ANTI-INFLAMMATORY EFFECTS OF GINGER AND ROSEMARY EXTRACTS OBTAINED BY SUPERCRITICAL FLUID TECHNOLOGY

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ABSTRACT

In this work, we investigated the effects of ginger and rosemary extracts, obtained through supercritical fluid technology, on the production of inflammatory mediators by murine macrophages. For ginger and rosemary, the extractions were carried out using the following conditions, 1.42×10^{-4} kg/s of CO_2, 30°C and 300 bar and 1.13×10^{-4} kg/s of CO_2, 40°C and 250 bar, respectively. The production of inflammatory cytokines and nitric oxide was studied using an *in vitro* system in which peritoneal macrophages and J774 cells were exposed to the plant extracts, in the presence or absence of lipopolysaccharide (LPS) from *Escherichia coli* plus interferon-γ (IFNγ), and cultured for 48 h. Then, cell viability was evaluated by a colorimetric method for the determination of cell densities using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Culture supernatants were collected to analyze the production of nitric oxide using the Griess reaction assay, and the cytokines TNF-α and IL-1, using ELISA tests. The results show that ginger and rosemary extracts were capable of inhibiting LPS+IFN-γ-induced TNF-α, IL-1 and NO production. Comparatively, ginger extract showed the most prominent anti-inflammatory activity. Thus, we conclude that some of the beneficial effects attributed to ginger and rosemary extracts, related with their anti-inflammatory activity, could be associated with inhibition of mediators in the inflammatory process.

INTRODUCTION

Inflammation is a complex immune process that can be defined by the sequential release of mediators such as pro-inflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor (TNF), anti-inflammatory cytokines, such as IL-10, and reactive oxygen and nitrogen species. These mediators initiate the inflammatory response, recruit and activate other cells to the site of injury and subsequently resolve the inflammatory process. The inhibition of the overproduction of such mediators, especially pro-inflammatory cytokines, may prevent or suppress a variety of inflammatory diseases [1].

In recent years, several products derived from plants have been investigated for therapeutical application due to their activity on inflammatory processes. Many herbs and spices, typically used for food flavoring, are excellent sources of phenolic compounds, which have been reported to show good antioxidant activity [2]. The anti-inflammatory activity of natural compounds has been frequently associated to their antioxidant activity, as well as to their capacity to suppress nitric oxide production in macrophages [3].

Ginger (*Zingiber officinale* Roscoe) and rosemary (*Rosmarinus officinalis* Labiatae) are among the most important and extensively used spices worldwide. Ginger and rosemary extracts contain numerous biologically active constituents, including polyphenolic compounds such as 6-gingerol and its derivatives (in ginger extract), and carnosic acid and carnosol (in rosemary extract).

The purpose of the present study was to evaluate the *in vitro* effects of ginger and rosemary extracts obtained through supercritical fluid technology upon the production of inflammatory mediators, specifically cytokines and nitric oxide, by macrophages.

MATERIAL AND METHODS

Plant Extracts

Organic ginger (*Zingiber officinale* Roscoe) and rosemary (*Rosmarinus officinalis* Labiatae) were cultivated in the Experimental Farm of Lageado (São Paulo, Brazil) and in the Agronomic Institute of Campinas (IAC) (São Paulo, Brazil), respectively. The raw materials were triturated, dried, packed in plastic bags (1.5 kg each) and kept at -5°C.

Extracts of ginger and rosemary were obtained by supercritical fluid extraction (SFE) using an Applied Separations unit (Spe-ed SFE, model 7071, Allentown, USA). The extractor vessel (Thar Designs, Pittsburgh, USA) total volume was $300x10^{-6}$ m^3 (0.1286 m in height, with an internal diameter of 0.0545 m). The ginger and rosemary extractions were carried out using $1.42x10^{-4}$ kg/s of CO_2, at 30°C and 300 bar and $1.13x10^{-4}$ kg/s of CO_2 at 40°C and 250 bar, respectively. The extracts obtained were diluted in dimethylsulfoxide (DMSO) to achieve 0.7 mg/mL and maintained at -20°C.

Cell Culture

Murine peritoneal macrophages and cells of the lineage J774 were employed in the *in vitro* anti-inflammatory effects studies.

Thyoglicolate-elicited murine macrophages were harvested from peritoneal cavities of 8-weeks old BALB/c mice intraperitoneally (i.p) injected with 3 mL of 3% sodium thioglycollate medium 4 days before the assays. The cells were transferred to a sterile polypropylene conical centrifuge tube on ice and centrifuged at 1350 rev/min for 10 min at 5°C. The supernatant was discarded and the sediment was ressuspended in DMEM supplemented with 10% fetal bovine serum (FBS; complete medium). Cells were counted in hemocytometer and the suspension was adjusted to $1x10^6$ cells/mL. The mouse macrophage-like cell line J774 (4) was cultured until semi-confluence in a humidified incubator at a 5% CO_2, at 37°C. Then, J774 cells were harvested, washed with DMEM without FBS, and adjusted to $1x10^6$ cells/mL in complete medium.

Peritoneal macrophages and J774 cells were seeded at a density of $2x10^5$ cells/well in 96-well plates and cultured for 24 hours. The non-adherent cells were removed by washing with DMEM without FBS. Then, 50 µL/well of either complete medium or dilutions of plant extracts in culture medium were added to the remaining adherent cells, and plates were incubated for 1h in the same culture conditions. Subsequently, the wells were filled with

50 μL of culture medium or a solution of LPS/IFNγ (1 μg/mL/150 IU/mL), and the plates were incubated for additional 48 h before being used for the MTT, NO, IL-1 and TNF assays. Plant extracts were used only at concentrations in which cell viability, evaluated through the MTT assay, was not affected (equal to 0.875ug/mL for ginger extract and 1.75 ug/mL for rosemary extract).
For the MTT assay, 10 μL of MTT (5 mg/mL) dissolved in 0.02 M phosphate buffered saline (PBS), pH 7, were added to each well and plates were incubated with for 4 h at 37°C. The formazan crystals formed by MTT reduction by living cells were dissolved in 5% SDS in 0.01 N HCL and the optical density was measured using a Multiskan MS microplate reader (Labsystems Oy, Helsink, Finland) at 540 nm.
Culture medium and reagents used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO, USA), except for FBS, that was purchased from Cultilab, Campinas, Brazil.

Measurement of nitrite concentration
Nitric oxide (NO) released into the cultures supernatants was indirectly determined using a quantitative colorimetric assay based on Griess reaction. Triplicate 50 μL aliquots of cell culture supernatants were incubated with 50 μL of Griess reagent, consisting of 1% sulfanilamide, 0.1% N-(1-naphthylethylenediamine) dihydrochloride and 2.5% ortho-phosphoric acid. After 10 min at room temperature, the absorbance was measured in a Multiskan MS microplate reader (Labsystems Oy, Helsink, Finland) at 540 nm.

Cytokine Determination
Enzyme-linked immunosorbent assay (ELISA) kits (BD-OptEIA Mouse Set; BD Biosciences Pharmingen, San Diego, CA) were used for IL-1 and TNF-α cytokine determination in the culture supernatants.

Statistical analysis
The software Prisma (version 5.0, Graph Software, Inc. San Diego, CA, USA) was used for the statistical analysis. The data are presented as the mean±S.E.M. Statistical comparisons of the percentage relaxation under different treatments were performed by one-way ANOVA followed by post hoc Bonferroni test. A P value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In the last years several products derived from plants have been investigated due to their potential therapeutical activity on the inflammatory processes, and frequently extracts with high antioxidant activity are employed for this purpose. In this context, ginger (*Zingiber officinale* Roscoe) and rosemary (*Rosmarinus officinalis* Labiatae) have been traditionally used in the popular medicine for a wide range of health-related problems, including chronic inflammatory diseases.
In the present study, the global yields obtained in SFE were 2.4 and 2.9 % for ginger and rosemary, respectively. These values are in agreement with the ones reported by Zancan et al. [5] and by Carvalho et al. [6]. High free radical scavenging capacity was observed for both extracts through the ABTS and DPPH methods, as previously reported [7].
The plant extracts were evaluated on their ability to induce or inhibit the production of NO and pro-inflammatory cytokines in peritoneal macrophages and J774 cells. Figures 1 to 3

show the results achieved when testing the effects of ginger and rosemary extracts upon both cell types.

Regarding NO production (Figure 1), macrophage cultures showed significant levels of nitrite in the supernatants only when exposed to LPS plus IFNγ, and either ginger or rosemary extracts were capable to inhibit NO production when used at 0.875ug/mL and 1.75 ug/mL, respectively. Inflammatory cells produce large amounts of NO when properly stimulated by bacterial endotoxin and/or certain cytokines, such as IFN-γ and TNF-α. These stimuli lead to the expression of the inducible NO synthase (iNOS), an enzyme that metabolizes L-arginine in citruline and NO [8; 9]. Nitric oxide released by iNOS from murine macrophages is cytostatic and cytotoxic for protozoan parasites, fungal cells and bacteria. However, overproduction of NO can cause the destruction of adjacent healthy cells and is probably an important mediator of the acute and chronic signs of inflammation. Thus, selective inhibitors of iNOS may in the future be useful in the treatment of inflammatory conditions [10]. In this regard, the plant-derived substances evaluated in this work may be good candidates.

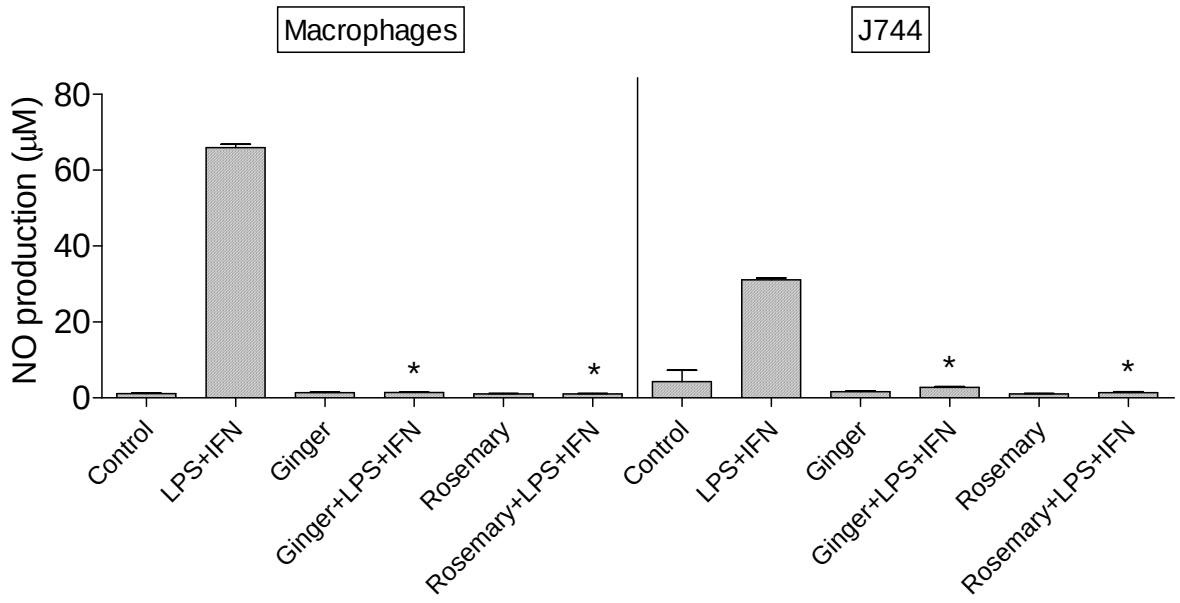

Figure 1 **-** Nitric oxide production by stimulated macrophages and J774 cells incubated for 48 h with ginger (0.875ug/mL) and rosemary (1.75 ug/mL) extracts or the extracts and LPS (1 μg/mL) plus IFN (150 IU/mL). The amount of NO released into the culture supernatants was expressed as nitrite. The columns represent the means ± SE (n = 3). *P < 0.05.

Both ginger and rosemary extracts were able to inhibit the production of TNF-α in peritoneal macrophages (Figures 2), but only rosemary extract was able to significantly reduce the production of IL-1 in peritoneal macrophages (Figure 3). TNF and/or IL-1 were not produced by J774 cells in response to any stimuli used here. TNF-α and IL-1 are the initial cytokines in inflammatory cascade. They share some biological properties and may act synergistically in several inflammatory disorders, such as rheumatoid arthritis (RA) and septic shock. Monoclonal antibodies against these cytokines have being successfully tested for therapeutic purposes in experimental models of RA [11]. However, the costs of antibody-based therapy are usually very high, thus justifying the search for alternative substances. Further

experiments are necessary to determine the feasibility of the use of products derived from rosemary or ginger in such conditions.

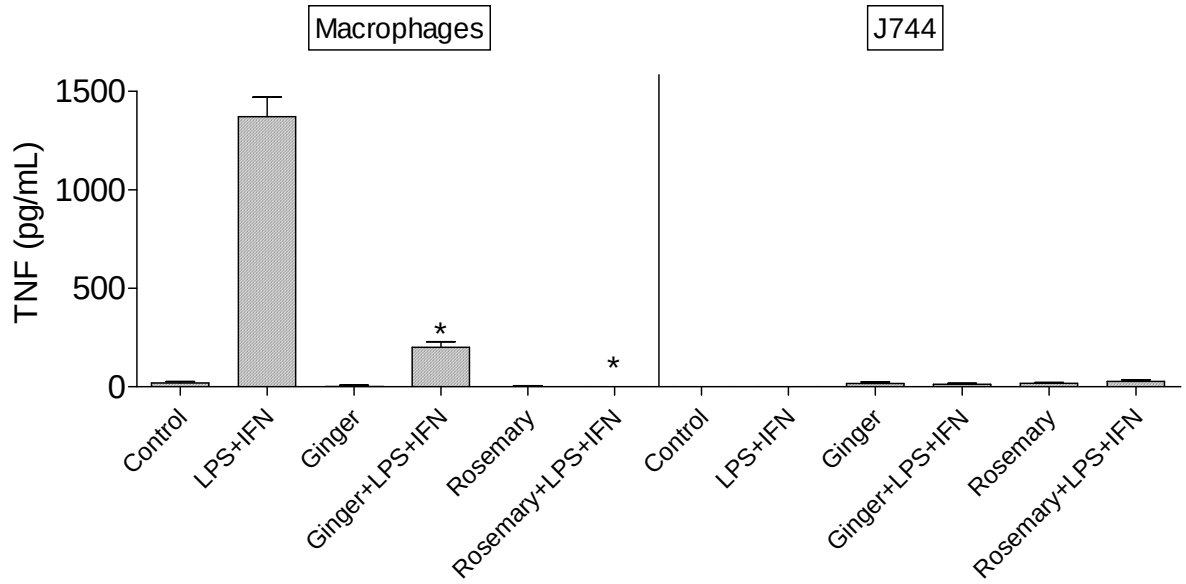

Figure 2 - Level of TNF cytokine in stimulated macrophages and J774 cell cultures incubated for 48 h with ginger and rosemary extracts. The columns represent the means ± SE (n = 4). *P < 0.05.

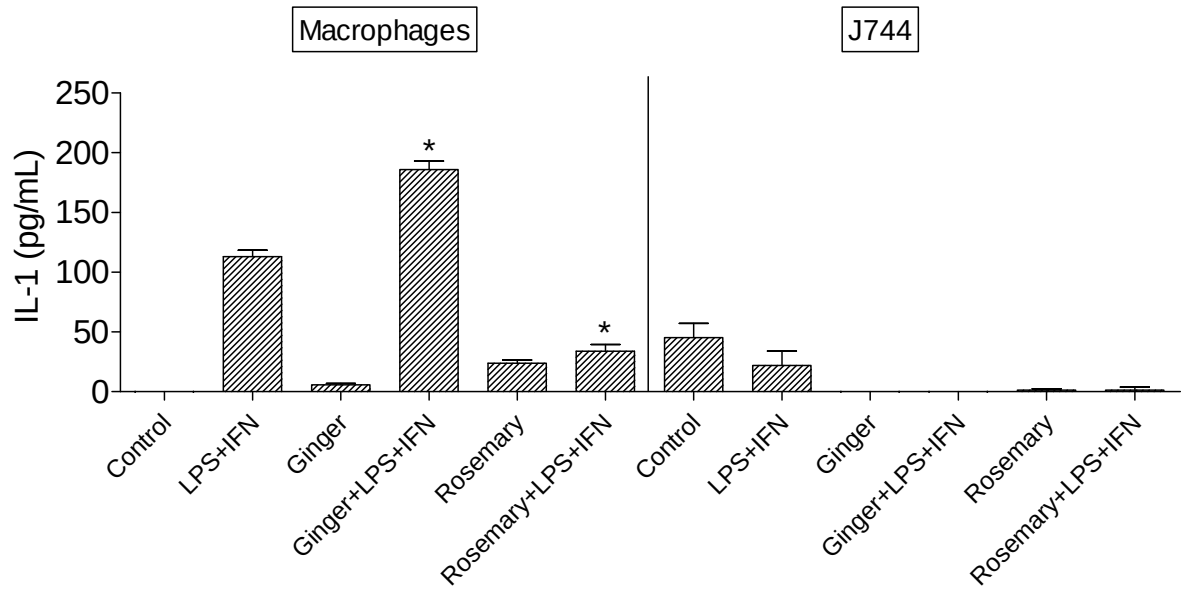

Figure 3 - Estimation of IL-1 cytokine in stimulated macrophages and J774 cell cultures incubated for 48 h with ginger and rosemary extracts. The columns represent the means ± SE (n = 4). *P < 0.05.

CONCLUSION

Ginger and rosemary extracts obtained through supercritical CO_2 extraction with high antioxidant activity and yields of 2.4 and 2.9%, respectively, were capable of inhibiting TNF-α, IL-1 and NO production. In comparison to rosemary, the ginger extract showed the highest anti-inflammatory activity.

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