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Kaempferitrin induces immunostimulatory effects *in vitro*

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ABSTRACT

Ethnopharmacological relevance: *Justicia spicigera* is a plant used as immunostimulatory in Mexican traditional medicine. Recently, we showed that *Justicia spicigera* extracts exerted immunostimulatory effects and the major component of this extract was kaempferitrin (KM). This work shows a correlation between the medical traditional use of *Justicia spicigera* and kaempferitrin, its active compound.
Materials and methods: The *in vitro* immunostimulatory effects of KM were evaluated on the proliferation of murine splenocytes and macrophages, and human peripheral blood mononuclear cells (PBMC). The effects of KM on NO production, lysosomal enzyme activity and neutral red uptake were assayed in murine macrophages RAW 264.7. The effects of KM on the NK cell activity were also assayed.
Results: KM at 25 μM, the highest concentration tested, increased the proliferation of murine macrophages (23%) and splenocytes (17%), and human PBMC (24%) in the absence of lipopolysaccharides (LPS), compared to untreated cells. KM also stimulated the pinocytosis (25%) and lysosomal enzyme activity (57%) in murine macrophages with a similar potency than LPS 1 μg/ml. In addition, KM induced the NK cell activity (11%).
Conclusion: KM exerts immunostimulatory effects on immune responses mediated by splenocytes, macrophages, PBMC and NK cells.

1. Introduction

The enhancement of host immune response has been recognized as a possible mechanism to treat tumor growth and some infections (Tu et al., 2008). Immunomodulatory agents from plant and animal origins have increased the immune responsiveness of the body against pathogens by activating immune cells (Patwardhan et al., 1990). However, the immunomodulatory effects of many plant extracts and compounds remain to be studied.

Justicia spicigera Schltdl (Acanthaceae), native from México and extending into South America, commonly known as muicle or mohuite, is an evergreen shrub with tubular orange flowers. ;In Mexican traditional medicine, *Justicia spicigera* is used as immunostimulatory (Herrera-Arellano et al., 2009). Recently, we showed that *Justicia spicigera* extracts exerted immunostimulatory effects *in vitro* (Alonso-Castro et al., 2012). In addition, the major component of this extract was the flavone kaempferol-3,7-bisrhamnoside (kaempferitrin, KM; Fig. 1). However, the immunomodulatory effects of this compound remain to be performed. This study shows, by the first time, that KM exerts immunostimulatory effects *in vitro* by stimulating the proliferation of murine macrophages and splenocytes, and human peripheral blood mononuclear cells. Furthermore, KM induced the lysosomal enzyme activity, neutral red uptake and NK cells activity.

2. Material and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM), RPMI medium and fetal bovine serum (FBS) were from GIBCO BRL (Grand Island, NY, USA). Lipopolysaccharides (LPS) and *p*-nitrophenyl phosphate (*p*-NPP) were purchased from Sigma Chem (St. Louis, MO, USA). KM, obtained from ChromaDex (Laguna Hills, CA, USA), was 98% purity according to the manufacturer. All other chemicals were from Sigma.

2.2. Cells and culture conditions

RAW 264.7 (murine macrophages) and K562 (human leukemia) cells, obtained from ATCC (Manassas, VA, USA), were cultured with DMEM supplemented with 7% FBS, penicillin 100 IU/ml and

Abbreviations: DMEM, Dulbecco's modified Eagle medium; FBS, Fetal bovine serum; KM, Kaempferitrin; LPS, Lipopolysaccharides; NK cells, Natural killer cells; *p*-NPP, *p*-nitrophenyl phosphate; MTT, Thiazolyl blue tetrazolium bromide

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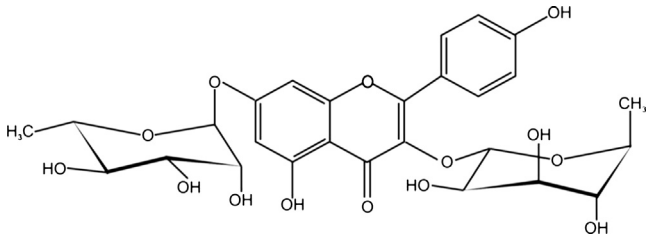

Fig. 1. Chemical structure of the flavonoid kaempferitrin (KM).

streptomycin 100 μg/ml. All cell cultures were grown at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Animals

C57BL/6 mice, weighing 18 to 23 g, from the Experimental Animal Center at the Instituto de Investigaciones Biomedicas, UNAM, México, were used. The experiments were performed following the NIH Guide for Treatment and Care for Laboratory Animals and by the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999). All the procedures carried out in this study were approved by the Research Ethic Committee from Instituto Nacional de Cancerología (Distrito Federal, México, number AGC1291). The mice, having free access to food and water, were housed in cages with filtered air in a climate and light controlled room with a 12 h light/dark cycle.

2.4. Proliferation assay with murine splenocytes and macrophages, and human PBMC

Murine splenocytes and human peripheral blood mononuclear cells, obtained as described by Jacobo–Salcedo et al. (2013), and RAW 264.7 macrophages were seeded in 96 wells plates at 5×10^4 cells/well. After 24 h of incubation, concentrations of KM between 1 and 25 μM were added to the cells in the absence or presence of LPS 1 μg/ml. LPS was used as positive control in all *in vitro* immunomodulatory experiments. The assay was carried out as described previously (Jacobo–Salcedo et al., 2013) and the optical density was measured at 590 nm using an ELISA reader (Biorad Laboratories, Hercules, CA, USA). The viability of treated cells was expressed as the relative growth inhibition as follows:

$$\text{relative viability} = \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \times 100$$

2.5. Nitric oxide (NO) production

Nitric oxide (NO) production was determined based on Griess reaction (Green et al., 1981). RAW 264.7 macrophages were seeded (5×10^4 cells/well) in 96 well plates. After 24 h of incubation, macrophages were treated with various concentrations of KM (1 to 25 μM) in the absence or presence of LPS 1 μg/ml. After 48 h of incubation, the assay was carried out as described by Jacobo-Salcedo et al., (2013).

2.6. Cellular lysosomal enzyme activity assay

The cellular lysosomal enzyme activity was used to determine acid phosphatase activity in macrophages (Suzuki et al., 1988). Briefly, RAW 264.7 macrophages were seeded (5×10^4 cells/well) in 96 well plates. After 24 h of incubation, macrophages were treated with various concentrations of KM (1 to 25 μM) in the presence or the absence of LPS 1 μg/ml during 48 h. Then, the assay was carried out as described by Jacobo-Salcedo et al., (2013). The percentage of lysosomal enzyme activity was calculated by the following equation:

$$\text{lysosomal enzime actiity } (\%) = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD negative control}} \times 100$$

2.7. Neutral red uptake

The pinocytic activity of KM was evaluated using the neutral red assay according to described by Cheng et al. (2008) with some modifications. RAW 264.7 macrophages were seeded (5×10^4 cells/well) in 96 well plates. After 24 h of incubation, macrophages were treated with various concentrations of KM (1 to 25 μM) in absence or presence of LPS 1 μg/ml for 48 h. Then, the assay was carried out as described by Jacobo-Salcedo et al., (2013). The neutral red uptake was calculated by the following equation:

$$\text{red naturaluptake } (\%) = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD negative control}} \times 100$$

2.8. NK cell activity

The activity of natural killer (NK) cells was measured as previously described Tu et al. (2008) with some modifications. Briefly, K562 cells used as target cells, were seeded in 96-well plates at a density of 2×10^4 cells/well in RPMI medium. After 24 h, murine splenocytes, used as the effector cells, were added at 1×10^6 cells/well to give effector/target cells a ratio of 50:1. After incubation for 4 h, cells were treated with KM concentrations between 1 and 25 μM during 48 h. Then, cells were subjected to MTT assay. NK cell activity was calculated as follows: NK activity (%)=(ODT−(ODS−ODE))/ODT × 100 where ODT, optical density value of target cells control, ODS, optical density value of test samples, and ODE, optical density value of effector cells control.

2.9. Statistical analysis

Experimental values are expressed as the mean ± the standard deviation of at least three experiments in hexaplicate. Statistically significant differences from the control group were identified by Student's *t*-test for paired data. The level of $P \leq 0.05$ was used to determine statistical significance. All calculations were performed using the Graph Pad Prisma V.3 software system (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. KM induces the proliferation of murine macrophages and human peripheral blood mononuclear cells

First, we evaluated the effects of KM on the viability of murine macrophages and splenocytes, and human PBMC. In the absence of LPS, KM increased significantly ($P \leq 0.05$) the viability of murine macrophages by 15% (10 μM) and 23% (25 μM), compared to untreated cells (Fig. 2A). In the presence of LPS, KM did not affect the viability, compared to LPS treatment (Fig. 2A). In murine splenocytes, KM increased the proliferation significantly ($P \leq 0.05$) by 13% (10 μM) and 17% (25 μM) in the absence of LPS. In the presence of LPS, KM did not affect the viability, compared to LPS treatment (Fig. 2B). KM tested at 10 μM and 25 μM increased significantly ($P \leq 0.05$) the proliferation of PBMC by 21% and 24%, respectively, but did not affect the viability in the presence of LPS (Fig. 2C).