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Antitumor activity of some natural flavonoids and synthetic derivatives on various human and murine cancer cell lines

Mariano Cárdenas, Mariel Marder, Viviana C. Blank and Leonor P. Roguin*

Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Junín 956, 1113, Buenos Aires, Argentina

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Abstract—The effect of various natural flavonoids, cinnamic acid derivatives, and a series of synthetic flavones on cell proliferation was evaluated in vitro in a panel of established human and murine tumor cell lines. The most potent antiproliferative agents were caffeic acid *n*-butyl ester (**12**) > 2′-nitroflavone (**26**) > caffeic acid ethyl ester (**11**) ~ 2′,6-dinitroflavone (**27**) > apigenin (**3**) > 3′-bromoflavone (**20**) ~ 2′-fluoro-6-bromoflavone (**31**). Some compounds showed a moderate effect, the order of cytotoxic activities being chrysin (**2**) > 2′-fluoro-6-chloroflavone (**30**) ~ 2′-chlorochrysin (**32**) > α-naphthoflavone (**7**) > β-naphthoflavone (**8**) ~6-chloroflavone (**14**) ~ 6-bromoflavone (**15**) ~ 4′-nitroflavone (**23**). A structure–activity relationship analysis of each group of compounds was performed. None of the natural or synthetic compounds tested affected the proliferation of epithelial cells derived from normal mammary gland of mice or fibroblastic cells from mouse embryo, suggesting a selective action against tumor cells.

1. Introduction

Flavonoids are an extensive group of polyphenolic compounds occurring in plants. They have been found in dietary components, including fruits, vegetables, olive oil, tea, and red wine.[1,2] Besides their physiological role in plants, they have been shown to possess antiallergic, antiinflammatory, antiviral, and anticarcinogenic activities.[1,3] In addition, some natural flavonoids as well as various synthetic derivatives obtained in our laboratory were identified as potent anxiolytic compounds in vivo.[4]

In a previous work, we determined the antiproliferative action of several flavonoids and related compounds in a cellular line derived from a human cervical carcinoma (WISH cells).[5] We found that the caffeic acid butyl ester and some synthetic nitroflavones obtained in our laboratory behaved as potent antimitogenic compounds. In this paper, we further explored the antitumor activities of various natural flavonoids, cinnamic acid derivatives, and synthetic flavones in different human (HeLa, cervix adenocarcinoma; KB, oropharyngeal carcinoma; MCF-7, breast cancer; SK-MEL-28, melanoma) and murine cell lines (F3II and LM3, mammary adenocarcinomas; LP07, lung tumor; B16-F0 melanoma).

2. Results and discussion

2.1. Chemistry

The synthesis of compounds **13**, **14**, **15**, **16**, **18**, **19**, **20**, **21**, **22**, **23**, **26**, **27**, **28**, **29**, **30**, and **31** was carried out according to procedures previously described.[6–10] Briefly, we used the Baker–Venkataraman transformation, the most common method of synthesizing flavones. In this process, a hydroxyacetophenone and a benzoyl chloride derivative are first converted into a benzoyl ester and this species is then treated with base, forming a 1,3-diketone. Treatment of this diketone with acid leads to generation of the desired flavone (Scheme 1). The 3-bromine-substituted flavones, compounds **24** and **25**, were obtained by treatment of the corresponding flavone with bromine in pyridine (Scheme 2).[7] Caffeic acid ethyl ester (**11**) and caffeic acid *n*-butyl ester (**12**) were prepared with caffeic acid and the corresponding alcohol.[9] The products obtained were recrystallized from ethanol/water and their identification was achieved on

Keywords: Natural flavonoids; Synthetic flavonoids; Antiproliferative activity; Tumor cell lines; Structure–activity relationship.
* Corresponding author. Tel.: +5411 4964 8290; fax: +54 11 4 962 5457; e-mail: rvroguin@qb.ffyb.uba.ar

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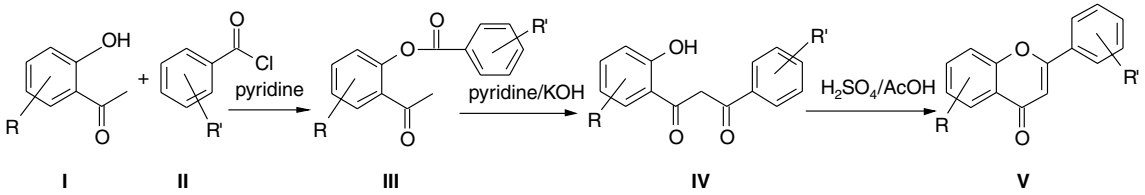
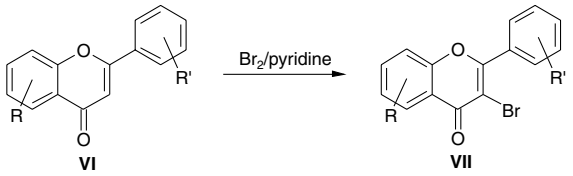


Scheme 1. Steps in the Baker–Venkataraman synthesis of flavones: a hydroxyacetophenone (**I**) and a benzoylchloride derivative (**II**) are first converted into a benzoyl ester (**III**) and this species is then treated with base, forming a 1,3-diketone (**IV**). Treatment of this diketone with acid leads to generation of the desired flavone (**V**).



Scheme 2. Synthesis of 3-bromine-substituted flavones (**VII**) by treatment of the corresponding flavone (**VI**) with bromine in pyridine.

the basis of ^{1}H NMR, ^{13}C NMR, and mass spectra.[6–10] The chemical structures of the compounds tested are described in Table 1.

2.2. Antiproliferative activity

The antiproliferative activity of eight natural flavonoids (**1**–**8**), four cinnamic acid derivatives (**9**–**12**), and 20 synthetic flavones (**13**–**32**) was initially tested in vitro at a 20 μM concentration against four human tumor cell lines (cervix HeLa adenocarcinoma; oropharyngeal KB carcinoma; breast MCF-7 cancer; SK-MEL-28 melanoma) and four murine cell lines (F3II and LM3, mammary adenocarcinomas; lung LP07 tumor; B16-F0 melanoma). Additionally, epithelial cells derived from normal mammary gland of mice (NMuMG) and fibroblastic cells from mouse embryo (3T3) were used as controls.

Compounds that inhibited ⩾50% cell proliferation (compounds **2**, **3**, **11**, **12**, **26**, and **27**) in at least four different cell lines, including results previously obtained in WISH cells,[5] are shown in Figure 1. The antiproliferative activities, expressed as IC_{50} values, are summarized in Table 2. This Table also includes IC_{50} values corresponding to those compounds that at a 20 μM concentration caused a reduction ⩾50% in the growth of only one or two tumor cell lines. However, IC_{50} values corresponding to compounds that inhibited tumor cell proliferation less than 50% at 20 μM were not evaluated. Remarkably, none of the compounds (**1**–**32**) at a 20 μM concentration exhibited a cytotoxic effect toward epithelial cells derived from normal mammary gland of mice (NMuMG) or fibroblastic cells from mouse embryo (3T3), suggesting a selective action against tumor cells (as an example, see Fig. 1 for compounds **2**, **3**, **11**, **12**, **26**, and **27**). In addition, when the cytotoxic effect of compounds included in Table 2 was studied in NMuMG cells up to a 80 μM concentration, we found that the majority of them inhibited cell proliferation less than 20%, except for compounds **14** and **15** that reduced near 40% cell growth, and compounds **30** and **31** that showed IC_{50} values ≅50 μM.

Based on the IC_{50} values, compounds were classified as:

Highly potent (IC_{50} < 10 μM): Compound **12** > **26** > **11** ∼ **27** > **3** > **20** ∼ **31**. The order of cytotoxic activity was established taking into account the number of cell lines affected with IC_{50} < 10 μM. Drugs included in this group also exhibited IC_{50} ∼ 10–20 μM in some cell lines (**3**, **11**, **12**, **26**, and **31**; see Table 2).

Moderately active (IC_{50} ∼ 10–20 μM): Compound **2** > **30** ∼ **32** > **7** > **8** ∼ **14** ∼ **15** ∼ **23**. In this case, the order was established taking into account the number of cell lines affected with IC_{50} < 15 μM (Table 2).

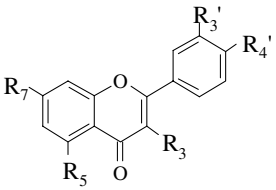
Among the natural flavonoids, compound **3**, which has been previously reported as an effective antitumor agent in vitro,[11] revealed to be the most active of the eight natural flavonoids tested. A comparative study of the cytotoxic activity of the cinnamic acid derivatives showed that compound **12** was the most potent antiproliferative agent without toxicity toward non-cancer cells (Fig. 1). In this sense, although different caffeic acid esters have been earlier examined in various types of tumor cell lines,[12,13] some of these derivatives appeared to be cytotoxic against healthy cells.[12] In addition, among the synthetic flavones, the 2′-nitroflavone (**26**), prepared in our laboratory, showed very strong antiproliferative potency and a lack of effect against non-tumor cells (Fig. 1). Based on these results, the molecular mechanisms involved in the generation of the antitumor action of the nitroflavone derivative are now being explored.

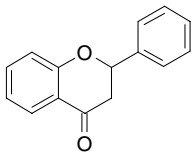
Among the tested cell lines, a differential behavior was observed for the previously characterized LM3 and F3II murine mammary cells.[14,15] LM3 cell line was obtained from primary cultures of a BALB/c mouse mammary tumor, whereas F3II cell line is a less differentiated and highly invasive variant established from a clonal subpopulation of the parental tumor. When the cytotoxic effect of the active compounds was comparatively analyzed in both lines, a higher susceptibility was found in LM3 cells (Fig. 1, Table 2). Thus, whereas LM3 cell proliferation was inhibited ⩾50% by compounds **2**, **3**, **7**, **11**, **12**, **26**, **27**, and **32**, only compounds **12** and **26** resulted to be cytotoxic in F3II cells.

We also compared the antitumor susceptibility of three related cell lines, such as HeLa, KB, and WISH cells.

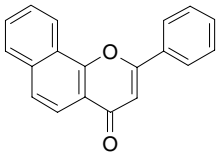
Table 1. Molecular structures of the compounds tested

	R_3	R_5	R_7	$R_{3'}$	$R_{4'}$
Natural flavonoids					
Flavone (**1**)	H	H	H	H	H
Chrysin (**2**)	H	OH	OH	H	H
Apigenin (**3**)	H	OH	OH	H	OH
Quercetin (**4**)	OH	OH	OH	OH	OH
5,7-Dimethoxyflavone (**5**)[a]	H	CH_3O	CH_3O	H	H

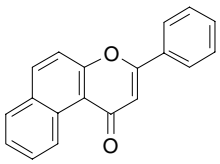




Flavanone (**6**)

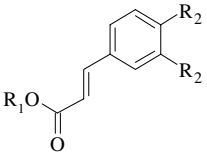


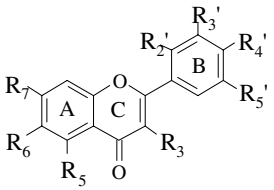
Alpha-naphthoflavone (**7**)



Beta-naphthoflavone (**8**)

	R_1	R_2
Related derivatives		
Cinnamic acid (**9**)	H	H
Caffeic acid (**10**)	H	OH
Caffeic acid ethyl ester (**11**)[b]	Ethyl	OH
Caffeic acid *n*-butyl ester (**12**)[b]	1-Butyl	OH





	R_3	R_5	R_6	R_7	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
Synthetic flavones								
6-Fluoroflavone (**13**)[c]	H	H	F	H	H	H	H	H
6-Chloroflavone (**14**)[c]	H	H	Cl	H	H	H	H	H
6-Bromoflavone (**15**)[a,c]	H	H	Br	H	H	H	H	H
6-Nitroflavone (**16**)[c]	H	H	NO_2	H	H	H	H	H
6-Methylflavone (**17**)	H	H	CH_3	H	H	H	H	H
3′-Bromo-6-methylflavone (**18**)[d]	H	H	CH_3	H	H	Br	H	H
3′-Methyl-6 bromoflavone (**19**)[b]	H	H	Br	H	H	CH_3	H	H
3′-Bromoflavone (**20**)[c]	H	H	H	H	H	Br	H	H
3′-Nitroflavone (**21**)[c]	H	H	H	H	H	NO_2	H	H
4′-Bromoflavone (**22**)[c]	H	H	H	H	H	H	Br	H
4′-Nitroflavone (**23**)[c]	H	H	H	H	H	H	NO_2	H
3-Bromoflavone (**24**)[a]	Br	H	H	H	H	H	H	H
3,6-Dibromoflavone (**25**)[a]	Br	H	Br	H	H	H	H	H
2′-Nitroflavone (**26**)[c]	H	H	H	H	NO_2	H	H	H
2′,6-Dinitroflavone (**27**)[c]	H	H	NO_2	H	NO_2	H	H	H
2′,6-Dinitro-5′-bromoflavone (**28**)[c]	H	H	NO_2	H	NO_2	H	H	Br
2′,6-Difluoroflavone (**29**)[e]	H	H	F	H	F	H	H	H
2′-Fluoro-6-chloroflavone (**30**)[e]	H	H	Cl	H	F	H	H	H
2′-Fluoro-6-bromoflavone (**31**)[e]	H	H	Br	H	F	H	H	H
2′-Chlorochrysin (**32**)[a]	H	OH	H	OH	Cl	H	H	H

[a] Ref. 7.
[b] Ref. 9.
[c] Ref. 6.
[d] Ref. 8.
[e] Ref. 10.

As indicated by American Type Culture Collection, oropharyngeal KB carcinoma cells and WISH cells were established via HeLa contamination. A comparative analysis between KB and HeLa cells showed that both cell lines behaved similarly, except for compound **32**, which was basically active in HeLa cell line. On the other hand, WISH cells were less responsive to the antiproliferative action of various compounds including natural flavonoids **2**, **3** and synthetic compounds **30**, **31**, and **32**, which were effective only in HeLa cells. Hence, the examination of these results emphasizes the notion that cancerous tumors usually contain cell subpopulations with different biological properties.

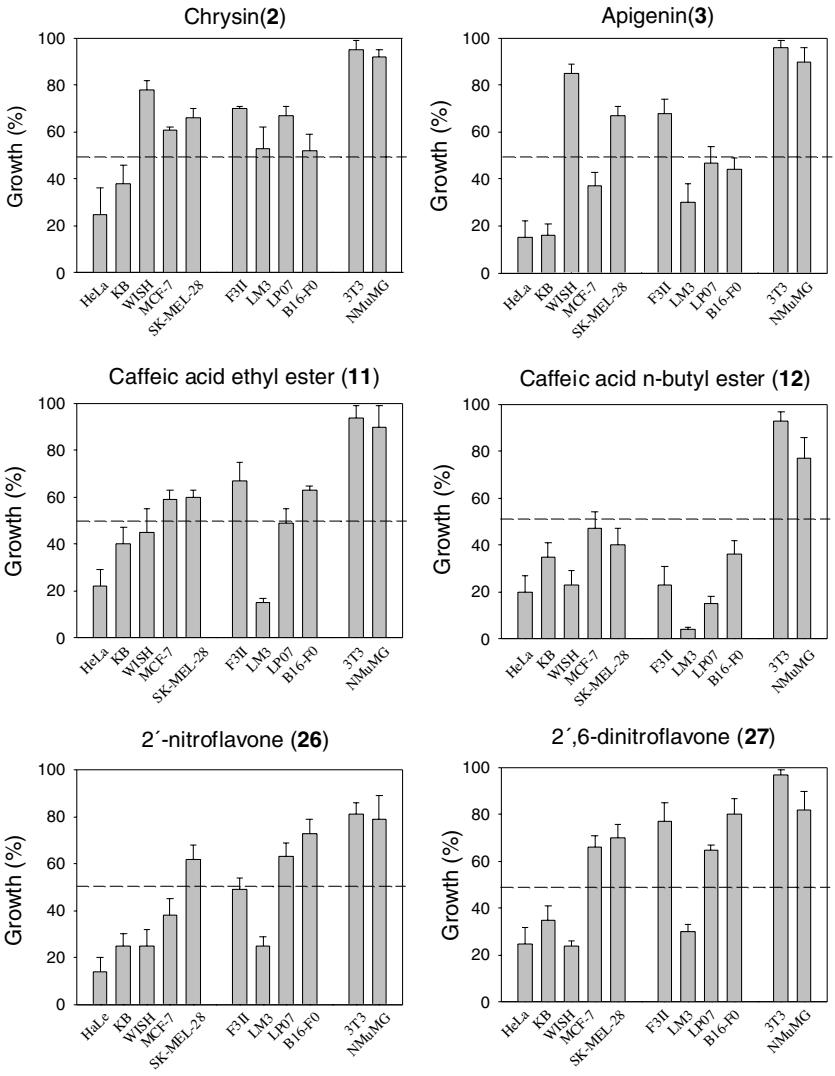


Figure 1. Effect of some natural flavonoids and synthetic derivatives on the proliferation of different human and murine cell lines. 1×10^4 cells/well (KB, WISH, SK-MEL-28, and B16-F0) or 2×10^4 cells/well (HeLa, MCF-7, F3II, LM3, LP07, NIH-3T3, and NMuMG) were incubated in the presence or absence of 20 μM of different compounds for 72 h at 37 °C. Cell proliferation was determined by colorimetric determination of hexosaminidase levels.[25] Results are expressed as the percentage of growth obtained in the absence of flavonoids (control) and represent means ± SE of three different experiments.

2.3. Structure–activity relationship

The following structure–activity relationships (SARs) were obtained by analyses of the information gathered from Tables 1 and 2.

2.3.1. Natural flavonoids. Since apigenin (**3**) displayed cytotoxic activity stronger than that of chrysin (**2**), mainly in HeLa, KB, MCF-7, and LP07 cells, a hydroxyl group in carbon 4′ would improve the antitumor effect. In a similar way, it has been previously shown that apigenin behaves as a more potent antiproliferative agent than chrysin.[16]

Methoxylation of compound **2** at positions 5 and 7 (compound **5**) rendered it inactive. However, it should be taken into account that a different behavior was observed when the anticancer effect of this compound was tested in other tumor cell lines. Thus, it has been previously reported that compound **5** showed cytotoxicity higher than that of compound **2** towards both human gastric and colorectal adenocarcinoma cells.[17] In addition, compound **32**, a chrysin derivative obtained after the introduction of a chlorine atom in position 2′ (**32**), was only active in HeLa and LM3 cells.

Comparison of compounds **3** and **4** indicated that hydroxyl groups in carbons 3 and/or 3′ would diminish the effect. In spite of these results, an antiproliferative effect of compound **4** has been previously described in various human cancer cells, including lymphoblastoid, mammary, colon, laryngeal, leukemia, and lung cell lines, although its antitumor potency depends on the cell line tested.[18–23] In addition, an in vivo study demonstrated that both apigenin and quercetin were similarly effective in inhibiting melanoma growth.[24]

Naphthoflavones are moderately more active than the mother compound flavone. Furthermore, α-naphthofla-

Table 2. Antiproliferative activities of different natural flavonoids and synthetic derivatives

Flavonoids and related compounds	IC_{50} (μM)[a]								
	Human cell lines					Murine cell lines			
	HeLa	KB	WISH	MCF-7	SK-MEL-28	F3II	LM3	LP07	B16-F0
Chrysin (**2**)	13 ± 2	13 ± 2	—	—	—	—	17 ± 6	—	22 ± 2
Apigenin (**3**)	6 ± 1	4 ± 1	—	17 ± 2	—	—	11 ± 4	18 ± 3	16 ± 4
α-Naphthoflavone (**7**)	—	—	—	—	—	—	14 ± 2	—	22 ± 3
β-Naphthoflavone (**8**)	—	—	—	20 ± 1	—	—	—	—	—
Caffeic acid ethyl ester (**11**)	3 ± 1	3 ± 1	10 ± 3	—	—	—	3 ± 1	15 ± 3	—
Caffeic acid *n*-butyl ester (**12**)	2 ± 1	4 ± 1	3 ± 1	16 ± 5	11 ± 3	5 ± 1	2 ± 1	3 ± 1	6 ± 2
6-Chloroflavone (**14**)	—	—	—	22 ± 7	—	—	—	—	—
6-Bromoflavone (**15**)	—	—	—	19 ± 2	—	—	—	—	—
3′-Bromoflavone (**20**)	—	—	—	10 ± 2	—	—	—	—	—
4′-Nitroflavone (**23**)	—	—	—	20 ± 1	—	—	—	—	—
2′-Nitroflavone (**26**)	3 ± 1	5 ± 1	3 ± 1	6 ± 1	—	19 ± 4	4 ± 1	—	—
2′,6-Dinitroflavone (**27**)	3 ± 1	6 ± 1	3 ± 1	—	—	—	9 ± 3	—	—
2′-Fluoro-6-chloroflavone (**30**)	13 ± 3	16 ± 3	—	—	—	—	—	—	—
2′-Fluoro-6-bromoflavone (**31**)	9 ± 3	16 ± 5	—	—	—	—	—	—	—
2′-Chlorochrysin (**32**)	12 ± 2	—	—	—	—	—	15 ± 3	—	—

The symbol (—) indicates those compounds that inhibited cell growth less than 50% at a 20 μM concentration.

[a] The molar drug concentrations required to cause 50% growth inhibition (IC_{50}) were determined from dose–response curves. Results represent means ± SE of at least three different experiments.

vone (**7**) inhibited the proliferation of LM3 and B16-F0 cells, while β-naphthoflavone (**8**) was active only in MCF-7 cells, suggesting a differential selective action in the panel of tested cell lines.

2.3.2. Related compounds. In accordance with previous results,[13] the cytotoxic action would depend on esterification of caffeic acid, being enhanced by the length of the ester moiety. Thus, caffeic acid *n*-butyl ester (**12**) was the most active one, showing a significant antiproliferative activity toward all the tested cell lines, whereas the caffeic acid ethyl ester (**11**) was effective in only five of the nine tested cell lines.

2.3.3. Synthetic flavones. Among derivatives substituted in carbon 6 of the flavone nucleus (A ring) (compounds **13**–**17**), only those with a chlorine or bromine atom produced moderately active compounds (**14**, **15**) in MCF-7 cell line. Besides, the introduction of fluorine atom in carbon 2′ (compounds **30**, **31**) changed their selective action toward other cell lines, being 2′-fluoro-6-chloroflavone (**30**) and 2′-fluoro-6-bromoflavone (**31**) effective in HeLa and KB cells.

Analyses of compounds mono-substituted in B and C rings (compounds **20**–**24** and **26**), showed that a bromine atom in position 3′ (**20**) and a nitro group in carbon 4′ or 2′ (**23** and **26**) yielded active compounds. However, the most potent synthetic flavone was 2′-nitroflavone (**26**), which significantly reduced the growth of HeLa, KB, WISH, MCF-7, F3II, and LM3 cells. The incorporation of another nitro group at the 6-position on the A ring rendered a less active derivative, showing the 2′,6-dinitroflavone (**27**) an antiproliferative effect in HeLa, KB, WISH, and LM3 cells.

Any other additional substitution on the synthetic flavones mentioned above produced inactive compounds.

3. Conclusion

We examined the in vitro antitumor activity of eight natural flavonoids, four cinnamic acid derivatives, and 20 synthetic flavones in a panel of established cancer cell lines. Analyses of each group of compounds indicated that natural flavonoid **3**, caffeic acid ester **12**, and the synthetic nitroderivative **26** possess the most potent and selective antiproliferative activities against at least six different tumor cell lines. The synthesis of novel substituted 2′-nitroderivatives and investigation of molecular mechanisms responsible for the antiproliferative activity of the most active compounds are currently in progress.

4. Experimental

4.1. Chemicals

Flavanone (**6**) was obtained from Extrasynthese, Genay, France. Flavone (**1**), chrysin (**2**), apigenin (**3**), quercetin (**4**), α-naphthoflavone (**7**), β-naphthoflavone (**8**), cinnamic acid (**9**), caffeic acid (**10**), 6-methylflavone (**17**), ethanol, 1-butanol, L-glutamine, penicillin, streptomycin, sodium pyruvate, non-essential amino acids and Hepes were from Sigma Chem. Co., St. Louis, MO, USA. Culture mediums and fetal bovine serum were purchased from Gibco-BRL, Gaithersburg, MD, USA.

4.2. Cell lines and culture conditions

WISH (ATCC CCL-25), HeLa (ATCC CCL-2), and F3II cells (generously provided by the Laboratory of Molecular Oncology, Quilmes National University, Buenos Aires, Argentina) were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. The same conditions, but with the addition of 1 mM sodium pyruvate/4 mM

sodium bicarbonate or 1 mM non-essential amino acids, were employed to maintain KB (ATCC CCL-17) and SK-MEL-28 cells (ATCC HTB-72), respectively. NIH-3T3 cell line (ATCC CRL-1658) was cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FBS, 4 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. In addition, a supplement of 1 mM sodium pyruvate and 0.1 mM non-essential amino acids was also included to maintain MCF-7 cells (ATCC HTB-22). B16-F0 cell line, kindly provided by the Laboratory of Molecular Oncology (Quilmes National University, Buenos Aires, Argentina), was grown in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. NMuMG (ATCC CRL-1636), LM3, and LP07 cells, gently provided by the Institute of Oncology Angel H. Roffo (Buenos Aires, Argentina), were cultured in DMEM-F12 containing 10% FBS, 2 mM L-glutamine, 0.6% Hepes, 50 U/mL penicillin, and 50 μg/mL streptomycin.

4.3. Cell proliferation assay

Cells were placed in 96-well microplates at a density of 1×10^4 cells/well (KB, WISH, SK-MEL-28, and B16-F0) or 2×10^4 cells/well (HeLa, MCF-7, F3II, LM3, LP07, NMuMG, and NIH-3T3) and incubated for 72 h at 37 °C in the presence or absence of 20 μM of the different compounds in a total volume of 0.2 ml of culture medium. Flavonoids were dissolved in dimethylsulfoxide (DMSO) as 10 mM stock solutions and stored at −70 °C. Prior to use, the compounds were diluted 1:10 in ethanol and added at the indicated concentrations to the culture medium. Control cells were treated under similar conditions and a final concentration of 20 μL vehicle/mL of culture medium was used in all experiments. Total cell number was evaluated by colorimetric determination of hexosaminidase levels, a ubiquitous lysosomal enzyme.[25] Briefly, cells were washed twice with phosphate-buffered saline and then incubated at 37 °C with 60 μL of 3.25 mM *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide dissolved in 50 mM citrate buffer, pH 5, 0.25% Triton X-100. After 45–120 min, the color reaction was developed and the enzyme activity was blocked by adding 90 μL of 50 mM glycine buffer, pH 10.4, containing 5 mM EDTA. Absorbance values were measured at 405 nm in a visible plate reader (Biotrak II, Amersham Biosciences, USA). The molar drug concentrations required to cause 50% growth inhibition (IC_{50}) were determined from dose–response curves ranging from 1.25 to 80 μM.

Acknowledgments

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