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Investigation of Anti-Proliferative Effects of Natural Products Quercetin Hydrate and Catechin Hydrate on Leukemia Lymphocytes

DONIKA IVANOVA[1]*, ZVEZDELINA YANEVA[1], DESSISLAVA LAZAROVA[2]
[1]Trakia University, Faculty of Veterinary Medicine, Department of Pharmacology, Animal Physiology and Physiological Chemistry, Chemistry Unit, Students campus, 6000 Stara Zagora, Bulgaria
[2]Sofia University "St. Kliment Ohridski", Faculty of Medicine, Department of Physics, Biophysics and Roentgenology, 1 Koziak Str., 1407 Sofia, Bulgaria

Abstract. *The major problem of conventional cancer therapy is lack of selectivity and induction of harmful side-effects on normal (healthy) cells and tissues. In the recent years, scientific efforts are focused to find a proper approach for highly selective influence on cell viability, as well as induction of cell death in cancer cells only. In this regard, natural herbal products are of great interest due to their low cytotoxicity to normal cells and tissues and their potential as supplements to conventional chemotherapeutics. It is well known that flavonoids exhibit various biological activities, such as anti-oxidative, anti-bacterial, anti-inflammatory, anti-viral and anti-cancer, and may play a role in cancer prevention. In the present study, the effects of low concentrations of quercetin hydrate and catechin hydrate on cell viability of leukemia lymphocytes were investigated, in order to provide an experimental basis for their future incorporation into newly-synthesized biocompatible nano-formulations.*

Keywords*: cancer, natural products, quercetin hydrate, catechin hydrate, cell viability*

1.Introduction

It is well known that the efficacy of conventional anti-cancer drugs and their side-effects are a result of disruption of cellular redox homeostasis and induction of oxidative stress [1, 2]. Our previous studies have shown that natural compounds in combination with chemotherapeutics increase selectivity, display synergistic cytotoxic effect and induce apoptosis in cancer cells only. At the same time, no side-effect has been observed on normal (healthy) cells, after exposure to the combination treatment [3-6].

Flavonoids (including flavonols, flavones, flavanones, etc.), are a group of natural substances with different phenolic chemical structures that attract scientific and therapeutic interest. These compounds are widely distributed in nature and have diverse multiple biological activities, including antioxidant, anti-microbial, anti-inflammatory, anti-viral, anti-allergic and anticancer [7-9]. Over 4,000 flavonoids have been identified, most of which are found in fruits, vegetables and beverages [9]. Epidemiological studies have demonstrated that the daily consumption of natural foods (vegetables, fruits, and tea), containing flavonoids, correlates with a decreased risk of cancer [10].

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) is one of the main representatives of flavonoid subclass of flavonols [9]. As a chemotherapeutic agent, quercetin has been widely studied due to its beneficial effects on several cancer models [11]. There are also a large number of studies on the bioactivity and health benefits of naturally derived catechins. For example, the natural product catechin (flavan-3-ol monomer) has been reported to scavenge free radicals, reduce the rate of LDL oxidation, inhibit lipid peroxidation, and participate in the modulation of immune response in biological systems [12-17].

Studies published so far about the anticancer effects of flavonoids in leukemia cells are focused on their application at relatively high concentrations. The aim of our study was to evaluate the anti-proliferative effects of quercetin hydrate and catechin hydrate applied alone on leukemia lymphocytes in low/tolerable concentrations in order to be used as a slow-released matrix, incorporated into micro- and nanocarriers.

**email: donika_georgiewa@abv.bg*

In addition, low/tolerable concentrations of quercetin and catechin allow detection of synergistic, additive and antagonistic effects in combination with conventional anticancer drugs.

2. Materials and methods

UV/Vis spectrophotometry

Catechin hydrate and quercetin hydrate spectral characteristics were determined by an adapted UV-Vis spectrophotometric methodologies [18,19] using DR 5000 UV-Vis Spectrophotometer (Hach Lange, Germany), supplied with 10 mm quartz cuvette cells. All spectra were recorded in the UV region at λ = 280 nm and λ = 373 nm, respectively, with 2 nm slit width, 900 nm min^{-1} scan speed and very high smoothing.

Cells and treatment protocol

The experiments were performed on leukemia lymphocytes (Jurkat; RIKEN Bioresource Center, Saitama, Japan), derived from patients with acute lymphoblastic leukemia. The cells were cultured in RPMI-1640 medium (Sigma-Aldrich, Steinheim, Germany), supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Auckland, New Zealand) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (Gibco) in a humidified atmosphere at 37°C with 5% CO_2. All cells were collected by centrifugation (1000 × g for 10 min) and replaced in a fresh medium without antibiotics, before treatment. Cells were in a logarithmic phase. Quercetin hydrate and catechin hydrate (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO; suitable for cell cultures; Sigma-Aldrich). The final concentration of DMSO in the cell suspension did not exceed 1%. At this concentration, DMSO did not influence cell viability. One hundred μL of the sell suspension (containing 1×10^5 cells) were placed in 96-well plates and incubated with quercetin hydrate and catechin hydrate at the following concentrations: 0.5, 1, 10, 20, and 50 μM for 24-, 48-, and 72h.

Cell proliferation and viability assays

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich) was used for detection of cell viability and proliferation. Fifty μL of MTT was added to 50 μL of cells in fresh medium (placed in 96-well plates) and incubated at 37°C for 1h. After incubation, 150 μL of MTT solvent was added to each well and incubated for 15 min in dark with shaking to fully dissolve MTT formazan. The absorbance at 590 nm was recorded immediately after that, using a microplate reader (TECAN Infinite® M1000, Austria).

Statistical analysis

All results are expressed as mean ± standard deviation from two independent experiments with three parallel samples for each experiment (n=6). Comparisons between the groups were performed using Student's *t*-test. A value of $p < 0.05$ was considered significant.

3. Results and discussions

The standard UV/Vis calibration curves of catechin hydrate and quercetin hydrate at *p*H = 4 are presented in Figure 1.

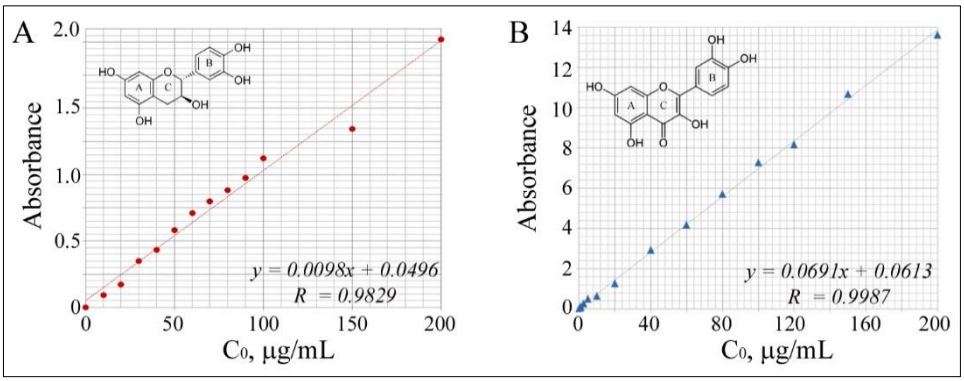

Figure 1. Standard calibration curves of: (A) catechin hydrate at λ = 280 nm; (B) quercetin hydrate at λ = 373 nm; measured at *p*H = 4

Cells were incubated with different concentrations of quercetin hydrate and catechin hydrate. The cell viability was measured after 24, 48, and 72h of incubation, using MTT assay. The effect of each concentration was calculated as a percentage of control (untreated cells), where the proliferation activity of the cells was considered 100%. The aim of this study was to select the optimal concentration of quercetin hydrate and catechin hydrate, which induce about 20-30% cytotoxicity on leukemia lymphocytes after 48h incubation time. These levels of cytotoxicity will enable future assessment of the synergistic, additive or antagonistic effect, after their combined application with chitosan carriers.

The data on Figure 2 represent the anti-proliferative effects of the two flavonoids on leukemia lymphocytes. At concentrations up to 20 µM, quercetin manifested a poor anti-proliferative effect even at 72h of incubation (Figure 2A). However, at 50 µM this flavonoid suppressed significantly the proliferation activity of leukemia lymphocytes - about 50 and 60% at 48 and 72h of incubation, respectively (Figure 2A). At the same doses and experimental conditions, catechin did not affect significantly the viability of leukemia lymphocytes (Figure 2B).

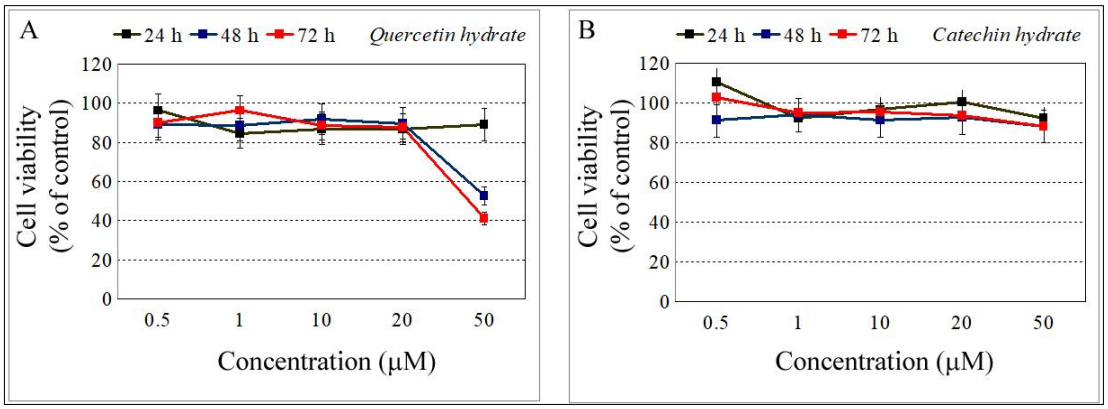

Figure 2. Anti-proliferative effect of quercetin hydrate (A) and catechin hydrate (B) on leukemia lymphocytes (Jurkat), analyzed by MTT assay at different incubation times. The data are mean±SD from six values at each concentration

Our data confirm the effects of the two flavonoids published by other authors. For example, Ren et al. have demonstrated a time-dependent inhibitory effect of high concentrations of quercetin (from 12 to 30 mg/mL) on ovarian cancer cell growth (SKOV 3 cells) [20]. Amorim et al. have reported that quercetin (at concentrations up to 200 µM) inhibits viability of colon cancer cells HCT-15 and RKO at 24h of incubation [21]. Another study conducted on breast cancer and ovarian cancer cell lines has demonstrated that quercetin inhibits their proliferation [22]. The reduction of cell viability and growth of B-lymphoma cells (BC3, BCBL1 and BC1) by quercetin has been observed, when it was applied in wide range of concentrations (from 12 to 100 µM for 24h of incubation). At the same time, no cytotoxic

effect in normal B-lymphocytes is detected [23]. Similar results about the anti-proliferative effect of quercetin at physiologically relevant concentration (about 10 μM for 4 days of incubation) on breast cancer cell lines (SK-Br3 and MDA-MB-453) have been observed [24].

The cytotoxic activity of different catechins, including epigallocatechin-3-gallate, catechin, on human cervix adenocarcinoma cells (SiHa cells) had been analyzed by Payen et al., using MTT assay. The authors have studies a wide concentration range of catechins (from 100 nM to 100 μM) for 16h of incubation. Their data indicate a significant cytotoxic activity of epigallocatechin-3-gallate at dose ≥ 10 μM than other studied compounds at the same conditions. The significant reduction of MTT by catechin was observed at concentration 100 μM [25]. Kürbitz et al. have investigated anticancer effects of epigallocatechin gallate on human pancreatic ductal adenocarcinoma (PDAC) cells, in comparison with the effects of two minor components of green tea catechins, catechin gallate and epicatechin gallate, at concentration range from 20 μM to 80 μM for 24 and 72h of incubation. Their experimental data indicate that the three catechins inhibit the proliferation of PDAC cell lines (PancTu-I, Panc1, Panc89 and BxPC3) in a dose- and time-dependent manner [26].

The data in Figure 3 represents a comparative analysis of cytotoxic activity of both flavonoids used in our study, after 48h of incubation. Quercetin shows a superior activity compared to catechin. The effect of quercetin is dose-dependent, while the effect of catechin is not dose-dependent in the selected concentration interval (0.5 - 50 μM). Both flavonoids have similar chemical structures with a slight difference at B-ring and C-ring (Figure 1). This structural difference seems crucial for the anti-proliferative effect of these two flavonoids.

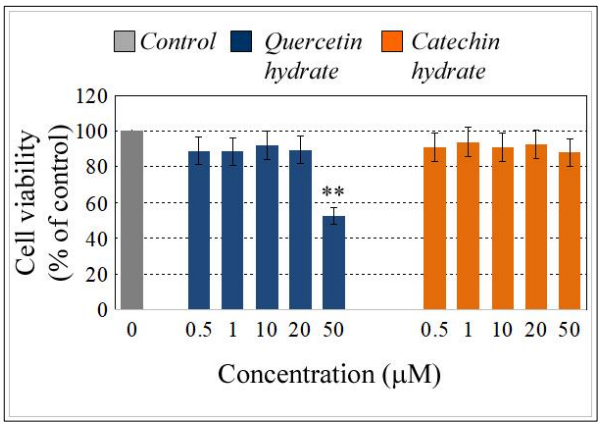

Figure 3. Comparative analysis of anti-proliferative activity of quercetin hydrate and catechin hydrate on leukemia lymphocytes (Jurkat) after 48h of incubation – comparative analysis. The data are mean±SD from six values at each concentration **p<0.01 versus control grou (untreated cells)

The differences between the cytotoxic effect of quercetin hydrate and catechin hydrate could be due to different molecular mechanism of action of these compounds. It is generally accepted that quercetin hydrate induces a direct induction of cell cycle arrest on cancer cells and/or initiation of cancer cell death [27, 28], while the mechanism of catechin hydrate is linked to the modulation of immune response [12-17]. Recent article shows that anticancer effects of quercetin are due to modulation of PI3K/Akt/mTOR, Wnt/-catenin, and MAPK/ERK1/2 activities [29]. Studies conducted on nasopharyngeal carcinoma cell lines [30] and human breast cancer cell lines [24, 31] have shown that quercetin causes cell cycle arrest at the G2/M or G1 phases. At low doses (0 - 10 μM for 4 days incubation), quercetin leads to mild cytotoxic effect and cell cycle arrest at the G1 phase. This anti-proliferative effect of quercetin is mediated by down-regulation of B1 and cyclin-dependent kinase 1 (CDK1), essential components of G2/Mcell cycle progression, as well as by induction of phosphorylation of the retinoblastoma tumor suppressor protein, pRb [24]. Hypophosphorylated Rb binds to and sequesters the transcription factor E2F1, which is essential for the expression of cell proliferation-associated genes, resulting in cell cycle arrest at the G1 phase [32]. Experiments conducted on SKOV-3 ovarian cancer cells [33], as well as on MCF-7 breast cancer cells [34] indicate that at high concentrations (50-130 μM) quercetin inhibits cell cycle progression from G0/G1 to G2/M phase at time- and dose-dependent manner due to increased levels of pro-apoptotic biomarker surviving [33, 34]. On the other hand, quercetin is implicated to the

modulation of cellular redox status due to increased reactive oxygen species (ROS) generation and induction of DNA damage. Metodiewa et al. have demonstrated that peroxidase-catalyzed one electron oxidation of quercetin leads to production of quercetin radical [35]. These quercetin radicals undergo redox cycling with production of ROS and subsequent oxidative DNA damages [35].

$$\text{Quercetin-OH} + RO_2^{\cdot} \rightarrow \text{Quercetin-O}^{\cdot} + RO_2H$$

It is proposed that the anti-inflammatory and immunomodulating activity of flavonoids including catechin hydrate is due to regulation of NF-kB signaling (nuclear factor kappa-light-chain-enhancer of activated B cells). In this context, T-cell activation is an important step in the initiation of immune response [12]. It is widely accepted that in norm, T cells do not contain constitutive levels of IL-2 [36]. But up-regulation of transcription factors, such as NF-κB and NF-AT (nuclear factor of activated T-cells), leads to transcription and secretion of IL-2 [37, 38]. Recent studies have noticed that the regulation of IL-2 at the level of transcription is critically involved in the control of T-cell expansion and native immune response [36, 39]. Some flavonoids, including catechin, modulate phorbol 12-myristate 13-acetate (PMA)-induced NF-κB activation in Jurkat T-cells. It has been found that pre-incubation of leukemia T-lymphocytes for 24h with catechin at concentrations from 1.7 μM to 17.2 μM decreases PMA-induced NF-κB binding activity through a direct interaction of flavonoid with NF-kB proteins and subsequent inhibition of NF-κB-driven IL-2 gene [12]. Thus, catechin can influence the immune response by modulating NF-κB activation. Kürbitz et al. have also established that epicatechin gallate inhibits TNFα-induced activation of NF-kB and a consequence suppresses the secretion of pro-inflammatory and invasion promoting proteins like IL-8 and urokinase plasminogen activator (uPA) [26].

4. Conclusions

In summary, our data provide evidence that quercetin hydrate at low/tolerable concentration of 50 μM manifests a good anti-proliferative activity on leukemia lymphocytes, while catechin has poor anti-proliferative effect at the same experimental conditions. However, catechin is known as immuno-modulator, which suggests that it has a potential for in vivo application. Therefore, both flavonoids are suitable for incorporation in chitosan carriers to increase their efficacy as supplements in anticancer therapy. The anticancer and anti-inflammatory effects of these flavonoids, incorporated in chitosan micro- and nanoparticles, need to be evaluated on different cancer cell lines in our forthcoming study.

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