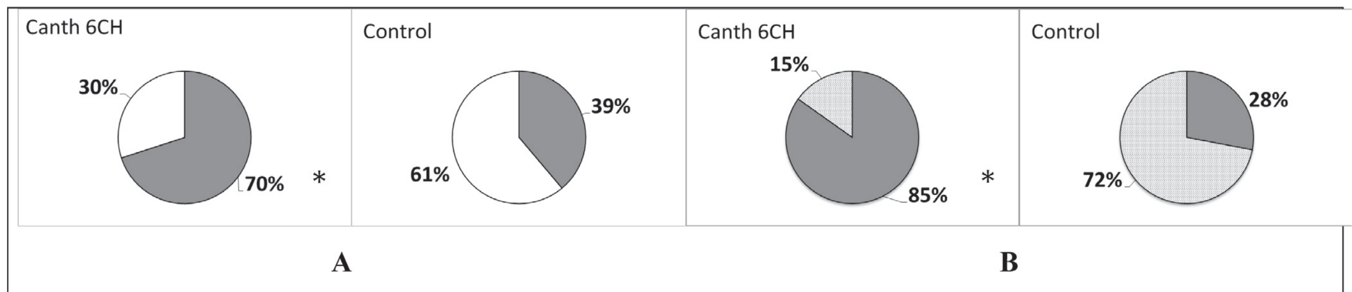
	Bladder		Renal pelvis		Kidney	
	Control	Cantharis 6cH	Control	Cantharis 6cH	Control	Cantharis 6cH
T Ly	11	12	10	15	0	10
B Ly	7	28*	19	5*	0	5
NK	31	7	3	1	1	0
MIF +	18	5	7	11	1	13
VEGF +	13	0	24	4#	0	0

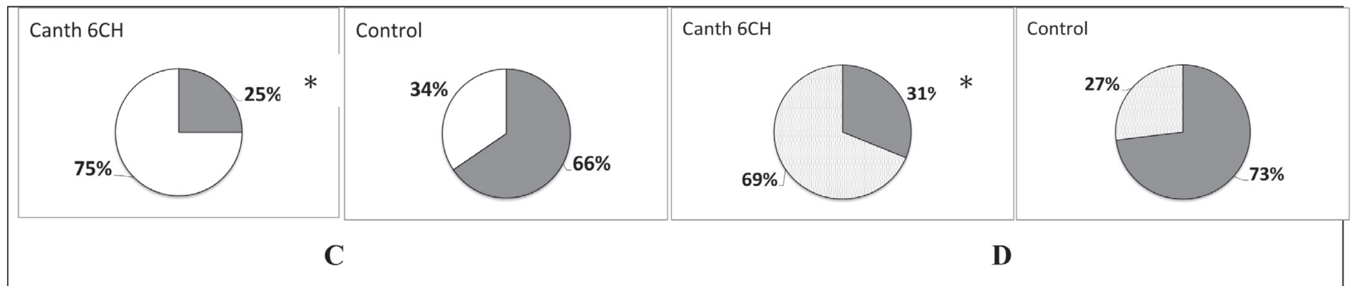
* Fisher Exact test, p = 0.05.
# Fisher Exact test, p = 0.01.

Table 3
Median and interval by group. Cells were identified from tissue sections labeled with specific antibodies, by immunohistochemistry. Bladder, renal pelvis and kidney were represented, from samples obtained from Cantharis 6 CH and vehicle group.

	Bladder		Renal pelvis		Kidney	
	Control	Cantharis 6cH	Control	Cantharis 6cH	Control	Cantharis 6cH
LT	0 (0–6)	0,5 (0–5)	0 (0–5)	0 (0–7)	0 (0–0)	0 (0–5)
LB	0 (0–7)	2,5 (0–11)*	0 (0–11)	0 (0–3)*	0 (0–0)	0 (0–5)
NK	1,5 (0–13)	0 (0–7)	0 (0–2)	0 (0–1)	0 (0–1)	0 (0–0)
MIF+	1,0 (0–7)	0 (0–3)	0,5 (0–3)	0 (0–7)	0 (0–1)	0 (0–12)
VEGF+	0 (0–9)	0 (0–0)	1 (0–12)	0 (0–2) #	0 (0–0)	0 (0–0)

* Fisher Exact test, p = 0.05.





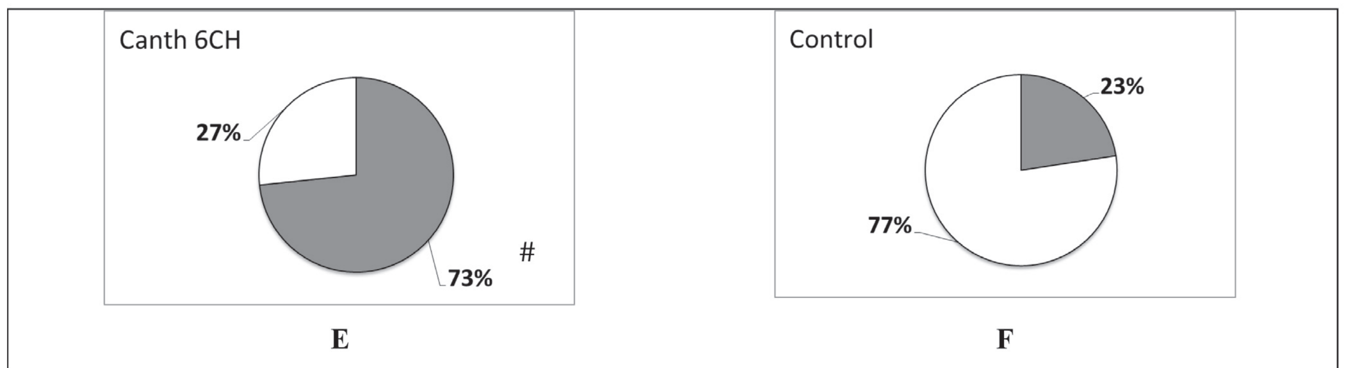


Fig. 2. B-lymphocytes/T-lymphocytes ratio and B-lymphocytes/MIF+ macrophages ratio in the bladder mucosa and renal pelvis, comparing Cantharis 6 CH and vehicle group. In the bladder mucosa, mice treated with Cantharis 6CH presented a shift toward B lymphocytes (grey) in relation T lymphocytes (white) [A] and MIF+ macrophages (light grey) [B] (Fisher exact test, $^{*}p \leqslant 0.05$). In the renal pelvis, treated mice presented reduction of B lymphocytes (grey) in relation to T lymphocytes (white) [C] and MIF+ macrophages (light grey) [D] (Fisher exact test, $^{*}p \leqslant 0.05$); moreover, in the pelvis, treated mice presented a shift toward MIF+ macrophages (grey) in relation to VEGF+ macrophages (white) [E, F] (Fisher exact test, $^{\#}p \leqslant 0.001$).

with Cantharis 6cH and the major concentration of T lymphocytes is found in the pelvis. This balance suggests a possible protective pattern of inflammatory and immune cells regarding to ascendance of the infection toward the kidney, since no difference between groups was seen in relation to the inflammatory cell populations present in the renal tissues. The same rationale could be applied to the balance between MIF+ and VEGF+ macrophages in the pelvis. Active MIF+ phagocytes are predominant in the pelvis of Cantharis 6cH treated mice, showing a higher phagocytic activity in this case. On the other hand, VEGF + macrophages usually

Table 4
Cytokines concentration (pg/ml) in the bladder washing fluid, represented by mean ± standard deviation. Mann Whitney test, *$p \leqslant 0.05$, **$p \leqslant 0.02$ compared to the vehicle group.

Cytokines	Control	Cantharis 6 cH
ϒ-IFN	4,55 ± 2,29	11,85 ± 7,47**
IL1a	84,15 ± 36,17	111,5 ± 54,77
IL1b	3,54 ± 3,70	7,00 ± 6,17
IL6	2,08 ± 0,74	6,25 ± 9,13
IL10	8,0 ± 3,76	4,13 ± 2,41**
IL12p40	5,53 ± 6,24	11,97 ± 8,47*
IL12p70	2,99 ± 1,91	3,56 ± 2,42
MCP1	7,91 ± 9,80	14,41 ± 17,86
MCP1b	4,35 ± 4,50	6,22 ± 6,58
RANTES	4,25 ± 1,27	4,22 ± 1,75
VEGF	1,82 ± 0,43	1,93 ± 0,37
TNF α	1,82 ± 0,33	1,99 ± 0,42

* Mann Whitney test, $p \leqslant 0.05$ compared to the vehicle group.
** Mann Whitney test, $p \leqslant 0.02$ compared to the vehicle group.

present an anti-inflammatory behavior [11,39]. A hypothesis could be proposed, in which active macrophages situated in the pelvis could orchestrate a Th2 response to activate the migration of B lymphocytes toward the lowest segments of the urinary tract, impairing the progression of the infection to the upper structures. However, this hypothesis needs supplementary studies to be confirmed.

Such changes are accompanied by significant increase of γIFN and IL12p40 in the bladder washing fluid, concomitant to the decrease of IL10. Hannan et al. [34] observed that the development of chronic cystitis is preceded by biomarkers of local and systemic inflammation 24 h after infection, which corroborates our findings. Moreover, the IL-10 regulates the immune response in potentially excessive infections having an anti-inflammatory role but, in some cases, the IL-10 production can damage the elimination of infectious agents, leading to the latency of the infection [34,40]. In co-culture of uroepithelial cells, B-lymphocytes can secrete the double the amount of IL-10 after a challenge [41]. In this work, since the cytokines were measured from the fluid only, it was impossible to distinguish the local variations of them (from bladder to pelvis). The balance of soluble cytokines measured in the fluid is, thus, the average of the whole urinary tract behavior, face to the treatment or not. In this sense, a possible more active and faster immune response was held in Cantharis 6cH treated mice in relation to the control, which could explain the improvements seen in the gross pathology.

5. Conclusion

In the present study, the treatment of mice with Cantharis 6 cH induced an inverse relation between the concentrations of IL12 p40 / γIFN and IL-10 in the bladder washing fluid, together with increase of B/T lymphocytes balance in the bladder mucosa associated to the predominance of MIF+ macrophages in the pelvis. These orchestrated changes probably lead to a more refined and narrowed inflammatory response against the UPEC infection, that seems to be more efficient to promote the infection remission.

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