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A Fraction Containing Kaempferol-3,4′-dimethylether from *Larrea divaricata* Cav. Induces Macrophage Activation on Mice Infected with *Candida albicans*

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***Larrea divaricata* Cav. is a plant growing in South America. Both the infusion and a derived fraction (F1) of *L. divaricata* have proved to have immunomodulatory properties. Moreover, F1 can activate macrophages obtained from mice infected with *Candida albicans*. In this work, F1 was administrated to infected animals, and the state and type of activation of resident macrophages were studied. Results showed that F1 was able to activate macrophages obtained from infected mice by both classical and alternative pathways, probably by inducing a translocation of nuclear factor kappa-B. F1 increases not only the lysosomal activity of macrophages but also the production of phagosomal superoxide anion as a consequence of the activation of the Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) complex. F1 induced an increase in the macrophage capacity to kill the fungus, which was reflected in a decrease in the levels of colonization of organs. A main flavonoid, kaempferol-3,4′-dimethylether, was identified in F1 by HPLC. This compound increased *in vitro* production of nitric oxide in heat-killed *C. albicans*-stimulated macrophages. The flavonoid could thus be considered one of the responsible molecules mediating the overall effects of F1 on the immune system in infected animals. Copyright © 2013 John Wiley & Sons, Ltd.**

Keywords: Larrea divaricata; *Candida albicans*; macrophages; kaempferol-3,4′-dimethylether.

INTRODUCTION

Larrea divaricata Cav. (Zygophyllaceae) is an evergreen shrub distributed in northwest, center and southeast of Argentina. The infusion prepared with its leaves is used in folk medicine to treat several diseases. Over the past years, the number of reports claiming its folk uses and properties has increased, such as antimicrobial (Stege *et al*., 2006; Vogt *et al*., 2013), antiproliferative (Anesini *et al*., 2001; Davicino *et al*., 2010; Davicino *et al*., 2011a, 2011b), antioxidant (Turner *et al*., 2011) and immunomodulatory (Davicino *et al*., 2006; Davicino *et al*., 2007; Davicino *et al*., 2008).

It has previously been demonstrated that a free nordihydroguaiaretic acid fraction obtained from an aqueous extract, called fraction 1 (F1), can induce the activation of macrophages and lead to both *in vitro* (Martino *et al*., 2010) and *in vivo* effects (Martino *et al*., 2011a). Moreover, this fraction is capable of enhancing the macrophage antimicrobial activity against the opportunistic fungus *Candida albicans* in an infection model (Martino *et al*., 2011b; Martino *et al*., 2012). However, the compounds present in F1 and its mechanism of action in this model have not been elucidated.

Despite the fact that *C. albicans* is a fungus of the normal flora of the oropharynx and the gastrointestinal tract of healthy individuals (Richardson, 2005), under predisposing conditions, colonization may lead to mucocutaneous and also invasive infection. This microorganism is the most frequent cause of systemic fungal infections in immunocompromised patients (Steinshamn and Waage, 1992). Besides, phagocytes such as neutrophils and macrophages are crucial to prevent systemic candidiasis by means of phagocytosis, generating reactive oxygen species (ROS), and by increasing the lysosomal activity (Ferrari *et al*., 2011). It has been established that after *C. albicans* is recognized, an induction of both M1 (classical) and M2 (alternative) phenotypes of macrophages is observed. These events lead to the elimination of the fungus in the first case and to the extracellular matrix repair in the second one (Paraje *et al*., 2009).

Taking these observations into account, the aim of this work was to evaluate in an *in vivo* model the effects of F1 on the mechanism developed by macrophages in the anti-*C. albicans* response.

MATERIALS AND METHODS

Plant material and preparation of fraction 1. *Larrea divaricata* leaves were collected in autumn in San Luis, Argentina. Plant specimens were identified at the herbarium of the National University of San Luis (voucher no. 467 UNSL). F1 was obtained from the aqueous extract as previously described (Martino *et al*., 2010;

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Martino *et al.*, 2011a). F1 was endotoxin free, as evaluated by the Laemmli's test.

Identification of kaempferol-3,4′-dimethylether by HPLC. The identification of the main flavonoid present in F1 was performed by HPLC analysis in a Varian Pro Star Instrument (CA, USA) equipped with a Rheodyne injection valve (20 µL) (Chromtech, Apple Valley, MN, USA) and photodiode array detector set at 260 nm. The chromatographic procedure was performed at room temperature (18–25 °C). Samples were analyzed with the Varian Star Chromatography Workstation version 6.x. A reversed-phase column Phenomenex C18(2) Luna (CA, USA; 250 mm × 4.6 mm and 5 µ dp) was used. Four different methods were used. Method 1: The mobile phase used was (A) water and phosphoric acid (0.5%) and (B) methanol and phosphoric acid (0.5%). The gradient was from 60% A to 20% A in 30 min, 20% A to 0% A in 2 min, and back to the initial conditions. Method 2: (A) water and (B) methanol, applying the same elution gradient as in method 1. Method 3: (A) water and acetic acid (98:2) and (B) methanol and acetic acid (98:2), and the gradient was from 15% B to 40% B in 30 min, 40% B to 75% B in 10 min, 75% B to 85% B in 5 min and 100% B in 5 min, 100% B for 10 min, and back to the initial conditions. Method 4: (A) water and (B) acetonitrile, employing the same elution gradient as in method 3. The retention time of each method and the UV spectra were compared with a pure kaempferol-3,4′-dimethylether standard.

Mouse infection and treatment. For all experiments, female Rockland mice (average weight 20 g) were used. Animals were housed and cared at the Animal Resource Facilities, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis. Mice were handled according to the Institutional Committee for the Care and Use of Laboratory Animals guidelines. *C. albicans* was grown on Sabouraud's glucose agar for 48 h at 37 °C. Cells suspensions were prepared in sterile phosphate buffered saline (PBS) and adjusted to the appropriate cell concentration before injection. In this work, a clinical isolate of *C. albicans* strain was used.

Mice were challenged intravenously with 10^6 yeasts (Villamón *et al.*, 2004). F1 was administered by orogastric gavage in a dose of 15 mg/kg during 3 days, beginning 24 h after the infection day. This dose had previously been demonstrated to induce an important oxidative response against *C. albicans* on macrophages *in vivo* (Martino *et al.*, 2011a). Control animals only received PBS. Mice were divided into four groups of at least five mice per group, and a minimum of two independent experiments were carried out. The treatment groups were as follows: (1) healthy mice without any treatment (control); (2) healthy mice treated with F1; (3) infected mice, without treatment (infected control); and (4) infected mice treated with F1.

Mice were sacrificed 4 days after infection, and peritoneal cells obtained. Peritoneal macrophages were purified by adherence onto 96-well flat bottomed tissue culture plates in Dulbecco's Modified Eagle Medium supplemented with 20 µg/mL gentamicin and 5% fetal calf serum. Non-adherent cells were removed after incubating for 2 h at 37 °C. All experiments were performed immediately after the obtainment of macrophages, with the exception of nitric oxide (NO) and arginase activity, which were performed 48 h after the obtainment of cells. All culture reagents were endotoxin free, as assessed by the Laemmli's test.

Determination of cell activation. Cell activation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Madesh and Balasubramanian, 1997). The purple formazan product formed was solubilized by the addition of acidic isopropanol and measured at 570 nm. Cells from untreated mice were used as control of reduced MTT (100%), and results were expressed as percent of reduced MTT related to control.

Anti-*Candida albicans* assay. Macrophages from all groups were incubated with *C. albicans* blastoconidia (multiplicity of infection 1:10) for at least 4 h at 37 °C in order to evaluate the killing of the phagocytosed fungus. The culture medium was removed, and the adherent macrophages were lysed with cold lysis buffer. The lysates were serially diluted and plated onto Sabouraud's agar to determine viable counts. Results were expressed as macrophage candidicidal activity (%) according the following formula: % of growth inhibition = 1 – [(CFU of test wells/CFU of *C. albicans* without macrophages)] × 100 (Tavanti *et al.*, 2006).

Determination of tissue fungal burden. Organs (liver, spleen and kidneys) were obtained from infected mice treated or not with F1 and homogenized in 2 mL PBS. Serial dilutions of homogenates were plated onto Sabouraud's agar and incubated at 37 °C. After 48 h, yeast colonies were counted. The fungal load was expressed as log colony forming units (CFU) per organ (Held *et al.*, 2008).

Effect of fraction 1 on reactive oxygen species production and NADPH oxidase activation. The production of ROS was evaluated with the nitroblue tetrazolium (NBT) assay. ROS reduces NBT to formazan, an insoluble blue salt (Schopf et al., 1984). In order to stimulate the oxidative burst, 1 mg/mL of opsonized zymosan (OPZ) was employed. In all groups, macrophages were incubated with NBT or NBT-OPZ and left for 60 min. The reaction was stopped with 1 N HCl. Formazan was extracted with dioxane, and the absorbance was measured at 525 nm. Results were expressed as nanomolar of reduced NBT per 10^6 cells.

The activation of macrophage NADPH oxidase was evaluated by the membrane expression of the cytosolic subunit $p47^{phox}$ by western blot. Briefly, cells were lysed and resuspended in relaxation buffer. Cell debris were removed by centrifugation at 1000 ×**g** for 10 min. Supernatants were ultracentrifuged at 100,000 ×**g** for 30 min to separate cytosol and membrane fractions. Pellets (membranes) were resuspended in relaxation buffer, and protein quantification was carried out. Samples were run in 10% Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) and transferred to

a nitrocellulose membrane. Membranes were incubated with an antiserum against mouse $p47^{phox}$ and followed by a goat anti-mouse immunoglobulin G antiserum conjugated to peroxidase. Immunological reactions were revealed with a solution of H_2O_2-TMB. $p47^{phox}$ expression from cytosolic fraction was also detected (Deng *et al.*, 2012).

Lysosomal activity. The lysosomal activity was evaluated by analyzing the activity of tartrate-resistant acid phosphatase. The enzyme activity was measured by the cleavage of *p*-nitrophenylphosphate (pH 5.8). The *p*-nitrophenol released after 1 h of incubation at 37 °C was converted to *p*-nitrophenolate by addition of 1 M NaOH. The absorbance was measured at 405 nm. Results were expressed as percent lysosomal activity compared with control (Zenger *et al.*, 2007).

Nitric oxide and arginase activity/inducible nitric oxide synthase and Arginase-1 expression. Cells of all groups were cultured for 48 h at 37 °C. Culture supernatants were mixed with Griess' reagent (sulfanilamide and *N*-(1-naphthyl) ethylenediamide dihydrochloride). Absorbance was measured at 540 nm, and the nitrite concentration was derived from a sodium nitrite standard curve. Results were expressed as micromolar of NO per 10^6 cells.

For the determination of arginase activity, cell lysates were incubated with L-arginine, and the production of urea was quantified spectrophotometrically by the α-isonitrosopropiophenone reagent. Results were expressed as units of arginase per 10^6 cells. The expressions of inducible nitric oxide synthase (iNOS) and Arginase-1 were evaluated by SDS-PAGE and western blot analysis from the whole macrophages lysate. Bands were identified by using specific antibodies (α-mouse iNOS and α-mouse Arginase-1). The expression of β-actin was used as loading control (Reiner Neil, 2009).

Nuclear factor kappa-B activation assay. The effect of F1 on nuclear factor kappa-B (NF-κB) translocation in macrophages was determined by ELISA by using the Cayman Chemical NF-κB (p65) transcription factor kit (MI, USA). DNA binding activity in nuclear extracts was prepared according to the manufacturer's instructions. Results were expressed as nuclear extract absorbance (Davicino *et al.*, 2010).

Effect of kaempferol-3,4′-dimethylether on macrophage activation. Resident peritoneal macrophages obtained from healthy animals were incubated with or without heat-killed *C. albicans* (HKCa) and with different concentrations of kaempferol-3,4′-dimethylether (1 and 10 to 20 μM) for 2 h. Cells were washed, and their viability and NO production were measured as described earlier.

Statistical analysis. Values are shown as mean ± SD. Significance testing was performed using analysis of variance in order to compare all groups. Values of $p \leq 0.05$ were considered significant.

RESULTS

Effect of fraction 1 on the elimination of *Candida albicans* by macrophages

First, the effect of F1 on the activation of macrophages was assessed. As shown in Fig. 1, F1 was able to increase the activation of macrophage in infected mice when compared with infected control animals ($p < 0.05$). Moreover, F1 increased the candidicidal activity of macrophages both in healthy mice and in infected mice ($p < 0.01$, Fig. 2A). Furthermore, the effect of F1 on *in vivo* fungus clearance was assessed. Results showed that the treatment of mice with F1 induced the elimination of the fungus from the liver and kidneys ($p < 0.001$). Besides, a decrease in viable counts in the spleen was observed ($p < 0.001$) after the treatment (Fig. 2B).

Activation of macrophages

In order to evaluate the candidicidal mechanisms involved in fungus elimination by macrophages, the production of ROS, the expression of $p47^{phox}$ on the cell surface and lysosomal activity were measured. Fig. 3A shows that F1 induced a significant increase in the production of ROS by macrophages obtained from infected animals, as compared with infected controls ($p < 0.001$). It was also observed that after infection, the expression of membrane $p47^{phox}$ decreased (Fig. 3B). Nevertheless, F1 was able to induce an increase in the membrane expression of this cytosolic NADPH oxidase subunit in both healthy and infected animals. Moreover, both the infection and F1 treatment were capable of increasing the lysosomal activity, as compared with controls ($p < 0.05$; Fig. 3C). In addition, macrophages obtained from infected and treated mice were those with the highest lysosomal activity ($p < 0.05$ vs infected control).

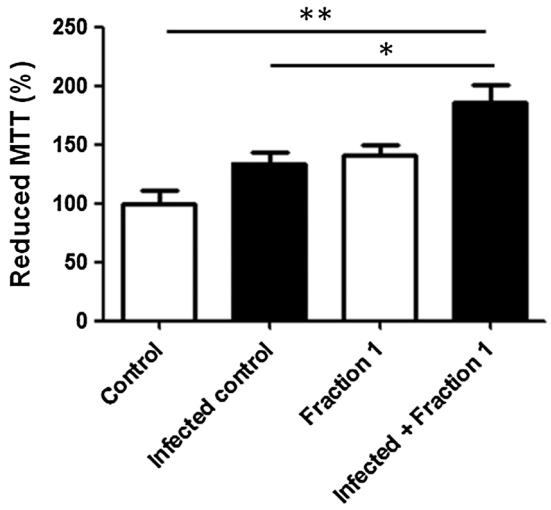

Figure 1. Effect of fraction 1 on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in peritoneal macrophages. Macrophages from all groups were obtained and incubated with MTT dye for 4 h. The formazan salt production was measured at 570 nm in a multiwell spectrophotometer reader. Results were expressed as reduced MTT (%) with respect to healthy control. Values represent the mean ± SD of three experiments made in triplicate. $*p < 0.05$; $**p < 0.01$.

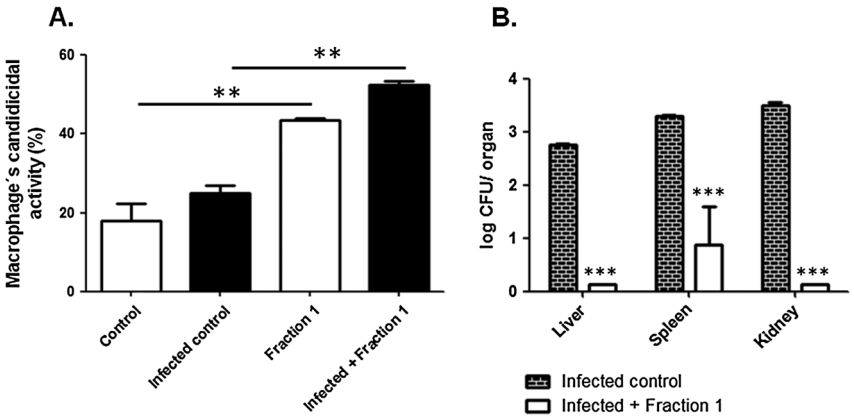

Figure 2. (A) Effect of fraction 1 on macrophage anti-*Candida albicans* activity: macrophages were incubated with live *Candida albicans*. After 4 h, phagocytic cells were lysed, and viable counts were performed. Results were expressed as macrophages candidicidal activity (%). Values represent the mean ± SD of three experiments made in triplicate. (B) Determination of fungal burden in organs: liver, spleen and kidneys from infected animals were homogenized, plated onto Sabouraud's agar and incubated for 48 h. Colony forming units (CFU) were counted. Results were expressed as log CFU per organ. Values represent the mean ± SD of three experiments made in triplicate. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

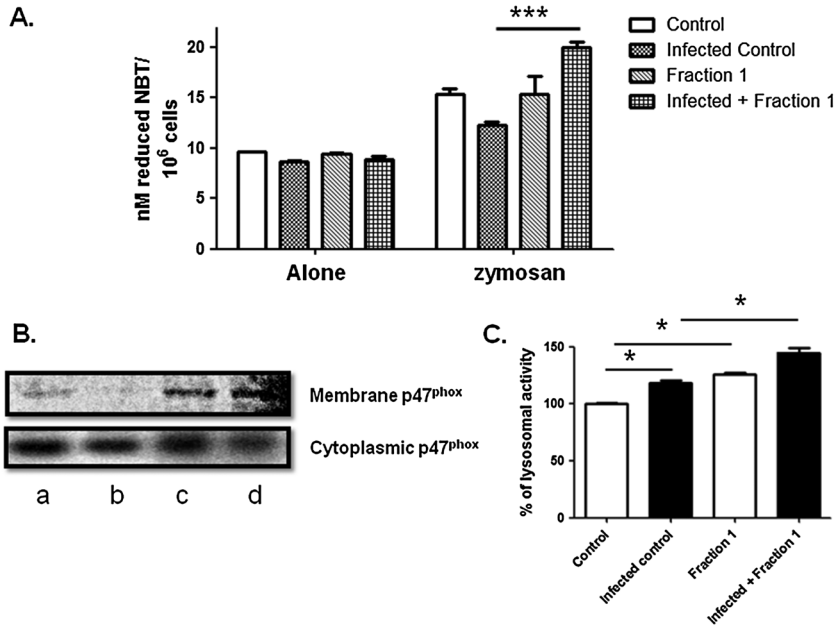

Figure 3. (A) Effect of fraction 1 on superoxide anion production by the nitroblue tetrazolium (NBT) test: macrophages from all groups were obtained and incubated with zymosan for 60 min in order to stimulate the oxidative burst. A control without zymosan was also performed in order to evaluate the basal levels of superoxide anion. Cells were also incubated with the NBT dye. Results were expressed as nanomolar of reduced NBT per 10^6 cells. (B) Membrane and cytosolic $p47^{phox}$ expression: macrophage membranes and cytosolic proteins were obtained, and the expression of $p47^{phox}$ (47 kDa) was analyzed by western blot—(a) healthy control; (b) infected control; (c) fraction 1; and (d) infected + fraction 1. (C) Macrophage lysosomal activity: macrophages were obtained, and the tartrate-resistant acid phosphatase activity was analyzed by the incorporation of *p*-nitrophenylphosphate as substrate (pH 5.8). The *p*-nitrophenol released after 1 h of incubation was measured in a multiwell spectrophotometer reader. Results were expressed as percent lysosomal activity compared with control. Values represent mean ± SD of three experiments made in triplicate. $*p < 0.05$; $***p < 0.001$.

Taking into account that the classical and/or alternative pathways of activation can modulate the immune response against this fungus, it was interesting to evaluate whether F1 could activate macrophages by one of these two pathways. To this end, the production of NO and the expression of iNOS were determined in order to characterize the M1 phenotype (classical activation), whereas arginase activity together with Arginase-1 expression were determined to recognize the M2 phenotype (alternative activation). Results showed that *C. albicans* decreased both NO levels ($p < 0.01$) and arginase activity ($p < 0.001$) when compared with macrophages obtained from healthy animals (Fig. 4A,B). In infected mice, the treatment with F1 reverted the effect induced by *C. albicans* by inducing an increase of NO levels ($p < 0.05$) and arginase activity ($p < 0.001$). These results were confirmed by the increase of iNOS and Arginase-1 expression induced by F1 in infected animals. The latter results were found to be related to the increase of NF-κB nucleus translocation also induced by F1 in infected animals ($p < 0.001$).

Kaempferol-3,4′-dimethylether identification

The identification of the main flavonoid present in F1 was performed by HPLC analysis as described earlier. The retention times of the flavonoid for each

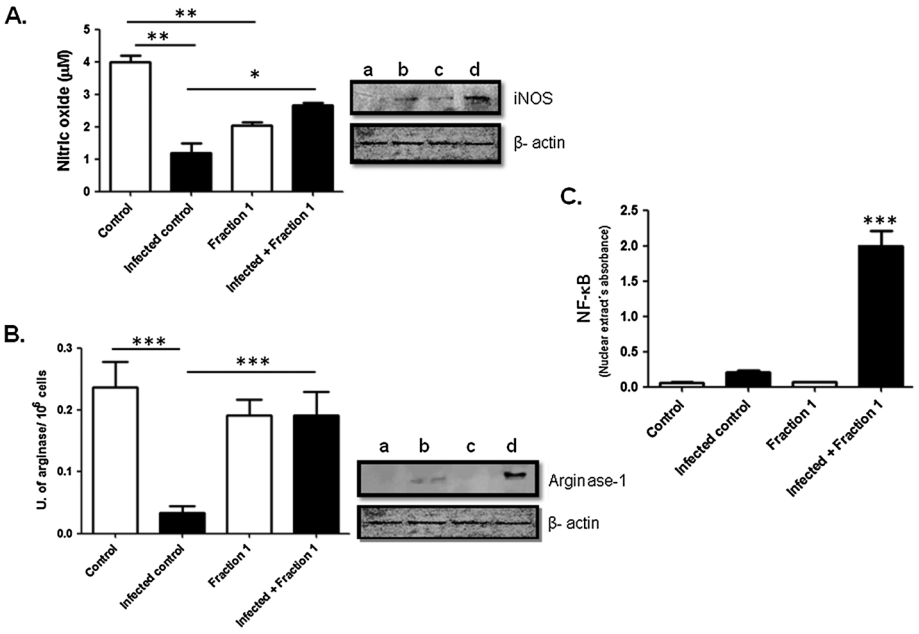

Figure 4. Evaluation of classical and alternative activation of macrophages and nuclear factor kappa-B (NF-κB) nucleus translocation. Cells were incubated in 96-well culture plates for 48 h. After incubation, supernatants and cellular pellets were obtained. (A) Production of nitric oxide and expression of inducible nitric oxide synthase (iNOS): the production of NO was determined in cell supernatants by the Griess' reaction. Results were expressed as micromolar of NO by using a standard curve made with sodium nitrite. Values represent mean ± SD of three experiments made in triplicate. The expression of iNOS (131 kDa) was determined by western blot analysis. The expression of β-actin was evaluated as loading control. (a) Healthy control; (b) infected control; (c) fraction 1; and (d) infected + fraction 1. (B) Arginase activity and expression of Arginase-1: arginase activity was measured in cell pellets after 48 h of culture by a spectrophotometric method. Results were expressed as units of arginase per 10^6 cells. Values represent mean ± SD of three experiments made in triplicate. The expression of Arginase-1 (37 kDa) was determined by western blot analysis. The expression of β-actin was evaluated as loading control. (C) Activation of NF-κB in macrophages: macrophage nuclear extracts were obtained, and the levels of NF-κB were determined by using an ELISA kit. Results were expressed as nuclear extract absorbance. Values represent mean ± SD of three experiments made in triplicate. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

method performed were as follows: method 1, 21.6 min; method 2, 31.7 min; method 3, 47 min; and method 4, 40.82 min. For each method, both the retention time and the UV spectra coincided with the kaempferol-3,4′-dimethylether standard used ($\lambda_{band\ I}$: 348.46 nm, $\lambda_{band\ II}$: 266.35 nm; Fig. 5).

Kaempferol-3,4′-dimethylether and macrophage activation

In order to evaluate whether kaempferol-3,4′-dimethylether was one of the compounds responsible for the effects observed with F1, the ability of cells to reduce MTT

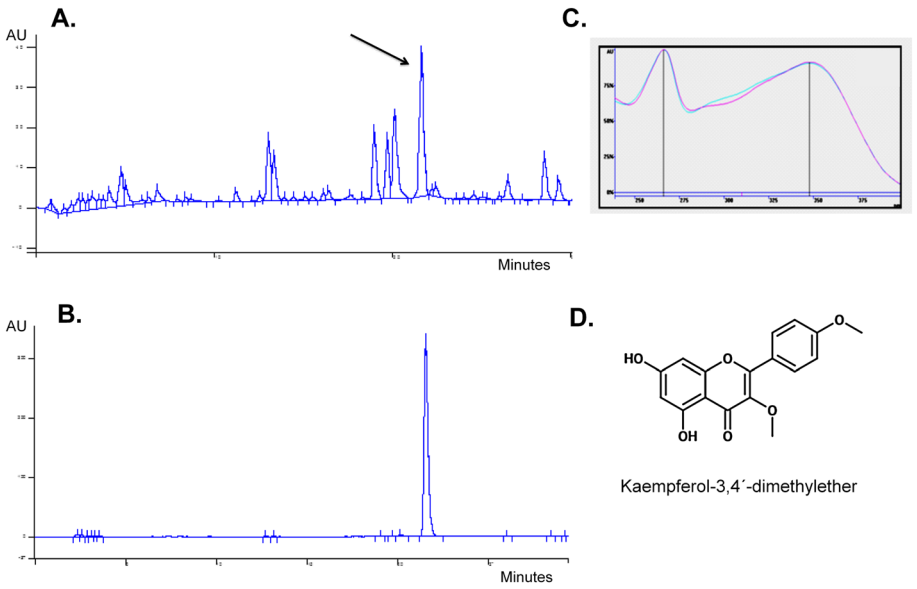

Figure 5. Fraction 1 (F1) HPLC analysis and kaempferol-3,4′-dimethylether identification. (A) Representative F1 chromatographic profile described in the Materials and Methods section as 'method 1', where a main peak with a retention time of 21.6 min can be observed (indicated with a arrow), corresponding to kaempferol-3,4′-dimethylether. (B) Kaempferol-3,4′-dimethylether standard run under the same condition as F1. (C) Overlay of the ultraviolet spectra of the 21.6 min peak of A and the kaempferol-3,4´-dimethylether standard. (D) Chemical structure of kaempferol-3,4′-dimethylether. This figure is available in color online at wileyonlinelibrary.com/journal/ptr

and NO production was measured. The range of concentration tested of the flavonoid was chosen according to the concentration of F1 previously assessed *in vitro* (Martino *et al.*, 2010). Fig. 6 shows that kaempferol-3,4′-dimethylether did not affect the ability of macrophages to reduce MTT at the concentrations assessed. Nevertheless, when macrophages were co-incubated with HKCa and treated with 1 and 10 μM of kaempferol-3,4′-dimethylether, they produced higher levels of NO than the control ($p < 0.05$).

DISCUSSION

In the present work, the effect of F1 obtained from *L. divaricata* on the activation of macrophages in a *C. albicans* infection model was studied.

The effect of F1 was determined on animals that had been previously infected with *C. albicans*. Because macrophages play a pivotal role in the immune clearance of *C. albicans*, we investigated the activation status of these cells upon infection with the fungus. Because MTT reduction, which is considered an activation parameter, was not observed, it can be concluded that the infection by itself is not capable of activating peritoneal macrophages. This result is in agreement with the fact that the infection per se did not increase the candidicidal activity of macrophages *in vitro*. Nevertheless, F1 was able to increase MTT reduction levels in infected macrophages. This phenomenon could be related to the activation of phagocytosis exerted by F1, as previously observed by Martino *et al.* (2011b). Thus, the increased phagocytosis of *C. albicans* would explain the enhanced candidicidal activity observed.

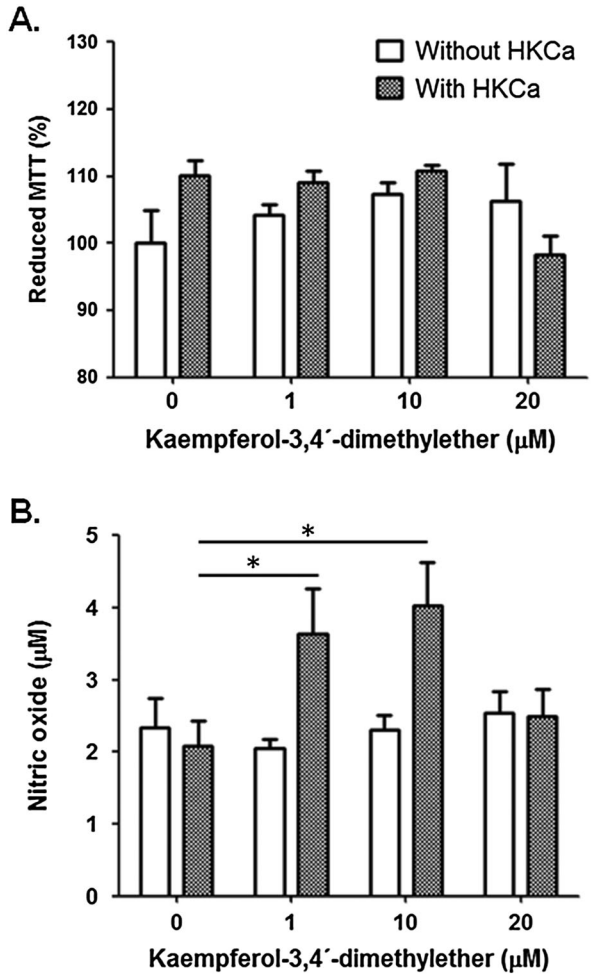

Figure 6. Effect of kaempferol-3,4′-dimethylether on macrophage activation. Resident peritoneal macrophages obtained from healthy animals were incubated with heat-killed *Candida albicans* (HKCa) and different concentrations of the flavonoid for 2 h. Cells were washed, and their viability (A) and the production of NO (B) were determined as described in the Materials and Methods section. Results are expressed as mean ± SD of three experiments made in triplicate. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. *$p < 0.05$; **$p < 0.01$.

In addition, the decreased fungal load in spleen, liver and kidneys observed after treatment of animals with F1 (Fig. 2B) could be related to the fact that F1 was capable of enhancing the ability of macrophages to kill *C. albicans* (Fig. 2A), leading to the clearance of the pathogen.

The candidicidal activity of mononuclear phagocytic cells has been associated with the production of superoxide anion, one of the products of reactive oxygen intermediate metabolism that is essential for 'oxidative killing' (Marodi *et al.*, 1991). The superoxide anion is produced when extracellular signals activate specific kinases (such as Syk and Src), which in turn phosphorylate the cytosolic NADPH oxidase complex subunits (i.e., $p47^{phox}$) producing large amounts of this ROS in the phagosome membrane (Elsori *et al.*, 2011). It was observed that zymosan-stimulated macrophages isolated from infected and treated animals were able to exert the highest phagosomal superoxide anion production. This result was confirmed by the F1-mediated increase in $p47^{phox}$ expression on the macrophage membrane, together with a decreased expression in the cytosolic fraction and related to the activation of NADPH oxidase. It is likely that the increase in the phagocytosis of *C. albicans* exerted by F1 be a consequence of the activation of macrophages. This activation would be accomplished by activating intracellular signaling pathways, which lead to the assembly of the NADPH oxidase complex and to the production of large amounts of superoxide anion, which finally attacks the fungus wall (Soloviev *et al.*, 2011). Furthermore, when *C. albicans* is phagocytosed, the phagosome fuses with the lysosome, and different proteolytic enzymes are activated such as the tartrate-resistant acid phosphatase and defensins, which have proved to be important in *C. albicans* killing in an 'oxygen-independent mechanism' (Vázquez-Torres and Balish, 1997). F1 was capable of increasing the lysosomal activity, in both healthy and infected animals. This latter effect could be related to the activation of the NADPH oxidase, which in turn produced ROS implicated in the activation of proteolytic enzymes (Newman *et al.*, 2005). Thus, this mechanism would contribute to the candidicidal activity previously observed.

In order to determine the type of activation induced by F1, the effects of this fraction on the production of NO (classical activation or M1) and on arginase activity (alternative activation or M2) were evaluated. The decrease of both NO production and arginase activity observed in infected animals could be related to the resistance exerted by *C. albicans* on macrophages, which in turn can induce a status of immunosuppression by the secretion of soluble factors (Chinen *et al.*, 1999). Although F1 decreased NO production in healthy animals, it was able to revert the decrease observed

after the infection (Fig. 4). The same effect was observed when arginase activity was measured. This phenomenon was accompanied by an increase in the expression of the enzymes involved in L-arginine metabolism (iNOS and Arginase-1). Moreover, the increase in iNOS expression by F1 could be related to the activation of NF-κB observed. These results suggested that F1 could act as an immunomodulatory agent, by the induction in macrophages of both phenotypes (M1 and M2) in the presence of *C. albicans*. This 'dual activation' exerted by F1 could be helpful not only to kill *C. albicans* but also to avoid the immunopathological consequences due to the high levels of NO in the microenvironment where the immune response takes place (Muller and Rudel, 2001). In fact, even though alternative activation had been associated with an impaired response against *C. albicans*, it is known that this pathogen requires L-arginine to trigger the morphogenetic switch from yeast to hyphae (which is a critical factor in escaping from macrophages after phagocytosis) (Ghosh *et al*., 2009). In this context, the increase of L-arginine metabolism exerted by F1 could be helpful in controlling the latter virulence factor.

The chromatographic profile of F1 was also evaluated. One of the major compounds found by HPLC analysis was identified as kaempferol-3,4′-dimethylether (a flavonoid commonly named as ermanin), which had been previously identified in *L. divaricata* (Rivero-Cruz *et al*., 2005). Finally, the ability of kaempferol-3,4′-dimethylether to activate macrophages was also assessed. As shown in Fig. 6, this flavonoid significantly increased NO production at lower concentrations when macrophages were co-incubated with HKCa, without affecting MTT reduction and probably without changing cell viability. These results suggested that, in this context, this flavonoid act as a pro-inflammatory agent. On the other hand, the antiinflammatory activity of this flavonoid, also present in *Tanacetum microphyllum*, has also been reported in lipopolysaccharide-stimulated RAW 264.7 macrophages (Guerra *et al*., 2006). It is noteworthy that kaempferol-3,4′-dimethylether could be exerting either proinflammatory or antiinflammatory effects depending on the micro-environmental conditions where macrophages are exposed (lipopolysaccharide, *C. albicans* antigens). Consequently, and depending on the membrane receptors involved, kaempferol-3,4′-dimethylether would exert different modulatory activities on macrophages. In addition, the effects observed with this flavonoid could also be concentration dependent. This phenomenon has been reported for other flavonoids. For example, it is known that at low doses, catechin has an antiinflammatory effect, whereas at higher concentrations, it induces an inflammatory response (Dames *et al*., 1985). Finally, it could be stated that kaempferol-3,4′-dimethylether could be one of the compounds responsible for the overall effect observed with F1 in this fungal infection model. The results presented herein could widen the pharmacological targets of flavonoids as antiinflammatory or proinflammatory drugs.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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