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Original article

In vivo effect of three fractions of *Larrea divaricata* Cav. (jarilla) on the innate immune system: macrophage response against *Candida albicans*

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Summary

Larrea divaricata Cav. (jarilla) is a plant with well-documented applications in folk medicine in Argentina. In this study, we aimed to evaluate functional parameters of peritoneal macrophages isolated from mice injected with three fractions (F1, F2 and F3) of *L. divaricata*. The response of macrophages against *Candida albicans* was evaluated. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, apoptosis was evaluated using Giemsa, acridine orange/ethidium bromide and ladder assay, oxidative burst was assayed using nitroblue tetrazolium test and nitrite production using Griess assay. Cell stimulation and their ability to kill *C. albicans in vitro* were measured. The number and cell viability were similar to controls. However, we found that F1 induces pre-activation of macrophages, and this pre-activation is enhanced by *C. albicans*. The effects exerted by F1 make it more important than F2 and F3 for the treatment of disseminated candidiasis in patients with immunodeficiency diseases such as AIDS and chronic granulomatous disease, among others.

Key words: *Larrea divaricata* Cav., fractions, innate immune response, *in vivo* effects, *Candida albicans*.

Introduction

Larrea divaricata Cav. (Zygophyllaceae) is a plant with well-documented applications, such as immunomodulatory and antitumoral properties, in Argentinean folk medicine.[1] This plant is also widely used to treat diseases such as healing sores and wounds, rheumatism, inflammation, gastric disturbance, venereal diseases, arthritis, tuberculosis and common cold.[2–4] In previous studies, we have demonstrated antifungal and antibacterial activities of different extracts of the plant.[5,6] We also demonstrated that *L. divaricata* decoction has immunomodulatory activity both *in vitro* and *in vivo*.[7,8] Moreover, fractions isolated from the decoction induced the activation of the innate immune system *in vitro*.[9] On the other hand, Davicino *et al.* [10] showed the effect of an aqueous extract of this plant on proliferation, apoptosis, activation of Mn^{2+}–dependent superoxide dismutase, and production of H_2O_2 and nitric oxide in the tumour cell line BW5147.

In healthy individuals, phagocytic immune cells such as macrophages (MØs), monocytes and neutrophils[11–13] are pivotal in systemic infections. Innate immunity serves as an essential first line of defence against microbial pathogens and may also have influence on the nature of the subsequent adaptive immune response. Macrophages play a key role in innate immunity because of their ability to recognise, ingest and destroy many pathogens by oxidative and non-oxidative mechanisms.[14] Selective stimulation of this cellular population with non-toxic compounds could be important not only to elucidate the function of activated MØs in immune defence, but it may also contribute to

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develop therapeutic applications.[15] It is important to note that while ethanolic extracts of *L. divaricata* contain nordihydroguairetic acid (NDGA), with well-known hepatotoxic and nephrotoxic effects, the aqueous extracts contain very low amounts of NDGA.[7,16]

The aim of this study was to evaluate the effects of *C. albicans* on peritoneal MØs from mice injected with fractions F1, F2 and F3 obtained from *L. divaricata* Cav.

Materials and methods

Preparation of plant extracts and fractions

Leaves and tender branches of *L. divaricata* were collected in San Luis, Argentina. The plant was identified in the herbarium of the National University of San Luis (voucher number: UNSL #467). Leaves and tender branches were dried in a stove at 45 °C for 5 days and reduced to fine dust. From this material, we prepared Decoction (D) as follows: 5 g of dust plus 100 ml of distilled water was heated to boiling temperature and allowed to stand for 20 min. Percentage yield was 21.01% (w/w). The extract was filtered and centrifuged at 1370 ***g*** for 15 min. The supernatant was filter-sterilised through a 0.22-μm filter, lyophilised, aliquoted and stored at 20 °C until use.[8]

Fractions were prepared as described previously.[9] Briefly, 7 g of lyophilised material was resuspended in chloroform and chromatographed in a column of silica gel (130 gr) using different chloroform/methanol ratios (97.5 : 2.5; 95 : 5; 92.5 : 7.5; 90 : 10; 80 : 20 and pure methanol) as the mobile phase as described. The eluted portions were evaporated up to a volume of 2 ml. Later, eluted portions were chromatographed by thin layer and the band profile was observed using UV light. All the portions with similar profiles were pooled. Five fractions (F1–F5) were obtained and dried.[9] The dry weights of the fractions were: F1: 118 mg; F2: 288.11 mg; F3: 56 mg, F4: 72 mg and F5: 315 mg. Only F1–F3 were used in cell studies, as in previous reports.[9] Before assays, all drugs and extracts used were subjected to the *Limulus* test for the detection of lipopolysaccharide (LPS) contamination. The LPS was either low (0.01 EU ml^{-1}) or not detectable.

Treatment of mice with *Larrea divaricata* extracts

Male and female Rockland mice (average weight 20 g) were used in this study. Animals were housed and cared for at the Animal Resource Facilities, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, in accordance with institutional guidelines. Groups of four to five mice were injected i.p. twice in a 48 h period with 0.5, 5 and 15 mg kg^{-1} of fractions. Animals received a total volume of 10 ml kg^{-1} of fractions diluted in PBS. Control animals were inoculated only with phosphate-buffered saline (PBS).

Cell preparation

Peritoneal cells (PC) were harvested as described previously by us[8], i.e. 48 h after the second i.p. injection by sterile lavage with 20 ml Hank's Buffered Salt Solution (Sigma, San Diego, CA, USA) supplemented with 20 μg ml^{-1} gentamicin (Sigma) and heparin (Sigma) (50 U ml^{-1}) and adjusted to 1×10^6 cell ml^{-1}. Macrophages were purified by adherence onto 96-well flat-bottomed tissue culture plates in the cell culture medium Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) with 20 μg ml^{-1} gentamicin and 5% heat-inactivated fetal calf serum (FCS) (Gibco, Rockville, MD, USA). Non-adherent cells were removed after 2 h at 37 °C and complete medium was added. Under these conditions, the adherent Mϕ monolayers showed >90% of purity according to morphological analysis or non-specific esterase staining. All cells used in this study were obtained according to this procedure.[8]

Assessment of cell viability

Cell viability was analysed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[17] MTT is a membrane-permeable tetrazolium salt, which is reduced to formazan by superoxide anion. After incubation, 100 μl of DMEM and 10 μl of 5 mg ml^{-1} MTT was added to each cell suspension and then incubated for 4 h. The purple formazan product formed by the action of reactive oxygen compounds on mitochondria in living cells was solubilised by the addition of acidic isopropanol. The formazan produced was measured at 570 nm. Cells from untreated mice were used as control of viability (100%) and results were expressed as percentage of viability with respect to control.[18]

Assessment of apoptosis

Apoptosis was evaluated after staining with Giemsa and acridine orange/ethidium bromide. After incubation, treated and untreated cells were stained with Giemsa[7] or 10 μg ml^{-1} acridine orange/ethidium bromide.[9] Cells were counted with an Axiovert inverted microscope (Carl Zeiss MicroImaging, Jena, Germany) using an excitation filter of 480/30 nm. Live cells were

enumerated by the uptake of acridine orange (green fluorescence) and the exclusion of ethidium bromide (red fluorescence). Apoptotic cells were identified by condensation of chromatin stained by acridine orange and ethidium bromide and by the formation of apoptotic bodies. Necrotic cells were identified by uniform labelling of the cells with ethidium bromide and they were bigger and less stained. Live cells were green, necrotic cells were red and apoptotic cells were both green and red.[9]

To assess the DNA fragmentation, MØs were lysed by the addition of 50 mmol l^{-1} Tris–HCl (Promega, Fitchburg, WI, USA) pH 7.5; 1 mmol l^{-1} EDTA (Promega); 0.2% Triton X 100 (Promega) and centrifuged at 13 000 *g* for 10 min. Then, DNA was precipitated by adding 700 μl of isopropanol (Betsil, Buenos Aires, Argentine) and 100 μl of 5 mol l^{-1} NaCl (Merck, San Diego, CA, USA), and the samples were left overnight at −20 °C. Then, the samples were centrifuged at 13 000 ***g*** for 10 min and the dry pellets were resuspended in 10 mmol l^{-1} Tris–HCl, 1 mM EDTA, pH 7.5. Samples were diluted 1/5 (v/v) in the loading buffer, resolved on a 1% agarose gel and stained with 0.5 μg ml^{-1} ethidium bromide. DNA visualisation was accomplished under UV light. As positive control, we used 1.5% dimethylsulphoxide (DMSO) (Mallinckrodt, Nashville, TN, USA).[8]

Oxidative burst assay

The production of reactive oxygen species (ROS) was evaluated using the nitroblue tetrazolium (NBT) assay.[19] In all assays, MØs were incubated with NBT for 30 min; the reaction was stopped with 1 N HCl; the insoluble salt formazan was extracted with dioxane and the absorbance was measured at 525 nm.

Nitrite determination

Cells from treated and untreated mice were incubated for 48 h in stove at 37 °C. Cell-free supernatants (100 μl $well^{-1}$) were mixed with 200 μl of Griess reagent (sulphanilamide and *N*-(1-naphthyl) ethylenediamide dihydrochloride) and incubated for 10 min at room temperature.[8] Absorbance was measured at 540 nm and nitrite concentration was calculated with a sodium nitrite standard curve, generated for each experiment. Results were expressed as μmol l^{-1} of NO per 10^6 cells.

Stimulation with *Candida albicans*

Macrophages from mice were treated with the highest concentration (15 mg kg^{-1}) of each fraction or from untreated mice were co-cultured with *C. albicans* in a 1 : 10 cell: yeast ratio for 1 h.[20] Then, we evaluated (a) phagocytosis by Giemsa staining,[21] (b) oxidative burst, (c) nitrite levels and (d) *C. albicans* killing.

Killing of *Candida albicans*

Macrophages from treated and untreated mice were incubated for a further 1.5 h at 37 °C in DMEM on 96-well flat-bottomed tissue culture plates to evaluate the killing of phagocytised *C. albicans* cells (ratio 1/10). The medium was removed and adherent MØs were lysed by cold diethylpyrocarbonated (DEPC) water plus Triton X-100 at 1%, removed by vigorous pipetting and pooled. The lysates were serially diluted and plated on Sabouraud agar (Britania, Buenos Aires, Argentina) to determine the amount of viable yeast after overnight incubation at 37 °C. The number of fungal cells was expressed as CFU per 10^6 MØs.[22]

Results

Cell viability, apoptosis and oxidative burst

The viability of peritoneal MØs from mice treated with *L. divaricata* fractions was similar to control (Fig. 1), except for 15 mg kg^{-1} F2 that showed an increased viability ($P < 0.05$). As shown in Fig. 2a, at different doses, the three fractions tested induced a significant increment of apoptosis evaluated by Giemsa staining compared with control: 15 mg kg^{-1} F1 ($P < 0.01$), 0.5 mg kg^{-1} F2 at ($P < 0.01$), and 5 mg kg^{-1} ($P < 0.05$) or 15 mg kg^{-1} F3 ($P < 0.01$). The apoptotic phenomenon was further assessed by acridine orange/ethidium bromide staining (Fig. 2b). After inoculation with *L. divaricata* fractions, MØs showed chromatin condensation in the nuclear membrane and apoptotic bodies in the cytoplasm. The apoptotic phenomenon was confirmed by the ladder assay. As can be seen, 15 mg kg^{-1} F1 induced apoptosis in MØs similar to positive control (Fig. 2c).

The superoxide production of MØs was evaluated by the NBT assay (Fig. 3). F2 at 5 mg kg^{-1} and F3 at 15 mg kg^{-1} increased significantly the superoxide anion levels ($P < 0.05$).

Phagocytosis and killing of *Candida albicans*

After co-cultures of MØs from treated and untreated mice, we evaluated the phagocytosis of *C. albicans* (Fig. 4a). As can be seen, F1, F2 and F3 (15 mg kg^{-1}) stimulate the phagocytosis of *C. albicans* ($P < 0.01$), but

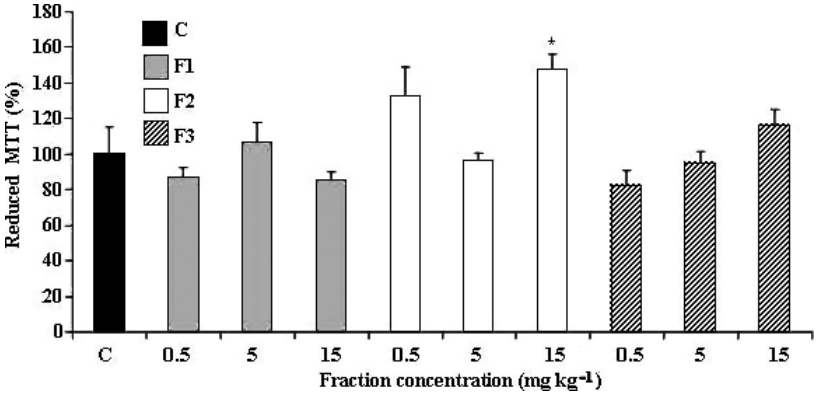

Figure 1 Effect of *Larrea divaricata* Cav fractions (F1, F2 and F3) on the viability of peritoneal macrophages from treated and untreated mice. The viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are expressed as reduced MTT relative to control (%), $^{*}P < 0.05$.

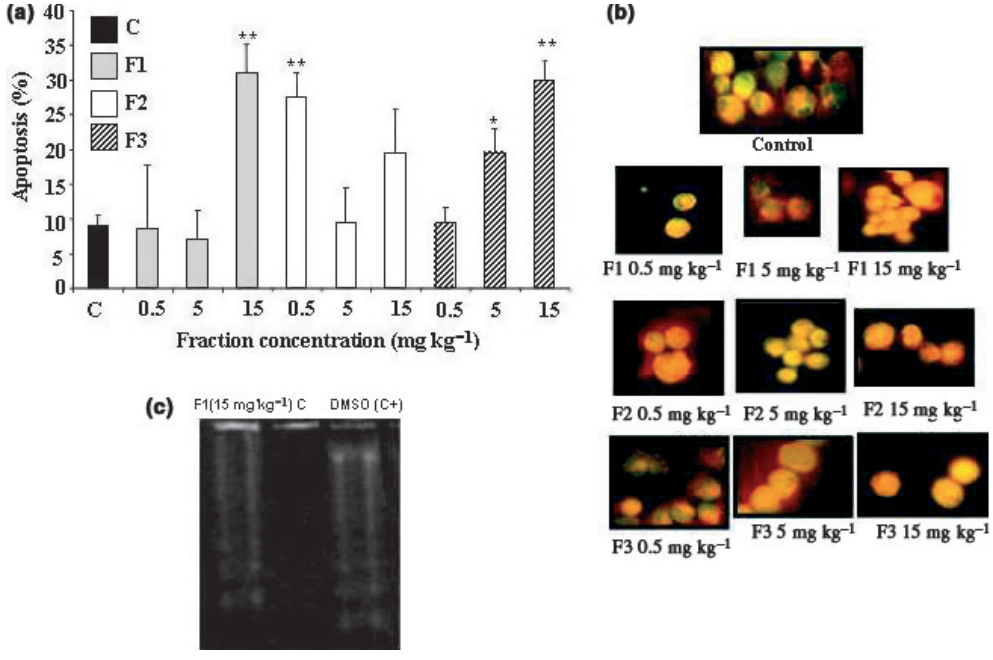

Figure 2 Apoptosis of peritoneal macrophages (Mϕs) after treatment of mice with *Larrea divaricata* fractions. (a) Apoptosis was evaluated after Giemsa staining. A minimum of 100 cells each was counted. Results are expressed as percentage (%) of apoptotic cells ($^{*}P < 0.01$; $^{**}P < 0.001$); (b) Representative microphotographs of acridine orange/ethidium bromide staining of each treatment. Mϕs from treated and untreated mice were incubated for 1 min with acridine orange/ethidium bromide and observed by fluorescence microscopy. Magnification ×100. (c) Mϕs from F1 and Control groups were lysed and centrifuged at 13 000 ***g***. Then, DNA was precipitated, centrifuged and pellets re-suspended. Samples were resolved on a 1% agarose gel and stained with ethidium bromide. DNA visualisation was accomplished under UV light. As positive control, we used 1.5% of dimethylsulphoxide (DMSO).

only F2 and F3 were able to stimulate the killing of the fungus (Fig. 4b) ($P < 0.05$).

Release of ROS and nitric oxide by MØs

When MØs from treated and untreated mice were co-cultured with *C. albicans* yeast, ROS production was significantly increased compared with unstimulated controls (Fig. 5a). Interestingly, F1 also showed a slight but consistent increment of ROS compared with control cells co-cultured with the yeast ($P < 0.05$). On the other hand, the production of total nitrites was evaluated after *C. albicans* stimulation. Compared with un-stimulated cells (Fig. 5b), the nitric oxide production was reduced in controls cultured with the fungus ($P < 0.05$). However, in

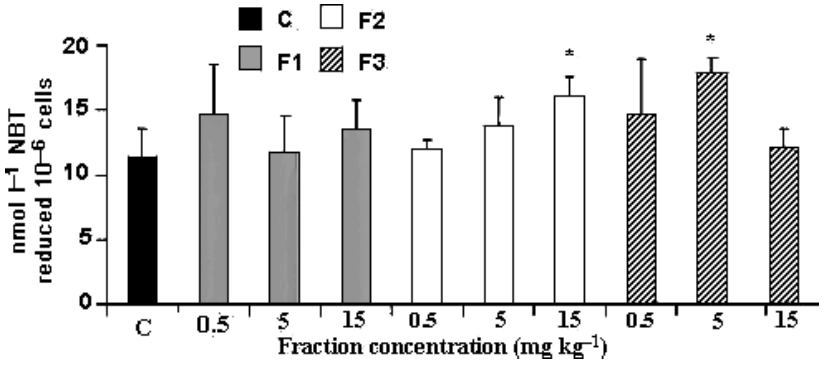

Figure 3 Effect of *Larrea divaricata* Cav fractions (F1, F2 and F3) on the production of superoxide anion. The reduction of nitroblue tetrazolium (NBT) to an insoluble formazan salt was assessed in macrophages from treated or untreated mice. Results are expressed as nmol l^{-1} reduced NBT/10^6 cells, $^*P < 0.05$.

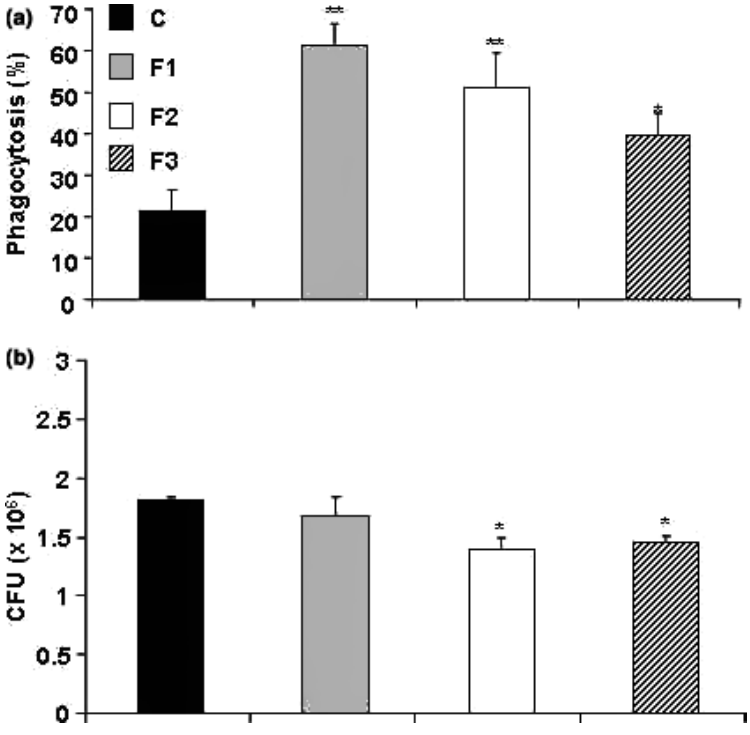

Figure 4 Effect of *Larrea divaricata* Cav fractions (F1, F2 and F3) on the phagocytosis of *Candida albicans*. Macrophages (Mϕs) from treated and untreated mice were incubated for 1.5 h at 37 °C with *C. albicans* cells (ratio 1/10). (a) Phagocytosis was evaluated by Giemsa staining; (b) After incubation, Mϕs were lysed, removed by vigorous pipetting, serially diluted and plated on Sabouraud agar to determine the amount of viable yeast scored as CFU after overnight incubation at 37 °C. The number of fungal cells was expressed as CFU per 10^6 cells. $^*P < 0.05$; $^{**}P < 0.01$.

MØs from F1- and F3-treated mice, the inhibition was abolished ($P < 0.05$).

Discussion

In this study, the effects of three fractions (F1, F2 and F3) obtained from *L. divaricata* decoction were tested on murine MØs. We found that F1 activates peritoneal MØs and enhances their candidicidal activity.

At the highest dose tested, *L. divaricata* F2 was able to stimulate an increased reduction of MTT (Fig. 1). When MTT reduction decreases, the viability is reduced.[8] However, it is difficult to interpret the increment in MTT reduction observed as an increase of viability. MTT is a membrane-permeable tetrazolium salt, which is reduced to formazan by superoxide anion in living cells,[18,23] either in the mitochondria or in intracellular vesicles identified as endosomes and lysosomes.[24] We have already demonstrated that *L. divaricata* decoction produces mitochondrial superoxide.[10] We believe that the high levels of reduced MTT could be indicating an increased production of superoxide anion.[25]

With some doses of F1 and F3 instead, we detected a trend to lower MTT reduction although without significance. This tendency correlated with results obtained in assays evaluating apoptosis (Fig. 2). In fact, the

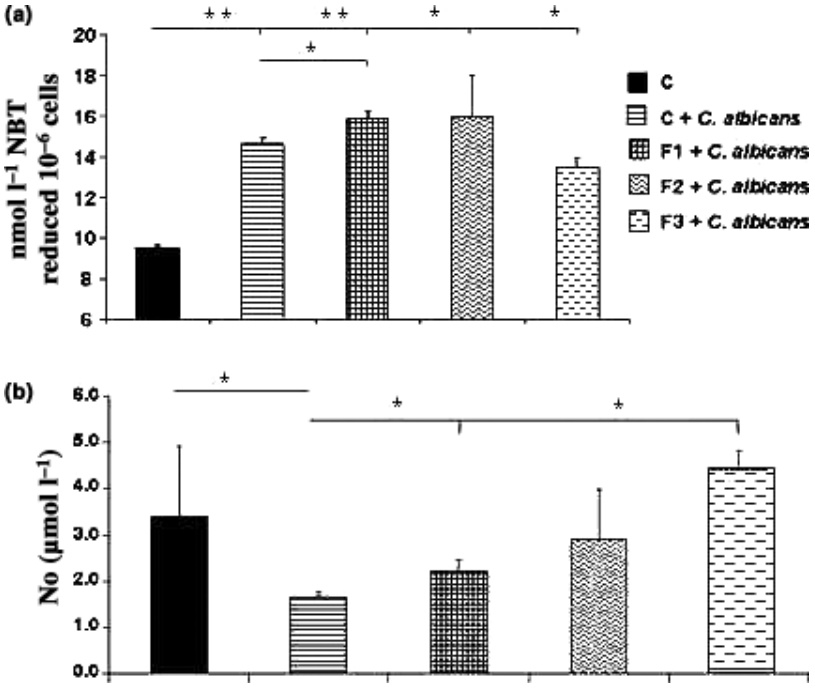

Figure 5 Production of superoxide anion and NO. Peritoneal macrophages from treated and untreated mice were incubated with or without *Candida albicans*. (a) The production of superoxide anion was assessed by the reduction of nitroblue tetrazolium (NBT) to insoluble formazan salt. Results are expressed as nmol l^{-1} reduced NBT/10^6 cells. (b) The content of nitric oxide in supernatants was determined by a colorimetric assay. The results are expressed as µM of NO. $^*P < 0.05$; $^{**}P < 0.01$.

decreased viability observed in MØs when mice were treated with F1, F2 and F3 is due to the apoptotic phenomenon, as demonstrated by acridine orange/ethidium bromide staining and ladder assay (Fig. 2b, c). As apoptosis is related to MØs activation[7], we also investigated the activation status of MØs from treated mice. We found that at the highest doses evaluated, F2 and F3 were able to stimulate superoxide anion (Fig. 3).

Finally, the ability of treated MØs to phagocytose and kill *C. albicans* was enhanced by the three fractions at 15 mg kg^{-1}, with the highest effect after F1 administration (Fig. 4a). The treatment could up-regulate the expression of receptors that mediate the phagocytosis of the yeast stimulating different mechanisms to uptake and destroy *C. albicans*. For instance, CR1 and CR3 are able to bind efficiently *C. albicans* promoting the production of ROS.[26,27] The yeast also binds to a β-glucan receptor, (dectin-1) and stimulates the production of ROS.[28] Recent study has reported the existence of a mannose receptor (MR), essential for *C. albicans* phagocytosis.[29] Supporting our hypothesis, we have demonstrated that *L. divaricata* decoction up-regulates dectin-1.[25] While the three fractions stimulated the phagocytosis, F2 and F3 showed stronger ability to kill the fungus (Fig. 4b). This could be due to the increased NO production produced by F1 and F3, as this metabolite is particularly important to destroy *C. albicans* and to control the infection.[30] The superoxide contribution should be not discarded as F2 and F3 stimulated significantly its production in MØs from treated mice at 15 mg kg^{-1} and 5 mg kg^{-1}, respectively, although the NBT reduction was not enhanced after *C. albicans* stimulation. The opposite effect was observed when MØs were treated with F1 at 15 mg kg^{-1}, in which a potentiating effect of the production of superoxide anion was observed in the presence of *C. albicans*.

In summary, the effects observed with the F1 treatment at the higher dose were: increased superoxide anion production, with an increase in the phagocytosis of *C. albicans*, and an higher production of NO compared with control. All these effects were observed in the presence of *C. albicans*.

In conclusion, F1 may induce a state of pre-activation on MØs, which is enhanced by the presence of *C. albicans*. This, along with the fact that this is the only fraction that does not contain NDGA could make F1 more important than F2 and F3 for the treatment of disseminated candidiasis in patients with immunodeficiency diseases such as AIDS, chronic granulomatous disease, among others.

Differential activation of the innate immune system with compounds obtained from plants is especially important to treat drug-resistant candidiasis (among other diseases) in immunocompromised patients.[31] The isolation and characterisation of compounds present in F1 are in process in our laboratory.

Acknowledgments

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Conflict of Interest

The authors report no conflicts of interest.

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