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# Kaempferitrin induces apoptosis via intrinsic pathway in HeLa cells and exerts antitumor effects

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ABSTRACT

*Ethnopharmacological relevance:* *Justicia spicigera* is used for the empirical treatment of cervical cancer in Mexico. Recently, we showed that *Justicia spicigera* extracts exerted cytotoxic and antitumoral effects and the major component of this extract was kaempferitrin (KM).
*Materials and methods:* The cytotoxic and apoptotic effect of KM on human cancer cells and human nontumorigenic cells were evaluated using MTT and TUNEL assays, and Annexin V/Propidium iodide detection by flow cytometry. The effect of KM on cell cycle was analyzed by flow cytometry with propidium iodide. The apoptotic and cell cycle effects were also evaluated by western blot analysis. Also, different doses of KM were injected intraperitoneally daily into athymic mice bearing tumors of HeLa cells during 32 days. The growth and weight of tumors were measured.
*Results:* KM induces high cytotoxic effects in vitro and in vivo against HeLa cells. The general mechanisms by which KM induces cytotoxic effects include: cell cycle arrest in G1 phase and apoptosis via intrinsic pathway in a caspase dependent pathway. Also, KM exerts chemopreventive and antitumor effects.
*Conclusion:* KM exerts cytotoxic and antitumor effects against HeLa cells.


## 1. Introduction

Cancer, a multifactorial disease, represents a serious public health problem since it accounts for substantial portions of national health expenditures worldwide (Zambrana et al., 2008; McKoll, 2009; Bergstrom, 2012; Marino et al., 2012). The early diagnostic is the first step to reduce the epidemic proportion of cancer. During 2007, 11.3 million cases of cancer were reported in the world and this rate is expected to rise 15.5 million of cases by 2030 (WHO, 2012). In 2008, 7.6 million people died by cancer, representing 13% of deaths worldwide, and this rate is projected to double in 2030 (WHO, 2012). Particularly, cervical cancer accounted for 530,000 cases and 275,000 deaths in 2008 (WHO, 2012).

Plants are an important source of currently antitumoral drugs (Gordaliza, 2007). However, many compounds remain to be studied. Flavonoids constitute a large series of compounds found in many dietary plants and, therefore, they make up an important part of the diet. Flavonoids play a broad spectrum of biological activities such as cytotoxic and antitumor effects (Ren et al., 2003). It has been reported that some flavonoids exert chemopreventive effects in cancer development (Ren et al., 2003). Therefore, this class of secondary metabolites could be a promising source of future anticancer drugs.

*Justicia spicigera* Schltdl (Acanthaceae), native from México and extending into South America, commonly known as muicle or

*Abbreviations:* (CDDP), Cisplatin; (CM-H2DCFDA), 5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate; (DMEM), Dulbecco's modified Eagle's medium; (DMSO), Dimethyl sulfoxide; (EDTA), Ethylenediaminetetraacetic acid; (FBS), Fetal bovine serum; (HRP), Horseraplate peroxidase; (KM), Kaempferitrin; (MTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; (PBS), Phosphate buffer solution; (PCX), Paclitaxel; (ROS), Reactive oxygen species; (TUNEL), Terminal deoxynucleotidyl transferase dUTP nick end labeling

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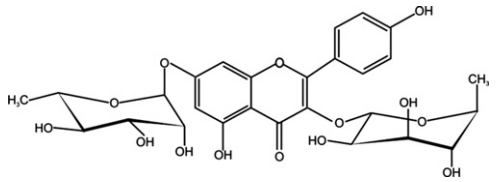

**Fig. 1.** Chemical structure of the flavonoid kaempferitrin.

mohuite, is used for the empirical treatment of cervical cancer (Márquez et al., 1999). Recently, we showed that extracts from the plant *Justicia spicigera* Schltdl (Acanthaceae) exerted cytotoxic and antitumoral effects against HeLa cells (Alonso-Castro et al., 2012). In addition, the major component of this extract was the flavone kaempherol-3, 7-bisrhamnoside (kaempferitrin, KM; Fig. 1) (Alonso-Castro et al., 2012). However, it is unknown whether kaempferitrin exerts cytotoxic and antitumoral effects.

KM has been isolated from several plants (Hamzah et al., 1994; Fang et al., 2005; Pinheiro et al., 2006; De Melo et al., 2009; Pereira et al., 2011; Alonso-Castro et al., 2012) and the fungus *Annulohypoxylon boveri var. microspora* (Cheng et al., 2011). KM has shown antibacterial (Dalmarco et al., 2010), antinociceptive and anti-inflammatory properties (De Melo et al., 2009), as well as a significant hypoglycemic effect in diabetic rats and antioxidant properties with similar potency than quercetin (de Sousa et al., 2004). This work shows, by the first time, that kaempferitrin exerts apoptosis through intrinsic pathway dependent of caspases. Here, it is also shown that KM induces the arrest of HeLa cells in G1 phase. Finally, KM induces antitumoral and chemopreventive effects in a murine xenographt tumor model.

## 2. Materials and methods

### 2.1. Materials

DMEM, RPMI and fetal bovine serum (FBS) were obtained from GIBCO BRL (Grand Island, NY, USA), whereas paclitaxel (PCX), catalase from bovine liver and *tert*-butyl hydroperoxide (TBHP) were obtained from Sigma Chem (St Louis, MO, USA). Cisplatin (CDDP) was from Accord Farma (Distrito Federal, México), Vybrant Apoptosis Assay Kit no.2 and 5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (CM-$H_2$DCFDA) were from Invitrogen (Carlsbad, CA, USA). Kaempferitrin (KM), obtained from ChromaDex (Laguna Hills, CA, USA), was 98% purity according to the manufacturer. The primary antibodies for phospho-Akt (pAkt $Ser^{473}$; sc7985-R), Puma (sc28226), Bcl2 (sc7382), Bad (sc-8044), p53 (sc-126), p16 (sc81156), anti-cleaved caspase-3 (sc-22171), p21 (sc51689), Cyclin D1 (sc-718) and $\beta$ actin (sc1616) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), whereas the primary antibody for Akt (P-2482) was from Sigma. Proliferating Cell Nuclear Antigen (PCNA; M0879) was from Dako cytomation (Glostrup, Denmark). The secondary antibodies for Western blot donkey antirabbit IgG-HRP (sc-2313) and donkey antigoat IgGHRP (sc-2020) were from Santa Cruz Biotech. The secondary antibody goat antimouse IgG- HRP was from Zymed laboratories (San Francisco, CA, USA). The secondary antibody for immunofluorescence assays was donkey anti-goat IgG FITC (sc-2024 Santa Cruz Biotechnology). Sevoflurane was from Abbot Laboratories (Chicago, IL, USA). Dead End Fluorometric TUNEL system and zVAD fmk were from Promega Corporation (Madison, WI, USA).

### 2.2. Cell lines and culture conditions

Human tumor-derived cell lines from cervical (HeLa), colorectal (SW-480), breast (MDA-MB-231) and liver (HepG2) carcinomas as well as nontumorigenic human immortalized keratinocytes (HaCaT) were maintained in DMEM supplemented with 7% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Ovarian (SKOV-3) and prostate (DU-145) carcinoma cell lines were maintained in RPMI medium supplemented with 7% FBS and antibiotics. All cell lines were obtained from ATCC (Manassas, VA, USA). All cell cultures were grown at 37 °C in a 5% $CO_2$ atmosphere.

### 2.3. Animals

Six week old nu/nu mice, weighing 18 to 23 g, from the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, were used. The experiments were performed following the NIH Guide for Treatment and Care for Laboratory Animals and 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). 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. Cell viability assay

Cells were seeded in 96-well microplates at 5000 cells/well. After 24 h, cells were incubated with concentrations of KM, CDDP or PCX between 0.01 to 200 μM. DMSO at final concentration of 0.01% was used as vehicle control. After 48 h of treatment, the MTT assay was performed and relative viability was calculated as described previously (González-Sánchez et al., 2011). The concentration leading to 50% inhibition of viability ($IC_{50}$) was also calculated by regression analysis (percent survival versus log concentration).

### 2.5. Apoptosis detection assays

The in situ DNA fragmentation was detected by Dead End Fluorometric TUNEL system according to the manufacturer's instructions. HeLa cells were seeded at $5 \times 10^5$ on 60 mm Petri plates and were allowed to adhere and grow on glass coverslips for 24 h. HeLa or HaCaT cells were treated with KM 45 μM or PCX 0.1 μM, or the vehicle DMSO 0.01% for 24 or 48 h. After incubation, cells were treated as described by González-Sánchez et al. (2011). The samples were rinsed with PBS for three times and analyzed by fluorescence microscopy.

In additional experiments, HeLa o HaCaT cells were seeded in 60 mm culture plates at $1 \times 10^5$ cells per plate. After 24 h of incubation, control vehicle (DMSO 0.01%), KM 45 μM or PCX 0.1 μM were added to cells. After 48 h of treatment, cells were stained with annexin Alexa 488 and propidium iodide (PI) and apoptosis assay was carried as described by Ortiz-Sánchez et al. (2009) using FACScan analytic flow cytometer (BD Bioscience, Mountain View, CA, USA). A total of 30,000 events were recorded. The level of apoptosis of cells treated with KM or PCX is calculated by the subtraction of the total percentage of apoptosis of untreated cells from the percentage of apoptosis of cells treated with KM or PCX. The software Weasel cytometry analysis v.2.6.1 (Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia) was used for data analysis. Moreover, the effects of KM, in the presence of absence of catalase 2000 U/ml, or in the presence or absence of the caspase inhibitor zVAD fmk 50 μM were evaluated on the apoptosis of HeLa cells stained with Annexin V and PI as described above.