Full Length Research Paper

Larrea divaricata Cav. enhances the innate immune response during the systemic infection by *Candida albicans*

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Larrea divaricata Cav. is a plant used in Argentinean folk medicine. The aim of this work was to evaluate the effect of a purified fraction (F1) obtained from an extract of the plant on murine macrophages (MØ) against a systemic infection by *Candida albicans*. Mice were divided in four groups: 1) healthy without treatment; 2) healthy with F1 treatment; 3) infected, without treatment; and 4) infected with F1 treatment. Peritoneal MØ were harvested and the following tests were performed: Apoptosis, phagocytosis, hydrogen peroxide (H_2O_2) production, nitric oxide (NO) production, determination of seric TNF-α and IL-10 and toxicity of F1. Results showed that F1 increased phagocytosis, H_2O_2 production, superoxide anion and NO levels in presence of *C. albicans*. These effects are probably due to a modulate mechanism of diverse receptors, such as TLR-2, Dectin-1, mannose receptor and CRs. Apoptosis and TNF-α levels were also increased by F1. The use of this fraction did not induce toxicity on mice. In conclusion, F1 induces a state of pre- activation of MØs, which is enhanced by the presence of *C. albicans*. The lack of toxicity in addition with the immunomodulatory action becomes F1 a potential safe treatment of disseminated candidiasis.

Key words: *Larrea divaricata,* fraction, macrophages, disseminated candidiasis, immunomodulatory activity.

INTRODUCTION

Over 60% of the world's population (80% in developing countries) depends directly of plants for medical purposes (Dhillion et al., 2002). Traditional medicine is still recognized as the primary health care system in many rural communities because of its effectiveness, lack of medical alternatives, and cultural preferences (Tabuti et al., 2003). Moreover many drugs with therapeutic properties employed in modern medicine are isolated from plants (Menichini et al., 2009; Simirgiotis et al., 2009). *Larrea divaricata* Cav. (Zygophyllaceae) is a plant with well documented applications in Argentinean folk medicine. This plant is widely used as immunomodulatory and antitumoral agent (Ratera et al., 1980), and to treat several diseases such as healing sores and wounds, rheumatism, inflammation, gastric disturbance, venereal diseases, arthritis (Lambert et al., 2002), tuberculosis and common cold (Tyler and Foster, 1999; Waller and Gisvold, 1945). In previous works, we demonstrated the antifungal (Davicino et al., 2007a) and antibacterial activities (Stege et al., 2006) of different extracts of *L. divaricata*. We also demonstrated that *L. divaricata*

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decoction has immunomodulatory effects both *in vitro* (Davicino et al., 2006) and *in vivo* (Davicino et al., 2007b). Moreover, fractions obtained from *L. divaricata* decoction are able to induce the activation of the innate immune system (Martino et al., 2010a). Notably, *L. divaricata* aqueous extracts contain very low amounts of nordihydroguaiaretic acid (NDGA) (Davicino et al., 2006; Obermeyer et al., 1995). This compound is present in ethanolic extracts and exhibits hepatotoxic and nephrotoxic effects.

Candida albicans is a common fungus of the normal flora of the oropharyx and the gastrointestinal tract of healthy individuals (Richardson, 2005). However, under predisposing conditions their colonization may lead to mucocutaneous and also invasive infection. *C. albicans* is the most frequent cause of systemic fungal infections in immunocompromised patients (Steinshamn et al., 1992). Phagocytes such as neutrophils and macrophages (MØ) are crucial to prevent systemic candidiasis, as these cells can clear the pathogen via phagocytosis. Furthermore, MØ activation leads to the release of several key mediators such as pro-inflammatory cytokines. TNF-α is pivotal in the host defense against disseminated candidiasis (Edwards, 1991). In immunocompromised patients who have drugs-resistant candidiasis, the differential activation of the innate immune system with compounds obtained from plants could be a very important alternative (Loyola et al., 2002). In this work the *in vivo* effect of fraction (F1) from *L. divaricata* was evaluated on *C. albicans* infected mice.

MATERIALS AND METHODS

Purification of F1

L. divaricata was collected in San Luis, Argentina. The plant was identified in the herbarium of the National University of San Luis (voucher number: UNSL # 467). Seven grams of *L. divaricata´s* lyophilized decoction was re-suspended on chloroform and chromatographied in a column of silica gel (130 g) by using a chloroform/methanol mix in a ratio 97,5:2,5 as mobile phase. The eluted fraction F1 was evaporated up to a volume of 2 ml and chromatographed by thin layer (TLC) to observe the band profile. For the assay an activated silica gel plate and 80:20 chloroform/methanol mix as stationary and mobile phase, respectively were used. F1 was re-suspended on sterile phosphate-buffered saline (PBS) for treatments (Martino et al., 2010).

Mouse infection

Female rockland mice (average weight 20 g) were used. Animals were housed and cared for at the Animal Resource Facilities, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis and handled according to the Comité Institucional de Cuidado y Uso de Animales (CICUA) guidelines. *C. albicans* was grown on Sabouraud medium for 48 h. Cells were resuspended in sterile PBS, washed twice and adjusted to 5×10^6 yeast/ ml before injection. *C. albicans* stain was isolated from an immunocompromised patient suffering a systemic infection. Mice were divided in four groups: 1) uninfected and without treatment (Control group); 2) uninfected with F1 treatment (F1 group); 3)

infected without treatment (Infected Control group); and 4) infected with F1 treatment (Infected + F1 group). Mice were challenged i.v. with 10^6 yeasts in a volume of 0.2 ml (Villamón et al., 2004). F1 treatment was carried by gastric administration, in a 15 mg.kg^{-1} concentration for 3 days, beginning 24 h after the infection. Controls received only PBS. All the experiments were carried out by using four mice per group. On day 4 after infection mice were killed and blood and peritoneal cells were obtained and processed for further studies.

Differential counting

Leukocytes were counted in all groups. For differential counting, smears were fixed for 5 min in methanol and stained with May-Grümwald Giemsa for 15 min.

Cell preparation

MØ were purified from peritoneal cells by adherence onto 96-well flat bottomed tissue culture plates in Dulbecco's Modified Eagle Medium (DMEM) with 20 µg/ml of gentamicin and 5% heat-inactivated fetal calf serum. Non-adherent cells were removed after 2 h at 37°C.

Toxicity assays

Serum creatinine and glutamic-oxalacetic transaminase (GOT) were determined with commercials kits according to the manufacturer instructions. Concentrations were expressed as mg/dl for creatinine and IU/L for GOT values.

TNF-α and IL-10 determination

TNF-α and IL-10 concentrations were quantified in serum of all groups of mice by an ELISA kit, according to the manufacturers' instructions. Cytokine concentrations were determined with a standard curve and expressed in pg/ml.

Assessment of apoptosis

Apoptosis was evaluated after staining with acridine orange/ ethidium bromide. Cells were stained with 10 µg/ml acridine orange/ ethidium bromide (Martin et al., 2004; Velardez et al., 2004) and counted using an Axiovert inverted microscope with an excitation filter 480/30 nm. Live cells were identified by the uptake of acridine orange (green fluorescence) and the exclusion of ethidium bromide (red fluorescence). Apoptotic cells showed condensation of chromatin stained by acridine orange and ethidium bromide and formation of apoptotic bodies. Necrotic cells were bigger, less stained and showed uniform labeling with ethidium bromide. The live cells were green, necrotic cells were red and cells in apoptotic process were both green and red (Robertson et al., 1999).

Effect of F1 on phagocytosis and ROS production

To evaluate phagocytosis MØ were fixed on slides. After the first incubation cells were exposed to *C. albicans* blastoconidia in a 1:10 MØ: yeast ratio at 37°C for 1 h (Loyola et al., 2002). Monolayers were washed twice with sterile PBS. Fungal uptake was evaluated by using Giemsa (10%) reagent for 20 min. A minimum of 150 MØ were scored. Cells containing four or more yeasts were considered as positive. Phagocytosis index was calculated as the number of
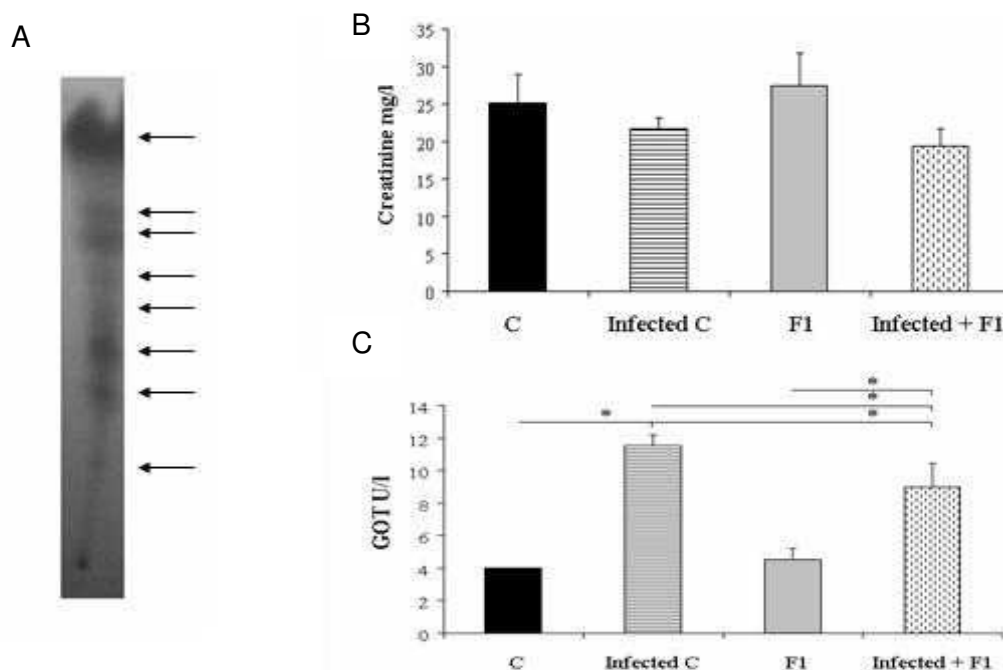
Figure 1. (A) Thin layer chromatography of Fraction 1 (F1) obtained from decoction of *L. divaricata* Cav. **(B)** Determination of serum Creatinine concentration. Concentration was expressed in mg/dl. **(C)** Determination of serum glutamic- oxalacetic transaminase (GOT) activity. Concentration was expressed in IU/L. Results were expressed by Mean ± SD of two experiments made by duplicate. *p< 0.05.

positive cells /total number of phagocytic cells (Blasi et al., 2006).

The production of reactive oxygen species (ROS) was evaluated with the nitroblue tetrazolium (NBT) assay. ROS reduces NBT to formazan, an insoluble blue salt (Schopf et al., 1984). We used 1 mg/ml opsonized zymosan (OPZ) to stimulate the oxidative burst. In all groups, MØ were incubated with NBT or NBT-OPZ and left 60 min. The reaction was stopped with 1N HCl. Formazan was extracted with dioxane and the absorbance was measured at 525 nm. Results were expressed as nM of reduced NBT/ 10^6 cells.

Determination of hydrogen peroxide

Cells from different groups were washed and a solution of phenol red containing 140 mM NaCl, 10 mM potassium phosphate pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 0.01 mg/ml type II horseradish peroxidase was added. After 1 h incubation the reaction was stopped with 10 ml of 4 N NaOH and absorbance was measured at 650 nm. A standard curve was prepared with known molar concentrations of H_2O_2 in buffered phenol red (Santos et al., 2004). The results were expressed as μM of H_2O_2/ 10^6 cells.

Nitrite determination

Cells isolated from different groups were cultured 48 h at 37°C. Then, free cell culture supernatants (100 μl/well) were mixed with 200 μl of Griess reagent (sulfanilamide and N-(1-naphthyl) ethylenediamide dihidrochloride) and incubated for 10 min at room temperature (Niknahad and O´Brien, 1996; Zhuang et al., 1998). Absorbance was measured at 540 nm and nitrite concentration was calculated with a sodium nitrite standard curve, generated for each experiment. Results are expressed as μM of NO per 10^6 cells.

Statistical analysis

Values are shown as mean ± SD. Differences between group means were assessed using analysis of variance (ANOVA), followed by the Student-Newman-Keuls test for multiple comparisons. A P value < 0.05 was considered significant.

RESULTS

Chromatographic analysis of the fraction 1 (F1)

The F1 from an aqueous extract of *L. divaricata* was analyzed by TLC, and revealed by ultraviolet light. As shown in Figure 1A, the F1 presents almost 8 bands that correspond probably to eight different compounds. For comparative purposes, a NDGA standard was chromatographed in parallel (data non show). This compound was absent in F1 as previously shown (Martino et al., 2010).

Toxicity assays

In a first step we evaluated the toxicity of F1 on mice. As shown in Figure 1B, the serum creatinine concentration was similar in all groups. GOT activity, a liver damage marker instead, showed an increment on infected groups compared with uninfected mice (Figure 1C). Interestingly,

Table 1. Total blood leukocytes count and blood leukocytes` number was measure in all groups by mixing 380 µl of Türk reagent and 20 µl of blood. Counting was carried out by using Neubauer chamber. Results were express as number of leukocytes/ mm3.

	Leukocytes/ mm^3
Control	2700 ± 141
Infected control	2125 ± 742
Fraction 1	4000 ± 353
Infected + Fraction 1	2875 ± 106

Table 2. White blood cells differential count. The WBC differential count was performed by using blood smears. Leukocytes were differentiated by the nucleus and cytoplasm morphologies after May-Grümwald Giemsa staining as neutrophils, eosinophils, basophils, lymphocytes and monocytes. A total of 100 cells were counted and the results were express as percentage of total cells counted.

%	Control	Infected Control	Fraction 1	Infected + Fraction 1
Neutrophils	23.25 ± 4.2	15.7 ± 3.1	13.25 ± 0.07	55.85 ± 0.9
Eosinophils	0 ± 0	0 ± 0	1.15 ± 1	4.35 ± 3
Basophils	0 ± 0	0 ± 0	0 ± 0	2.85 ± 1.8
Lymphocytes	71.7 ± 1.7	79.8 ± 1.7	73.85 ± 0.8	36.95 ± 9.2
Monocytes	5.05 ± 2.5	4.5 ± 1.4	11.75 ± 0.9	0 ± 0

in mice infected and treated with F1 the GOT activity was significantly reduced compared with infected controls ($p < 0.05$).

Hematological parameters

We also evaluate the variation of hematological parameters. The leukocytes counting showed a decrease in infected mice, compared to healthy controls (Table 1). Although F1 treatment increases the leukocytes number there are no significant differences. On the other hand, WBC differential count shows a formula inversion with an increase of neutrophils and the concomitant decrease of lymphocytes on infected F1 treat group ($p < 0.01$), compared with infected control (Table 2). Fraction 1 also increased monocytes counting in healthy animals ($p < 0.05$).

TNF-α and IL-10 production

Serum levels of TNF-α and IL-10, which are representative of an inflammatory or anti-inflammatory response respectively, were evaluated on day 4 after infection. A significant increment in TNF-α levels was observed at that time in F1 group ($p < 0.05$), while IL-10 concentration remained below 12 pg/ml, the detection limit of the technique (Figure 2).

Assessment of apoptosis

The apoptotic phenomenon was assessed with acridine orange /ethidium bromide staining. After treatment with F1, peritoneal MØ from both infected and uninfected groups showed chromatin condensation in the nuclear membrane and apoptotic bodies in the cytoplasm. Similar cell morphology was observed in infected control. On the control group mainly viable cells were observed. Representative photographs are shown in Figure 3.

Phagocytosis assays and ROS production

F1 increased phagocytosis *in vitro* in MØ from mice with and without infection, compared with control (Figure 4B). The phagolisosomal superoxide production of MØ was evaluated by the NBT assay. Zymosan, a constituent of the fungal wall, increased significantly the NBT reduction of all groups. As shown in Figure 4A, the production of ROS in F1 treated infected group was higher compared to uninfected control ($p < 0.05$). Moreover, compared with the control infected group, the infected + F1 mice showed a higher NBT reduction value ($p < 0.05$) (Figure 4A). Moreover, when infected mice were treated with F1, the H_2O_2 production by MØ was increased ($p < 0.05$) compared to infected and uninfected controls (Figure 4C).

Release of nitric oxide by macrophages

Finally, the production of nitrites released *ex vivo* was evaluated (Figure 5). A significant increment in NO levels was observed only in supernatants of MØ isolated from infected +F1 group compared to control ($p < 0.05$).
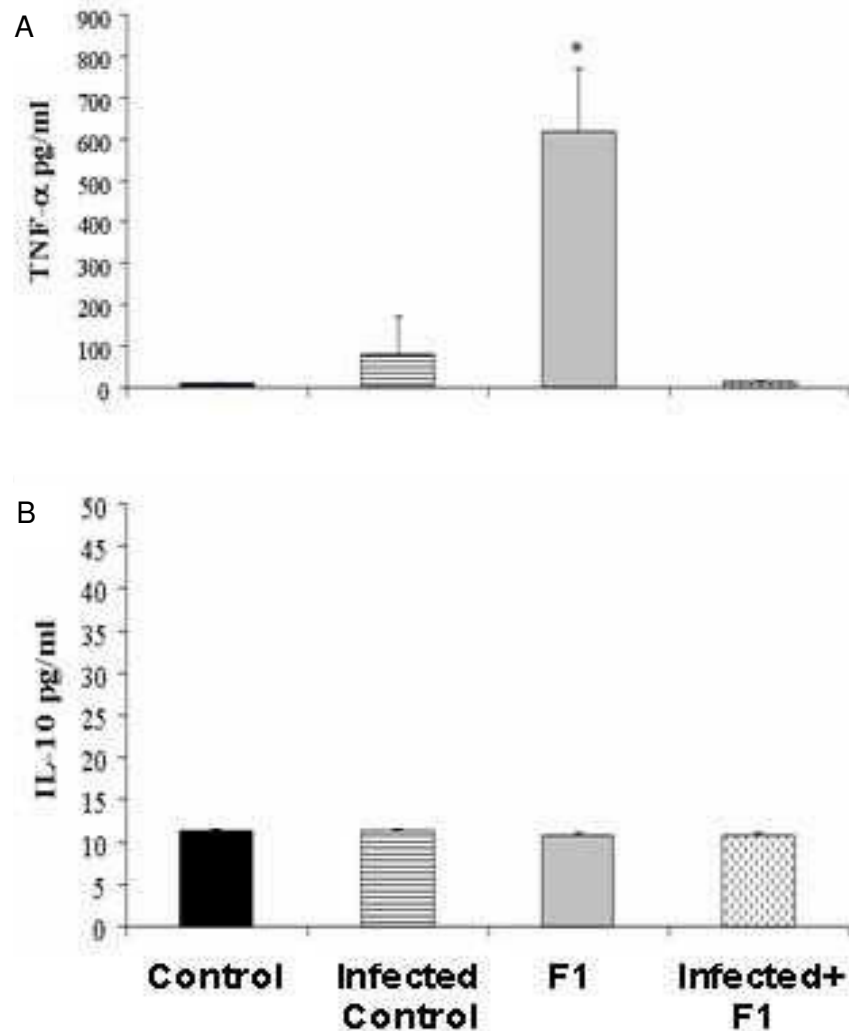
Figure 2. Determination of serum TNF-α (A) and IL-10 (B) on mice. Cytokines concentrations were expressed in pg/ml. Results were expressed by Mean ± SD of two experiments made by duplicate. *p< 0.05.

DISCUSSION

In this study, we tested the immunomodulatory activity of F1 obtained from *L. divaricata* decoction in *C. albicans* systemic infection. We choose F1 because this fraction does not contain NDGA. This phenolic compound is present in other fractions (F2 and F3) tested previously and shows hepatotoxic and nephrotoxic effects (Martino et al., 2010). Herein, we found that F1 does not produce toxic effects. The increment in GOT activity on infected groups is likely due to liver damage caused by the fungus. In fact, the treatment with F1 reversed the hepatic damage exerted by *C. albicans*. This fraction contains very low amounts of phenolic compouns. However cumarins could be the major compounds present in F1 (data non show).

The blood leukocyte count shows an increase when uninfected and infected mice were treated with F1 (Table 1). Moreover, F1 *per se* was able to invert the leukocyte differential count, by increasing the percentage of neutrophils, that is as we can see, cells that have internalized yeast on first days of infection are increased (Loyola et al., 2002). F1 exerts a pro-inflammatory effect. The absence of toxicity and its pro-inflammatory effect, make it the fraction chosen for study its effects in a mice model infected with *C. albicans*.

Experimental models evaluating host defense against *C. albicans* have shown that both, innate and acquired cell-mediated immunity are involved in the anti *Candida* response (Van't et al., 1992). Essential components of the immune defense against infections by *C. albicans* are phagocytic cells, especially MØ (Netea et al., 1999). These cells are able to kill *Candida* by internalization and fusion of the phagosome with a lysosome followed by
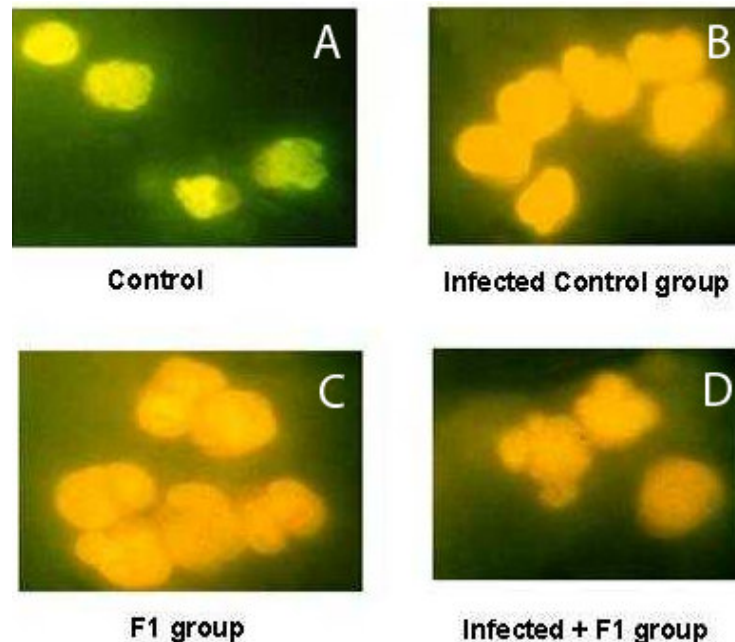
Figure 3. Apoptosis phenomenon evaluated by acridine orange/ ethidium bromide staining. Cells were removed from animals with different treatment: A: control, B: infected, C: treated with fraction, D: infected and treated with fraction. Magnification x 100.

degranulation of the fungicidal content into the immediate microenvironment (Alieke et al., 2002). For this reason, the effect of F1 on phagolisosomal superoxide (O_2^-) release was evaluated by using NBT reduction assay. We observed that macrophages stimulated with zymosan and treated with F1 showed an increased phagolisosomal O_2^- both, on infected and healthy mice. The same effect was observed in phagocytosis assay. These results suggest that F1 may condition MØ in presence of *C. albicans* (infected group), and when these cells are exposed again to this β-glucan constituent of the fungal wall (zymosan), phagocytic activity are enhanced. Possibly, the treatment with F1 up-regulate the expression of receptors such as CR1 and CR3 which bind efficiently zymosan and *C. albicans* wall (Brown et al., 2002). These receptors could promote the production of ROS (Marzocchi-Machado et al., 2000). This is an important mechanism involved in the ingestion and destruction of the yeast. Zymosan also binds to a β-glucan receptor, dectin-1 (Reid et al., 2009) and stimulates the production of ROS. Previously we have demonstrated that *L. divaricata* decoction up-regulated dectin-1 (Davicino et al., 2008), and the compound(s) responsible for this activity could be present in F1. Another receptor involved is TLR-2. According to Blasi et al. (2005) TLR-2 -/- MØ are significantly more effective than MØ wild type to phagocyte and kill *C. albicans*. Recent work reported the existence of a mannose receptor (MR), essential for *C. albicans* phagocytosis

(Vasquez-Torres and Balish, 1997). Possibly, both F1 and *C. albicans* antigens contribute to the up-regulation of MØ receptors such as CR1, CR3, dectin-1 or MR.

In order to evaluate other ROS than O_2^-, H_2O_2 was determined. As show Figure 4C, F1 *per se* decreased H_2O_2 level, but in presence of *C. albicans* a significantly increase in H_2O_2 production was observed ($p< 0.05$). In this case H_2O_2 probably comes from the phagolysosome. The increase of H_2O_2 production, but not of O_2^- on infected mice, could be due to the activity of Mn^{2+} superoxide dismutase from *C. albicans*. This enzyme plays an important role in the protection of yeast from oxidative damage of O_2^- (Rhie et al., 1999). However, F1 in presence of *C. albicans*, reverses this effect by increasing the overall production of ROS (both, O_2^- and H_2O_2), being available more free radicals to attack and destroy *C. albicans*. Other reactive species produced by oxidative stress is NO. F1 was only able to increase NO production in presence of infection, but not in uninfected mice (Fig 5). The same effect was observed in previous works, when *in vivo* treated MØ were co-cultivated with *Candida* (Martino et al., 2010b, unpublished). NO synthesis is the primary candidacidal mechanism of the murine peritoneal cells activated by *C. albicans* infection (Rementería et al., 1995). Thus, the increase of NO production induced by F1 on infected mice is particularly important to kill and destroy *C. albicans* yeast and for resolution of the infection.
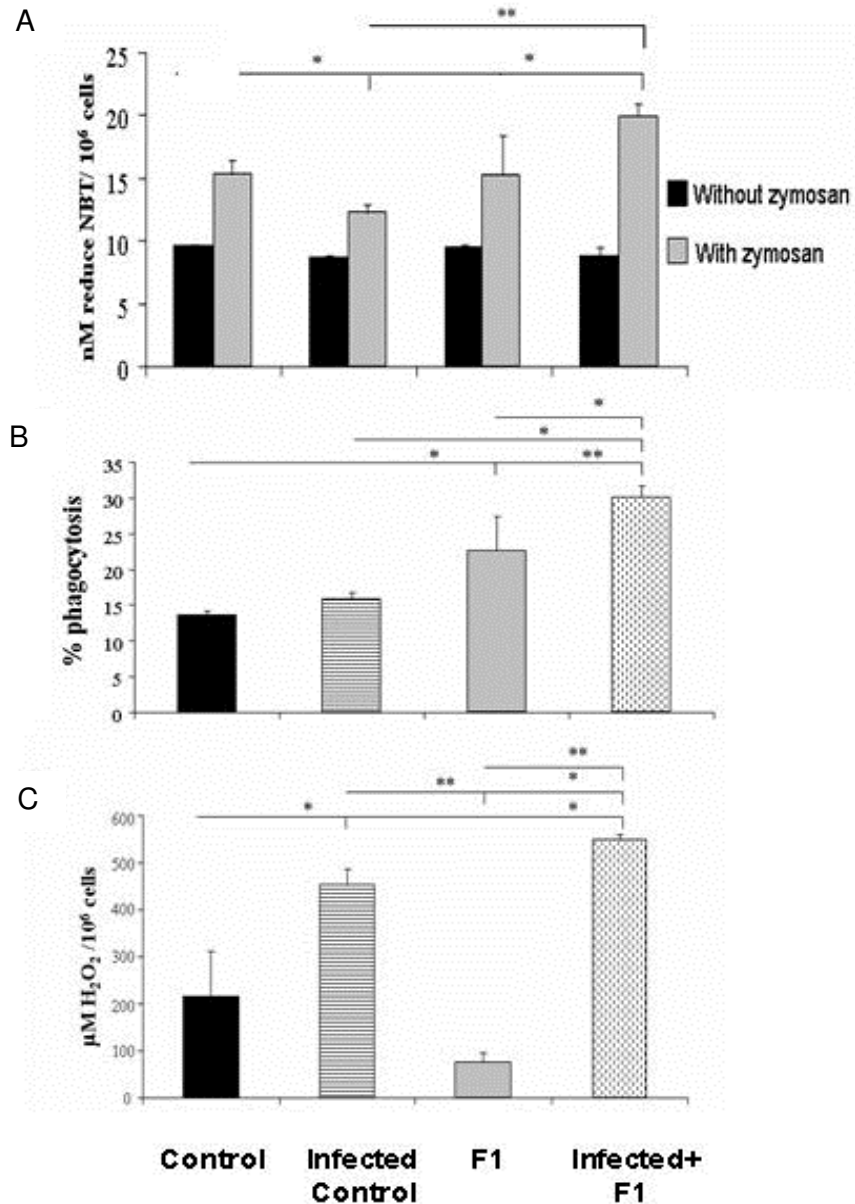
As apoptosis is a phenomenon related to MØ

A

B

C

Figure 4. (A) Effect of fraction 1 on ROS production by the NBT test. Results were expressed as concentration of NBT reduced (nM)/10^6 cells. *p< 0.05; ** p< 0.01. (B) Effect of F1 on the phagocytic activity by peritoneal macrophages after Giemsa staining. The phagocytosis index was calculated as the number of positive cells /total number of phagocytic cells. *p< 0.05; ** p< 0.01. (C) Production of H_2O_2 by peritoneal macrophages triggered by F1. Results were expressed as µM of H_2O_2 / 10^6 cells. *p< 0.05; ** p< 0.01. All results were expressed by Mean ± SD of two experiments made by duplicate.

activation, the possibility that F1 increase apoptosis was study. It is known that TNF-α mediates apoptotic signal through activation of initiator caspase-8 and then effector caspases-3, caspases-6 and caspases-7 (Gupta and Gollapudi, 2005). This phenomenon has been observed by using *Acalypha wilkesiana* on lymphocytes and granulocytes (Büssing et al., 1999). We observed that, as shown Figure 3, apoptosis occurs on all groups, except

on uninfected control group. *C. albicans* induced early apoptotic changes in MØ that internalized the yeast *in vivo* (Gasparoto et al., 2004). Moreover, F1 *per se* induces apoptosis *in vivo*. This mechanism is not due to an activation process because F1, in absence of *C. albicans*, does not produce an increase of ROS (Figures 4 A and C). Probably, apoptosis is due to the increase observed of TNF-α (Figure 2a).
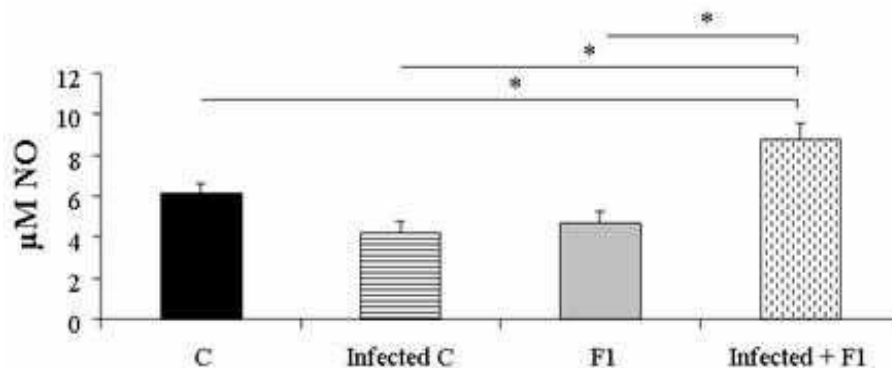
Figure 5. Production of nitric oxide (NO) triggered by F1, results was expressed as µM of NO per 10^6 cells by Mean ± SD of two experiments made by duplicate. *$p < 0.05$.

With the aim to evaluate the type of response that F1 induce *in vivo*, TNF-α and IL-10, inflammatory or anti-inflammatory cytokines respectively, were evaluated. TNF-α plays an important role on host protection against *C. albicans* infection (Netea et al., 2004). As previously stated, F1 increased TNF-α concentration, which triggers a protective response to infection. However, when infected mice were treated with F1, a decrease of TNF-α at similar levels of those of control mice was observed. This behavior could be due to a direct effect of *Candida* on mice. These results are according to Van der Graaf et al. (2005) who observed that after 96 h of infection, *C. albicans* decrease TNF-α production to values close to control. In fact, they demonstrated that TNF-α production by MØ depends directly of TLR-2 stimulation by *C. albicans*. Furthermore, the increase of serum TNF-α by F1 probably is due to different pathway stimulation, or to the production by another cell type. IL-10 is produce by LTh-2 and MØ. It acts as a potent suppressor of MØ function. As show Figure 2B, there is no variation on IL-10 concentration four days after infection. This is probably due because the determination was made four days after infection and IL-10 could appear later.

In short, we observed that F1 triggers several defense mechanisms in MØ, as increased phagocytosis along with reactive oxygen compounds and nitric oxide production, apoptosis induction, and an increase of serum TNF-α. In conclusion, we observed that F1 induces a state of pre- activation of macrophages, which makes more effective the response against *C. albicans* infection. The lack of toxicity in addition with the immunomodulatory action becomes F1 a potential safe treatment of disseminated candidiasis.

ACKNOWLEDGEMENTS

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