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RESEARCH ARTICLE

"*In vivo*" murine macrophages activation by a dichloromethane extract of *Tilia x viridis*

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Abstract

Macrophages are involved in the host defense against infectious pathogens and tumors. *Tilia* species have been used in folk medicine for the treatment of infectious diseases, previously it was demonstrated that a dichloromethane (DM) extract possess antiproliferative action "*in vitro*" on a lymphoma cell line. The aim of this work was to study the "*in vivo*" effect of DM extract upon mice peritoneal macrophages. DM extract–activated macrophages phagocytosis through hydrogen peroxide (H_2O_2) and nitric oxide (NO) production (phagocytosis (%): basal 16.93 ± 0.18, DM extract 25.93 ± 2.8; H_2O_2 (M): basal 0.0022 ± 0.00016, DM extract 0.0036 ± 0.0005; NO (mM): basal 0.0052 ± 0.0007, DM extract 0.0099 ± 0.0004). These actions were mediated by cell superoxide dismutase activation. On the other hand, DM extract decreased tumor necrosis factor α but increased interleukin-10 in serum. These results suggest that the modulation activity exerted by the extract on immune system cells could be an important mechanism to acquire resistance to tumors and infectious diseases.

Keywords: *Hydrogen peroxide; macrophages; nitric oxide; superoxide dismutase;* Tilia x viridis

Introduction

It is well known that the immune system plays a major role against tumors and infectious diseases.[1] Among immune system cells, macrophages play an important role in innate immunity. Macrophages exert its function through the production of effectors molecules such as nitric oxide (NO).[2,3] Macrophages can be activated by using different molecules. Various agents, including interferon-γ (IFN-γ), lipopolysaccharide (LPS), lectins, and vegetal extracts, are known to stimulate the macrophages.[4,5]

On the other hand, some plants are used in popular medicine for the treatment of several diseases. In this sense, *Tilia* species have been used for many years in Europe to treat anxiety and as a psychological depressor[6] and, in addition, the extracts have been used for the treatment of colds, bronchitis, fever, inflammations, and influenza infections. In this regard, the tea prepared with *Tilia* flowers is listed in German Pharmacopeia.[7]

Tilia x viridis (Bayer) Simonk nothosubspecie moltkei (Dippel) Xifreda is a plant widely distributed in Argentina. A previous study has shown that the dichloromethane (DM) extract of *Tilia x viridis* presents an antiproliferative action on a lymphoma cell line (BW 5147) without affecting the viability of normal lymphocytes. Moreover, one fraction rich in coumarins and another fraction rich in monoterpenes were obtained from DM extract. The fractions most active against tumor cells were the fractions containing monoterpenes including limonene.[8] The immunomodulatory effects of limonene have already been reported.[9,10]

The aim of this work was to study the "*in vivo*" effect of a DM extract from *Tilia x viridis*, rich in monoterpenes, on the viability and activation of mouse peritoneal macrophages. The action of the extract upon the intracellular molecules involved in macrophage activation was also studied. Moreover, in order to evaluate the safety of the extract, the effect of DM on mice hepatic enzymes was analyzed.

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Materials and methods

Plant material and extract preparation

Tilia x viridis (Bayer) Simonk nothosubspecie moltkei (Dippel) Xifreda flowers were collected in the province of Buenos Aires in January and authenticated by Dr Gustavo Giberti (IQUIMEFA-CONICET). A voucher specimen was deposited at Museum of Pharmacobotanic, Faculty of Pharmacy and Biochemistry, University of Buenos Aires. To prepare the DM extract, dried flowers (9 g) were macerated twice overnight with 200 mL of DM, filtered, evaporated, and the residue was used for the experimental studies.

Animals

Ten female C3H mice with an initial body weight of about 20 g (3 months old) were used. The animals were kept at a controlled temperature of 20 ± 2°C with a photoperiod cycle of 12 h light and 12 h darkness. The animals were fed *ad libitum* with Purina Chow and water. During the whole process, the animals were handled in accordance with the guidelines of care and experimental use of animals described in DHEW Publication, NHI 80-23.

Five animals were treated with 1× phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and the other five animals were treated intraperitoneally with three doses of the DM extract (0.0225 g/mL) in 1× PBS with intervals of 48 h. Forty-eight hours after the administration of last doses, the animals were killed by cervical dislocation and the peritoneal macrophages were obtained.

Cells preparation, viability, and apoptosis assays

Peritoneal cells (PCs) were harvested by sterile lavage with 20 mL of PBS sterile, from DM treated animals and untreated animals, and adjusted to 1×10^6 cell/mL. Macrophages were purified from PC by adherence on to 96-well flat bottomed tissue culture plates in RPMI 1640 AQ1 medium (Sigma, San Diego, CA, USA), supplemented with penicillin/streptomycin and glutamine and 10% heat-inactivated fetal calf serum (Gibco, Rockville, MD, USA). Nonadherent cells were removed after 2 h at 37°C and complete medium was added. The adherent macrophages monolayer showed 90% of purity according to morphologic analysis or nonspecific esterase staining. Macrophages viability was determined by the reduction of MTT (Sigma, St. Louis, MO, USA), immediately (0 h) and after 48 h of incubation. Briefly, 1×10^6 cells/mL, isolated from treated and untreated animals, were incubated with 100 µL of RPMI 1640 containing 10 µL of 5 mg/mL MTT (Sigma). Untreated cells were used as control of viability (100%) and results were expressed as % of viability relative to control.[11] To determine apoptosis status of macrophages, isolated cells from untreated or DM treated animals were assayed immediately (0 h) or after 48 h of incubation, then the cells were processed with annexin V–FITC and propidium iodide (Sigma) and analyzed by using flow cytometry.[12]

Macrophages phagocytic activity and spreading assay

Macrophages were incubated at 37°C for 1 h with 0.5 mL of 1% active carbon particles. Number of particles ingested by 100 macrophages was counted under light microscope (×100) and the mean value was calculated. This value represents phagocytosis (%), as much as greater the quantity of ingested particles, greater is the phagocytosis activity.[13] Moreover, an aliquot of cells was layered on glass cover slips and incubated for 1 h at 37°C in a humid atmosphere with 5% CO_2, and the shape was observed with phase contrast microscope at ×400, accordingly to Rabinovitch et al.[14]

Release of hydrogen peroxide

Macrophages (1×10^6 cells/mL) were incubated with medium alone for 1 h, then a solution of 0.56 mM of diaminobencidine tetrahydrochloride (DAB) containing 140 mM NaCl, 10 mM potassium phosphate, 5.5 mM dextrose, and 0.01 mg/mL type II horseradish peroxidase (Px) (Sigma) was added. After 1 h incubation, the reaction was stopped by the addition of 10 mL of 4 N NaOH and the absorbance was measured at 650 nm by using a microplate reader (Microplate Reader Benchmark; Bio-Rad, CA, USA). Results were expressed as hydrogen peroxide (H_2O_2) M/10^6 cells. A standard curve of known molar concentrations of H_2O_2 in buffered DAB was run in each test.[12]

Total nitrite determination

The total production of nitrites by macrophages was determined using the Griess reagents.[15] Briefly, cells were collected and centrifuged at 800 *g* for 10 min, then were incubated with the Griess reagent for 20 min in the dark and measured at 540 nm. Total nitrites were calculated by interpolation in a standard curve made with known concentrations of nitrites.[12]

Cell suspensions

Macrophages (1×10^6 cells/mL) were incubated for 1 h with culture medium. After the addition of 10 µL of 10^{-4} M PMSF and 5 µL of Triton X-100, these cells were disrupted by pipetting and then centrifuged at 1500 *g* for 15 min at 4°C. After centrifugation, supernatant was used for the determination of enzymatic activity.

Px and superoxide dismutase activities assays

The Px activity was determined by the method of Herzog and Fahimi.[16] Briefly, 25 µL of each sample were incubated with 950 µL of DAB (5×10^{-4} M) and 25 µL of H_2O_2 (30%) reaching a final volume of 1000 µL. A DAB solution without H_2O_2 was used as reaction blank. Absorbance readings were recorded at 30 s intervals for 5 min by using a Shimadzu recording spectrophotometer UV-240 (graphic printer PR-1) set at 465 nm. The variation in the absorbance (△)/min was calculated. A calibration curve of peroxidase concentration versus △/min was plotted by using horseradish peroxidase obtaining a linear relationship in the range of 1.95×10^{-3} to 2.5×10^{-5} U/mL. The activity of samples was calculated by interpolation in the standard curve. Superoxide dismutase (SOD) activity was determined by its ability to inhibit the spontaneous oxidation of adrenaline to adrenochrome, which is measured by spectrophotometer at 480 nm. Results were expressed as units (U) of SOD activity/mL, where 1 U of SOD inhibits the auto-oxidation of adrenaline by 50%.[17]

TNF-α, IL-6, and IL-10

Blood samples from animals were taken from retro-ocular vein and serum was obtained as follows: blood samples were incubated at 37°C for 10 min, and then centrifuged at 800*g* for 10 min. Interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) were determined in the supernatant of cultured macrophages. IL-10 and TNF-α were measured in serum. The cytokines were determined by using the commercial kits, such as mouse IL-6-ELISA kit (Pierce Biotechnology, Rockford, IL, USA), mouse TNF-α ELISA kit (Pierce Biotechnology), and IL-10 TiterZyme EIA kit (Assay Designs Inc., Ann Arbor, MI, USA), in accordance with the manufacturer's recommendations.

Hepatic enzymes determination

The hepatic enzymes were determined in serum obtained from the exudates of the blood gathered in tubes without EDTA, by using kits from Biosystems (Madrid, Spain), and were assayed for the following enzymes: serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, and γ-glutamyl transpeptidase.

Statistical analysis

Data were analyzed by using Student's *t*-tests. Significant difference was determined when $P \leq 0.05$.

Results

DM did not affect cell viability neither at 0 h nor at 48 h of cell culture (control macrophages viability (%): 90 ± 9; DM treated macrophages viability (%) (0 h): 89 ± 8 (NS: not significant); DM treated macrophages viability (%) (48 h): 80 ± 8 (NS) (Figure 1A). To analyze whether DM could produce apoptosis without any changes in cell viability, an apoptosis assay was performed. At 0 h, DM did not modify viable cells in a significant manner, nevertheless, some cells in early and late apoptosis were shown: at 0 h, basal cells: viable (%): 96 ± 9, early apoptosis (%): 1.50 ± 0.09, late apoptosis (%): 2 ± 0.19, necrosis (%): 1 ± 0.9; DM treated cells: viable (%): 81 ± 8 (NS with respect to control), early apoptosis (%): 9.50 ± 0.9 ($P < 0.05$ with respect to control), late apoptosis (%): 9 ± 0.8 ($P < 0.05$ with respect to control). By other way, at 48 h, DM increased cell viability in comparison with basal, decreasing cells in late apoptosis: at 48 h, basal cells: viable (%): 42 ± 4, early apoptosis (%): 18 ± 1.5, late apoptosis (%): 40 ± 4, necrosis (%): 4 ± 0.3; DM treated cells: viable (%): 55 ± 2 ($P < 0.05$ with respect to control), early apoptosis (%): 19 ± 0.19 (NS with respect to control), late apoptosis (%): 28 ± 1 ($P < 0.05$ with respect to control), necrosis (%): 2 ± 0.15 ($P < 0.01$ with respect to control). Results obtained at 0 h and 48 h are resumed in Figure 1B and 1C. With the intention to study whether DM could activate macrophages functionality, first the percentage of cells with spreading ability were recorded, and treated macrophages presented an increase in the spreading ability: spreading (%): control macrophages: 10 ± 1; DM treated macrophages: 34 ± 4. By other way, phagocytosis was analyzed. It has been shown in Figure 2 that DM increased phagocytosis in a significant manner not only at 0 h ($P < 0.05$ with respect to control) but also at 48 h ($P < 0.01$ with respect to control). It is important to note that the phagocytosis exerted by control macrophages, without DM treatment, was at 48 h, lower than at 0 h ($P < 0.05$) (Figure 2). As the treatment with the extract produced an increase in macrophages phagocytosis, the mechanism involved in this effect was studied. First, the effect on the production of macrophages TNF-α and IL-6 was assayed. It has been shown in Figure 3A,B that DM decreased TNF-α ($P < 0.01$) and IL-6 ($P < 0.01$) levels significantly. Also in serum, DM decreased TNF-α level ($P < 0.01$) (Figure 3C). As macrophages phagocytosis can be stimulated by two ways, one through TNF-α and the other through reactive oxygen species (ROS), the participation of ROS, principally H_2O_2 and NO, was studied. AQ2 DM increased H_2O_2 ($P < 0.05$) significantly (Figure 4A) and also total nitrites ($P < 0.01$) level in macrophages (Figure 4B). To study the mechanism by which DM increased H_2O_2 level, the participation of the enzymes commonly involved in H_2O_2 metabolism was assayed. It is shown in Figure 4C and 4D that DM increased cell SOD activity ($P < 0.05$) significantly but did not modify cell Px activity.

By other way, as DM decreased the number of cytokines normally involved in inflammation response, it was thought that DM could affect cytokines, implicated in

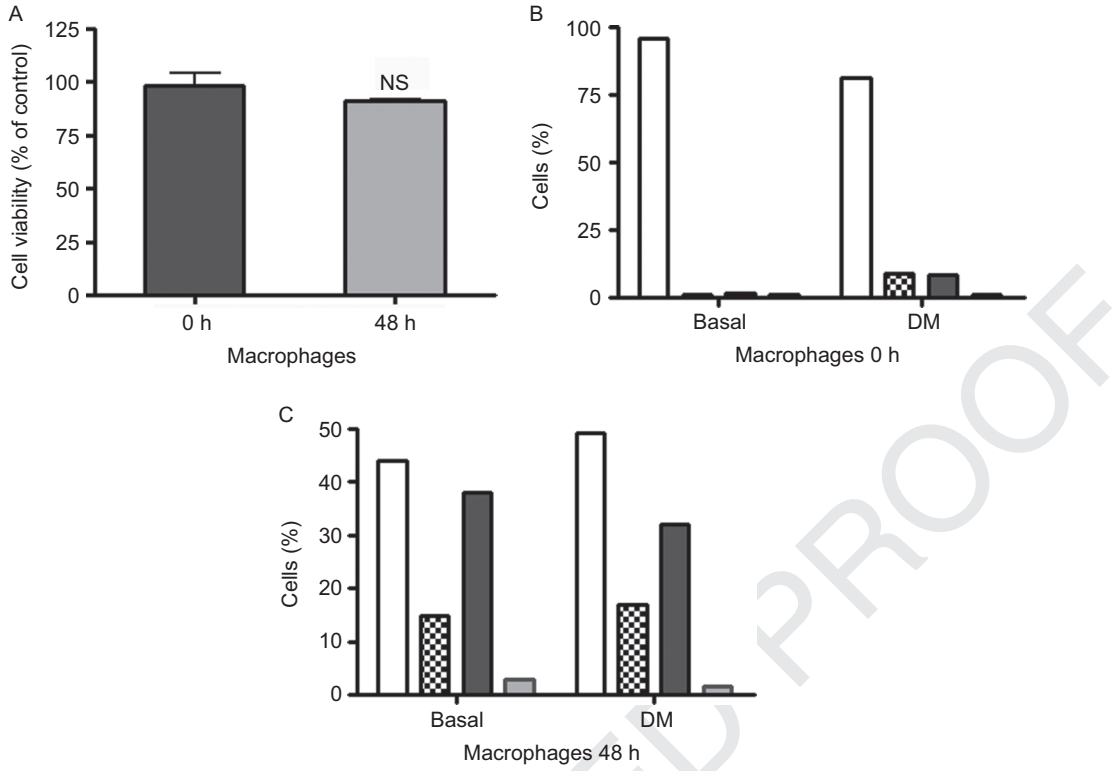

Figure 1. Effect of DM extract on cell viability. Cells were isolated from DM treated animals. (A) Cell viability was determined immediately or after an incubation time of 48 h, by MTT assay. Results were expressed as cell viability (% of control) and represented the mean ± SEM of three determinations performed by triplicate. (B and C) Analysis of apoptosis by IP versus annexin obtained from flow cytometry analysis (a representative analysis). (White column: viable cells; column with square lines: early apoptosis; black column: late apoptosis; gray column: necrosis.)

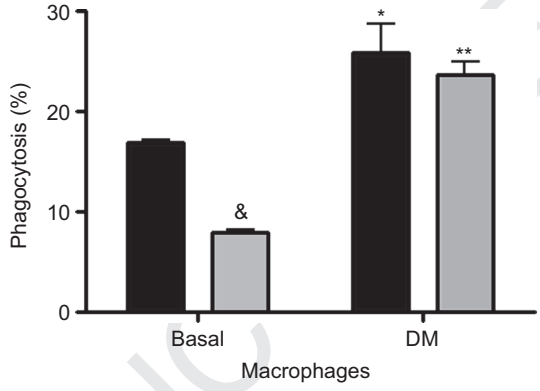

Figure 2. Effect of DM on phagocytosis. Phagocytosis was determined on macrophages immediately isolated from animals (black column) and on macrophages cultured during 48 h (gray column). Results represent the mean ± SEM of three experiments made by triplicate. Basal values, activity of macrophages from control animals; DM values, activity of macrophages from treated animals. $^{*}P<0.05$, $^{**}P<0.01$ significant differences between basal and treated and $^{\&}P<0.05$ significant differences between basal 0 h and 48 h, in accordance to Student's *t*-test.

anti-inflammatory effects. In this way, the level of IL-10, a cytokine with a well-documented anti-inflammatory action, was measured in the serum of DM treated animals. It has been shown that DM increased the level of IL-10 in serum significantly ($P<0.01$) (Figure 5).

Moreover, to study the safety of DM extract, the hepatic enzymes, which are markers of good hepatic functionality, were studied. DM did not modify the serum hepatic enzymes in comparison with control mice, NS differences were found, as shown in Table 1.

Discussion

In this work, the "*in vivo*" immunomodulatory effect of a DM from *Tilia x viridis* on macrophages was demonstrated. The extract did not affect macrophages viability. Previously it was shown that a DM extract from this plant did not modify normal murine lymphocytes viability.[8] In addition, cell viability of some cells in early and late apoptosis did not change, immediately after they were isolated from the animals (Figure 1A–C). It is important to remark that cells in early apoptosis could maintain membrane integrity, not modifying cell viability. In a previous study it was shown, with a DM extract from another related species such as *Tilia cordata*, that the extract does not affect normal murine lymphocytes cell viability and does not produce apoptosis from 5 to 100 µg/mL, but a significant decrease in cell viability and apoptosis were observed with a concentration of 1000 µg/mL.[18] In the present

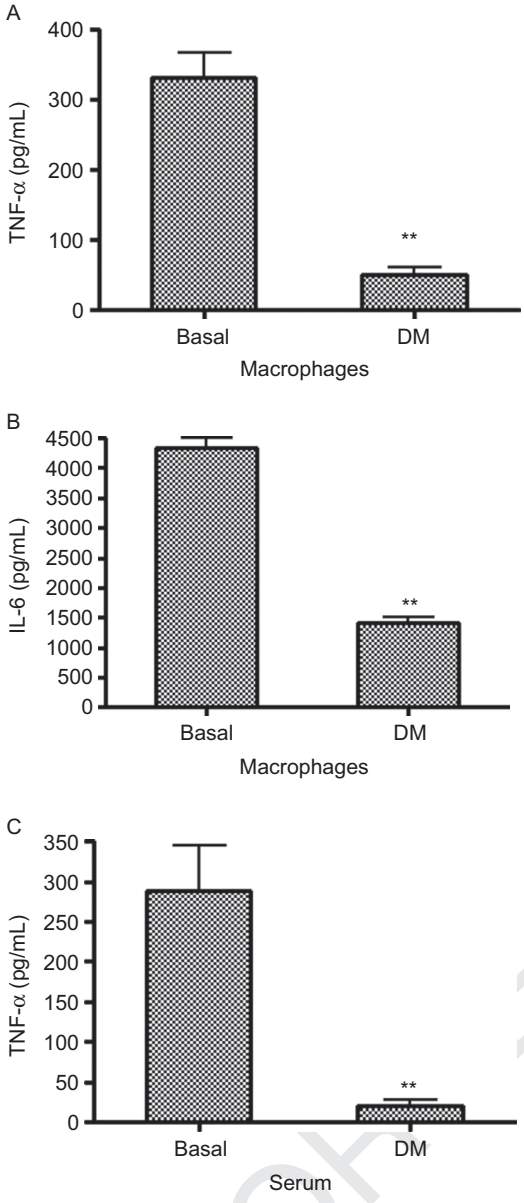

Figure 3. Effect of DM on (A) TNF-α, (B) IL-6 in macrophages and (C) TNF-α in serum. Cytokines were determined in macrophage supernatants from treated animals or in serum of treated animals. Basal values, cytokines of untreated animals. Results represent the mean ± SEM of three experiments made by triplicate. $*P < 0.05$, $**P < 0.01$ significant differences between basal and treated in accordance to Student's *t*-test.

study, a dose of 0.0225 g of the extract corresponded to a concentration of 150 µg/mL in animal tissues, this concentration was selected because it produces an inhibition between 80% and 90% of lymphoma cell line proliferation and did not affect normal lymphocytes viability, so it was selected to study its effect on macrophages, cells related to innate host defense against tumors cells.[8] By other way, when cells were cultivated, during 48 h "*in vitro*," the number of viable cells increased, as well as, decreased cells in late apoptosis in comparison with basal. All these results together indicated that the extract was not cytotoxic on macrophages.

Furthermore, on macrophages functionality, the extract was capable of inducing cell phagocytosis (Figure 2). It could be possible that the extract induced some cells to apoptosis in relation to cell activation. It is already known that the activation of macrophages induces an increase in molecules which can conduce cells to apoptosis; among these molecules, TNF-α and H_2O_2 can be mentioned. In this respect, it was demonstrated that H_2O_2 induces lymphocytes to late and early apoptosis,[19] also TNF-α is capable of producing cell death by apoptosis.[20]

Activation of macrophages phagocytosis was also observed with other compounds and plant extracts. Various agents, including IFN-γ, LPS or other microbial products, lectins, and vegetal extracts, are known to stimulate macrophages.[4,5] It was demonstrated that an extract of Babassu, the popular name of *Orbignya phalerata* Mart., is able to activate "*in vitro*" and "*in vivo*" peritoneal macrophages through the production of NO, TNF-α, and H_2O_2.[21]

In our work, the molecular mechanism involved in macrophages activation was the production of ROS and not the induction of TNF-α or IL-6, as DM increased H_2O_2 and NO levels significantly (Figure 4A,B). On the other hand, DM decreased TNF-α and IL-6 in supernatants and TNF-α serum (Figure 3A–C). In this regard, it was shown that the activation of macrophages can be exerted by two types of molecules: (1) cytokines (TNF-α, IL-6) and (2) ROS (H_2O_2, NO). NO and H_2O_2 are secreted at high levels by activated macrophages and constitutes important cytotoxic effectors molecules in the defense against tumor cells, parasitic fungi, protozoa, helminthes, mycobacterium, and virus.[22,23] The mechanism of the extract involved in the increase of H_2O_2 was the activation of SOD enzyme, which normally involves in H_2O_2 production (without modifying Px activity) and in H_2O_2 elimination (Figure 4C,D).

By other way, the DM extract also induced NO level (Figure 4B). The expression of inducible NO synthase and NO production usually requires one signal to prime and another to trigger macrophage activation. One of these signals can be the TNF-α and also H_2O_2 released by the macrophages themselves. In this work, the compound that stimulated the release of NO could be H_2O_2 and not TNF-α. In fact, the production of NO is a result of the induction and gene expression of an enzyme, which is not constitutively present in macrophages. Some plant-derived products have been shown to be NO-inducers or inhibitors by different mechanisms.[24,25]

On the other hand, proinflammatory effects of NO and H_2O_2 seem to be mediated by its exacerbated production and have been associated with a range

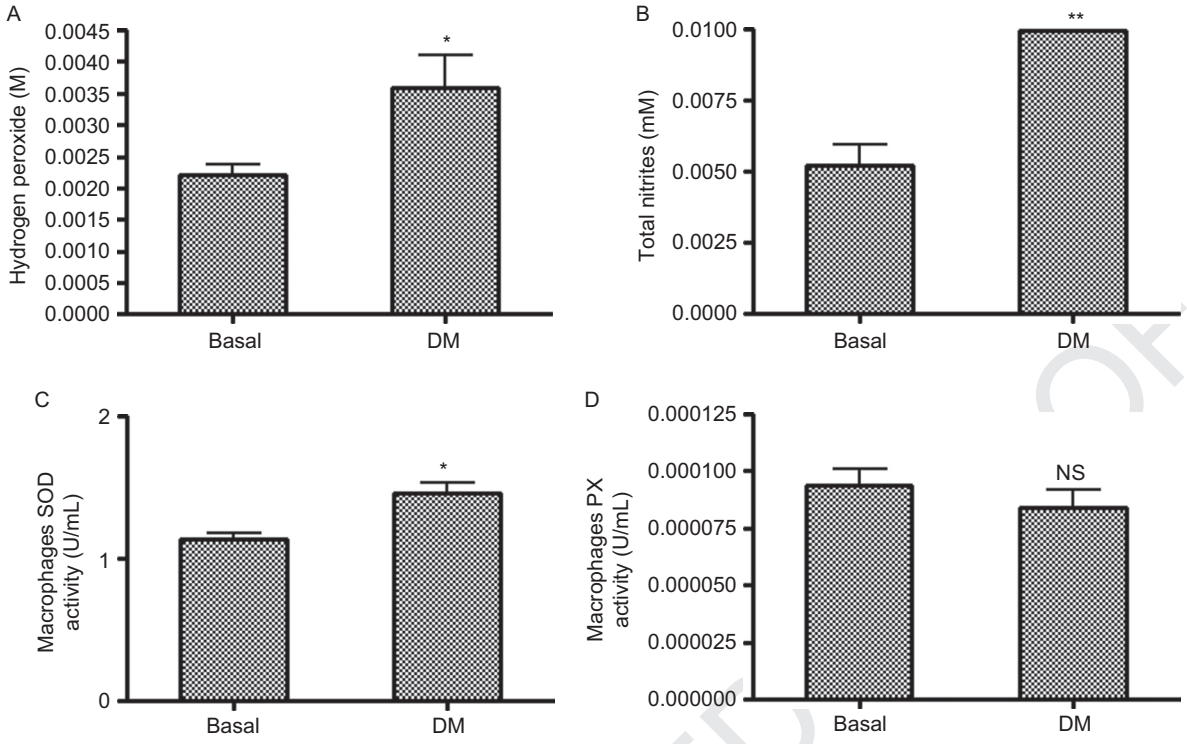

Figure 4. Effect of DM on (A) hydrogen peroxide production, (B) total nitrites, (C) SOD activity and (D) Px activity. The determinations were carried out on macrophages isolated from untreated (basal values) and DM treated animals. Results represent the mean ± SEM of three experiments made by triplicate. *$P < 0.05$, **$P < 0.01$ significant differences between basal and treated in accordance to Student's *t*-test.

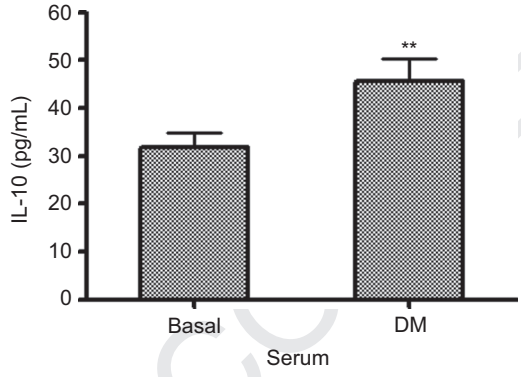

Figure 5. Effect of DM on IL-10 production. IL-10 was determined on serum of untreated (basal values) and treated animals. Results represent the mean ± SEM of three experiments made by triplicate. **$P < 0.01$ significant differences between basal and treated in accordance to Student's *t*-test.

of inflammatory diseases including atherosclerosis, ischemic reperfusion, hypertension, and septic shock.[26] But it is important to note that the extract appeared to present anti-inflammatory action increasing IL-10 level in serum (Figure 5). It has been observed that IL-10, also known as human cytokine synthesis inhibitory factor, acts as an anti-inflammatory cytokine. This cytokine is produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation.[27] It downregulates the expression of TH1 cytokines, major histocompatibility complex (MHC) antigens,[28] and costimulatory molecules on macrophages. It also enhances B-cell survival, proliferation, and antibody production. This cytokine can block NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway. Knockout studies in mice have suggested that this cytokine functions as an essential immunoregulator in the intestinal tract. A study in mice has shown that IL-10 is also produced by mast cells, counteracting the inflammatory effect that these cells have at the site of an allergic reaction.[29] In contrast, TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. The primary role of TNF-α is in the regulation of immune cells. TNF-α is also capable of inducing apoptotic cell death, inducing inflammation, and inhibiting tumorigenesis and viral replication. TNF-α is produced mainly by macrophages, but also by a broad variety of other cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neuronal tissue. Large amounts of TNF-α are released in response to LPSs, other bacterial products, and IL-1.[30–33]

The participation of the macrophages in host defense against tumor cells and microorganisms is well known.

Table 1. Effect of DM upon hepatic enzymes.

Animals	GPT	GOT	GGT
Control	26.60 ± 2	72.20 ± 7.4	6.75 ± 0.25
Treated with DM	23.50 ± 1.7^{NS}	78 ± 12.5^{NS}	7.50 ± 0.60^{NS}

The enzymes were assayed in serum of control animals, without any treatment and in DM treated mice. Results were expressed as mean ± SEM of two determinations. NS: no significant differences between control and treated, according to Student's *t*-test.

Some of the inflammatory mediators are produced by macrophages. It can be said that macrophages are very important in cell-mediated and humoral immunity, and they have an important role in the immune system as part of the host defense mechanism. It was demonstrated that activated macrophages can inhibit the growth of various tumor cells and microorganisms due to an increase in spreading and phagocytic ability; NO, H_2O_2 and cytokine production; and MHC expression.[34,35]

As the extract was administered by a systemic method, the toxicity of the extract was studied principally on the liver, an organ that is very sensible to toxic compounds. The DM extract did not modify the hepatic enzymes TGO and TGP; these enzymes are marker of liver functionality (Table 1). This fact implicated that DM was not hepatotoxic.

In relation to the compounds present in DM extract, a previous study found monoterpenes such as limonene, α-pinene, and β-pinene.[8] By other way, the coumarin scopoletin was found in a DM extract obtained from *Tilia cordata*, another species related to *Tilia x viridis*.[16] Barreiro Arcos et al.[18] demonstrated that scopoletin possess stimulatory action on murine lymphocytes; it could be possible that this compound is present in DM extract from *Tilia x viridis* and exerts an action on macrophages. Moreover, Manuele et al.[8] demonstrated that limonene can also stimulate immune cells similar to murine lymphocytes. Furthermore, the activity of D-limonene on macrophage activation has already been studied by Hamada et al.,[9] who demonstrated that D-limonene administered to rats enhanced the phagocytic activity of alveolar macrophages. Moreover, it was found that D-limonene modulates *in vivo* some aspects of immune response, such as delayed hypersensitivity reaction, macrophage phagocytosis, and microbicidal activity in BALB/c mice bearing L-5178-Y, a model characterized by defective immune response.[10] Moreover, enhance in the production of NO induced by DM extract could be attributed to limonene as, it was shown that, D-limonene increased NO production in peritoneal macrophages obtained from tumor-bearing mice.[10] Also D-limonene is capable of inducing NO production in a lymphoma cell line.[8] By other way, it was shown that scopoletin (6-methoxy-7-hydroxycoumarin), a coumarin compound, affects the production of inflammatory cytokine in macrophages, inhibiting the way in which phorbol 12-myristate 13-acetate plus A23187 induces the production of inflammatory cytokines such as TNF-α, IL-6, and IL-8.[36]

So, by this way, monoterpenes and coumarins could be the active compounds present in DM extract. Nevertheless, further studies with regard to scopoletin and monoterpenes on immune system, and especially on macrophages activation, shall be carried out.

The immunomodulatory activity on macrophages found with DM extract, besides its antiproliferative action, on lymphoma cell line could contribute to a potential antitumoral activity. In this respect, other plant extracts such as Dandelion leaf and some mushrooms were shown to stimulate the proliferation of lymphocytes, activate macrophages, and inhibit the growth of tumor cells.[37,38]

The present study provides the first results on the effect of *Tilia x viridis* DM extract on macrophage activation, exerting a modulator action on proinflammatory/cytotoxic molecules such as H_2O_2 and NO. Further studies are needed in order to characterize the bioactive compounds involved in the biological action of DM.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J., Schreiber, R.D. Cancer immunoediting: from immunosurveillance to tumor escape. Nat. Immunol. 2002, 3, 991–998.
2. Santoni, G., Cantalamessa, F., Spreghini, E., Sagretti, O., Staffolani, M., Piccoli, M. Alterations of T cell distribution and functions in prenatally cypermethrin-exposed rats: possible involvement of catecholamines. Toxicology. 1999, 138, 175–187.
3. Okamura, M., Lillehoj, H.S., Raybourne, R.B., Babu, U.S., Heckert, R.A., Tani, H., Sasai, K., Baba, E., Lillehoj, E.P. Differential responses of macrophages to Salmonella enterica serovars Enteritidis and Typhimurium. Vet. Immunol. Immunopathol. 2005, 107, 327–335.
4. Ma, J., Chen, T., Mandelin, J., Ceponis, A., Miller, N.E., Hukkanen, M., Ma, G.F., Konttinen, Y.T. Regulation of macrophage activation. Cell. Mol. Life Sci. 2003, 60, 2334–2346.
5. Fujihara, M., Muroi, M., Tanamoto, K., Suzuki, T., Azuma, H., Ikeda, H. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacol. Ther. 2003, 100, 171–194.
6. Font Quer, R. *Plantas Medicinales: el Dioscórides renovado*. Madrid: EditorialLlabor, 1976.

7. Thomson, A. *Healing Plants (A Modern Herbal)*. London: Editorial Mc Graw Hill Book Company, 1987.
8. Manuele, M.G., Ferraro, G., Anesini, C. Effect of Tilia x viridis flowers, on the proliferation of a lymphoma cell line and on normal murine lymphocytes: Participation of monoterpenes specially limonene. Phytother. Res. 2008, 22, 1520–1526.
9. Hamada, M., Uezu, K., Matsushita, J., Yamamoto, S., Kishino, Y. Distribution and immune responses resulting from oral administration of d-limonene in rats. J. Nutr. Sci. Vitaminol. (Tokyo) 2002, 48, 155–160.
10. Del Toro-Arreola, S., Flores-Torales, E., Torres-Lozano, C., Del Toro-Arreola, A., Tostado-Pelayo, K., Guadalupe Ramirez-Dueñas, M., Daneri-Navarro, A. Effect of D-limonene on immune response in BALB/c mice with lymphoma. Int. Immunopharmacol. 2005, 5, 829–838.
11. Davicino, R., Martinez, C., Mattar, M.A., Casali, Y., Correa, S.G., Aragon, L., Saidman, E.A., Messina, G., Micalizzi, B. Larrea divaricata Cav (Jarilla): production of superoxide anion, hydrogen peroxide and expression of zymosan receptors. Immunopharmacol. Immunotoxicol. 2008, 30, 489–501.
12. Davicino, R., Manuele, M.G., Turner, S., Ferraro, G., Anesini, C. Antiproliferative activity of *Larrea divaricata Cav*. on a lymphoma cell line: participation of hydrogen peroxide in its action. Cancer Invest. 2009, In press.
13. Izgut-Uysal, V.N., Agar, A., Yargicoglu, P., Apaydin, K.C. The effect of *Ginkgo biloba* extract on macrophage phagocytic activity in experimental diabetes. J.I.A.S. 1993, 6, 302–305.
14. Rabinovitch, M., Manejias, R.E., Russo, M., Abbey, E.E. Increased spreading of macrophages from mice treated with interferon inducers. Cell. Immunol. 1977, 29, 86–95.
15. Bécherel, P.A., Chosidow, O., Le Goff, L., Francès, C., Debré, P., Mossalayi, M.D., Arock, M. Inducible nitric oxide synthase and proinflammatory cytokine expression by human keratinocytes during acute urticaria. Mol. Med. 1997, 3, 686–694.
16. Herzog, V., Fahimi, H.D. A new sensitive colorimetric assay for peroxidase using 3,3'-diaminobenzidine as hydrogen donor. Anal. Biochem. 1973, 55, 554–562.
17. Carrillo, M.C., Kanai, S., Nokubo, M., Kitani, K. (-) deprenyl induces activities of both superoxide dismutase and catalase but not of glutathione peroxidase in the striatum of young male rats. Life Sci. 1991, 48, 517–521.
18. Barreiro Arcos, M.L., Cremaschi, G., Werner, S., Coussio, J., Ferraro, G., Anesini, C. Tilia cordata Mill. Extracts and scopoletin (isolated compound): differential cell growth effects on lymphocytes. Phytother. Res. 2006, 20, 34–40.
19. Davicino, R., Manuele, M.G., Ferraro, G., Micalizzi, B., Anesini, C. Modulatory effect of hydrogen peroxide on tumoral lymphocytes proliferation. Immunopharmacol. Immunotoxicol. 2009, 31, 130–139.
20. Aggarwal, S., Gollapudi, S., Gupta, S. Increased TNF-alpha-induced apoptosis in lymphocytes from aged humans: changes in TNF-alpha receptor expression and activation of caspases. J. Immunol. 1999, 162, 2154–2161.
21. Nascimento, F.R.P., Barroqueiro, E.S.B., Azevedo, A.P.S., Lopes, A.S., Ferreira, S.C.P., Silva, L.A., Maciel, M.C.G., Rodriguez, D., Guerra, R.N.M. Macrophage activation induced by *Orbignya phalerata* Mart. J. Ethnopharmacol. 2006, 103, 53–58.
22. Croen, K.D. Evidence for antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. J. Clin. Invest. 1993, 91, 2446–2452.
23. Macmicking, J.D., Nathan, C., Xie, Q.W. Nitric oxide and macrophage function. Annu. Rev. Immunol. 1997, 15, 323–350.
24. Ignácio, S.R., Ferreira, J.L., Almeida, M.B., Kubelka, C.F. Nitric oxide production by murine peritoneal macrophages *in vitro* and *in vivo* treated with Phyllanthus tenellus extracts. J. Ethnopharmacol. 2001, 74, 181–187.
25. Calixto, J.B., Otuki, M.F., Santos, A.R. Anti-inflammatory compounds of plant origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor kappa B (NF-kappaB). Planta Med. 2003, 69, 973–983.
26. Moncada, S., Higgs, E.A. Endogenous nitric oxide: physiology, pathology and clinical relevance. Eur. J. Clin. Invest. 1991, 21, 361–374.
27. McClain, M.A., Gatson, N. N., Powell, N.D., Papenfuss, T. L., Gienapp, I. E., Song, F., Shawler, T.M., Kithcart, A., Whitacre, C.C. Pregnancy Suppresses Experimental Autoimmune Encephalomyelitis through Immunoregulatory Cytokine Production. J. Immunol. 2007, 179, 8146–8152.
28. Kundu, N., Fulton, A.M. Interleukin-10 inhibits tumor metastasis, downregulates MHC class I, and enhances NK lysis. Cell. Immunol. 1997, 180, 55–61.
29. Grimbaldeston, M.A., Nakae, S., Kalesnikoff, J., Tsai, M., Galli, S.J. Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. Nat. Immunol. 2007, 8, 1095–1104.
30. Old, L.J. Tumor necrosis factor (TNF). Science. 1985, 230, 630–632.
31. Beutler, B., Greenwald, D., Hulmes, J.D., Chang, M., Pan, Y.C., Mathison, J., Ulevitch, R., Cerami, A. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. Nature. 1985, 316, 552–554.
32. Wajant, H., Pfizenmaier, K., Scheurich, P. Tumor necrosis factor signaling. Cell Death Differ. 2003, 10, 45–65.
33. Gaur, U., Aggarwal, B.B. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. Biochem. Pharmacol. 2003, 66, 1403–1408.
34. Nascimento, F.R., Ribeiro-Dias, F., Russo, M. Cytotoxic activity of BCG-activated macrophages against L929 tumor cells is nitric oxide-dependent. Braz. J. Med. Biol. Res. 1998, 31, 1593–1596.
35. Nascimento, F.R., Calich, V.L., Rodríguez, D., Russo, M. Dual role for nitric oxide in paracoccidioidomycosis: essential for resistance, but overproduction associated with susceptibility. J. Immunol. 2002, 168, 4593–4600.
36. Phil-Dong, Moon., Byung-Hee, Lee., Hyun-Ja, Jeong., Hyo-Jin, An., Seok-Jae, Park., Hyung-Ryong, Kim., Seong-Gyu, Ko., Jae-Young, Um., Seung-Heon, Hong., Hyung-Min Kim. Use of scopoletin to inhibit the production of inflammatory cytokines through inhibition of the IκB/NF-κB signal cascade in the human mast cell line HMC-1. Eur. J. Pharmacol. 2007, 555, 218–225.
37. Lee, S.H., Park, J.B., Park, H.J., Park, Y.J., Sin, J.I. Biological properties of different types and parts of the dandelions: comparisons of anti-oxidative, immune cell proliferative and tumor cell growth inhibitory activities. Kor. J. Food. Sci. Nutr. 2005, 10, 172–178.
38. Park, J.M., Lee, S.H., Kim, J.O., Park, H.J., Park, J.B., Sin, J.I. *In vitro* and *in vivo* effects of extracts of *Lentinus edodes* on tumor growth in a human papilloma virus 16 oncogenes-transformed animal tumor model—apoptosis mediated tumor cell growth inhibition. Kor. J. Food. Sci. Technol. 2004, 36, 141–146.

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