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# Biological Effects of Epicuticular Flavonoids from *Primula denticulata* on Human Leukemia Cells

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The biological effects of epicuticular substances in farinose exudates accumulated on inflorescence shafts and calyces of *Primula denticulata* on human acute myeloid leukemia cells (HL-60) were analyzed. The crude material possessed little antioxidative capacity but strong cytostatic properties. Some of its known components (5-hydroxyflavone, 2′-hydroxyflavone, 5,2′-dihydroxyflavone, and 5,8-dihydroxyflavone) were further tested to identify the biologically active compounds. The effects of these flavones on cell cycle progression, mitochondrial membrane potential, and reactive oxygen species have been investigated by flow cytometry. The flavonol quercetin was included in the study as reference compound because of its known cytostatic properties and its activity as radical scavenger. Compared to quercetin the flavones induced little apoptosis (up to 40 $\mu$M), but despite their low toxicity, the *Primula* flavonoids possessed strong cytostatic properties even at low concentrations. The cell cycle distribution showed a characteristic time-dependent shift, giving evidence of a generally short-lived effect of the test compounds in the exposed cells. The antioxidative properties quantified according to two different methods correlated with the number of hydroxyl groups. Whereas quercetin strongly affected the mitochondrial membrane potential, none of the *Primula* flavones showed a comparable effect.

**KEYWORDS: Flavones; cell cycle; apoptosis; mitochondrial membrane potential; reactive oxygen species**

## INTRODUCTION

*Primula* is a plant genus with some 400 species, most of which are endogenous to the temperate zones. Some of them are popular garden plants because of their colorful early blossoms. The farinose leaf exudate of *Primula* species consists of a number of flavones with different biological activities (*1*).

The induced activity of exudate compounds is illustrated dramatically by *Primula* dermatitis, which was first described by White more than 100 years ago (*2*). Allergic reactions were described mostly after contact with *P. obconica.* In most cases women and persons above the age of 35 were affected (*3*). The main sensitizer is the quinone primin, which is predominantly found in the distal cells of the microscopic glandular trichomes surrounding the calyx, but other allergens have been postulated (*1*, *4*). Hausen et al. (*4*) isolated primetin (5,8-dihydroxyflavone) from *P. mistassinica* Michaux, the "birds eye primrose", and demonstrated that this flavone had strong sensitizing properties. The substance has been detected in several other *Primula* species such as *P. auricula*, *P. halleri*, *P. malacoides*, *P.marginata*, and the species analyzed in this paper, *P. denticulata* (*5*).

Many flavonoids have cytostatic properties (*6*) and may induce apoptosis (*7*). These activities have prompted numerous investigations to explore possible pharmaceutical applications. The cytostatic and apoptotic activities of the flavonoids have been attributed to their modulation of several biological processes. Flavonoids are potent inhibitors of processes involved in mitogen signaling or DNA synthesis, and this is thought to be the reason for the $G_1$/S and/or $G_2$/M arrests seen in some cell types following flavonoid exposure (*8*).

Another sensitive and quite different indicator for cell toxicity is the induced reduction of the mitochondrial inner transmembrane potential ($\Delta\Psi$m). Recently, mitochondria have been shown to play a key role in apoptosis through the release of cytochrome *c* in response to many anticancer drugs and cellular stress (*9*). In many apoptotic pathways, the mitochondrial transmembrane potential collapses (see, for example, refs *10* and *11*), indicating the opening of the so-called mitochondrial permeability transition pore. Even when a temporary reduction of the transmembrane potential does not result in apoptosis, the effect on the potential dependent proton pump presumably lowers adenosine 5′-triphosphate (ATP) production (*12*, *13*). In this study we examined the effects of the flavonoids on the mitochondrial membrane potential using the fluorescent potential-sensitive dye JC-1.

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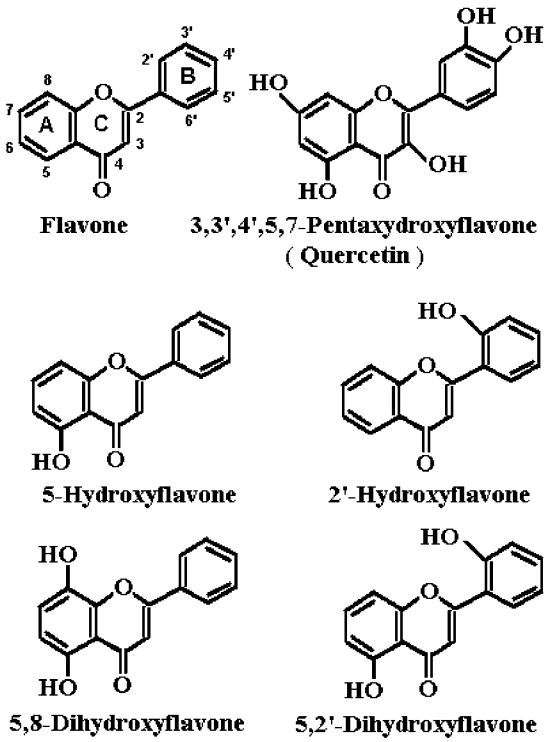


**Figure 1.** Chemical structures of the analyzed flavonoids.

The property of quercetin and of many flavonoids to act as scavengers for reactive oxygen species (ROS) is thought to produce the well-known beneficial effects of dietary flavonoids. For this reason, we have also quantified the antioxidative potency of the studied flavonoids using the known radical scavenging activity of quercetin (*14*, *15*) as a reference.

## MATERIALS AND METHODS

**Extraction and Purification of Flavones from *P. denticulata*.** Freshly collected leaves of *P. denticulata (*Botanical Gardens of the TU Darmstadt and Botanical Gardens of the TU Dresden, Germany) were rinsed with acetone to dissolve the exudate, which was concentrated in a vacuum and fractionated by column chromatography on silica gel (Kieselgel N, Machery-Nagel, Düren, Germany) and on polyamide (DC11, Machery-Nagel) as described previously (*16*). Flavone, 5-hydroxyflavone (5-HF), 2′-hydroxyflavone (2′-HF), 5,2′-dihydroxyflavone (5,2′-DF), and 5,8-dihydroxyflavone (5,8-DF) were isolated from a minor fraction as trace constituents by column chromatography and repeated preparative thin-layer chromatography (TLC) on silica gel 60 (40−63 $\mu$m, Merck, Darmstadt, Germany) according to ref *1*. The structures of analyzed substances are shown in **Figure 1**.

**Cell Culture.** Acute myeloid leukemia cells (HL-60, DSMZ) were maintained in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (Gibco). Cells were grown at 37 °C in a humidified 5% $CO_2$ atmosphere and maintained at a density of $2 \times 10^5$ to $1 \times 10^6$ cells/mL by resuspending the cells in fresh culture medium every 2 days.

**Effects on Cell Proliferation.** HL-60 cells were stained with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) as described by Lyons (*17*) and Parish (*18*). Cells were incubated at 37 °C in PBS containing 10 $\mu$M CFSE for 10 min, washed, and placed in culture medium ($\sim 2 \times 10^5$ cells/mL) 1 h before exposure to the test compounds (5, 10, 20, 40, and 80 $\mu$M) or total epicuticular extracts (0.3, 1.5, 3, 6, and 9 $\mu$g/mL). Because the flavonoid stock solutions were prepared in DMSO, all cultures were made 0.1% in DMSO including the control sample. The same volume of the suspension cultures was harvested after 6, 12, 18, 24, 30, 48, and 96 h, fixed in 70% ethanol, and stored overnight at −20 °C. The cells were spun down again and the pellet resuspended in PBS containing 50 $\mu$g/mL propidium iodide (PI) and 0.2 mg/mL RNase (Sigma) and incubated for at least 45 min. Between 1 and $5 \times 10^5$ cells per sample were analyzed by flow cytometry (CyFlow, Partec). For each variable (exposure conditions, culture periods, etc.) a minimum of six samples were quantified. The fraction of cells present in different cell generations and their representation in the respective cell cycle phases were calculated using CyFlow software (Partec).

**Antioxidative Potential.** Free radical scavenging activities of leaf extracts from *P. denticulata* were determined in a chemical reaction using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, Fluka) as described by ref *19*. The flavonol quercetin was analyzed in parallel as positive control. The assays were performed in a 300 $\mu$L reaction mixture containing 200 $\mu$L of freshly made 0.1 mM DPPH solution in 96% ethanol, 90 $\mu$L of 50 mM Tris-HCl buffer (pH 7.4), and 10 $\mu$L of test compounds in DMSO at final concentrations of 0 (control), 0.75, 1.5, 3.0, 4.5, 6.0, 9.0, 12, 24, and 30 $\mu$g/mL. After 30 min of incubation at room temperature, the absorbance was measured in 96 well plates at 517 nm using a Spectra Rainbow photometer (Labinstruments GmbH).

A more detailed study on the radical scavenging potential of the flavonoids in HL-60 cells was carried out by flow cytometry. We used a well-established detection system based on the induced fluorescence of 2′,7′-dichlorodihydrofluorescein diacetate (DCF; Fluka) to quantify the generation of ROS including hydrogen peroxide and nitric oxide (*20*, *21*). The compound diffuses passively into the cells and is deacetylated and subsequently oxidized to yield the highly fluorescent 2′,7′-dichlorofluorescein diacetate (*22*). The staining procedure was basically carried out as described by ref *23*. HL-60 cells were incubated in medium containing the flavonoids (20 $\mu$M) for 120 min and then incubated in 10 $\mu$M DCF for an additional 30 min. The cells were centrifuged again to replace the medium with PBS, and the fluorescence was determined by flow cytometry (Cyflow; Partec).

**Mitochondrial Membrane Potential.** The mitochondria-selective indicator for the membrane potential, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR). This dye forms at high mitochondrial membrane potential ($\Delta\Psi m > 140$ mV) characteristic J-aggregates with an emission maximum at 590 nm (*24*). A decrease of $\Delta\Psi$ can be monitored as a reduction of red fluorescence and an increase in yellow-green JC-1 monomers (emission maximum = 527 nm). The dye was dissolved in DMSO (2 mg/mL), and the effect of each flavonoid was tested at a concentration of 20 $\mu$M. Cells were adjusted to a density of $(0.5-3) \times 10^6$/mL, incubated in medium containing the test substances for 120 min, and stained with a final concentration of 2 $\mu$g/mL JC-1 for an additional 30 min at 37 °C. Cells were washed with PBS and the yellow-green and red fluorescences quantified by flow cytometry (CyFlow, Partec).

**Statistics.** Statistical analysis was performed using Student's *t* test. Significance levels were set at $P < 0.05$.

## RESULTS

**Toxicity and Radical Scavenger Activity of Epicuticular Extracts.** The externally accumulated flavones of *P. denticulata* showed a remarkable effect on the cell cycle of asynchronously proliferating HL-60 cells. The DNA profiles of cell cultures treated with 30 $\mu$g/mL extract and a control culture are compared in **Figure 2a**. In control cultures most cells are in the $G_{0+1}$ phase, and typically about 6 ± 3% of apoptotic cells are present in exponentially growing cultures. In cultures that were treated with 9 $\mu$g/mL *Primula* extract for 24 h, the DNA distribution changed considerably. While the fraction of cells in $G_{0+1}$ decreased, more cells were present in the S and, in particular, in $G_2$ phases of the cell cycle (**Figure 2a**). In addition, the number of apoptotic (hypodiploid) cells increased to 19 ± 5% ($P < 0.05$). The percentage of cells in the respective cell cycle phases was quantified at different concentrations of the extract (**Figure 2b**). At the highest concentrations tested, 30 ± 3% of cells were in $G_2$ compared to 14 ± 2% in controls (significant at $P < 0.05$). This rather strong cytostatic property of the

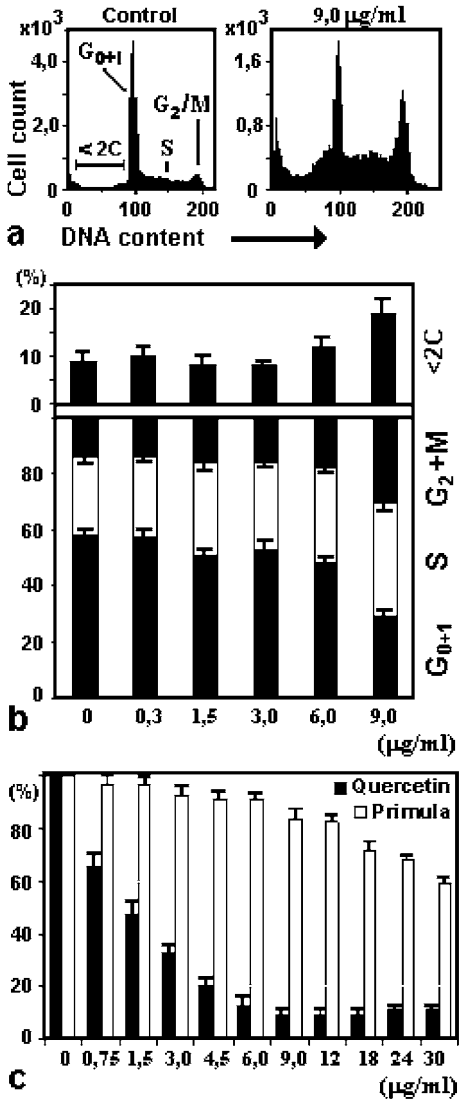


**Figure 2.** Toxicity and free radical (DPPH) scavenger activity of total epicuticular flavone from the leaves of *P. denticulata:* (**a**) example of DNA distribution of HL-60 cells before (left) and 24 h after treatment with 80 $\mu$g of *P. denticulata* exudate (right); (**b**) cell cycle effect of HL60 cells 24 h after treatment with *P. denticulata* exudates (dose dependence) (data represent the arithmetic mean ± SD of four separate experiments); (**c**) reduction of DPPH by epicuticular *Primula* flavones and quercetin (reference compound) in comparison to untreated cells (=100%) (data represent the arithmetic mean ± SD of four separate experiments).

*Primula* extract prompted us to initiate a thorough study using isolated compounds from the extract.

The radical scavenging activity of the flavonoid exudate of *P. denticulata* was quantified using the DPPH assay. At a concentration of 12−30 $\mu$g/mL a significant protective effect was recorded (**Figure 2c**). However, in comparison to quercetin, which served as positive control with known antioxidative properties, the effect was weak. Under the chosen experimental conditions quercetin had a significant antioxidative effect at the lowest concentration tested (0.75 $\mu$g/mL). However, because the *Primula* exudate consists of several compounds, we cannot exclude the possibility that single compounds have considerable antioxidative capacity but others do not. This question will be addressed below using isolated compounds.

**Effects of Isolated Flavones.** *Cell Cycle Arrest and Apoptosis.* Five representative members of the flavones (**Figure 1**) that are present in the farinose exudate of *Primula* leaves and inflorescences were examined with respect to their biological effects on exponentially growing HL-60 cells. Cell cultures were exposed to different flavones for 24 h at a concentration range of 5−80 $\mu$M (**Figure 3a**). All tested substances affected cell cycle progression to different degrees. In the case of the most active compounds, 5,8-DF and quercetin, the highest concentration tested was 40 $\mu$M because at higher concentrations a large fraction of cells became apoptotic.

The cell cycle of asynchronously proliferating HL-60 cells was easily disturbed even at low concentrations of the tested flavonoids. Whereas 14 ± 2% of the cells in control cultures were in the $G_2$/M phase, significantly more cells ($P < 0.05$) were present in this phase of the cell cycle after exposure to 5 $\mu$M concentration of the respective compounds; the percentage of cells in the $G_2$/M phase reached 24 ± 3% (flavone), 23 ± 2% (5-HF), 25 ± 2% (5,2′-DF), and 28 ± 3% (5,8-DF, 5 $\mu$M). The comparatively strong reaction is best illustrated by comparison with the effect of quercetin, a well-known cytostatic compound. In parallel experiments with this flavonoid 28 ± 2% were in the $G_2$/M phase at 10 $\mu$M concentration. When the concentration of the flavones was increased (>20 $\mu$M), the cell cycle was lengthened at early S stage and the fraction of cells in the S phase strongly increased. For example, at a concentration of 80 $\mu$M the percentage of cells in the S phase reached 59 ± 3% (flavone), 62 ± 4% (2′-HF), 53 ± 3% (5-HF), and 60 ± 2% (5,2′-DF) compared to 39 ± 3% in untreated control cultures. At the same time such a high concentration resulted in increased apoptosis, although the flavones were clearly less toxic than the reference compound quercetin (**Figure 3a**).

Changes in the cell cycle distribution during 30 h following treatment with different flavonoids (20 $\mu$M) are shown in **Figure 3b**. After 6 h of incubation, the cell cycle of the exposed cultures showed remarkable changes and cells accumulated at the $G_2$+M phase. Cells seem to be relieved from the block later because samples analyzed after 6 h typically showed an increasing fraction of cells in the S phase. The effect of quercetin was longer lasting than that of flavones. Interestingly, 12 h later a maximum of apoptosis was observed and in the following the cell cycle changes became smaller; at 30 h the phase distribution resembled that of control cultures (0 h). In this single-parameter analysis (DNA content) no distinction can be made between proliferating and arrested cells.

However, these two cell populations can be studied by two-parameter analysis. The identification of proliferating and cell cycle arrested cell populations is possible by labeling with CFSE. The technique allows the dynamics of proliferation for several cell cycles to be monitored and quantified (*25*) due to the fact that the CFSE fluorescence is roughly halved at every division (*17*, *18*). The reduction of CFSE fluorescence of cycling cells after 2 days of culture is illustrated in **Figure 4a**. The control population (labeled "0" division) divides one to three times during the 48 h culture period. Among cells of the same generation $G_2$ cells fluoresce most strongly due to their larger size compared to $G_1$ cells. The populations of apoptotic (hypodiploid) and cell cycle arrested cells with high CFSE content can clearly be defined as indicated in **Figure 4a**.

Quantification of the respective cell populations is shown in **Figure 4b**. The data represent the means of five cultures consisting of 100000 cells each treated in the same way. About 90 ± 4% of the cells in control cultures (treated with 0.1% DMSO) divided two times within 2 days of culture. The remaining cells were not cycling (6 ± 3%) or became apoptotic (4 ± 2%). The percentage of hypodiploid cells increased after 2 days of culture with the tested flavonoids (except flavone) at

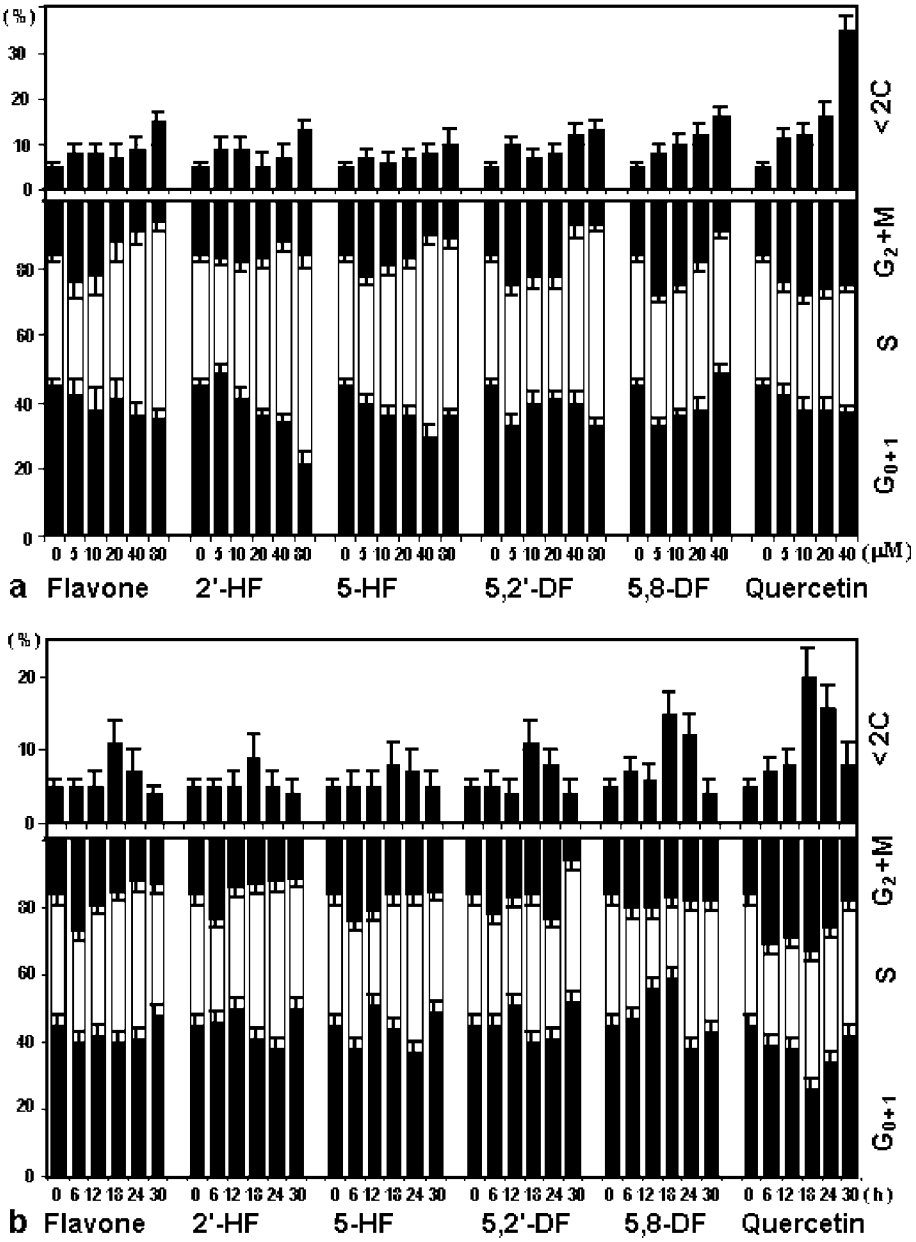


**Figure 3.** Cell cycle effect of flavones from *P. denticulata* and quercetin as a reference compound: (**a**) dose-dependent effect of flavonoids on the cell cycle progression and apoptosis 24 h after treatment (data represent the arithmetic mean ± SD of six separate experiments); (**b**) time dependence of effect after exposure to different flavonoids (20 $\mu$M) (data represent the arithmetic mean ± SD of four separate experiments).

a concentration of 20 $\mu$M. The strongest effect was observed for 5,2′-DF (28 ± 2% hypodiploid cells). The population of noncycling cells was largest in the cases of 5,8-DF (31 ± 3%) and quercetin (59 ± 3%). An interesting difference between flavone, 2′-HF, 5-HF, and 5,2′-DF, on the one hand, and 5,8-DF and quercetin, on the other hand, became apparent after 2 days of culture. Whereas the cell cycle arrested cells in the samples treated with 5,8-DF (or quercetin) never re-entered the cell cycle, the cultures treated with the other flavonoids showed only temporary effects and permanently arrested cells were not seen (data not shown).

*Radical Scavenging Activity.* Because of the medically relevant antioxidative property of many flavonoids, we studied the ROS scavenging activity of the flavone derivatives and included quercetin as positive control. Quercetin is a known scavenger of hydroxyl, peroxyl, and superoxide radicals (*26*−*28*). We used the cell permeant indicator DCF (see Materials and Methods for details), which after reaction with ROS becomes fluorescent and can be quantified by flow cytometry. The mean intensity of control cells was set to 100%, and deviation in the experimental samples was calculated with reference to the control. The data obtained with the selected flavonoids are shown in **Figure 5a**.

Exposure of HL-60 cells to 5,2′-DF, 5,8-DF< and quercetin (20 $\mu$M) for 60 min reduced the DCF fluorescence significantly ($P < 0.05$) to 70 ± 10, 58 ± 12, and 48 ± 6% of control level. Flavone, 2′-HF, and 5-HF showed no significant effect in this test system.

*Reduction of Mitochondrial Transmembrane Potential.* Changes in the mitochondrial membrane potential ($\Delta\Psi$m) can be reliably measured using the fluorescent indicator dye JC-1. A drop in the potential results in attenuated or abolished ATP production (*13*) and can, in some cases, indicate the initiation of an apoptotic process. In a series of experiments we treated HL-60 cells with each of the selected flavonoids (20 $\mu$M). For each sample 50000 cells were stained with JC-1 and analyzed by

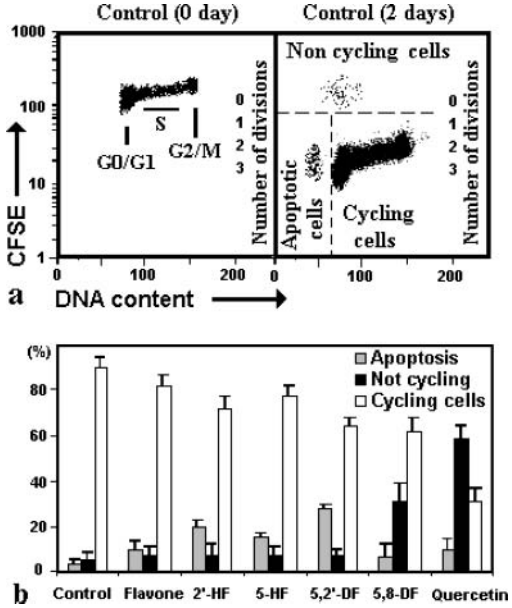


**Figure 4.** Effect of flavonoids from *P. denticulata* and quercetin on cell proliferation. (**a**) Asynchronously proliferating HL-60 cells were stained with CFSE (division 0). After 1 h and 2 days (left and right graphs, respectively), the cells were stained with PI and the fluorescence was analyzed in two-dimensional plots. The different cell generations labeled 1–3 can be distinguished on the basis of their CFSE contents. Cycling and arrested (noncycling and apoptotic) cells can be distinguished and the dynamics of proliferation determined in each sample. (**b**) Percentage of cycling, noncycling, and apoptotic cells was determined 20 days after treatment with the compounds (20 $\mu$M). Data represent the arithmetic mean ± SD of six separate experiments.

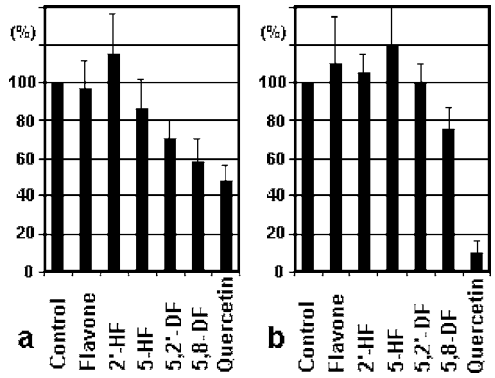


**Figure 5.** Analysis of ROS and $\Delta\Psi$m by flow cytometry. (**a**) The fluorescent probe DCF was used to measure the level of ROS in HL-60 cell cultures. The cells were treated for 60 min with the indicated flavonoids (20 $\mu$M). The fluorescence level in the cultures was determined with reference to the controls (=1) by flow cytometry. Data represent the arithmetic mean ± SD of three separate experiments. (**b**) $\Delta\Psi$m was assayed with the dye JC-1, which fluoresces red when the mitochondrial membranes are normally polarized and green at low potential. Quantitative data on the induced effects on the mitochondrial membrane potential were calculated by the intensity ratio of red to green fluorescence (control = 100). Data represent the arithmetic mean ± SD of three separate experiments.

flow cytometry. Whereas a high membrane potential results in red fluorescence, a shift to green fluorescence is observed as the potential drops (see Materials and Methods).

The membrane potential $\Delta\Psi$m was quantified by calculating the ratio of the fluorescence intensity distributions of red to green fluorescence (**Figure 5b**). The control value was set to 100%. None of the flavone derivatives had a strong effect on the mitochondrial membrane potential, in contrast to quercetin. Maximal reduction of $\Delta\Psi$m was recorded for 5,8-DF (58 ± 12%). The reference flavonoid quercetin reduced $\Delta\Psi$m by 10 ± 6% with reference to control cultures.

## DISCUSSION

A variety of micronutrients from plant products have been identified as compounds with potential anticarcinogenic properties, and among these substances phenolic compounds and especially flavonoids have been the center of attention. The cytostatic properties of flavonoids have been investigated using many different cell types and cell lines (for review see ref *29*). Quercetin produces antiproliferative effects in various cancer cell lines and, for example, strongly inhibits the proliferation of gastric and colon cancer cells (*31*), leukemia T-cells (*32*), and Ehrlich ascites tumor cells (*33*). These results suggest that flavonoids, particularly quercetin, may be useful for cancer chemoprevention (*34*).

Because genetic instability is a characteristic feature of tumor cells, it is reasonable to expect that the genetic constitution of a tumor cell line determines the response to therapeutic agents. As an example, mutations in the p53 tumor suppressor gene occur with unusually high frequency in a broad spectrum of human cancers (*35*) and may have a profound effect on drug sensitivity (*36*). The concentration of p53 is normally upregulated as a response to DNA damage, irradiation, chemotherapeutic agents, or other cellular stressors. The cell may then respond by cell cycle arrest or by initiating apoptosis depending on the cell type and pattern of activated oncogenes (*37*, *38*). Because HL-60 cells are mutant with respect to the p53 gene, the results obtained in this study cannot be generalized with respect to other cancer cell lines or cells with normal p53 status (*39*).

We have shown that the farinose exudate of *P. denticulata* induces a cell cycle delay in HL-60 cells. Treatment with 9 $\mu$g/mL of leaf extracts increased the percentage of cells in the $G_2$/M phase by 30 ± 3%. This strong cytostatic effect is not mirrored by an equally effective ROS scavenging activity. However, because the exudates contain several substances at different concentrations, we cannot exclude the possibility that single minor components have a particularly high activity which is masked by other inactive compounds.

The molecular structure of flavonoids may determine not only the extent of cytostatic activity but also the cell cycle phase that is preferentially affected. For example, in human OCM-1 melanoma cells the presence of a hydroxyl group at the 3′-position of the ring B in quercetin was shown to correlate with cell cycle arrest in $G_1$, whereas its absence in apigenin correlated with $G_2$/M block (*30*, *40*). Our analysis reveals further interesting properties of the flavonoids with respect to different concentrations and the time course of cell cycle effects. A block in the $G_2$/M phase of the cell cycle was typically observed at low concentrations (20 $\mu$M). At higher concentrations a larger fraction of cells were present in the S phase. This concentration-dependent shift is best illustrated in the case of flavone (**Figure 3a**), and a similar block at post-$G_1$ phase was also observed after human colon carcinoma cells were treated for 24 h with 150 $\mu$M flavone (*41*).

The same phenomenon can apparently be induced by exposing cells to hyperthermia and/or radiation. Under these stress conditions a cell cycle arrest between $G_1$ and $G_2$ was observed in HL-60 cells (*43*), which may formally be described as a block in the S phase, but DNA synthesis may not occur as shown in

other p53-deficient human cells such as melanoma cells (MeWo) and squamous carcinoma (4451) or glioma (U87) cells (*42*). However, cells with an intact p53 gene such as melanoma (Be11), carcinoma (4197), or glioma (EA14) cells typically show cell cycle arrest in the $G_1$ phase after being exposed to the stress conditions (*42*, *44*).

Analysis of the time course of the cell cycle effects over 30 h at 20 $\mu$M concentration reveals an interesting phenomenon. As early as 6 h after the exposure to the flavonoids, the $G_2$/M block was noticeable with all tested compounds. Because the accumulation in this cell cycle phase takes some hours, considering a doubling time of ~25 h, the flavonoids seem to act immediately on the cells without any delay. However, the effect does not seem to last long. While the block is relieved, the fraction of apoptotic cells increases ~12 h later (i.e., 18 h after the beginning of the experiment; **Figure 3b**). This reaction pattern suggests that, first, the flavonoids exert their effect instantaneously and lose their activity successively and, second, the cell cycle arrest at $G_2$/M at 20 $\mu$M concentration (and below) apparently leads to apoptosis with a 12 h delay.

The HL-60 cell line is a good model system to study the effects of flavonoinds on ROS production and the biological consequences because of significant ROS production in control cultures and because the ROS level can easily be changed experimentally (*23*). Not only may flavonoids act directly as ROS scavengers due to their low redox potentials or by way of their metal-chelating activity but, in addition, enzymes may become inhibited that are involved in ROS production or homeostasis. Examples for the inhibitory effects include enzymes such as xanthine oxidase (*45*), protein kinase C (*46*), and also cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione *S*-transferase, mitochondrial succinoxidase, and NADH oxidase, all involved in ROS generation (*47*, *48*).

The structural features of flavonoids determine their antioxidant properties and among the identified structural properties most relevant for the present discussion are an OH group in position 3 of the C-ring and the number of OH groups substituted in A- and B-rings (*49*). While quercetin possesses both of these properties, the flavones tested here do not. Among the tested flavonoids the number of OH groups varied over a wide range: quercetin has five OH groups, whereas flavone has no hydroxyl group at all. The relative level of decreasing scavenger activity of flavonoids from quercetin to 5,8-DF, 5,2′-DF, 5-HF, 2′-HF, and flavone suggested a possible correlation of hydroxyl groups number with reduced scavenger activity of investigated flavones.

Although the structural requirements of flavonoids for their radical scavenger activity have been studied in detail (reviewed in refs *47* and *49*−*51*), the molecular targets for the observed cell cycle effects are ill-defined and further work needs to address this problem. In some cases a correlation between the antioxidant activity of flavonoids and their cytostatic properties has been reported (*52*), but the situation is clearly complex and molecular studies on specific target molecules involved in the regulation of the cell cycle are called for to unravel the complex cellular reactions to flavonoids.

## ABBREVIATIONS USED

5-HF, 5-hydroxyflavone; 2′-HF, 2′-hydroxyflavone; 5,2′-DF, 5,2′-dihydroxyflavone; 5,8-DF, 5,8-dihydroxyflavone; ATP, adenosine 5′-triphosphate; CFSE, carboxyfluorescein succinimidyl ester; DCF, 2′,7′-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; HL-60, human acute myeloid leukemia; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; NADH, nicotinamide adenine dinucleotide phosphate; PI, propidium iodide; ROS, reactive oxygen species; TLC, thin-layer chromatography; $\Delta\Psi$m, mitochondrial inner transmembrane potential.

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