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Original article

# Synthesis and effects on cell viability of flavonols and 3-methyl ether derivatives on human leukemia cells

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ABSTRACT

Flavonoids are polyphenolic compounds which display an array of biological activities and are considered potential antitumor agents. Here we evaluated the antiproliferative activity of selected synthetic flavonoids against human leukemia cell lines. We found that 4′-bromoflavonol (flavonol **3**) was the most potent. This compound inhibited proliferation in a concentration-dependent manner, induced apoptosis and blocked cell cycle progression at the S phase. Cell death was found to be associated with the cleavage and activation of multiple caspases, the activation of the mitogen-activated protein kinase pathway and the up-regulation of two death receptors (death receptor 4 and death receptor 5) for tumor necrosis factor-related apoptosis-inducing ligand. Moreover, combined treatments using 4′-bromoflavonol and TRAIL led to an increased cytotoxicity compared to single treatments. These results provide a basis for further exploring the potential applications of this combination for the treatment of cancer.



## 1. Introduction

Flavonoids are naturally occurring polyphenolic compounds which may have beneficial effects for health and might be considered as potentially protective or therapeutic agents against cancer [1]. Among the different bioflavonoids, quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the best studied and is widely distributed in nature. This natural product induces apoptosis in a variety of tumors [2–4], including leukemia [5]. Antitumor properties of flavonoids are mediated by different types of cell cycle arrest and induction of apoptosis, an active process of cell death which displays an essential role in the development and survival. It is also an important response to many chemotherapeutic agents. Cells that suffer this kind of cell death display features such as internucleosomal DNA fragmentation, the translocation of phosphatidylserine to the outside of the plasma membrane, apoptotic bodies formation and chromatin condensation. Caspases are fundamental executioners of apoptosis. They comprise a family of cysteine proteases which are synthesized as zymogens and are activated by proteolysis [6]. There are two main apoptotic pathways [7]. In the extrinsic pathway, apoptosis is mediated by death receptors (such as receptors for tumor necrosis factor-α, Fas and TRAIL) and involves caspase-8 activation. In the intrinsic pathway pro-apoptotic signals induce mitochondrial cytochrome *c* release to the cytosol, and promote the apoptosome assembly and caspase-9 activation. Both caspase-8 and caspase-9 activate caspase-3 which is responsible for specific cellular protein destruction during apoptosis [8]. The cellular response to this chemically-induced apoptosis is associated with the inactivation of the protein kinases involved in cell survival and with the activation of protein kinases that promote apoptosis. One of the most important aspects of apoptosis regulation is that it requires mitogen-activated protein kinases (MAPKs). MAPKs are a family of serine/threonine protein kinases directed to proline and function as intracellular mediators in response to diverse stimuli. They are activated by phosphorylation in threonine and tyrosine residues [9]. In mammals the MAPKs are mainly represented by the cascades

*Abbreviations:* ERK, extracelular signal-regulated kinase; $IC_{50}$, 50% inhibition of cell growth; JNK/SAPK, c-*jun* N-terminal kinases/stress-activated protein kinases; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated extracellular kinases; MTT, 3-(4,5-dimethyl-2-thiazolyl-)-2,5-diphenyl-2*H*-tetrazolium bromide; $p38^{MAPK}$, p38 mitogen-activated protein kinases; TRAIL, tumor necrosis factor-related apoptosis-induced ligand.

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ERK (extracellular signal-regulated protein kinases) 1/2, the c-Jun $NH_2$-terminal kinases/stress-activated protein kinases (JNK/SAPK) and the p38 mitogen-activated protein kinases ($p38^{MAPK}$). The activation of ERK can trigger both anti-apoptotic and pro-apoptotic effects, depending on the stimuli and cell type [10]. In contrast, JNK/SAPK and $p38^{MAPK}$ are mainly activated by cytotoxic stimuli and are associated with pro-apoptotic actions in many cell types [11]. However, there are multiple exceptions to this rule.

Recent studies indicate that the combination of conventional cytotoxic compounds and new drugs directed at molecular targets can enhance the cell death induced by the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in tumor cells [12]. The identification of compounds that activate death receptors for TRAIL or that block anti-apoptotic effectors might constitute an important therapeutic advance. However, very little is known about the synergy between semi-synthetic compounds derived from natural products and apoptosis-inducing ligands such as tumor necrosis factor α (TNF-α), Fas and TRAIL. The search for compounds that can enhance TRAIL sensitivity in human leukemia cells is therefore of undoubted biomedical interest.

TRAIL induces selective apoptosis in several tumor cell lines and shows low toxicity against normal cells [13]. This distinctive feature has inspired the development of drugs to combat malignant diseases. Recombinant TRAIL and agonist antibodies that bind to death receptors have been used in clinical trials in phase I–II [14]. TRAIL can bind to the death receptors DR4 (death receptor 4) and DR5 (death receptor 5), which contain a cytoplasmic death domain, and to the decoy receptors DcR1 and DcR2, which lack a functional death domain. Decoy receptors compete with death receptors for the ligands and therefore block the apoptotic signals.

The results from several studies suggest that flavonoids with a hydroxy group at carbon 3 are potential anticancer agents [15] and also the presence of 3′-hydroxy-4′-methoxy groups on the B ring enhances cytotoxicity [16]. The justification for investigating methoxylated derivatives is provided by their chemopreventive properties, which are much greater than those seen in the more common unmethylated flavonoids, and the fact that they show increased metabolic stability [17]. In a previous study of naturally occurring and semi-synthetic flavonoids, we showed that methylation of hydroxyl groups of quercetin yields a compound with a higher antiproliferative activity against human cancer cell lines [18]. Moreover, it has recently been reported that molecules with halogen substituents in the B ring show improved anticancer activity relative to methoxylated, methylated or hydroxylated analogs [19].

Comparative studies that examine methylation of hydroxy group at C3 and the introduction of different substituents on the B ring and minor changes on the A ring, will greatly enhance knowledge of their impact on cytotoxicity. Here we synthesized a series of flavonols and methyl ether derivatives carrying varying substitutions at positions 5, 7, 2′, 3′, 4′, 5′. The substitutions included different electron-donating and electron-withdrawing groups, such as Cl, Br, $CH_3O$, $CH_3$, and OH, and allowed us to compare their anticancer activity with similar compounds tested against other tumor cell lines. We studied the effects of these synthetic flavonoids on viability of human leukemia cells and then examined the effects of the most potent, 4′-bromoflavonol, on apoptosis induction. This semi-synthetic flavonoid has been recently assessed for cytotoxicity against the human colorectal carcinoma cell line HCT116 [19] but, so far, its potential use in antileukemia therapy is largely unexplored. We have evaluated whether caspase activation and the MAPK cascade are involved in the mechanism of action. Finally, we have investigated whether 4′-bromoflavonol induces death receptors expression and the potential modulation of cell death in combination with TRAIL.

## 2. Chemistry

A selected combination of nine commercially available aldehydes (containing halogen, methoxy, methyl or aromatic groups) with two hydroxyacetophenones provided nine flavonols (**1**–**9**) and ten 3-methyl ether flavonols derivatives. The synthetic compounds differ in terms of their substituents on the B ring (2-phenyl group) and minor changes on the A ring (Fig. 1).

The synthesis takes advantage of the well-known two steps procedure for flavonols which combines a Claisen–Schmidt condensation of 2-hydroxyacetophenones and benzaldehydes with NaOH as base followed by a cyclization under Algar–Flynn–Oyamada reactions conditions (Scheme 1) [20]. Treatment of flavonols with 5 equiv. of iodomethane led to new methyl ethers **11**, **13**, **15**, **17** and **18** in high yield. The spectroscopic data obtained for known compounds were compared with those described in the literature in order to confirm the structures **1**–**5** [19], **7** [21], **9** [22], **10** [19], **12** [23], **14** [24], **16** [25], **19** [19], **20** [26]. The structure of new synthetic flavonols (**6**, **8**) and the methyl ether derivatives (**11**, **13**, **15**, **17** and **18**) was determined using 1D and 2D NMR experiments, high resolution mass spectroscopy and IR. Compound **19** was synthesized from quercetin by partial methylation reaction as previously described [21].

## 3. Biological results

### 3.1. Effect of the synthetic flavonoids on the growth and cell viability of human leukemia cell lines

In the present study, the potential cytotoxic properties of a series of 20 flavonoids, including nine flavonols and eleven 3-methyl ethers with different substituents on the B ring (2-phenyl group) and minor changes on the A ring were evaluated using HL-60 cells (Table 1). The results indicated that 4′-bromoflavonol (flavonol **3**) was the most cytotoxic compound with an $IC_{50}$ value (the concentration that induces a 50% inhibition of cell growth) of 3.3 ± 0.7 μM. A 30-fold increase in antiproliferative activity was detected for 4′-substituted flavonols by increasing the size of the halogen from chlorine (flavonol **1**) to bromine (flavonol **3**). The properties of the bromine atom, including its higher volume and its polarizability together with the lower electron withdrawal at the benzene moiety compared with the chlorine atom, seem to play a key role in determining the potency of this compound on cell viability. The differences between flavonol **1** and flavonol **3** suggest that the higher potency of the latter might be due to steric and electronic properties conferred by the identity of the halogen on the B ring and are important in binding to the molecule target.

The introduction of a chlorine atom (flavonol **5**) or a methoxy group (flavonol **4**) on position 2′on the B ring improved the antiproliferative activity as compared with flavonol **1**, however, flavonol **5** was more potent than flavonol **4**. The position of chlorine on the B ring is important in conferring cytotoxicity, since 2′-chloroflavonol (flavonol **5**) exhibited a higher potency than 4′-chloroflavonol (flavonol **1**). The methylation of the hydroxyl group at position C3 of flavonol **1** to produce flavonol **12** improved the cytotoxicity. In contrast, methylation of the 3-hydroxy group of flavonol **3** to produce flavonol **13** did not enhance the potency against cell growth inhibition. The hydroxy group located at the C3 position seems to play a key role in determining the potency of flavonol **3** on cell viability since this is significantly more potent (7-fold) than the 3-methoxy derivative **13**. It thus seems unlikely that the C3 methoxy group is a major determinant of cytotoxicity. Although the exact target and binding site of this flavonoid has not

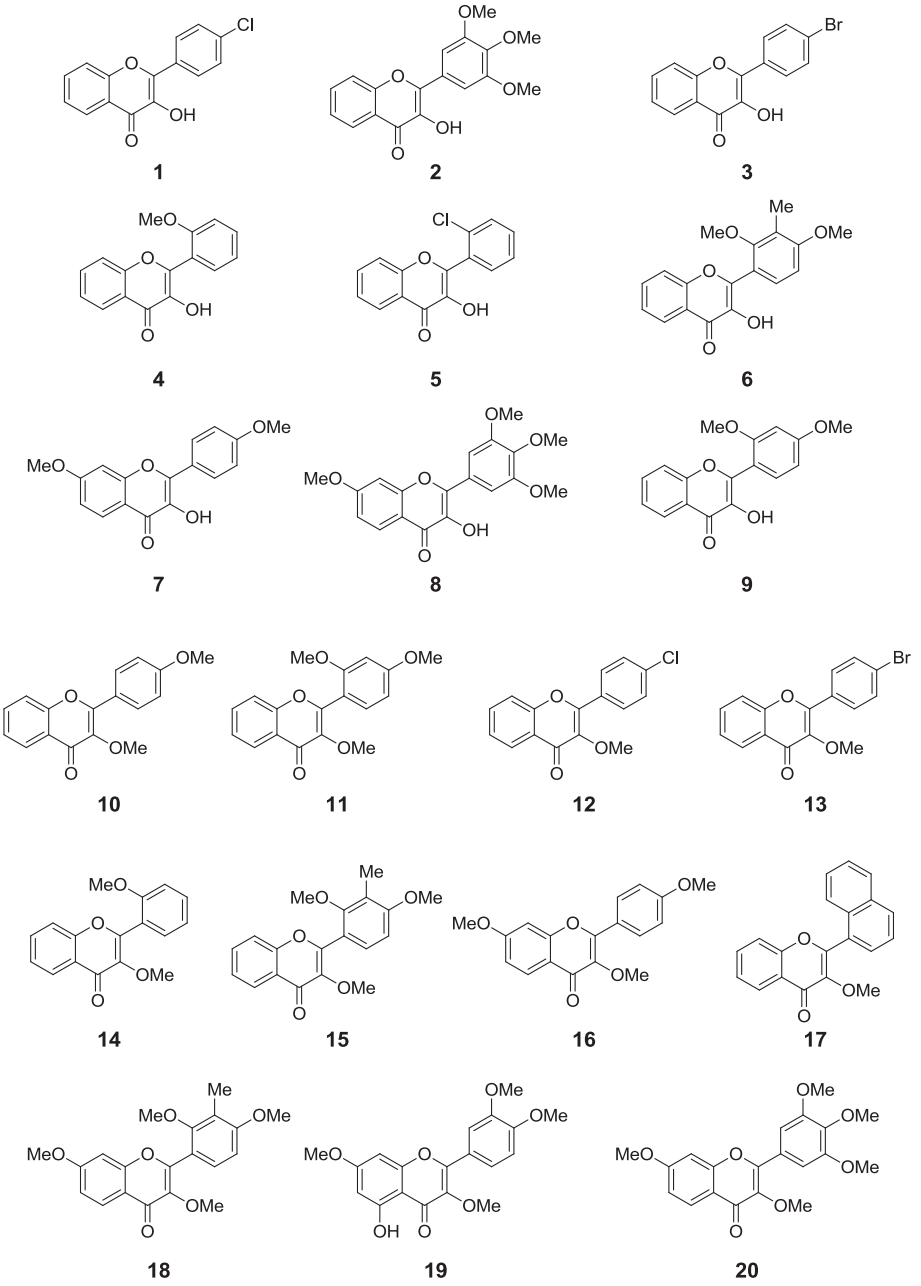



**Fig. 1.** Chemical structures of synthetic flavonoids.

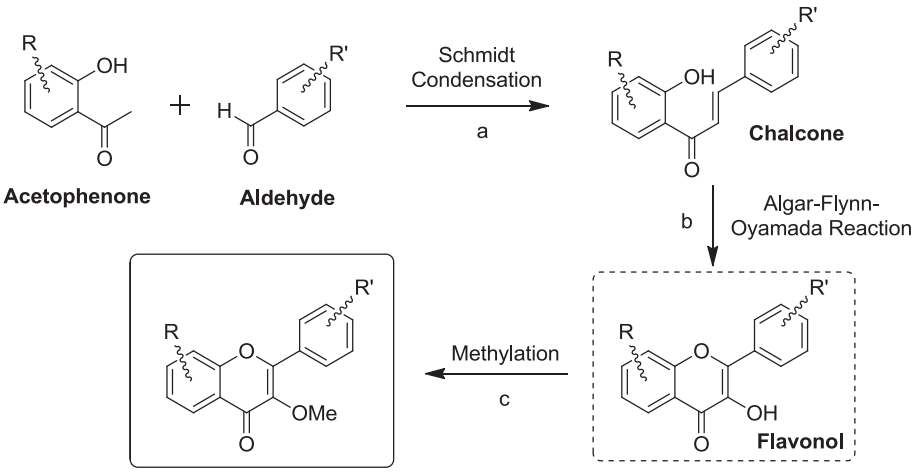
yet been determined, the hydroxy group could stabilize the binding by forming hydrogen bonds with the target.

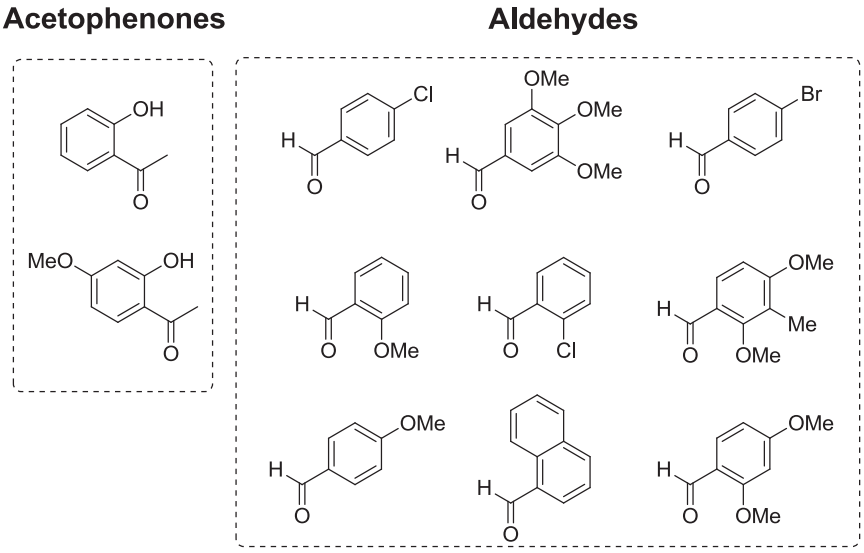
Among the 3-methoxy flavonols, the presence of an additional methoxy group at position 4′ on the B ring (flavonol **10**) improved the antiproliferative activity compared with the compound containing the methoxy group at position 2′ (flavonol **14**). The most potent 3-methoxy flavonols were those containing a 4′-chlorophenyl (flavonol **12**), a 4′-bromophenyl (flavonol **13**) and a naphthyl group (flavonol **17**) at position 2 of the phenylbenzo-γ-pyrone core. Differences in effects on cell growth might derive from differences in drug uptake, retention, and/or metabolism by the cells or from differences in compound stability in tissue culture medium.

The effect of the introduction of a methoxy group at C7 in 3-methoxy flavonols depends on the substitution pattern on the B ring: flavonol **16** was less potent than flavonol **10**, but flavonol **18** was more potent than flavonol **15**. Among the 3,7-dimethoxy derivatives, the least potent was flavonol **16** containing only an additional methoxy group at position 4′ on the B ring, although the introduction of two (flavonol **19**) or three (flavonol **20**) additional methoxy groups on the B ring increased the antiproliferative activity (Table 1). These results are in accordance with the fact that higher lipophilicity can facilitate penetration through the cell membrane and increase the cytotoxicity *in vitro*.

The antitumor agent etoposide and the flavonoid quercetin were used as positive controls in the cytotoxicity assays, and the $IC_{50}$ values were 0.6 ± 0.1 μM and 47 ± 8 μM, respectively.

The $IC_{50}$ value for flavonol **3** in HL-60 cells was similar to the values obtained for the human leukemia cell line resistant to the antitumor compound mitoxantrone, HL-60/MX1




**Scheme 1.** Synthesis of the intermediate chalcones and methyl ethers.

($IC_{50}$ = 6.5 ± 0.1 μM) and for the Burkitt lymphoma cell line Raji ($IC_{50}$ = 5.8 ± 0.1 μM) (Fig. 2A). Flavonol **5** contains a chlorine atom at position 2′ (2′-chloroflavonol) and displayed similar cytotoxic potency against HL-60 cells ($IC_{50}$ = 10.7 ± 2.9 μM) and low cytotoxicity against HL-60/MX1 and Raji ($IC_{50}$ > 30 μM). However, 4′-bromoflavonol (flavonol **3**) had a greater apoptotic effect than 2′-chloroflavonol (flavonol **5**), as determined by evaluation of the number of hypodiploid cells by flow cytometry. The percentage of apoptotic HL-60 cells increased approximately 14-fold (51.8 ± 3.9% vs 3.8 ± 0.6%), and 4-fold (14.9 ± 3.0% vs 3.8 ± 0.6%) after 24 h exposure to 4′-bromoflavonol (flavonol **3**) and 2′-chloroflavonol (flavonol **5**), respectively. Similar results were obtained for U937 cells (Fig. 2B). These results are in accordance with the qualitative assessment of DNA damage on agarose gels (Fig. 2C) and the fluorescence microscopy experiments (Fig. 2D). For this reason, 4′-bromoflavonol (flavonol **3**) was selected for further experiments.

**Table 1**
Effects of flavonoids on the growth of human tumor cell line HL60.

| Compound | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
|---|---|---|---|---|---|---|---|---|---|---|
| $IC_{50}$ (μM) | 102 ± 7 | 138 ± 18 | 3.3 ± 0.7 | 30 ± 6 | 10.7 ± 2.9 | 32 ± 3 | 148 ± 14 | 34 ± 1 | 53 ± 7 | 39 ± 1 |
| Compound | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** |
| $IC_{50}$ (μM) | 34 ± 7 | 26 ± 3 | 21 ± 3 | 98 ± 2 | 96 ± 13 | 122 ± 18 | 16 ± 4 | 27 ± 4 | 28 ± 2 | 21 ± 4 |

Cells were cultured for 72 h in presence of the indicated compounds and the $IC_{50}$ values were calculated as described in the Experimental Section. The data shown represent the mean ± SEM of 2–3 independent experiments with three determinations in each.

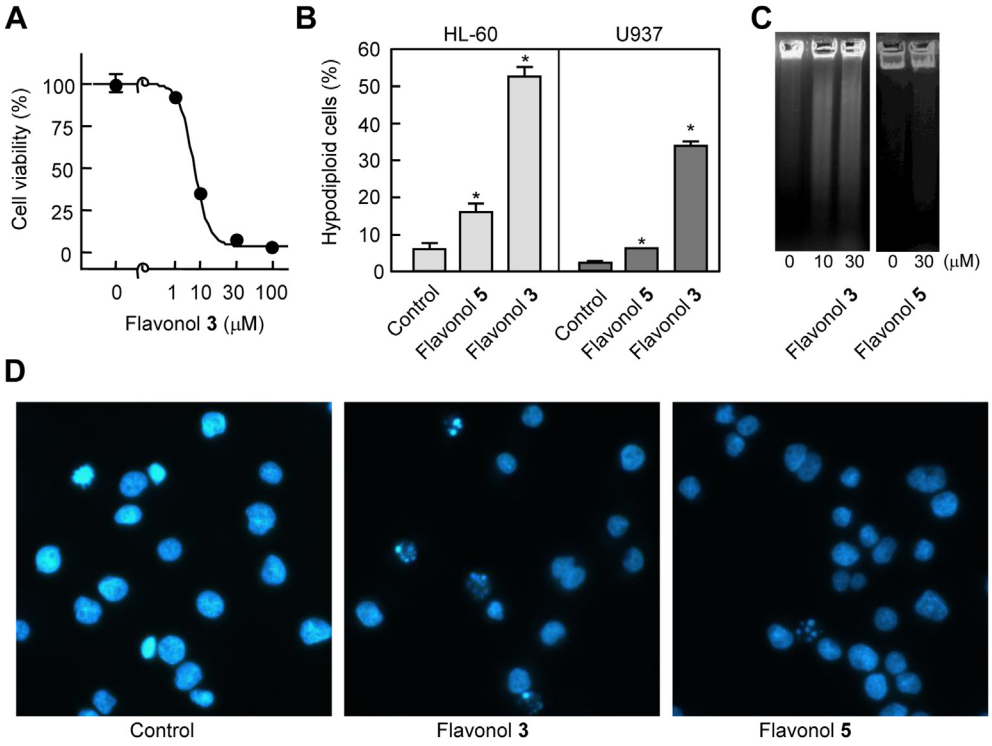



**Fig. 2.** (A) Dose–response of flavonol **3** on human HL-60 cell viability. The results of a representative experiment are shown. Each point represents the mean of three replicates. Similar results were obtained with HL-60/MX1 cell line and Raji cells. (B) Cells were incubated with 30 μM of the indicated flavonol and the percentage of cells in the sub-$G_1$ region was quantified using flow cytometry after propidium iodide staining. (C) HL-60 cells were incubated in the presence of the indicated concentrations of compounds for 24 h and genomic DNA was extracted, separated on an agarose gel and visualized under UV light by ethidium bromide staining. (D) Photomicrographs of representative fields of HL-60 cells stained with Hoechst 33258 to evaluate nuclear chromatin condensation (i.e. apoptosis) after treatment for 24 h with 30 μM of the indicated compounds.

### 3.2. *Flavonol **3** induces apoptosis in human leukemia cells*

To determine whether apoptosis was involved in the cytotoxic effects of flavonol **3**, we used complementary methods such as fluorescence microscopy after DNA staining with Hoechst 33258, the evaluation of DNA fragmentation and the quantification of hypodiploid cells by flow cytometry. When cells were incubated with flavonol **3**, nuclei displayed condensation of chromatin and the appearance of apoptotic bodies. The DNA showed the typical fragmentation patterns caused by internucleosomal hydrolysis of chromatin (Fig. 2C and D). To determine whether the inhibition of cell growth triggered by flavonol **3** is mediated by alterations in the progression of the cell cycle, we studied the effect of this compound on the distribution of the different phases of the cell cycle at different time periods in HL-60 and U937 cell lines by flow cytometry (Fig. 3 and Table 2). The treatment of HL-60 cells with 10 μM flavonol **3** for 24 h caused an arrest of ~33% cells in S phase of the cell cycle which was accompanied by a concomitant decrease in the proportion of cells in the $G_2$-M and $G_1$ phases of the cell cycle. The treatment of HL-60 cells with 3 μM flavonol **3** for 48 h caused an arrest of ~27% cells in S phase which was accompanied by a concomitant decrease of cells in the $G_1$ and $G_2$-M phases of the cell cycle. Flavonol **3** (3 μM) showed a similar trend in S arrest in U937 cells and this effect was sustained for 48 h. There was no arrest of HL-60 or U937 cells at the S phase of the cell cycle for flavonol **3** at concentrations below 3 μM. In HL-60 cells, the percentage of apoptotic cells increased to 20% (6-fold) and 56% (15-fold) in response to 10 μM flavonol **3** after 24 and 48 h of treatment, respectively (Table 2, Fig. 3). In U937 cells, the percentage of apoptotic cells increased to ~14% (~4-fold) and ~46% (~7-fold) after 24 and 48 h of treatment, respectively. These results indicate that this synthetic flavonoid induces cell cycle arrest in the S phase and apoptosis on human myeloid leukemia HL-60 and U937 cells.

### 3.3. *Flavonol **3** induces cell death mediated by caspases activation*

Caspases are cysteine aspartate-specific proteases which are essential executioners of apoptosis. To determine whether caspases were involved in the cell responses to flavonol **3**, this compound was examined for the production of proteolytic processing of caspases. Initial experiments were performed to determine the hierarchical relationship between the initiator caspases, caspases-8 and -9. To this end, kinetic assays were performed and analyzed by Western blots using antibodies which bind both the proenzyme (caspase precursors) and the cleaved caspases. The results show that pro-caspase-9 was processed before procaspase-8 (Fig. 4).

In order to identify the caspases activated and early mechanism of action of flavonol **3** on human leukemia cells, we performed dose–response experiments using concentrations up to 10-fold higher than the antiproliferative $IC_{50}$ value. To this end, HL-60, U937 and Molt-3 cells were treated with increasing concentrations of flavonol **3** for 24 h, and initiator caspases (caspase-8 and -9) were determined by Western blots (Fig. 5). The results show that flavonol **3** stimulated the cleavage of procaspase-9 to the 35–37 kDa fragment in HL-60 and Molt-3 cells, and also induced pro-caspase-9 processing in U937 cells. As shown in Fig. 5, flavonol **3** also induced pro-caspase-8 cleavage in all three leukemia cells. Moreover, this compound stimulated the proteolytic processing of executioner caspases-7 and -6, while the hydrolysis of pro-caspase-3 was only observed in HL-60 and Molt-3, but not in U937 cells. We also evaluated the capacity of flavonol **3** to induce cleavage of pro-caspase-4, which is involved in endoplasmic reticulum stress [27].

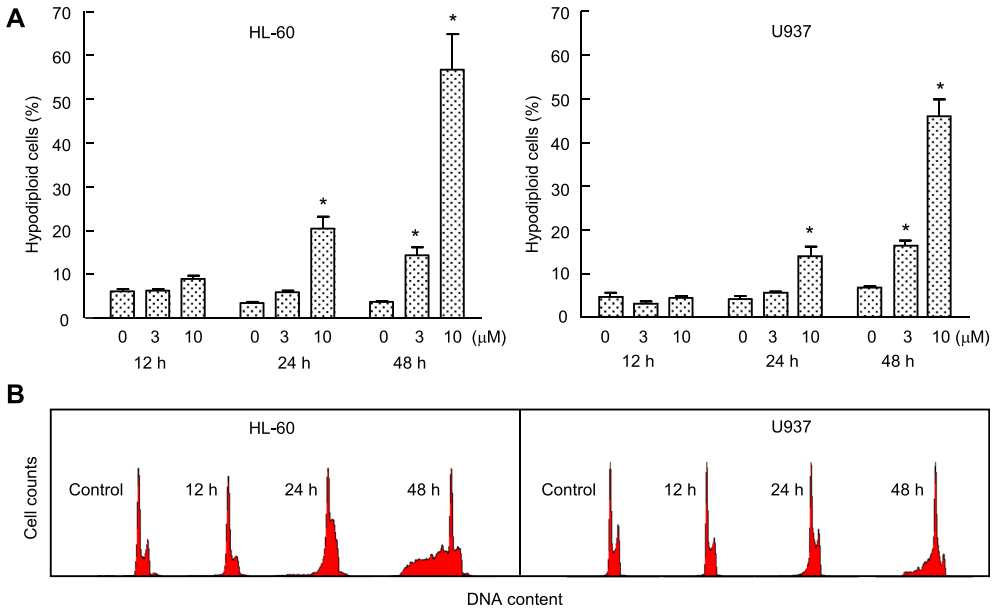



**Fig. 3.** Effects of flavonol **3** on apoptosis induction and on cell cycle phase distribution of HL-60 and U937 cells. (A) Cells were incubated with the indicated concentrations of flavonol **3** and apoptotic cells were quantified as percentage of cells in the sub-$G_1$ region using flow cytometry. Values represent means ± SEs of three independent experiments each performed in triplicate; * indicates $P < 0.05$ for comparison with untreated control. (B) Representative histograms of cell cycle. Cells were cultured with 10 μM flavonol **3** for the indicated periods of time and subjected to DNA flow cytometry.

We observed a reduction (Molt-3) and processing (HL-60 and U937) of this zymogen after 24 h of treatment (Fig. 5).

The expression of the Bcl-2 family members which control the intrinsic pathway of apoptosis was also investigated. Although no change in the expression of Bcl-2 and Bax was observed, in Molt-3 cells an 18 kDa cleavage fragment generated from the full length 23 kDa Bax was detected even after treatment with a very low concentration (3 μM) of flavonol **3**. Moreover, the flavonoid induced a decrease (HL-60 and Molt-3) or processing (U937) of Bid in accordance with caspase-8 processing (Fig. 5).

The enzymatic activities of caspases-3/-7, -8 and -9 were also evaluated since they are not always associated with pro-caspase processing. To this end, HL-60, U937 and Molt-3 cells were treated with 10 μM flavonol **3** for 24 h, and cell lysates were assayed for cleavage of the tetrapeptides DEVD-*p*NA, IETD-*p*NA, and LEHD-*p*NA as specific substrates for caspase-3/7, caspase-8, and caspase-9, respectively. In HL-60 cells, caspase-3/7 activity increased 4.5-fold while caspases-9 and -8 activities increased 1.8-fold and 1.7-fold over the control, respectively. A similar trend in caspase activity was observed in U937 and Molt-3 cells (Fig. 6).

**Table 2**
Effect of different durations of treatment with flavonol **3** on cell cycle phase distribution of human leukemia cells.

| | | | % Sub-$G_1$ | %$G_1$ | %S | %$G_2$-M |
|---|---|---|---|---|---|---|
| HL-60 | 12 h | Control | 6.1 ± 0.5 | 57.2 ± 0.9 | 14.7 ± 0.3 | 18.5 ± 0.5 |
| | | 1 μM | 5.6 ± 0.2 | 56.2 ± 0.7 | 15.6 ± 0.3 | 19.4 ± 0.2 |
| | | 3 μM | 6.3 ± 0.3 | 56.7 ± 0.6 | 14.8 ± 0.4 | 18.2 ± 0.3 |
| | | 10 μM | 8.9 ± 0.8 | 58.2 ± 1.0 | 15.9 ± 1.0 | 13.4 ± 0.6 |
| | 24 h | Control | 3.4 ± 0.3 | 53.0 ± 1.1 | 17.9 ± 0.6 | 22.5 ± 0.9 |
| | | 1 μM | 4.1 ± 0.5 | 49.5 ± 0.5 | 20.7 ± 0.9 | 22.5 ± 0.9 |
| | | 3 μM | 5.9 ± 0.4 | 50.1 ± 1.9 | 17.6 ± 0.7 | 21.8 ± 1.8 |
| | | 10 μM | 20.4 ± 2.8* | 37.3 ± 1.8* | 24.1 ± 0.9* | 12.6 ± 0.7* |
| | 48 h | Control | 3.7 ± 0.1 | 60.4 ± 0.4 | 15.3 ± 0.1 | 17.9 ± 0.7 |
| | | 1 μM | 3.4 ± 0.4 | 57.6 ± 1.1 | 15.5 ± 0.5 | 20.8 ± 0.7 |
| | | 3 μM | 14.3 ± 1.9* | 40.4 ± 1.2* | 22.1 ± 0.4* | 18.0 ± 0.8 |
| | | 10 μM | 56.7 ± 8.2* | 18.4 ± 5.4* | 7.3 ± 1.8* | 5.5 ± 1.6* |
| U937 | 12 h | Control | 4.6 ± 0.9 | 45.8 ± 0.8 | 20.5 ± 0.8 | 24.2 ± 1.2 |
| | | 1 μM | 3.3 ± 0.5 | 45.8 ± 0.6 | 21.5 ± 0.4 | 25.1 ± 0.3 |
| | | 3 μM | 3.1 ± 0.4 | 46.6 ± 0.7 | 22.4 ± 0.2 | 23.9 ± 0.4 |
| | | 10 μM | 4.3 ± 0.5 | 47.6 ± 0.5 | 24.9 ± 0.4 | 19.6 ± 0.6 |
| | 24 h | Control | 3.9 ± 0.8 | 49.8 ± 1.5 | 18.6 ± 0.3 | 22.8 ± 1.6 |
| | | 1 μM | 3.7 ± 0.4 | 47.4 ± 0.5 | 18.9 ± 0.3 | 25.2 ± 1.0 |
| | | 3 μM | 5.5 ± 0.3 | 42.3 ± 2.0 | 24.7 ± 0.3 | 21.7 ± 0.8 |
| | | 10 μM | 13.8 ± 2.2* | 40.2 ± 0.7* | 23.7 ± 1.6 | 18.7 ± 0.6 |
| | 48 h | Control | 6.7 ± 0.3 | 51.9 ± 0.9 | 18.9 ± 0.2 | 19.1 ± 1.1 |
| | | 1 μM | 7.0 ± 0.2 | 49.0 ± 0.7 | 19.6 ± 0.2 | 19.9 ± 0.1 |
| | | 3 μM | 16.3 ± 1.2* | 38.5 ± 1.0* | 21.2 ± 0.1 | 19.0 ± 0.7 |
| | | 10 μM | 45.7 ± 3.9* | 41.4 ± 18.0 | 11.9 ± 2.4* | 7.0 ± 0.8* |

Cells were cultured with the concentrations indicated of flavonol **3** for the indicated period of times and the cell cycle phase distribution was determined by flow cytometry. The values are means ± SEs of two independent experiments with three determinations in each. Asterisks indicate a significant difference ($P < 0.05$) compared with the corresponding controls.

### 3.4. *Flavonol **3** activates mitogen-activated protein kinases*

Since MAPKs pathways play a crucial role in a diverse array of cellular functions, including apoptosis, we evaluated the effect of this flavonoid on the activation of this signal transduction pathway. The results show a fast phosphorylation (2 h) of these MAPKs, phospho-ERK 1/2 remained elevated for at least 6 h, while the level

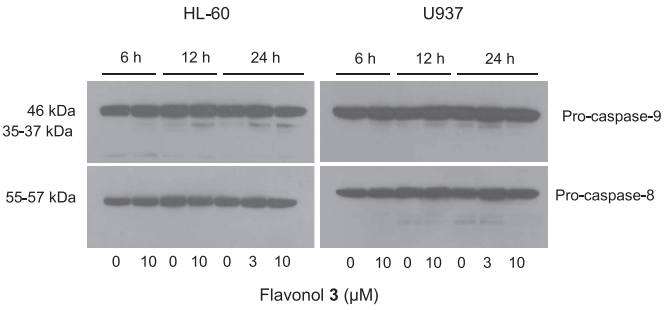



**Fig. 4.** Kinetics and dose–response of pro-caspases-8 and -9 processing in response to flavonol **3**. Cells were treated with the indicated concentrations, harvested at the indicated times and the cleavage of caspases was determined by immunoblotting.

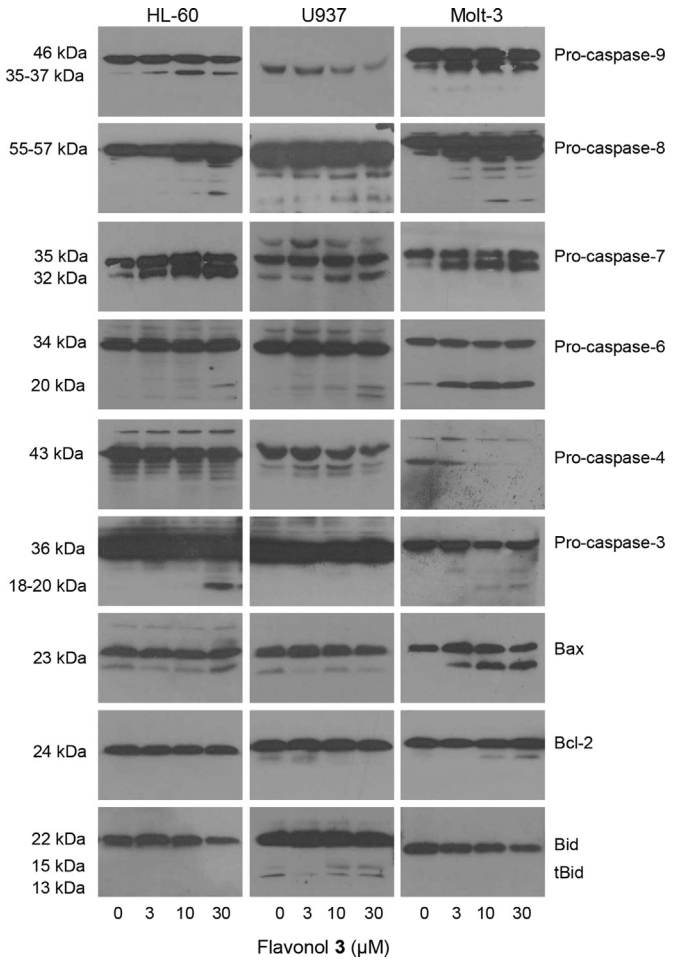



**Fig. 5.** Effect of flavonol **3** on caspases cleavage and on Bcl-2 family members expression. The cells were incubated in the presence of the indicated concentrations of flavonol **3**, harvested at 24 h and cell lysates were assayed by immunoblotting.

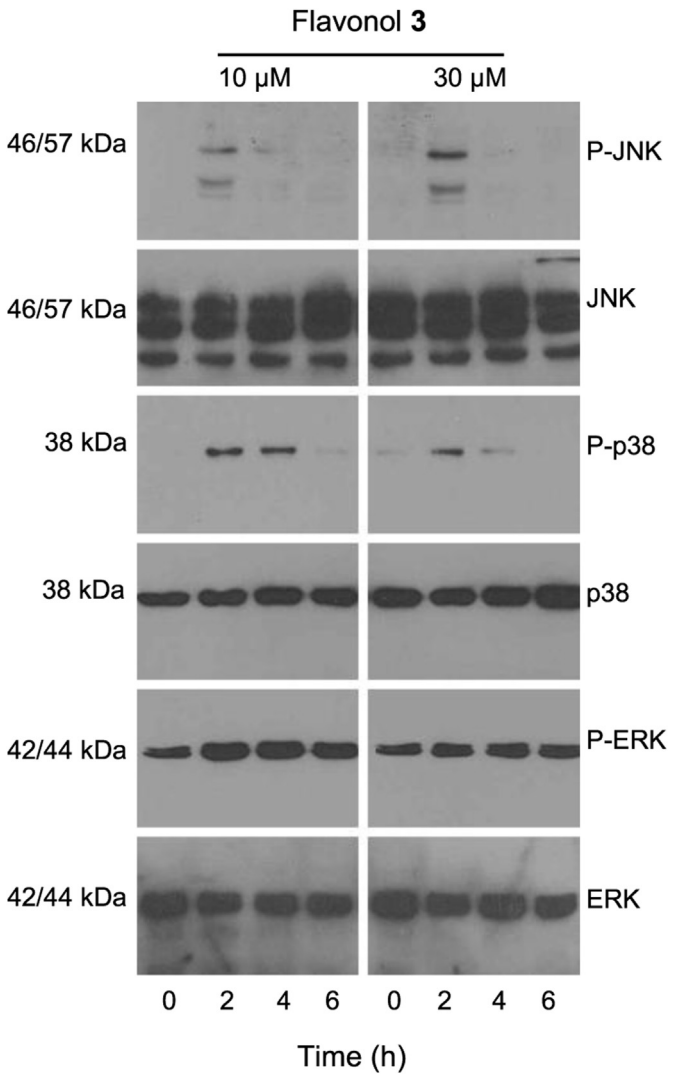



**Fig. 7.** Flavonol **3** induces phosphorylation of MAPKs. Representative Western blots show the time-dependent phosphorylation of JNK/SAPK, p38$^{MAPK}$ and ERK 1/2 by flavonol **3**. HL-60 cells were incubated with flavonol **3** for the indicated time points and protein extracts were analyzed by Western blots probed with specific antibodies to ascertain the phosphorylation of MAPKs. Membranes were stripped and reprobed with total JNK/SAPK, p38$^{MAPK}$ and ERK 1/2 antibodies as loading controls.

of phosphorylated JNK/SAPK and phospho-p38$^{MAPK}$ was transient (Fig. 7). This indicates that treatment of cells with flavonol **3** leads to activation of ERK 1/2, JNK/SAPK and p38$^{MAPK}$ following different kinetics.

To determine whether the phosphorylation of MAPK plays a key role in flavonol **3**-induced apoptosis, we examined the effects of specific inhibitors. Neither the selective JNK/SAPK inhibitor SP600125 nor the p38$^{MAPK}$ inhibitor SB203580 attenuated or influenced cell death (results not shown) suggesting that activation of these MAPKs is not required for apoptosis. In contrast, the mitogen-activated extracellular kinases (MEK) 1/2 inhibitors, PD98059 and U0126, enhanced the apoptosis induced by this flavonoid. In HL-60 cells, combination of flavonol **3** and PD98059

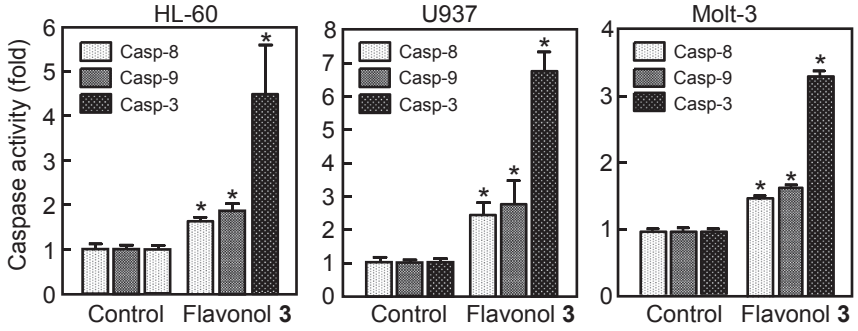



**Fig. 6.** Activation of caspases in response to flavonol **3**. Cells were incubated with 10 μM flavonol **3** for 24 h and cell lysates were assayed for caspase-3/7, -8 and -9 activities. Results are expressed as *n*-fold increases in caspase activity compared with control. Values represent the means ± SEs of three independent experiments each performed in triplicate; * indicates $P < 0.05$ for comparison with untreated control.

(10 μM) or U0126 (10 μM) at least doubled the rate of cell death when compared with flavonol **3** alone (results not shown).

### 3.5. Flavonol **3** up-regulates death receptors DR4 and DR5 and the combination of flavonol **3** and TRAIL enhances apoptosis

Since flavonol **3** also induces activation of caspase-8, it is possible that death receptors and/or their ligand might be involved in this cell death pathway. Therefore we first explored the effects of flavonol **3** on the expression of TRAIL, DR4 and DR5, in human leukemia cells. HL-60 and U937 were treated during 24 h and cell lysates were subjected to immunoblot analysis. As shown in Fig. 8A, flavonol **3** up-regulates expression of TRAIL, DR4 and DR5 in both cell lines.

Additional experiments were performed to determine whether the up-regulation of death receptors by flavonol **3** could contribute to the amplification of cell death in the combination treatment with TRAIL. To this end, U937 leukemia cells were treated with TRAIL (0.5 μg $mL^{-1}$), flavonol **3** (30 μM), and TRAIL and flavonol **3** simultaneously for 48 h or were incubated with flavonol **3** for 24 h, followed by TRAIL for 24 h. Cell viability was assessed by the MTT assay. The results show that the combination treatment, as well as the pretreatment with flavonol **3** followed by TRAIL, enhances cell death in comparison with both non-combination treatments (Fig. 8B).

## 4. Discussion

In previous studies with naturally occurring and semi-synthetic phenylbenzo-γ-pyrones, we showed that methylation of the hydroxy group at position C3 of the quercetin yields a compound with a higher antiproliferative activity against several cancer cell lines [18]. In this study, we have synthesized a series of 20 flavonoids and evaluated their potential cytotoxic properties using the human HL-60 tumor cell line, which is a useful model to study cell growth inhibition of leukemia cells by chemical agents. The antiproliferative studies indicate that flavonols containing chlorine or bromine atoms at positions 2′ (flavonol **5**) and 4′ (flavonol **3**) of the B ring, respectively, were the most cytotoxic compounds against HL-60 cells. Although both flavonols displayed similar $IC_{50}$ values in this cell line, additional experiments revealed that flavonol **3** was also cytotoxic against the mitoxantrone resistant, HL-60/MX1, and against the Burkitt lymphoma cell line Raji. Moreover, flavonol **3** was a more apoptotic inducer than flavonol **5** in HL-60 and U937 cells lines. Cell cycle analysis performed in both cell lines showed that inhibition of cell viability by flavonol **3** was caused by a significant cell cycle arrest at the S phase and accompanied by an increase in sub-$G_1$ fraction indicating apoptotic cell death.

The results also show that flavonol **3** induced the processing of initiator caspases, the caspase-8 and the caspase-9, suggesting that this synthetic flavonoid stimulates the two major pathways of apoptosis, the extrinsic and intrinsic pathways of cell death. Moreover, the endoplasmic reticulum stress signaling pathway appears to be involved since flavonol **3** induced caspase-4 cleavage.

Induction of caspase-8 and -9 as well as caspase-3 like activities was significantly increased in response to flavonol **3** in accordance with the processing of the corresponding pro-caspases as determined by Western blots. The results clearly demonstrated that this flavonoid also stimulates the proteolytic processing of other executioner caspases, namely caspase-7 and caspase-6 to form activated enzymes. Although flavonol **3** induced the lowest activation of caspase-3 in U937 cells, as determined by Western blot, it induced a clear caspase-3/7 activation as determined by enzymatic activity. It appears that the substrate used (DEVD-*p*NA) also determines caspase-7 activity, and, as shown, flavonol **3** also induced a processing of caspase-7 in these cells.

The intrinsic pathway of apoptosis is tightly regulated by B-cell lymphoma 2 (Bcl-2) family proteins that control the permeabilization of the outer mitochondria membrane [28]. Previous studies have shown that flavonoids such as luteolin [29] and astragalin heptaacetate [30] induce cleavage of Bcl-2. However, flavonol **3** did not induce any Bcl-2 cleavage or any change in Bcl-2 levels. There were no changes in Bax levels in HL-60 and U937 cells but this protein was cleaved in Molt-3 cells. Previous studies have shown that a fragment of 18 kDa generated by Bax cleavage is more potent at promoting mitochondrial membrane permeabilization than the full-length protein [31]. The levels of Bid were reduced and Bid processing occurred which is consistent with the finding of caspase-8 activation.

We have previously described the potentiation of cell death by the combined treatment of a quercetin derivative and specific mitogen-activated extracellular kinases 1/2 inhibitors [32]. Here we

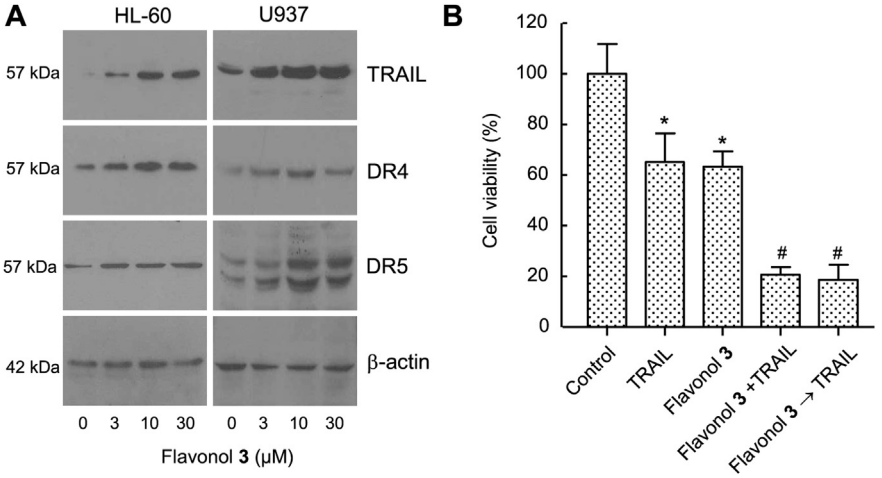



**Fig. 8.** (A) Flavonol **3** up-regulates the expression of the TRAIL and TRAIL receptors DR4 and DR5 in human leukemia cells. Cells were treated with increasing concentrations of flavonol **3** for 24 h and cell lysates were assayed by immunoblotting. (B) Increase in TRAIL sensitivity by flavonol **3** in U937 cells. Cells were incubated for 48 h with TRAIL (0.5 μg $mL^{-1}$), flavonol **3** (30 μM), **3** + TRAIL (simultaneous addition TRAIL and flavonol **3**), or TRAIL (0.5 μg $mL^{-1}$) was added after incubation with flavonol **3** (30 μM) for 24 h (**3** → TRAIL). Bars represent the mean ± SE of three independent experiments each performed in triplicate; * indicates $P < 0.05$ for comparison with untreated control, # indicates $P < 0.05$ for comparison with TRAIL treatment alone.

found that flavonol **3** induced the phosphorylation and activation of ERK 1/2, $p38^{MAPK}$ and JNK/SAPK following different kinetics. Our results also indicate that MEK 1/2 inhibitors PD98059 and U0126 enhanced the apoptotic effects of flavonol **3**.

Reactive oxygen species (ROS) have been implicated as second messengers in multiple signaling pathways, including cell death via MAPK activation [33–35]. However the cytotoxic and/or apoptotic effects of flavonol **3** seem to be ROS independent since neither *N*-acetyl-L-cysteine (an antioxidant agent) nor trolox (an analog of vitamin E acting specifically as ROS scavenger) blocked cell death (data not shown).

Since we demonstrated that apoptosis induced by flavonol **3** is associated with caspase-8 activation and Bid cleavage, it is possible that in the mechanism of action of this flavonoid might involve death receptors. The results demonstrate that flavonol **3** stimulated the expression of TRAIL, DR4 and DR5 and, at least in U937 cells, potentiating the sensitivity to TRAIL. Although further studies are necessary, these results are of potential importance by demonstrating the effect of this flavonoid against tumor cells that are resistant to TRAIL.

## 5. Conclusions

In this paper a series of 20 flavonoids, including nine flavonols and eleven 3-methyl ethers were synthesized and assessed for cytotoxicity against human leukemia cells. A flavonol derivative, containing an atom of bromine at position 4′ of the B ring, was selected since it displayed cytotoxic properties showing $IC_{50}$ values of approximately 5 μM, in tumor cells including the mitoxantrone resistant HL-60/MX1 cell line. The cytotoxic effects of this flavonol were accompanied by a concentration- and time-dependent appearance of apoptosis, associated with the activation of multiple caspases and the mitogen-activated protein kinase pathway and potentiated the sensitivity to TRAIL. The studies shown here open new avenues of research into the roles of signal transduction pathways in the death of the human leukemia cells, and suggest that this compound or derivatives might be useful in the development of new therapeutic strategies in the fight against cancer.

## 6. Experimental protocols

### 6.1. Chemistry

#### 6.1.1. Materials and methods

$^1H$ and $^{13}C$ NMR spectra were obtained on a Bruker model AMX-400 spectrometer with standard pulse sequences operating at 400 MHz in $^1H$ and 100 MHz in $^{13}C$ NMR. Chemical shifts ($\delta$) are given in ppm upfield from tetramethylsilane as internal standard, and coupling constants ($J$) are reported in hertz. IR spectra were recorded using a Bruker model IFS-55 spectrophotometer. Melting points were determined on a Büchi B-540 apparatus and are uncorrected. EIMS and HREIMS were taken on a Micromass model Autospec (70 eV) spectrometer. Column chromatography was carried out on silica gel 60 (Merck 230–400 mesh) and analytical thin layer chromatography (TLC) was performed using silica gel aluminum sheets.

#### 6.1.2. General procedure for the synthesis of flavonols

A mixture of the acetophenone (5–10 mmol, 1 equiv.) and the corresponding aldehyde (1 equiv.) in EtOH (20–40 mL) was stirred at room temperature and a 50% aqueous solution of NaOH (5–8 mL) was added. The reaction mixture was stirred at room temperature until all aldehyde had been consumed. HCl (10%) was then added until neutrality. Precipitated chalcones were generally filtered and crystallized from MeOH although in some cases the product was purified using column chromatography.

A solution of the corresponding 2-hydroxychalcone (2–4 mmol) in 3.0 M KOH in MeOH (20–30 mL) was cooled at 0 °C. An aqueous solution of $H_2O_2$ (30%) (5–8 mL) was added to the chalcone solution. The resulting mixture was stirred at room temperature, until the starting material was totally consumed (as evidenced by TLC). The reaction mixture was cooled in an ice bath and distilled water (2–4 mL) was added. HCl (2 M) was added until pH 2 and the precipitate was filtered and washed with distilled water and extracted with EtOAc. The organic layer was washed with brine until neutrality and dried with anhydrous $MgSO_4$. The solvent was evaporated in vacuo and the residue was purified using a chromatographic column ($SiO_2$, petroleum ether): EtOAc (1:0–7:3).

*6.1.2.1. 2′-Hydroxy-2,4-dimethoxy-3-methylchalcone (**chal 6**).* Orange solid, mp 118–120 °C (92%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3449, 3002, 2941, 2837, 1685, 1634, 1596, 1564, 1488, 1463, 1442, 1411, 1362, 1342, 1313, 1275, 1247, 1212, 1156, 1109, 1029, 990, 967, 863. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta$ 13.02 (s, OH); 8.15 (1H, d, $J$ = 15.4 Hz); 7.94 (1H, d, $J$ = 8.0 Hz); 7.72 (1H, d, $J$ = 15.4 Hz); 7.56 (1H, d, $J$ = 8.7 Hz); 7.50 (1H, ddd, $J$ = 1.4, 7.4, 7.4 Hz); 7.04 (1H, d, $J$ = 8.4 Hz); 6.95 (1H, dd, $J$ = 7.6, 7.6 Hz); 6.73 (1H, d, $J$ = 8.6 Hz); 3.91 (3H, s); 3.80 (3H, s); 2.20 (3H, s). $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta$ = 194.5, 163.9, 161.7, 160.0, 141.8, 136.3, 129.9, 127.8, 125.2, 121.3, 120.7, 119.2, 119.0, 118.9, 107.0, 61.8, 56.1, 9.2. HRMS (ESI-FT-ICR) $m/z$: 299.1286 $[M+H]^+$; calcd. for $C_{18}H_{20}O_4$: 299.1283.

*6.1.2.2. 3-Hydroxy-2′,4′-dimethoxy-3′-methylflavone (**6**).* Yellow solid, mp 208–210 °C (69%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3416, 3260, 2364, 1614, 1595, 1569, 1482, 1454, 1417, 1404, 1345, 1269, 1213, 1172, 1133, 1106, 1052, 1022, 940, 881, 809. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta$ 8.31 (1H, d, $J$ = 8.0 Hz); 7.70 (1H, dd, $J$ = 7.6, 7.3 Hz); 7.55 (1H, d, $J$ = 8.7 Hz); 7.52 (1H, d, $J$ = 8.8 Hz); 7.44 (1H, dd, $J$ = 7.1, 7.1 Hz); 6.81 (1H, d, $J$ = 8.5 Hz); 6.64 (1H, s, OH); 3.92 (3H, s); 3.74 (3H, s); 2.25 (3H, s). $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta$ = 173.5, 161.2, 157.8, 156.2, 146.9, 138.8, 133.6, 129.2, 125.9, 124.7, 121.8, 121.0, 118.7, 116.8, 106.4, 61.7, 56.2. HRMS (ESI-FT-ICR) $m/z$: 335.0886 $[M+Na]^+$; calcd. for $C_{18}H_{16}O_5Na$: 335.0895.

*6.1.2.3. 2′-Hydroxy-3,4,5,4′-tetramethoxychalcone (**chal 8**).* Orange solid, mp 89–91 °C (85%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3534, 3003, 2941, 2841, 2738, 2638, 1714, 1682, 1643, 1633, 1622, 1614, 1582, 1574, 1558, 1504, 1463, 1455, 1417, 1371, 1333, 1273, 1217, 1153, 1128, 1069, 1020, 1004, 976, 960, 837. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta$ = 13.47 (s, OH); 7.84 (1H, d, $J$ = 8.7 Hz); 7.80 (1H, d, $J$ = 15.4 Hz); 7.45 (1H, d, $J$ = 15.3 Hz); 6.87 (2H, s); 6.50–6.47 (2H, m); 3.93 (6H, s); 3.91 (3H, s); 3.86 (3H, s). $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta$ = 192.0, 167.0, 166.6, 153.9, 144.9, 132.6, 131.5, 130.6, 119.9, 114.5, 108.0, 107.9, 106.4, 101.5, 101.3, 61.3, 56.6, 55.9. HRMS (ESI-FT-ICR) $m/z$: 345.1337 $[M+H]^+$; calcd. for $C_{19}H_{21}O_4$: 345.1338.

*6.1.2.4. 3-Hydroxy-7,3′,4′,5′-tetramethoxyflavone (**8**).* Yellow solid, mp 177–180 °C (77%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3422, 3247, 2939, 1602, 1567, 1505, 1457, 1417, 1401, 1379, 1337, 1283, 1263, 1213, 1180, 1163, 1125, 1102, 1015, 848, 831, 820. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta$ = 8.04 (1H, d, $J$ = 8.9 Hz); 7.42 (2H, s); 6.91 (1H, dd, $J$ = 2.1, 8.9 Hz); 6.85 (1H, d, $J$ = 2.0 Hz); 3.88 (6H, s); 3.86 (3H, s); 3.86 (3H, s). $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta$ = 173.0, 164.7, 157.5, 153.6, 144.5, 138.2, 127.1, 126.7, 115.1, 114.9, 105.8, 105.1, 100.3, 61.3, 56.7, 56.2. HRMS (ESI-FT-ICR) $m/z$: 359.1128 $[M+H]^+$; calcd. for $C_{19}H_{19}O_7$: 359.1131; 381.0949 $[M+Na]^+$; calcd. for $C_{19}H_{19}O_7Na$: 381.0950.

*6.1.2.5. 2′-Hydroxy-2,4-dimethoxychalcone (**chal 9**).* Orange solid, mp 103–105 °C (89%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3011, 2966, 2940, 2836,

2747, 2628, 1634, 1606, 1573, 1565, 1553, 1504, 1488, 1465, 1443, 1419, 1367, 1343, 1306, 1278, 1204, 1157, 1119, 1024, 983, 940, 863, 831. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 13.1 (1H, s, OH); 8.18 (1H, d, $J$ = 15.5 Hz); 8.11 (1H, dd, $J$ = 2.1, 6.9 Hz); 7.05 (2H, dd, $J$ = 2.1, 6.9 Hz); 6.99 (1H, dd, $J$ = 2.3, 8.8 Hz); 6.93 (1H, d, $J$ = 2.3 Hz); 3.94 (3H, s); 3.92 (3H, s); 3.90 (3H, s). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 194.6, 163.9, 163.8, 161.1, 141.6, 136.1, 131.8, 129.9, 120.7, 118.9, 118.8, 118.5, 117.3, 106.0, 98.9, 56.0, 55.8. HRMS (ESI-FT-ICR) $m/z$: 285.1127 $[M+H]^+$; calcd. for $C_{17}H_{17}O_4$: 285.1127.

*6.1.2.6. 3-Hydroxy-2′,4′-dimethoxyflavone (**9**).* Yellow solid, mp 187–189 °C (72%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3396, 3311, 1609, 1569, 1507, 1467, 1425, 1411, 1284, 1209, 1162, 1138, 1114, 1041, 1029, 921, 893, 834. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 8.29 (1H, d, $J$ = 1.4, 8.0 Hz); 7.68 (1H, ddd, $J$ = 1.6, 7.0, 8.6 Hz); 7.54 (1H, dd, $J$ = 8.5, 11.6 Hz); 7.42 (1H, ddd, $J$ = 0.8, 7.0, 8.0 Hz); 6.66 (1H, dd, $J$ = 2.3, 8.5 Hz); 6.44 (1H, brs), 3.90 (3H, s); 3.88 (3H, s). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 194.6, 163.9, 163.8, 161.1, 141.6, 136.1, 131.8, 129.9, 120.7, 118.9, 118.8, 118.5, 117.3, 106.0, 98.9, 56.0, 55.8. HRMS (ESI-FT-ICR) $m/z$: 285.1127 $[M+H]^+$; calcd. for $C_{17}H_{17}O_4$: 285.1127.

*6.1.3. General method for the preparation of 3-methoxy-flavones*

$K_2CO_3$ (1.8 equiv.) and iodomethane (1.2 equiv.) were added to a solution of flavonol (0.10–0.20 mmol) at 40 °C in dry $CH_3CN$ (1–2 mL) until the starting material was totally consumed (as evidenced by TLC). The reaction mixture was then, partitioned between ethyl acetate and water. The ethyl acetate layer was washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography to yield methyl ethers.

*6.1.3.1. 3,2′,4′-Trimethoxyflavone (**11**) [36].* Pale yellow solid, mp 119–120 °C (70%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3441, 2097, 1643, 1624, 1507, 1466, 1436, 1381, 1307, 1267, 1211, 1162, 1122, 1029. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.20 (1H, dd, $J$ = 1.6, 8.0 Hz); 7.55 (1H, ddd, $J$ = 1.6, 7.0, 8.4 Hz); 7.36 (1H, d, $J$ = 8.0 Hz); 7.33–7.27 (2H, m); 6.53 (1H, dd, $J$ = 2.3, 8.3 Hz); 3.79 (3H, s); 3.75 (3H, s); 3.74 (3H, s). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 175.2, 163.2, 159.1, 156.5, 156.1, 142.3, 133.4, 132.0, 126.2, 125.0, 124.7, 118.4, 113.9, 105.2, 99.3, 60.6, 56.1, 55.9. HRMS (ESI-FT-ICR) $m/z$: 335.0904 $[M+Na]^+$; calcd. for $C_{18}H_{16}O_5Na$: 303.1021.

*6.1.3.2. 4′-Bromo-3-methoxyflavone (**13**) [19].* Pale yellow solid, mp 115–116 °C (69%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3446, 2360, 2341, 2066, 1643, 1615, 1487, 1468, 1439, 1399, 1379, 1340, 1291, 1238, 1213, 1147, 1110, 1072, 1009, 950, 898, 826. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 8.28 (1H, d, $J$ = 7.9 Hz); 8.02 (2H, d, $J$ = 8.6 Hz); 7.72–7.67 (3H, m); 7.55 (1H, d, $J$ = 8.3 Hz); 7.43 (1H, dd, $J$ = 7.8, 7.8 Hz); 3.93 (3H, s). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 175.3, 155.5, 154.6, 142.0, 133.9, 132.2, 130.3, 130.2, 126.2, 125.6, 125.1, 124.6, 118.3, 106.6, 60.4. HRMS (ESI-FT-ICR) $m/z$: 330.9966 $[M+H]^+$; calcd. for $C^{79}_{16}BrH_{12}O_3$: 330.9970; calcd. for $C^{81}_{16}BrH_{12}O_3$: 332.9949.

*6.1.3.3. 3,2′-Dimethoxyflavone (**14**).* White solid, mp 146–147 °C (74%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3449, 3002, 2938, 2840, 1642, 1624, 1581, 1569, 1492, 1466, 1437, 1386, 1339, 1300, 1278, 1257, 1233, 1212, 1171, 1147, 1111, 1048, 1023, 1008, 955, 900. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 13.1 (1H, s, OH); 8.18 (1H, d, $J$ = 15.5 Hz); 8.11 (1H, dd, $J$ = 2.1, 6.9 Hz); 7.05 (2H, dd, $J$ = 2.1, 6.9 Hz); 6.99 (1H, dd, $J$ = 2.3, 8.8 Hz); 6.93 (1H, d, $J$ = 2.3 Hz); 3.94 (3H, s); 3.92 (3H, s); 3.90 (3H, s). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = HRMS (ESI-FT-ICR) $m/z$: 283.0964 $[M]^+$; calcd. for $C_{17}H_{14}O_4$: 283.0970.

*6.1.3.4. 3,2′,4′-Trimethoxy-3′-methylflavone (**15**).* Pale yellow solid, mp 176–178 °C (71%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3710, 3014, 2970, 2944, 2843, 1643, 1623, 1610, 1591, 1488, 1463, 1438, 1406, 1386, 1297, 1274, 1258, 1222, 1172, 1148, 1107, 1052, 1002, 985, 920, 883. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.32 (1H, d, $J$ = 8.0 Hz); 7.67 (1H, dd, $J$ = 7.1, 8.3 Hz); 7.49 (1H, d, $J$ = 8.4 Hz); 7.42 (1H, dd, $J$ = 7.2, 7.3 Hz); 7.36 (1H, d, $J$ = 8.5 Hz); 6.77 (1H, d, $J$ = 8.5 Hz); 3.92 (3H, s); 3.84 (3H, s); 3.74 (3H, s); 2.23 (3H, s). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 175.2, 161.1, 158.0, 157.4, 155.9, 142.1, 133.5, 129.0, 126.2, 125.0, 124.8, 120.8, 118.4, 117.4, 106.1, 61.7, 60.7, 56.2. HRMS (ESI-FT-ICR) $m/z$: 349.1052 $[M+Na]^+$; calcd. for $C_{19}H_{18}O_5Na$: 349.1051.

*6.1.3.5. 3,7,4′-Trimethoxyflavone (**16**).* White solid, mp 142–144 °C (75%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3443, 2360, 2341, 2097, 1634, 1508, 1445, 1382, 1256, 1180, 1118, 1031, 1015, 953, 834. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 8.18 (1H, d, $J$ = 8.88 Hz); 8.11 (2H, d, $J$ = 6.9 Hz); 7.05 (2H, d, $J$ = 6.9 Hz); 6.99 (1H, dd, $J$ = 2.3, 8.8 Hz); 6.93 (1H, d, $J$ = 2.3 Hz) $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 174.8, 164.3, 161.7, 157.3, 133.4, 130.4, 127.5, 123.8, 118.5, 114.5, 114.3, 106.7, 100.3, 60.3, 56.1, 55.7. HRMS (ESI-FT-ICR) $m/z$: 313.1068 $[M+H]^+$; calcd. for $C_{18}H_{17}O_5$: 313.1076.

*6.1.3.6. 3-Methoxy-2-(1-naphthalenyl)-4H-1-benzopyran-4-one (**17**) [37].* Pale yellow solid, mp 165–166 °C (68%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3431, 3066, 3011, 1645, 1614, 1569, 1506, 1467, 1441, 1374, 1337, 1293, 1265, 1239, 1211, 1172, 1148, 1109, 1081, 1002, 942, 896, 805. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.39 (1H, d, $J$ = 7.9 Hz); 8.06 (1H, d, $J$ = 8.2 Hz); 7.97 (1H, d, $J$ = 7.5 Hz); 7.86 (1H, d, $J$ = 8.0 Hz); 7.76 (1H, d, $J$ = 7.0 Hz); 7.71 (1H, dt, $J$ = 1.0, 7.1, 8.4 Hz); 7.63 (1H, dd, $J$ = 7.6, 7.7 Hz); 7.60–7.46 (4H, m); 3.72 (3H, s). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 17, 157.8, 156.1, 133.5, 130.9, 128.5, 128.2, 127.0, 126.4, 126.0, 125.2, 124.9, 124.8, 118.2, 60.6, HRMS (ESI-FT-ICR) $m/z$: 303.1021 $[M+Na]^+$; calcd. for $C_{20}H_{15}O_3Na$: 303.1021.

*6.1.3.7. 2′-Hydroxy-2,4,4′-trimethoxy-3-methylchalcone (**chal 18**).* Orange solid, mp 161–163 °C (92%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3710, 3003, 2944, 2836, 1630, 1564, 1508, 1488, 1463, 1412, 1365, 1311, 1276, 1252, 1228, 1222, 1208, 1185, 1154, 1130, 1109, 1022, 989, 959, 857, 827. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 13.62 (s, OH); 8.10 (1H, d, $J$ = 15.5 Hz); 7.85 (1H, d, $J$ = 9.6 Hz); 7.64 (1H, d, $J$ = 15.5 Hz); 7.54 (1H, d, $J$ = 8.6 Hz); 6.73 (1H, d, $J$ = 8.7 Hz); 6.51–6.49 (2H, m); 3.90 (3H, s); 3.87 (3H, s); 3.80 (3H, s); 2.20 (3H, S). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 192.7, 167.0, 166.3, 161.5, 159.8, 140.7, 131.5, 127.6, 121.5, 120.9, 119.4, 114.7, 107.8, 107.0, 101.5, 63.2, 61.7, 56.1, 55.9, 9.2. HRMS (ESI-FT-ICR) $m/z$: 299.1286 $[M+H]^+$; calcd. for $C_{18}H_{20}O_4$: 299.1283.

*6.1.3.8. 3-Hydroxy-7,2′,4′-trimethoxy-3′-methylflavone (**flavonol-18**).* Yellow solid, mp 197–199 °C (70%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3734, 3005, 2942, 2839, 1682, 1610, 1505, 1490, 1452, 1408, 1273, 1256, 1226, 1203, 1174, 1132, 1109, 1022, 1000, 834. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.19 (1H, d, $J$ = 8.9 Hz); 7.49 (1H, d, $J$ = 8.6 Hz); 7.85 (1H, d, $J$ = 9.6 Hz); 7.01 (1H, dd, $J$ = 1.4, 8.9 Hz); 6.92 (1H, s); 6.80 (1H, d, $J$ = 8.6 Hz); 6.62 (H, s); 3.91 (6H, s); 3.71 (3H, s); 2.24 (3H, s). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 173.0, 164.4, 158.2, 157.8, 146.1, 138.5, 129.2, 127.2, 120.9, 116.8, 115.0, 106.4, 101.4, 100.3, 61.6, 56.1, 9.3. HRMS (ESI-FT-ICR) $m/z$: 343.1178 $[M+H]^+$; calcd. for $C_{19}H_{19}O_6$: 343.1182.

*6.1.3.9. 3,7,2′,4′-Tetramethoxy-3′-methylflavone (**18**).* Pale yellow solid, mp 153–154 °C (60%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3442, 1626, 1488, 1443, 1406, 1385, 1363, 1273, 1248, 1225, 1203, 1168, 1109, 1048, 1024, 1004, 989, 951. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.12 (1H, d, $J$ = 8.9 Hz); 7.25 (1H, d, $J$ = 8.8 Hz); 6.90 (1H, dd, $J$ = 2.3, 8.9 Hz); 6.78 (1H, d, $J$ = 2.3 Hz); 6.67 (1H, d, $J$ = 8.5 Hz); 3.83 (3H, s); 3.81 (3H, s); 3.74 (3H, s); 3.63 (3H, s); 2.14 (3H, s). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 174.7, 164.3, 161.0, 157.9, 157.7, 129.0, 127.5, 120.7, 118.9, 117.4, 114.6, 106.1, 100.4, 95.6, 61.6, 60.7, 56.1, 9.4. HRMS (ESI-FT-ICR)

*m/z*: 379.1151 $[M+Na]^+$; calcd. for $C_{20}H_{20}O_6Na$: 379.1158; 357.1333 $[M+H]^+$; calcd. for $C_{20}H_{21}O_6$: 357.1338.

*6.1.3.10. 3,7,3′,4′,5′-Pentamethoxyflavone (**20**).* Pale yellow solid, mp 125–127 °C (65%). IR (KBr, $cm^{-1}$) $\nu_{max}$: 3448, 3003, 2973, 2940, 2840, 1636, 1621, 1503, 1445, 1415, 1382, 1363, 1328, 1253, 1213, 1171, 1126, 1104, 1052, 1002, 953, 839. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta = 8.09$ (1H, d, $J = 8.8$ Hz); 7.31 (2H, s); 6.91 (1H, dd, $J = 2.3$, 8.9 Hz); 6.84 (1H, d, $J = 2.3$ Hz); 3.88 (6H, s); 3.87 (3H, s); 3.86 (3H, s). $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta$ = 174.7, 164.4, 157.2, 156.4, 155.2, 153.5, 141.6, 127.6, 126.4, 118.5, 114.6, 106.6, 100.4, 61.3, 60.5, 56.7, 56.2. HRMS (ESI-FT-ICR) *m/z*: 373.1280 $[M+H]^+$; calcd. for $C_{20}H_{21}O_7$: 373.1287; 395.1101 $[M+Na]^+$; calcd. for $C_{20}H_{20}O_7Na$: 395.1107.

### 6.2. Biological assays

#### 6.2.1. Reagents

Antibodies for caspases-3, -7, -8 and -9 were purchased from Assay Designs (Ann Arbor, MI, USA) and anti-caspase-6 was from Medical & Biological Laboratories (Nagoya, Japan). Antibodies for Bax and Bid were purchased from BD PharMingen (San Diego, CA, USA). Anti-Bcl-2 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-JNK/SAPK, anti-phospho-JNK/SAPK (T183/Y185), anti-p44/42 MAP kinase (ERK1/2), anti-Phospho-p44/42 MAP kinase (T202/Y204), anti-$p38^{MAPK}$ and a phosphorylated form (T180/Y182) of $p38^{MAPK}$ were purchased from New England BioLabs (Cell Signaling Technology, Inc, Beverly, MA, USA). Antibodies for DR4 and DR5 were from Abcam (Cambridge, UK). PVDF membranes were from Millipore (Temecula, CA, USA). Secondary antibodies were from GE Healthcare (Little Chalfont, UK). All other chemicals were obtained from Sigma (Saint Louis, MO, USA).

#### 6.2.2. Cell culture

HL-60, U937 and Molt-3 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). HL-60/MX1 and the Burkitt's lymphoma Raji cells were from the American Type Culture Collection (Manassas, VA). The human leukemia HL-60, U937, Molt-3 and Raji cells were grown as previously described [38].

#### 6.2.3. Cytotoxicity assays on human tumor cells

The cytotoxicity of flavonoids on human tumor cells was analyzed by colorimetric 3-(4,5-dimethyl-2-thyazolyl-)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay as previously described [39]. Concentrations inducing a 50% inhibition of cell growth ($IC_{50}$) were determined graphically for each experiment by a non-linear regression using the curve-fitting routine implemented in Prism 4.0 (GraphPad). Values are means ± SE from at least three independent experiments, each performed in triplicate.

#### 6.2.4. Evaluation of apoptosis

Fluorescent microscopy, flow cytometric analysis of propidium iodide-stained nuclei and DNA fragmentation assay were performed as described [39].

#### 6.2.5. Western blot analysis

Immunoblot analyses of caspase-9, caspase-8, caspase-7, caspase-6, caspase-3 and Bcl-2 family members were performed as previously described [39].

#### 6.2.6. Assay of caspase activity

Caspase activity was analyzed as described previously [38].

#### 6.2.7. Statistical analysis

Statistical significance of differences between means of control and treated samples were calculated using Student's *t*-test. *P* values of <0.05 were considered significant.

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