Determination of free radical scavenging activity of quercetin, rutin, luteolin and apigenin in H_2O_2-treated human ML cells K562*

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We investigated protective effects of four flavonoids against H_2O_2-induced DNA damage in human myelogenous leukemia cells (K562) using the comet assay. The structural difference of studied flavonoids – quercetin, rutin, luteolin and apigenin – are characterized by the number of hydroxyl groups on the B ring. The presence of an o-dihydroxy structure on the B-ring confers a higher degree of stability to the flavonoid phenoxyl radicals by participating in electron delocalization and is, therefore, an important determinant for antioxidative potential. The results correlate with earlier published data obtained in murine leukemia cell line L1210. Hydrogen peroxide induced in human K562 cells a concentration-dependent increase of single cell DNA strand breaks. The strongest inhibition against H_2O_2-induced DNA damage (44%, 42%) was found in a range of luteolin and quercetin concentrations of 20–100 μmol/l. Protective effect of rutin (100 and 1000 μmol/l) was only marginal (8–10%). Apigenin had no protective effect on DNA single strand breaks induced by H_2O_2. Luteolin and quercetin are therefore effective in the protection of human single cell DNA from oxidative attack.

Key words: q*uercetin, rutin, apigenin, luteolin, cytotoxicity, comet assay*

Flavonoids represent a large group of naturally occurring polyphenols with a wide range of chemical structures and pharmacological activities. To date, over 5000 flavonoids have been identified in the leaves, seeds, bark and flowers of plants. The antioxidant (free radical-scavenging) properties of the most of flavonoids may contribute to chemopreventive effect [11]. The results of the *in vitro* studies have shown that flavonoids can either inhibit or induce a large variety of mammalian enzyme systems [9].

Two flavonols, quercetin (QU) and rutin (RU), and two flavones, apigenin (AP) and luteolin (LU) (Fig. 1) have attracted attention several years ago and they are being still studied very intensively because of their potential in the prevention of cardiovascular diseases and cancer in humans [26]. Characteristics of these natural compounds were described in details in our previous paper [10].

QU (5,7,3',4'-tetrahydroxyflavonol) is one of the most common natural flavonoids occurring mainly in glycosidic forms such as RU (3-rhamnosyl-glucosyl-quercetin). QU and RU act as powerful antioxidants preventing oxidation of low-density lipoproteins. QU and its glycosides have been reported to exert numerous biochemical and pharmacological activities, such as free radical scavenging effects on immunity [2, 12], on inflammatory cell functions [18, 19], and even antitumor effects [1, 13]. QU inhibited the growth and viability of various acute myelogenous leukemia (AML) cell lines and AML blasts freshly isolated from patients with AML of various subtypes. The effects on inhibition of proliferation and decreased viability were also significant in normal CD34+ cells isolated from normal marrow donors [16]. AP (5,7,4'-trihydroxyflavone) is a flavonoid considered to have a bioactive effect on human health as antioxidant, radical scavenger, antiinflammatory, carbohydrate metabolism promoter, immunity system modulator. Results acquired by NAKAZAWA et al indicate the antidepressant properties of AP, which may be mediated by the dopaminergic mechanisms in the mouse brain [21]. AP inhibits endothelial-cell proliferation in G(2)/M [30].

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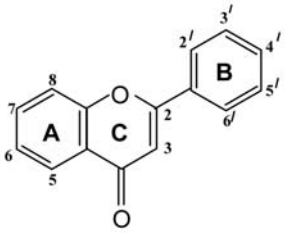

	3	5	7	3′	4′
Quercetin	OH	OH	OH	OH	OH
Rutin	Rham*	OH	OH	OH	OH
Luteolin	H	OH	OH	OH	OH
Apigenin	H	OH	OH	H	OH

* Rham - rhamnosyl-glucosyl

Figure 1. Chemical structures of flavonoids tested.

The cancer chemopreventive agent apigenin also has strong cytostatic and anti-angiogenic effects *in vitro* [6]. LU (5,7,3',4'-tetrahydroxyflavone) in the low micromolar range inhibits the proliferation of normal and tumor cells, as well as *in vitro* angiogenesis. Inhibition of extensive neovascularization can contribute to prevention of certain chronic diseases, including solid malignancies. Luteolin shows strong antiproliferative activity against different human cancer cell lines (in some hormone-dependent cancer lines such as breast, prostate, and thyroid cancer for instance) [8, 24] and has strong scavenging properties for superoxide radicals [5].

We reported previously that in H_2O_2-treated murine leukemia L1210 cells quercetin and luteolin possessed high protective effect, while apigenin and rutin had only marginal or no effect [10]. In the present study we have examined the protective effects of these compounds against H_2O_2-induced DNA damage in human myelogenous leukemia cells (K562) using the comet assay. Cytotoxic effects of QU, RU, AP and LU on human ML cells K562 were determined by the trypan blue exclusion technique.

Material and methods

Cell culture. The human myelogenous leukemia cell line (K562) was obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). K562 cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FCS and antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2: 95% air. All *in vitro* experiments were performed during the exponential phase of cell growth.

Chemicals. Na_2EDTA, NaOH, H_2O_2 and NaCl were purchased from Lachema Brno, Czech Republic. QU, RU, LU, AP, ethidium bromide, Triton X-100 and agarose electrophoresis reagent were obtained from Sigma-Aldrich Chemie, Steinheim, Germany. Tris(hydroxymethyl)-aminomethane was obtained from Serva Feinbiochemica, Heidelberg, Germany. Trypan blue solution (0.4%) was obtained from Fluka Chemie AG, Switzerland. Phosphate-buffered saline (PBS) was obtained from Sebak GmbH, Germany. Agarose II, RPMI (1640) medium without L-glutamine and $NaHCO_3$ was bought from Amresco-Biotechnology Grade, USA. Fetal calf serum (FCS) was purchased from Grand Island Biological Co., Grand Island, NY, USA.

Cytotoxicity assay. Cells were incubated with different concentrations of flavonoids for 30 min at 37 °C in a dark incubator together with untreated control samples. Samples were then centrifuged at 1000 x g for 5 min at 25 °C. After pretreatment, the cells were washed with PBS and again centrifuged at 1000 x g for 5 min at 25 °C. Cells were stained with the 0.4% trypan blue solution (TB) and the number of viable (uncolored) and dead (colored) cells was counted. The ratio of the number of viable cells to all cells gives the percentage of viable cells.

Single cell gel electrophoresis (the comet assay). The procedure of SINGH et al [27] modified by SLAMEŇOVÁ et al [28] was followed. Briefly: human myelogenous leukemia cells K562 were treated with different concentrations of flavonoids, QU (20, 50, 100 μmol/l), RU (100, 500, 1000 μmol/l), AP (300, 600, 1200 μmol/l), LU (20, 50, 100 μmol/l), for 30 min. $2x10^4$ of treated as well as control untreated cells were suspended in LMP agarose and spread on the base layer. Triplicate slides were prepared per sample. The slides were treated with 50 μl of 100 μmol/l H_2O_2 for 5 min on ice in the dark and they were washed twice with PBS. After lysis, electrophoresis and unwinding the slides were neutralized with Tris (0.4 mol/l, pH 7.5), and stained with 20 μl ethidium bromide (1 mg/ml). Ethidium bromide-stained nucleoids were evaluated with a Zeiss Jenalumar fluorescence microscope using the computerized image analysis (Komet 3.0, Kinetic Imaging, Ltd., Liverpool, UK). The percentage of DNA in the tail was determined as it is linearly related to the frequency of DNA breaks.

Statistics. The results were statistically evaluated using the Student's t-test and a statistically significant increase: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, resp. decrease of DNA strand breaks by H_2O_2 or flavonoids: $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ was indicated in A, B, C and D parts in Figure 3.

Results

Cytotoxicity of flavonoids. Cytotoxicity of studied compounds against K562 cells was evaluated by the trypan blue exclusion technique after 30 min treatment of cells with

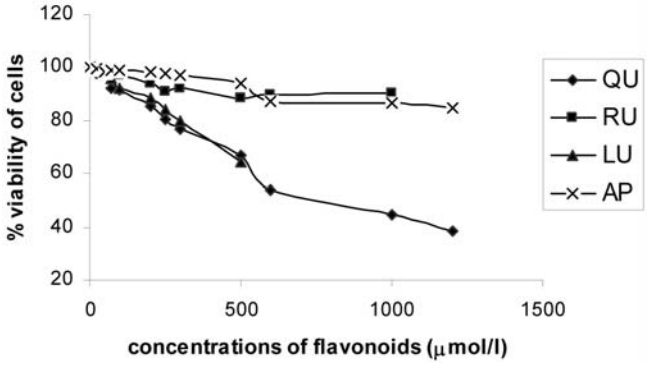

Figure 2. Percentage of viable cells after treatment of K562 cells with flavonoids evaluated by TB exclusion technique.

studied flavonoids. The results, depicted in Figure 2, showed that incubation of K562 cells in RPMI 1640 medium containing different concentrations QU (0–1200 μmol/l) and LU (0–500 μmol/l) caused cytotoxicity at the concentrations higher than 250 μmol/l. RU and AP (0–1200 μmol/l) did not have any cytotoxic effects even at substantially higher concentrations.

Effects of flavonoids on the level of single DNA strand breaks induced by H_2O_2. We measured the level of single strand DNA breaks in H_2O_2-treated K562 cells in order to test antioxidant effects of the studied flavonoids. The optimal concentration of H_2O_2 in our experiments was 100 μmol/l. The % of tail DNA corresponding to the level of DNA strand breaks at this H_2O_2 concentration, was 60. Figure 3 represents inhibitive effects of 30 min pretreatment of K562 cells with QU, LU (both 20–100 μmol/l), RU (100–1000 μmol/l) or AP (300–1200 μmol/l) in combination with a short-term treatment of cells with H_2O_2 (100 μmol/l). H_2O_2-induced DNA damage was significantly eliminated by QU and LU at the concentrations 20–100 μmol/l, while RU showed only marginal inhibition at higher concentrations (500 and 1000 μmol/l) (Fig. 3A, B, C). AP does not induce important changes in cellular DNA (Fig. 3D) at the concentration range 300–1200 μmol/l.

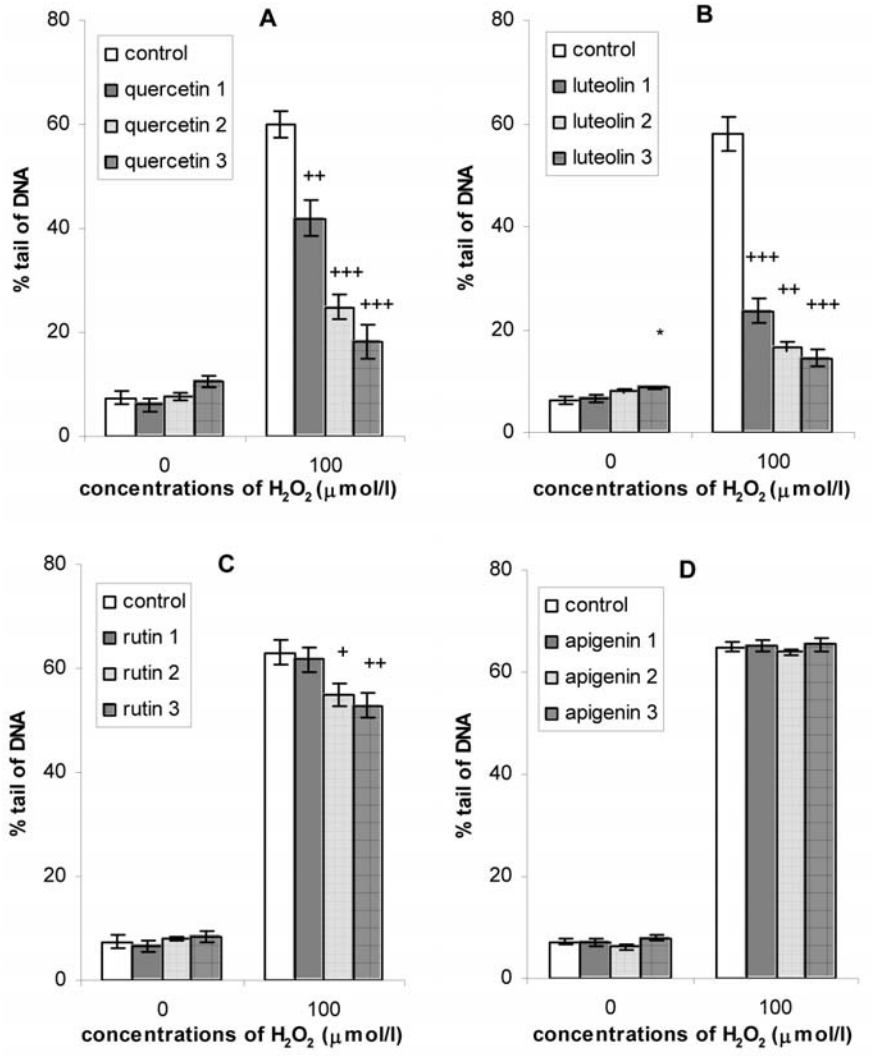

Figure 3. Incidence of DNA strand breaks in K562 cells treated with flavonoids, H_2O_2, or by combination of H_2O_2 and flavonoids. (A) quercetin 1 (20 μmol/l), quercetin 2 (50 μmol/l) and quercetin 3 (100 μmol/l), (B) luteolin 1 (20 μmol/l), luteolin 2 (50 μmol/l) and luteolin 3 (100 μmol/l), (C) rutin 1 (100 μmol/l), rutin 2 (500 μmol/l) and rutin 3 (1000 μmol/l), (D) apigenin 1 (300 μmol/l), apigenin 2 (600 μmol/l) and apigenin 3 (1200 μmol/l).

Discussion

The generation of reactive oxygen species (ROS) are implicated in the etiology of aging and diseases including cancer, multiple sclerosis, Parkinson disease, senile dementia and autoimmune diseases. H_2O_2 is mainly produced from the reaction of superoxide anion and dismutase of cells [23]. Furthermore, H_2O_2 has emerged as a pivotal molecule not only for cancer cell proliferation but also in determining the fate of cancer cells exposed to phytochemicals. H_2O_2 either promotes or inhibits the proliferation of cancer cells, and the phytochemicals either scavenge or generate H_2O_2 [17]. Structurally distinct antioxidants may partially prevent cells from degenerative damage of ROS. The structural assumptions of the flavonoids studied

are minutely referred in our previous work [10].

Many investigators worked in the past and are still working on the explanation of mechanism of antioxidant, resp. free radical scavenging effects of flavonoids [7, 20, 32]. In spite of that, inconsistent results have been obtained regarding their antioxidant and ROS scavenging properties [22]. BROWN's *in vitro* studies have demonstrated the antioxidant activities of the flavonoids as hydrogen-donating free radical scavengers and their structural dependence [4].

Biological effects of QU are attributed to its antioxidative activities in scavenging ROS [25]. However, QU can act as both antioxidant and prooxidant depending on the concentration and the source of the free radicals [14, 29]. RU is a glycoside of QU and represents the naturally occurring form of QU. RU and QU inhibited superoxide formation, hydroxyl radical formation in the Fenton reaction and lipid peroxyl radical generation. The structural difference between flavonoids studied (QU, RU, AP, LU) is in the number of hydroxyl groups on the B ring (Fig. 1). The presence of an o-dihydroxy structure on the B-ring confers a higher degree of stability to the flavonoid phenoxyl radicals by participating in electron delocalization and is, therefore, an important determinant for antioxidative potential [3]. LU dose-dependently inhibited O_2^- production by xanthine/xanthine oxidase and showed the same quenching effect of H_2O_2 as QU. Lack of 3-hydroxyl group on B ring showed that AP is much less active [31]. AP is mentioned as a less-toxic and non-mutagenic flavone with promising chemopreventive activity against skin cancer [15].

The results presented here relate to the previously reported study on protective properties of flavonoids QU, RU, AP and LU against H_2O_2 induced DNA damage of murine leukemia L1210 cells. The aim of our work was to compare their protective effect on human myelogenous leukemia K562 cells.

The cytotoxicity of QU and LU was five-fold more toxic to K562 cells compared to L1210 cells while AP and RU did not show toxicity to any of the cell lines studied [10].

The protective action against DNA damage induced by H_2O_2 of four flavonoids was followed in concentration-dependent manner. We found that LU possessed the highest DNA-protective effect among the flavonoids studied (34–44% at the concentrations 20–100 μmol/l). The free radical scavenging activity of QU was lower when compared to LU. The percentage of QU protective effect was 18% at the concentration 20 μmol/l, resp. 42% at the concentration 100 μmol/l and for RU was relatively low (8–10%) at the concentrations 500 and 1000 μmol/l in K562 cell line. AP did not protect cellular DNA against the formation of single DNA strand breaks induced by H_2O_2 under experimental conditions used.

When compared with our previous results [10], QU and LU have manifested the highest protective effects towards H_2O_2 induced damage in both leukemia cells used (L1210, K562). On the other hand, only marginal, resp. no protective effects were caused by RU on DNA single strand breaks induced by H_2O_2. AP has even affected as pro-oxidant at the rather high concentration, 1200 μmol/l, in L1210 cell line, whereas this flavonoid did not protect cellular DNA against damage by H_2O_2 in K562 cells.

The most abundant flavonoid in human diet is QU and it may play an important role in protecting murine and human DNA from oxidative attack. The results are in agreement with the structural assumptions that are responsible for scavenging activity against ROS.

References

[1] AGULLO G, GAMET L, BESSON C, DEMIGNE C, REMESY C. Quercetin exerts a preferential cytotoxic effect on active dividing colon carcinoma HT29 and Caco-2 cells. Cancer Lett 1994; 87: 55–63.

[2] ASGARY S, NADERI G, SARRAFZADEGAN N, GHASSEMI N, BOSHTAM M et al. Anti-oxidant effect of flavonoids on hemoglobin glycosylation. Pharm Acta Helv 1999; 73: 223–226.

[3] BORS W, HELLER W, MICHEL C, SARAN M. Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. Meth Enzymol 1990; 186: 343–355.

[4] BROWN JE, KHODR H, HIDER RC, RICE-EVANS CA. Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. Biochem J 1998; 330: 1173–1178.

[5] COTELLE N, BERNIER JL, CATTEAU JP, POMMERY J, WALLET JC, GAYDOU EM. Free antioxidant properties of hydroxyflavones. Radic Biol Med 1996; 20: 35–43.

[6] ENGELMANN C, BLOT E, PANIS Y, BAUER S, TROCHON V et al. Apigenin-strong cytostatic and anti-angiogenic action in vitro contrasted by lack of efficacy in vivo. Phytomedicine 2002; 9: 489–495.

[7] FERNANDEZ MT, MIRA ML, FLORÉNCIO MH, JENNINGS KR. Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. J Inorg Biochem 2002; 92: 105–111.

[8] FOTSIS T, PEPPER MS, AKTAS E, BREIT S, RASKU S et al. Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. Cancer Res 1997; 57: 2916–2921.

[9] HOLIMAN PCH, HERTOG MGL, KATAN MB. Analysis and health effects of flavonoids. Food Chemistry 1996; 57: 43–46.

[10] HORVÁTHOVÁ K, NOVOTNÝ L, VACHÁLKOVÁ A. The free radical scavenging activity of four flavonoids determined by the comet assay. Neoplasma 2003; 50: 120–124.

[11] HORVÁTHOVÁ K, VACHÁLKOVÁ A, NOVOTNÝ L. Flavonoids as the chemoprotective agents for civilization diseases. Minireview. Neoplasma 2001; 48: 435–441.

[12] KANDASWAMI C, MIDDLETON E. Free radical scavenging and antioxidant activity of plant flavonoids. Adv Exp Med Biol 1994; 366: 351–376.

[13] KAWAII S, TOMONO Y, KATASE E, OGAWA K, YANO M. Antiproliferative activity of flavonoids on several cancer cell lines. Biosci Biotechnol Biochem 1999; 63: 896–899.

[14] LAUGHTON MJ, HALLIWELL B, EVANS PJ, HOULT JR. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. Biochem Pharmacol 1989; 38: 2859–2865.

[15] LI B, ROBINSON DH, BIRT DF. Evaluation of properties of apigenin and [G-3H] apigenin and analytic method development. J Pharm Sci 1997; 86: 721–725.

[16] LIESVELD JL, ABBOUD CN, LU C, MCNAIR C, MENON A et al. Flavonoid effects on normal and leukemic cells. Leuk Res 2003; 27: 517–527.

[17] LOO G. Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation (Review). J Nutr Biochem 2003; 14: 64–73.

[18] MIDDLETON E, KANDASWAMI C, editors. Plant flavonoid modulation of immune and inflammatory cell function. In: Human Nutrition: a comprehensive treatise. New York: Plenum Press, 1993: 239–266.

[19] MIDDLETON E, KANDASWAMI C. Effects of flavonoids on immune and inflammatory cell function. Biochem Pharmacol 1992; 43: 1167–1179.

[20] MUROTA K, TERAO J. Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. Arch Biochem Biophys 2003; 417: 12–17.

[21] NAKAZAWA T, YASUDA T, UEDA J, OHSAWA K. Antidepressant-like effects of apigenin and 2,4,5-trimethoxycinnamic acid from perilla frutescens in the forced swimming test. Biol Pharm Bull 2003; 26: 474–480.

[22] NG TB, LIU F, WANG ZT. Antioxidative activity of natural products from plants. Life Sci 2000; 66: 709–723.

[23] PARK CH, SO HS, SHIN CH, BAEK SH, MOON BS et al. Quercetin protects the hydrogen peroxide-induced apoptosis via inhibition of mitochondrial dysfunction in H9c2 cardiomyoblast cells. Biochem Pharmacol 2003; 66: 1287–1295.

[24] POST JFM, VARMA RS. Growth inhibitory effects of bioflavonoids and related compounds on human leukemic CEM-C1 and CEM-C7 cells. Cancer Lett 1992; 67: 207–213.

[25] RICE-EVANS CA, MILLER NJ, PAGANGA G. Structure-antioxidant activity relationship of flavonoids and phenolic acids. Free Radic Biol Med 1996; 20: 933–956.

[26] SHEN SC, LEE WR, LIN HY, HUANG HC, KO CH et al. In vitro and in vivo inhibitory activities of rutin, wogonin, and quercetin on lipopolysacharide-induced nitric oxide and prostaglandin E_2 production. Eur J Pharmacol 2002; 446: 187–194.

[27] SINGH NP, MCCOY MT, TICE RR, SCHNEIDER EL. A simple technique for quantitation of low levels of damage in individual cells. Exp Cell Res 1988; 175: 184–191.

[28] SLAMEŇOVÁ D, GÁBELOVÁ A, RUŽEKOVÁ L, CHALUPA I, HORVÁTHOVÁ E et al. Detection of MNNG-induced DNA lesions in mammalian cells; validation of comet assay against DNA unwinding technique, alkaline elution of DNA and chromosomal aberrations. Mutat Res 1997; 383: 243–252.

[29] SUGIHARA N, ARAKAWA T, OHNISHI M, FURUNO K. Anti- and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with alpha-linolenic acid. Free Radic Biol Med 1999; 27: 1313–1323.

[30] TROCHON V, BLOT E, CYMBALISTA F, ENGELMANN C, TANG RP et al. Apigenin inhibits endothelial-cell proliferation in G(2)/M phase whereas it stimulates smooth-muscle cells by inhibiting P21 and P27 expression. Int J Cancer 2000; 85: 691–696.

[31] UDA Y, PRICE KR, WILLIAMSON G, RHODES MJC. Induction of the anticarcinogenic marker enzyme, quinone reductase, in murine hepatoma cells in vitro by flavonoids. Cancer Lett 1997; 120: 213–216.

[32] VITAGLIONE P, FOGLIANO V. Use of antioxidants to minimize the human health risk associated to mutagenic/carcinogenic heterocyclic amines in food. J Chromatogr B 2004; 802: 189–199.