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Immunomodulatory and Antioxidant Properties of Kaurenoic Acid on Macrophages of BALB/c *in Vitro*

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Abstract: Kaurenoic acid has been displaying anti-inflammatory effect described in different models. However, the per se immunomodulatory effects of kaurenoic acid remain to be investigated. Thus, the immunomodulatory and antioxidant effects of kaurenoic acid were investigated *in vitro* on peritoneal macrophages from BALB/c mice. Kaurenoic acid induced per se the production of pro-inflammatory cytokines such as TNFα, IL-1β and IFN-γ while also increased the levels of IL-10. There was also reduction of NO production and induction of anti-oxidant profile. Therefore, in addition to inhibiting inflammation, kaurenoic acid presents immunomodulatory effects per se.

Keywords: Immunomodulation, Kaurenoic Acid, Macrophages, *In Vitro* Study

Introduction

Kaurenoic Acid (ent-kaur-16-en-19-oic acid) (KA) is a tetracyclic diterpene that occurs naturally in several plants (Batista *et al*., 2005). Among the biological effects of this diterpene, studies have shown analgesic activity (Mizokami *et al*., 2012), anti-inflammatory effects in asthma models (Cho *et al*., 2010), antitumoral, antimicrobial and antiprotozoal actions (Costa-Lotufo *et al*., 2002; Wilkens *et al*., 2002; Izumi *et al*., 2012; Santos *et al*., 2013).

In fact, studies *in vitro* have demonstrated the ability of KA in inhibiting the expression of inducible nitric oxide synthase (iNOS) in RAW 264.7 macrophages stimulated with LPS and the expression of cyclooxygenase-2 (COX-2), consequently decreasing the nitric oxide (NO) and prostaglandin E2 (PGE2) production. The proposed mechanism for immunomodulatory properties was related to inhibition of nuclear factor κB (NF-κB) activation (Choi *et al*., 2011).

On the other hand, it was demonstrated that KA acts by inducing the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) which regulates expression of genes that are involved on antioxidant response without affecting NF-κB activation (Lyu *et al*., 2011).

As the modulation of cytokines affects many physiological and pathological functions, including innate immunity, acquired immunity and aspects of the inflammatory response, it is important to investigate the action of KA related to possible oxidative stress and immune regulation considering *in vitro* non inflammatory conditions.

Materials and Methods

Kaurenoic Acid

The KA used in this manuscript was obtained from *Sphagneticola trilobata*. The crude extract was obtained from dried roots pulverized and extracted with dichloromethane and partitioned with *n*-hexane and ethyl acetate, all solvents were dried under reduced pressure. The hexane fraction was subjected to VLC by increasing gradient polarity, on the second fraction amorphous compound were washed with cold methanol (200 mg), this compound were analyzed by high performance liquid chromatography (HPLC) methods yielding 96% of purity.

The identification was performed by 1H and 13 C NMR, EIMS and literature data (Da Costa *et al*., 1996).

The stock solution of KA was dissolved in DMSO (Invitrogen-Gibco®) at 2% in all experiments.

Cell Culture and Treatment In Vitro

Macrophages (5×10^5/mL) were obtained from the peritoneal cavity of BALB/c mouse by the injection of 2 mL of RPMI 1640 culture medium supplemented with fetal bovine serum 10% and cultured on 24 well plates for 2 h of adherence. The cells received KA (50, 70 or 90 µM) or medium for 24 h at 37°C and 5% CO_2. DMSO concentration did not exceed 0.01% in the wells. Reagents for cell cultures were purchased from Invitrogen-Gibco®. Female BALB/c mice used were obtained from the Fundação Osvaldo Cruz, FIOCRUZ, Curitiba, Brazil. Mice were kept under pathogen-free conditions and used according to protocols approved by Animals Ethics Committee of the Estate University of Londrina (protocol number 33064/2012.42).

Cytokine Production Determination by ELISA

The supernatants obtained from macrophage treated with KA (50, 70, 90 µM) by 24 hours were used for measurement of TNF-α, IFN-γ, IL-1β, TGF-β andIL-10using the technique of capture Enzyme-Linked Immune Sorbent Assay (ELISA) kit from Bioscience (USA). The concentration of cytokines was determined by reference to standard curve for serial dilutions and the optical absorbance measured at 450nm.

Total Antioxidant Capacity of Samples (Trapping Antioxidant Parameter-TRAP)

Samples (50 µL of cell supernatant) were analyzed by chemiluminescence method, for verifying the antioxidant profileas previously described by Repetto *et al*. (1996). Soluble vitamin E (Trolox) was employed as a standard antioxidant. The chemiluminescence curves were obtained using the Glomax luminometer (Promega) and the results are expressed in nM of Trolox.

Malondialdehyde (MDA)

MDA levels were determined using HPLC as previously described by Victorino *et al*. (2013) with slight modifications. The analyses were conducted with an Alliance e2695 HPLC (Waters, Milford, MA, EUA) equipped with a Security Guard ODS-C18 (4×3,0 mm, Phenomenex), C18 reverse phase column (Eclipse XDB-C18; 4,6×250 mm, 5 µm, Agilent) and a photo-diode array detector (Photodiode Array Detector (PDA, 2998)). Analyses were conducted in the Empower 2 software (Waters, Milford, MA, EUA). MDA standards were prepared using 1,1,3,3-Tetraetoxipropane (TEP). Aliquots containing 250 µL of the cells and supernatants were deproteinized by adding trichloric acid 20% and reacted with 1mL of thiobarbituric acid. The mobile phase was constituted with 70% 10 mM KH_2PO_4 buffer, pH 7.0 and 40% HPLC grade methanol. Readings were obtained at 532 nm, following an 8 min isocratic flow at the rate of 1 mL/min. Results were expressed in nM of MDA.

Determination of Nitrite Levels

The determination of nitrite supernatants collected from KA treated cells were used as estimates of the concentrations of NO by Griess reagent accordingly to Panis *et al*. (2012) with some modifications. Briefly, supernatant aliquots were recovered and diluted in glycine buffer solution (45 g/L pH 9.7). It was added Cadmium granules previously activated with $CuSO_4$ 5 mM solution to the samples for 10 minutes under stirring. Aliquots of 200 µL were recovered into suitable tubes for determination of nitrite and the same volume of Griess reagent was added. After 10 min incubation at room temperature, the tubes were centrifuged at 10,000 rpm, 2 min, 25°C and added to 96-well micro plates in triplicate. Calibration curve was prepared by dilution of $NaNO_2$ and the absorbance was determined at 550 nm in a microplate reader.

Statistical Analyses

Statistical differences among groups were analyzed using a one-way Analysis Of Variance (ANOVA) follow Tukey test. Data are shown as the means ± Standard Error of the Mean (SEM) and significance was defined as $p<0.05$.

Results

Initially, the KA concentrations tested were evaluated about toxicity by MTT assay of peritoneal macrophages and the concentrations did not present interference on cell viability (data not show).

In attempt of evaluating the immunomodulatory properties of KA, we analyze cytokine production (IFN-γ, IL-1β, TNF-α, IL-10 and TGF-β) in BALB/c peritoneal macrophages treated during 24 h with KA.

We verified that, KA treatment increased the IFN-γ and IL-1β production of concentration dependent manner, with a significant increase at 70 and 90 µM (Fig. 1A and 1B). The TNF-α levels were increased by KA treatment only at the concentration of 70 µM (Fig. 1C).

On the other hand, the IL-10 levels were increased in all concentration tested (Fig. 1D). Therefore, KA treatment did not affect the levels of TGF-β (Fig. 1E).

In order to evaluate the role of KA about some oxidative stress parameters, we observed that this diterpene diterpene promoted increasing of total antioxidant capacity (TRAP) (Fig. 2A) in all the concentration used. This capacity was also observed in reduction of lipid per oxidation (malondialdehyde assay) (Fig. 2B) as well as in the nitrite dosage (Fig. 3).

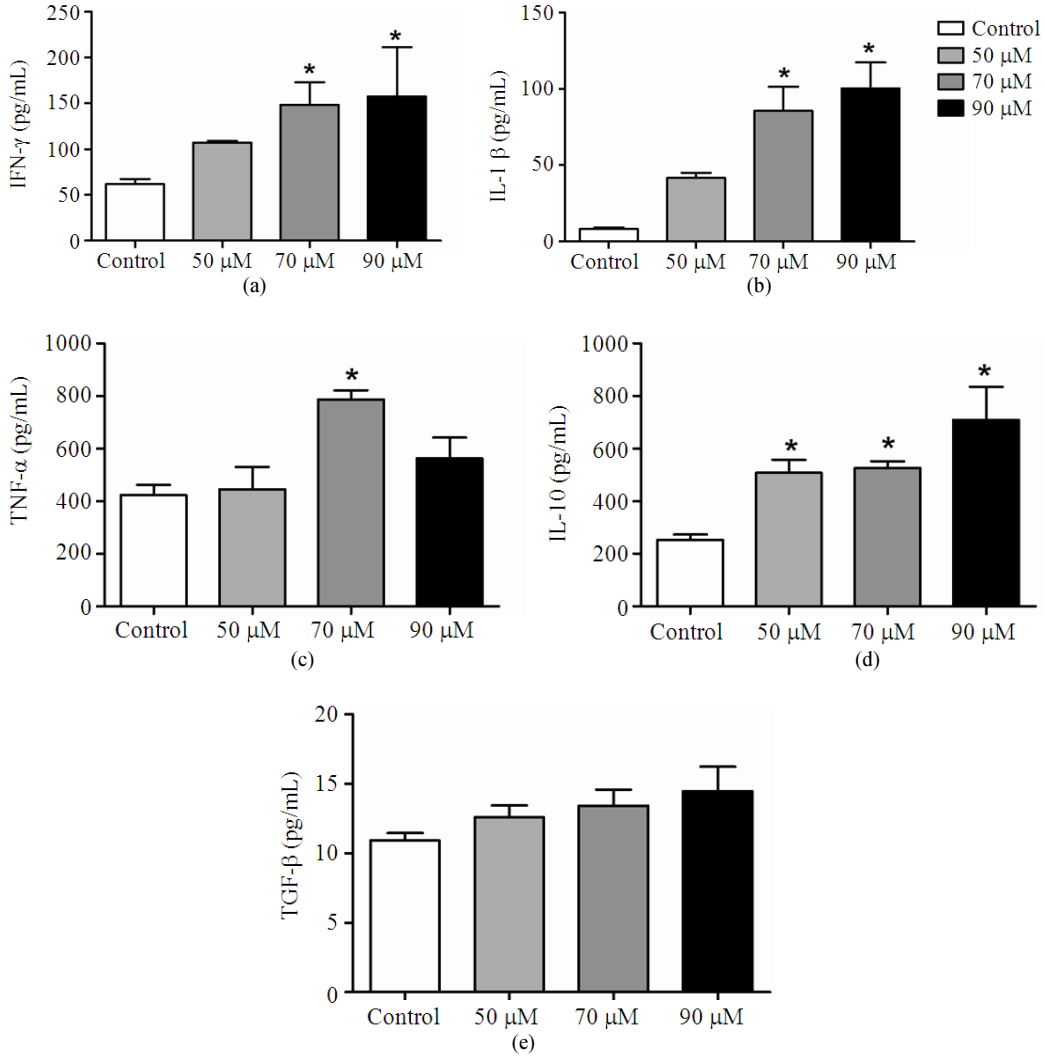

Fig. 1. Mapping the cytokine profile produced *in vitro* by macrophages treated with Kaurenoic acid (50, 70, 90 µM) for 24 h detected by ELISA. IFN-γ production (Panel A), IL-1βproduction (Panel B), TNF-α production (Panel C), IL-10 production (Panel D) and TGF-β production (Panel E). Data represent the mean ± SEM of three independent experiments

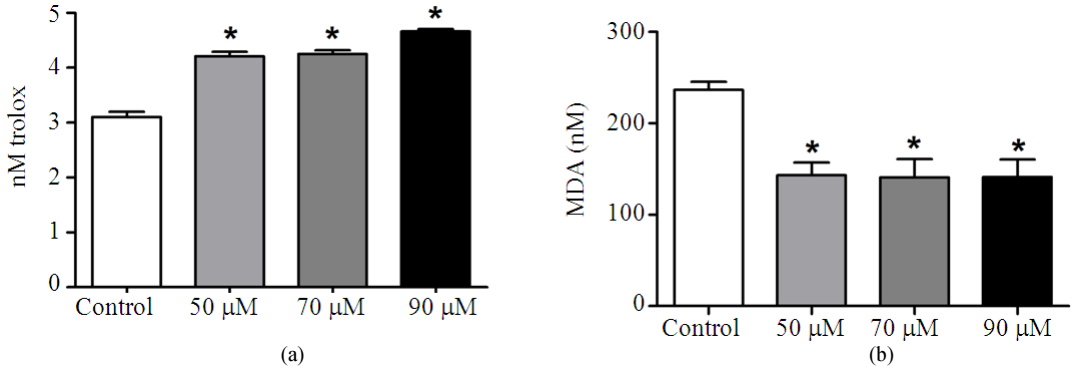

Fig. 2. Parameters about oxidative stress of macrophages treated with kaurenoic acid (50, 70, 90 µM) for 24 h. Total Antioxidant Capacity (TRAP) measurement by chemiluminescence (Panel A). Lipoperoxidation (malondialdehyde-MDA) levels measurement by High Performance Liquid Chromatography (HPLC) (Panel B). Data represent the mean ± SEM of three independent experiments

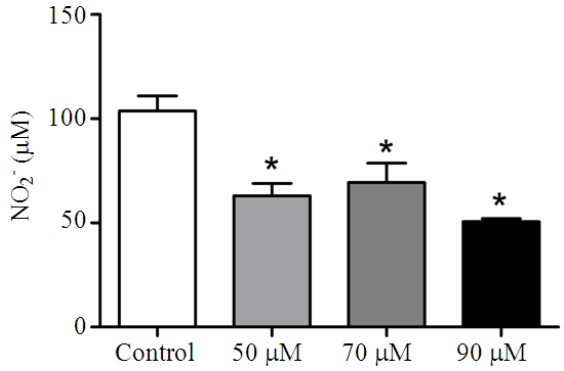

Fig. 3. Nitrite levels produced by macrophages treated with kaurenoic acid (50, 70, 90 µM) for 24 h. Data represent the mean ± SEM of three independent experiments

Discussion

Immunomodulation consists on the adjustment of the immune response by agents (endogenous or exogenous) that activate or suppress the immune response (Dutta, 2002). The class of immunomodulatory drugs presents a wide range of critical biological effects for a variety of therapeutic approaches including immunotherapies against cancer, infectious diseases, treatment of autoimmune disorders and allergies, transplant surgeries and regenerative medicine (Zimmerman, 2009; Purwada *et al*., 2013).

Macrophages, for being part of the first line of defense, play an important role in the early immune response mainly with the production of cytokines that will define the response pattern (Dinarello, 2000).

Therefore, one of the main targets of immunotherapy consists in modulating the secretion of cytokines that is responsible for the communication between cells that will determine the type, quality, amplitude, duration and outcome of the immune response (Bouabe, 2012).

In this present study, to evaluate the profile of immune response after the treatment of KA, we verified that this diterpene was able to activate the synthesis of pro-inflammatory cytokines: IFN-γ (Fig. 1A), IL-1β (Fig. 1B), TNF-α (Fig. 1C) demonstrating for the first time that KA presents its own effects independently of an inflammatory stimulus.

These results corroborate with earlier study that demonstrated that KA did not inhibit the expression of pro-inflammatory cytokines (Lyu *et al*., 2011) permitting to infer that KA presents immunomodulatory properties.

On the other hand, our results corroborate with the findings of Choi *et al*. (2011) that observed an inhibitory effect of KA on LPS induced in flammatory response.

Concerning of IL-10 function its known the ability to inhibit the production of other cytokines, such as TNF-α, IL-1β, IL-6 e IL-8 (Poole *et al*., 1995). In addition, the classic production of Reactive Oxygen Species (ROS) and Nitric Oxide (NO) is dependent of cytokines like IFN-, TNF-α and IL-1β. However, our findings reinforce this role of IL-10 since even with the IFN-γ, TNF-α and IL-1β synthesis, the parameters analyzed for ROS and NO were decreased, demonstrating the prevalence of IL-10 on pro-inflammatory cytokines (Fig. 1D).

Haddad and Fahlman (2002) demonstrated that IL-10 was described the anti-inflammatory cytokine with an antioxidant properties.

Additionally, Lyu *et al*. (2011) reported that KA induces the Nrf2 activation, which regulates inducible antioxidant responses attenuating oxidative stress, consequently the inflammatory response (Lee and Johnson, 2004; Kim *et al*., 2011).

This result suggested that KA can be used to control the damage provoked by inflammatory response by the modulation of the oxidants effects.

Corroborating that KA induces Nrf2 activation (Lyu *et al*., 2011), it induced an increase of total antioxidant capacity (TRAP) and reduced lipid peroxidation (malondialdehyde-MDA).

KA reduced the NO levels in LPS-stimulatedRAW264.7 (Choi *et al*., 2011). In the present study, KA reduced the basal NO levels in primary macrophage culture. Choi *et al*. (2011) data correlated with the inhibition of iNOS expression and activation of NF-κB.

The controversial effects on *in vitro* inflammatory pathways previously demonstrated can be explained by differences of strategy study adopted like time accessing of substances produced, stimulus, concentration and treatments used.

Conclusion

This study evidenced the immunomodulatory property of Kaurenoic acid on *in vitro* primary macrophages culture. Therefore, it is important to consider the immunomodulatory effects of KA during non-inflammatory conditions.

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Author's Contributions

Juliana Aparecida Macri: Conducted and analyzed the experiments of cell culture and treatment i*n vitro*; participated in the design of the study and manuscript writing.

Suelen Santos da Silva: Conducted and analyzed the experiments for cytokine determination by ELISA; participated in the design of the study and writing and discussion of the manuscript.

Milena Menegazzo Miranda: Conducted and analyzed the experiments of Griess assay and TRAP measurement; participated in the design of the study and writing and discussion of the manuscript.

Natalia Yoshie Kawakami: Assisted in the macrophage experiments and maintenance of animals.

Thiago Hideki Hayashida: Assisted in kaurenoic acid extraction and characterization.

Tiago Bervelieri Madeira: Performed experiments regarding MDA and data analysis.

Suzana Lucy Nixdorf: Performed experiments regarding MDA and data analysis.

Vinicius Ricardo Acquaro Junior: Performed experiments regarding MDA and data analysis.

Sérgio Ricardo Ambrósio: Assisted in kaurenoic acid extraction and characterization.

Waldiceu Aparecido Verri Junior: Contributed to financial support, data analysis, discussion and writing of the manuscript.

Rubens Cecchini: Contributed to data analysis regarding oxidative stress markers.

Ivete Conchon Costa: Contributed to the conception of the study, financial support and data discussion.

Nilton Syogo Arakawa: Designed the kaurenoic acid extraction experiments.

Wander Rogério Pavanelli: Coordinator of the study, contributed to the design of the study, financial support, data analysis and the writing and discussion of the manuscript.

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