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Selective Antiproliferative and Apoptotic Effects of Quercetin in Normal Versus Tumorigenic Hepatic Cell Lines

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Abstract – Quercetin is a dietary anticancer chemical that is capable of inducing apoptosis in tumor cells. However, little is known about its biological effect in nonmalignant hepatic cells. Using embryonic normal hepatic cell line (BNL CL.2) and its SV40-transformed tumorigenic cell line (BNL SV A.8), we evaluated the effects of quercetin on cell proliferation and apoptosis. As the results, our present study demonstrated that quercetin had a selective growth inhibition in normal versus tumorigenic hepatic cells such that BNL SV A.8 cells were very sensitive to the quercetin-mediated cytotoxicity. In particular, as evidenced by the increased number of positively stained cells in the TUNEL assay, the induction of characteristic nuclear DNA ladders, and the migration of many cells to sub-G1 phase in the BNL SV A.8 cells, quercetin treatment more sensitively induced apoptosis in BNL SV A.8 cells than in BNL CL.2 cells. Collectively, our findings suggest that quercetin can be approached as a potential agent that is capable of inducing selective growth inhibition and apoptosis of hepatic cancer cells.
Keywords – Quercetin, Hepatocytes, Selective growth inhibition, Apoptosis

Introduction

In recent years, there has been a global trend toward the use of natural bioactive substances as cancer chemopreventive or therapeutic agents (Pezutto, 1997; Christou *et al.*, 2001; Mukherjee *et al.*, 2001). Most of these substances exert their chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death. Therefore, cell growth arrest and apoptosis induction in tumor cells has become a prominent indicator of the tumor treatment response in employing a plant derived-bioactive substance to reduce and control human mortality due to cancer (Smets, 1994; Paschka *et al.*, 1998). However, further efficient-chemopreventive approaches are to use the biochemical differences between cancer cells and their normal counterparts. Namely, target organ specific agents that are capable of inducing selective apoptosis of cancer cells are receiving considerable attention in developing novel cancer preventive approaches (Mukherjee *et al.*, 2001).

Flavonoids are a group of naturally occurring compounds and commonly found in most plants. They have been recognized as a dietary chemopreventive agent that might block neoplastic inception or delay tumor progression (Gao *et al.*, 1999; Wong and McLean, 1999). In addition, many reports in current studies showed their biological activities affecting basic cell functions, such as proliferation, differentiation, and apoptosis (Formica and Regelson, 1995; Plaumann *et al.*, 1996; Caltagirone *et al.*, 2000). Among the flavonoids, quercetin (3,3',4,5,7-pentahydroxyflavone) is one of the most widely studied flavonoids and numerous experiments have been reported that quercetin had biological, pharmacological, and medicinal properties (Morel *et al.*, 1993; Hollman and Katan, 1999). The beneficial effects of quercetin are thought to be due to the inhibition of enzymes involved in cell cycling and its antioxidant property (Caltagirone *et al.*, 2000; Aligiannis *et al.*, 2001). Collectively, we postulated that quercetin is capable of inducing selective growth inhibition and apoptosis of cancer cells, although little is known about the effect of quercetin in nonmalignant hepatic cells. In order to elucidate this postulation, we evaluated whether quercetin has a cell specific effect in inhibiting cell growth and inducing apoptosis in normal and mouse tumorigenic hepatocytes.

Materials and Methods

Chemicals and laboratory wares – Unless otherwise specified, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) and all the laboratory wares were from Falcon Labware (Becton-

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Dickinson, Franklin Lakes, NJ). Quercetin was obtained from Sigma Chemical Co. and dissolved in dimethylsulfoxide (DMSO) prior to use, and the final concentration of DMSO did not exceed 0.1% (v/v) throughout the experiments.

Cell culture and treatment – Embryonic normal hepatic cell line, BNL CL.2 cells, and its SV40-transformed tumorigenic cell line, BNL SV A.8 cells, which are obtained from the same strain and stage of age, were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). Before quercetin treatment, cultures were switched to a fresh batch of the same medium.

Determination of DNA synthesis and cytotoxicity – The level of DNA synthesis by BNL CL.2 or BNL SV A.8 cells after quercetin treatment was measured by adding 1 μCi of [*methyl*-^{3}H] Thymidine (Amersham Pharmacia Biotech Inc., Piscataway, NJ) to each well of 96-well culture plates for the last 12 h of various culture periods. The cells were then collected with a cell harvester (Inotech Inc., Switzerland), and the tritium contents were measured using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

Cellular cytotoxicity induced by quercetin treatment was measured using trypan blue exclusion assay. Briefly, the hepatic cells were cultured in DMEM supplemented with 10% FBS in the presence of 1 to 200 μM quercetin for various times. After incubation, the cells were stained with 0.4% trypan blue and about 100 cells were counted for each treatment. Cytotoxicity was calculated as follows: % cytotoxicity = [(total cells - viable cells) / total cells×100.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay –After exposure to quercetin for various times, the hepatic cells were fixed with 1% buffered formaldehyde (pH 7.5) for 30 min on ice. The cells were then washed with PBS, resuspended in 70% ice-cold ethanol, and kept at –20°C for 1 h. The cells were rehydrated with PBS and incubated in TdT buffer containing 30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM $CoCl_2$, 0.05 mg/ml BSA, 0.1 mM DTT, 7.5 U/ml TdT, and 0.4 nmol/ml FITC-5-dUTP. After a 30-min incubation at 37°C, the reaction was blocked by transferring the cells to a buffer containing 300 mM sodium chloride, 30 mM sodium citrate, and 2% bovine serum albumin for 30 min. Finally, the cells were washed with PBS and observed under a fluorescence microscope (Axioskop 2, Carl Zeiss, Germany).

DNA fragmentation assay – After quercetin treatment, the hepatic cells were incubated with lysis buffer (1% NP-40 and 1% SDS in 50-mM Tris-HCl, pH 8.0) for 1 h at 65°C. DNA was extracted with phenol/chloroform/isoamyl alcohol and the degree of fragmentation was analyzed using 2% agarose gel electrophoresis followed by ethidium bromide staining.

Cell cycle analysis – Quercetin-induced DNA fragmentation was also determined by flow cytometric analysis after propidium iodide (PI) staining. Initially, the suspension (2×10^6 cells) of quercetin-treated cells was fixed for 24 h at 4°C with 80% ethanol and then incubated overnight at 4°C with 1 ml of PI staining mixture (250 μl of PBS, 250 μl of 1 mg/ml RNase in 1.12% sodium citrate, and 500 μl of 50 μg/ml PI in 1.12% sodium citrate). After the staining, 1×10^4 cells were analyzed with FACS Calibur® system (Becton Dickinson, San Jose, CA).

Statistical analyses – All data are expressed as mean ± standard error (SE). A one-way ANOVA using SPSS ver. 10.0 software was used for multiple comparisons. A value of $P<0.05$ was considered significant.

Results

Effect of quercetin on proliferation in normal and tumorigenic hepatic cells – The effect of quercetin on cell proliferation was determined by tritium incorporation using BNL CL.2 and BNL SV A.8 cells (Fig. 1). As shown in Fig. 1A, the addition of quercetin to cultured BNL CL.2 cells resulted in a slight inhibition of tritium incorporation by DNA of the cells. In contrast, quercetin treatment to BNL SV A.8 cells induced a dramatic inhibition of the tritium uptake by the cells such that only 35.4% of tritium uptake was observed when treated with 100 μM quercetin for 24 h, compared with untreated cells. In addition, quercetin-mediated inhibition of DNA synthesis in BNL SV A.8 cells was time dependent (Fig. 1B). These results indicated that BNL SV A.8 cells were more sensitive to quercetin-mediated inhibition of proliferation, which occurred at lower doses and times than in the BNL CL.2 cells.

Effect of quercetin on cytotoxicity of normal and tumorigenic hepatic cells – To determine how quercetin inhibited the hepatic cell proliferation, we determined if quercetin exerted a cytotoxic effect on hepatic cells by monitoring trypan blue exclusion after the quercetin treatment (Fig. 2). The added quercetin did not exert a substantial cytotoxic effect in BNL CL.2 cells and the cytotoxicity induced by a 24-h incubation of the cells with 100 μM quercetin was only 6.4% (Fig. 2A). However, more than 13% of BNL SV A.8 cells were stained positive with trypan blue when treated with 100 μM quercetin for 24 h. Moreover, following a 48-h incubation with 100 μM of quercetin in the BNL SV A.8 cells, cytotoxicity was determined to be 27.5% (Fig. 2B). A prominent aspect

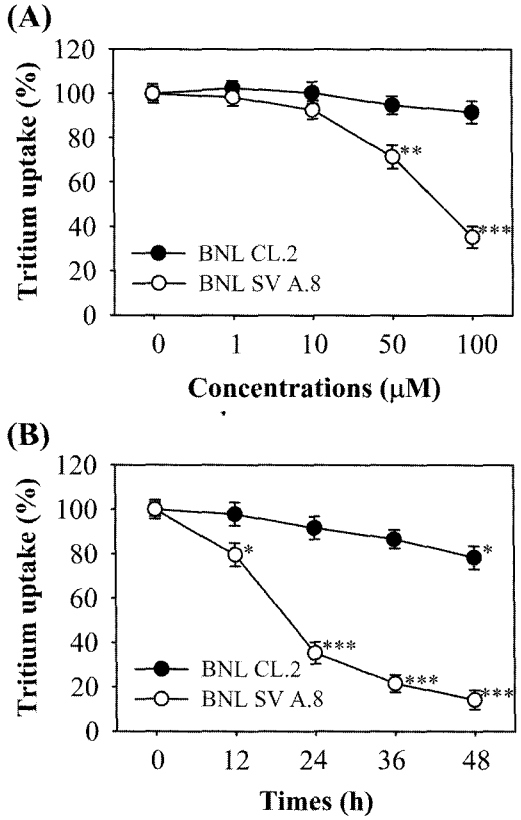

Fig. 1. Effects of quercetin on DNA synthesis in hepatic cells. (A) Cells were treated with the indicated concentrations of quercetin for 24 h and then incubated for another 12 h in the presence of [*methyl*-^{3}H] TdR. (B) Cells were treated with 100 μM quercetin for the indicated times and incubated with [*methyl*-^{3}H] TdR for the last 12 h of the incubation period. Representative results from three separate experiments are shown and the figures represent the mean ± SE of experiments performed in triplicate. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ represent significant differences between the experimental and control value.

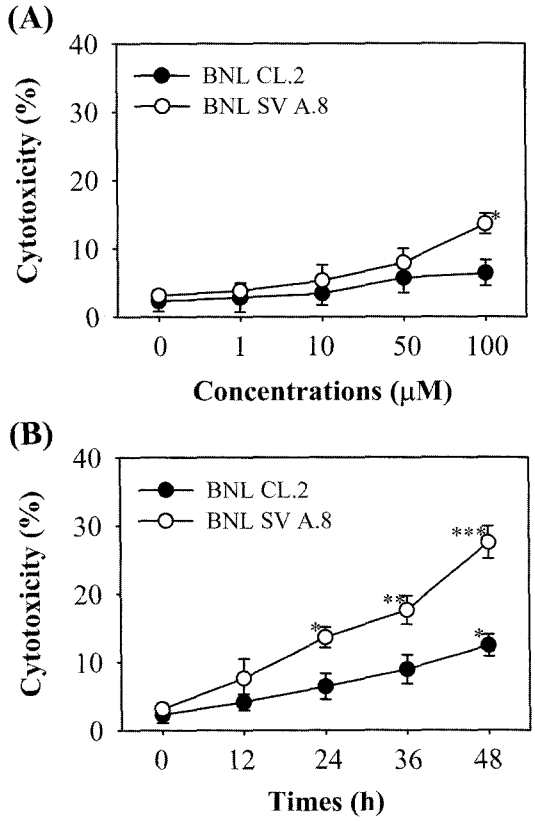

Fig. 2. Cytotoxic effects of quercetin in hepatic cells. Cells were treated with the indicated concentrations of quercetin for 24 h (A) or with 100 μM quercetin for the indicated times (B), and then processed for trypan blue staining. The figures represent the mean ± SE of three separate experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ represent significant differences between the experimental and control value.

from the time-course study was also that the BNL SV A.8 cells were further sensitive to RCMF-mediated cytotoxicity, as expected.

Quercetin-mediated apoptosis in normal and tumorigenic hepatic cells – To more understand the effect of quercetin on hepatic cells, quercetin-treated hepatocytes were subjected to apoptosis assays, including TUNEL assay, agarose gel electrophoresis of genomic DNA, and cell cycle analysis after PI staining (Figs. 3 & 4, Tables 1& 2). Initially, a dose-dependent increase in the number of positively stained BNL SV A.8 cells was observed in the TUNEL assay after quercetin treatment (Fig. 3). For example, after 24 h of exposure to 100 or 200 μM quercetin, apoptosis was seen in 29.7% and 56.4% of the cells, respectively. In contrast, BNL CL.2 cells showed a resistant against the quercetin-mediated apoptosis and thus only 15.1% of the cells were to be apoptotic even when treated with 200 μM

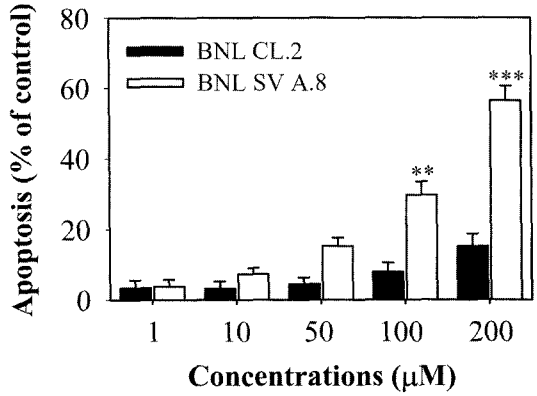

Fig. 3. Quercetin-mediated apoptosis in hepatic cell lines determined by TUNEL assay. BNL CL.2 and BNL SV A.8 cells were treated with various concentrations (1-200 μM) of quercetin for 24 h. After incubation, the cells were stained with FITC-conjugated dUTP and the degree of apoptosis was assessed. Each bar shows the mean ± SE of three separate experiments and $^{**}P < 0.01$ and $^{***}P < 0.001$ represent significant differences between the experimental and control value.

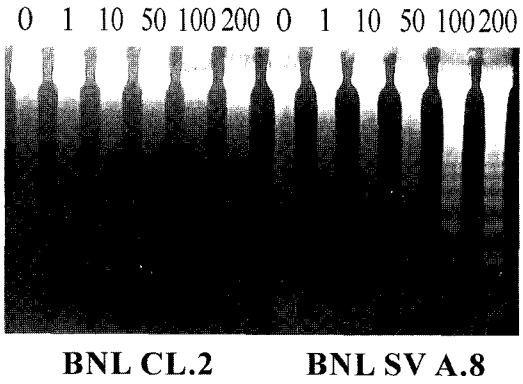

Fig. 4. Analysis of DNA fragmentation using agarose gel electrophoresis. BNL CL.2 and BNL SV A.8 cells were incubated in the presence of 1 to 200 μM quercetin for 24 h. Genomic DNA was prepared and analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining. A representative result from three independent experiments is shown.

Table 1. Effect of quercetin on the cell cycle distribution of BNL CL.2 cells

Concentrations (μM)	% Cell			
	Sub-G_0/G_1	G_0/G_1	S	G_2/M
0	3.3±0.6	56.2±3.1	12.4±1.6	28.1±1.5
10	3.4±0.3	52.1±4.1	11.6±1.4	32.9±2.3
50	4.1±0.7	54.3±3.8	12.9±2.4	28.7±1.6
100	5.7±0.5	53.1±2.9	14.1±1.6	27.1±1.2
200	30.8±2.7***	28.2±2.2***	20.2±1.9	20.8±2.2

BNL CL.2 cells were treated with the indicated doses of quercetin for 48 h. The percentage of cells in each phase of the cell cycle was calculated through WinMDI 2.8 program of PI fluorescence data. Data represent the mean ± SE of experiments performed in triplicate. $^{***}P < 0.001$ represents a significant difference between the experimental and control value.

Table 2. Effect of quercetin on the cell cycle distribution of BNL SV A.8 cells

Concentrations (μM)	% Cell			
	Sub-G_0/G_1	G_0/G_1	S	G_2/M
0	3.5±0.9	63.1±3.1	11.8±1.6	21.6±1.6
10	2.9±0.6	62.2±5.2	11.7±1.6	23.2±1.8
50	15.6±2.8**	38.8±2.2***	14.2±1.3	31.4±1.9*
100	79.3±5.5***	10.0±1.1***	2.6±1.2*	8.1±1.8**
200	89.3±6.7***	5.7±1.2***	1.3±0.1**	3.7±1.6***

BNL SV A.8 cells were treated with the indicated doses of quercetin for 48 h. The percentage of cells in each phase of the cell cycle was calculated through WinMDI 2.8 program of PI fluorescence data. Data represent the mean ± SE of experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ represent significant differences between the experimental and control value.

quercetin for 24 h. The induction of quercetin-mediated apoptosis was further examined by investigating DNA ladder formation after quercetin treatment (Fig. 4). As shown the figure, quercetin treatment dose-dependently induced apoptotic laddering of genomic DNA in BNL SV A.8 cells and the formation of DNA ladders was started from dose of 50 μM. However, quercetin did not induce a DNA ladder in BNL CL.2 cells even at the very high concentration (200 μM quercetin) added. The sensitive induction of apoptosis in the BNL SV A.8 cells by the quercetin treatment was further confirmed with PI staining (Tables 1 & 2). For example, following treatment with 50 or 100 μM quercetin for 48 h, 15.6% and 79.3% of the BNL SV A.8 cells became apoptotic, respectively (Table 2). Meanwhile, only 30.8% of apoptotic cells were observed in BNL CL.2 cells even when 200 μM quercetin was added for 48 h (Table 1). Furthermore, when the BNL CL.2 cells were treated with 200 μM quercetin, there was no a significant reduction of cell numbers in S and G_2/M phases, whereas BNL SV A.8 cells were very sensitive to quercetin-mediated inhibition of proliferation, which occurred at lower doses and times than in the BNL CL.2 cells. These results indicated a selective dose-response effect of quercetin to induce apoptosis in BNL SV A.8 cells, compared to BNL CL.2 cells.

Discussion

In recent years, a plant derived-bioactive substance that is capable of arresting cell growth and inducing apoptosis of tumor cells has received a considerable attention in cancer chemopreventive approaches (Smets, 1994; Paschka *et al.*, 1998). In those respects, bioflavonoid quercetin is thought to be an ideal substance for cancer chemoprevention. However, the effect of quercetin on cell cycle arrest in nonmalignant hepatic cells was not much reported.

We initially evaluated the growth inhibitory response of quercetin by determining tritium incorporation in BNL CL.2 and BNL SV A.8 cells. The choice of these two cell lines might give an ideal design, because the cells are obtained from same strain and age stage. Results from the tritium uptake assay showed that BNL SV A.8 cells were more sensitive to quercetin-mediated inhibition of proliferation (Fig. 1). In addition, the trypan blue staining experiment revealed that the viability of BNL SV A.8 cells was further sensitively reduced by quercetin treatment, compared to that of BNL CL.2 cells (Fig. 2). These results suggested a selective response of quercetin to the tumorigenic BNL SV A.8 cells compared to the BNL CL.2 cells.

To understand the selective response of quercetin on the cytotoxicity in the normal and tumorigenic cells, quercetin-treated hepatic cells were subjected to apoptosis assays. As evidenced by the increased number of positively stained cells in the TUNEL assay (Fig. 3) and the induction of characteristic nuclear DNA ladders (Fig. 4), quercetin

treatment sensitively induced apoptosis in BNL SV A.8 cells than in BNL CL.2 cells. This differed response of apoptosis induction by the quercetin treatment in the hepatic cells was further confirmed by the migration of many cells to sub-G_0/G_1 phase in the BNL SV A.8 (Tables 1 & 2).

Among the promising strategy that can be approached for cancer chemoprevention, selective apoptosis induction is regarded as one of the best ideal way to remove tumor cells. Since almost artificial agents currently used in cancer therapy are known to be toxic and produce severe damage to normal cells, in recent years naturally occurring agents that are capable of selective and preferential elimination of cancer cells by inhibiting cell cycle progression and/or causing apoptosis are further emphasized as promising agents for the therapy (Gupta *et al.*, 2001). Collectively, the results from apoptosis analyses suggested that quercetin could play a pivotal role as cancer chemopreventive and therapeutic substance. We assumed that the severe induction of apoptosis in the BNL SV A.8 cells is related to the inhibition of the activity of signal transduction molecules, since flavonoids have been reported to exert inhibitory effects on many kinds of protein kinases and some transcriptional factors (Yang *et al.*, 1998; Gamet-Payrastre *et al.*, 1999; Miranda *et al.*, 1999). However, additional experiments should be performed to determine the exact mechanism(s) by which quercetin induces a selective response on apoptosis in the normal versus tumorigenic hepatic cells. In addition, another experiments using other hepatic cells or tissues should be also carried out to compare and conform the selective effects of quercetin.

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References

Aligiannis, N., Mitaku, S., Mitrocotsa, D., and Leclerc, S., Flavonoids as cycline-dependent kinase inhibitors: Inhibition of cdc 25 phosphatase activity by flavonoids belonging to the quercetin and kaempferol series. *Planta Med.* **67**, 468-470 (2001).

Caltagirone, S., Rossi, C., Poggi, A., Ranelletti, F.O., Natali, P.G., Brunetti, M., Aiello, F.B., and Piantelli, M., Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. *Int. J. Cancer* **87**, 595-600 (2000).

Christou, L., Hatzimichael, E., Chaidos, A., Tsiara, S., and Bourantas, K.L., Treatment of plasma cell leukemia with vincristine, liposomal doxorubicin and dexamethasone. *Eur. J. Haematol.* **67**, 51-53 (2001).

Formica, J.V. and Regelson, W., Review of the biology of quercetin and related bioflavonoids. *Food Chem. Toxicol.* **33**, 1061-1080 (1995).

Gamet-Payrastre, L., Manenti, S., Gratacap, M.P., Tulliez, J., Chap, H., and Payrastre, B., Flavonoids and the inhibition of PKC and PI3-kinase. *Gen. Pharmacol.* **32**, 279-286 (1999).

Gao, Z., Huang, K., Yang, X., and Xu, H., Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. *Biochim. Biophys. Acta* **1472**, 643-650 (1999).

Gupta, S., Afaq, F., and Mukhtar, H., Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. *Biochem. Biophys. Res. Commun.* **287**, 914-920 (2001).

Hollman, P.C. and Katan, M.B., Dietary flavonoids: intake, health effects and bioavailability. *Food Chem. Toxicol.* **37**, 937-942 (1999).

Miranda, C.L., Stevens, J.F., Helmrich, A., Henderson, M.C., Rodriguez, R.J., Yang, Y.H., Deinzer, M.L., Barnes, D.W., and Buhler, D.R., Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (*Humulus lupulus*) in human cancer cell lines. *Food chem. Toxicol.* **37**, 271-285 (1999).

Morel, I., Lescoat, G., Cogrel, P., Sergent, O., Pasdeloup, N., Brissot, P., Cillard, P., and Cillard, J., Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. *Biochem. Pharmacol.* **45**, 13-19 (1993).

Mukherjee, A.K., Basu, S., Sarkar, N., and Ghosh, A.C., Advances in cancer therapy with plant based natural products. *Curr. Med. Chem.* **8**, 1467-1486 (2001).

Paschka, A.G., Butler, R., and Young, C.Y.F., Induction of apoptosis in prostate cancer cell lines by the green tea component, (−)-epigallocatechin-3-gallate. *Cancer Lett.* **130**, 1-7 (1998).

Pezutto, J.M., Plant-derived anticancer agents. *Biochem. Pharmacol.* **53**, 121-133 (1997).

Plaumann, B., Fritsche, M., Rimpler, H., Brandner, G., and Hess, R.D., Flavonoids activate wild-type p53. *Oncogene* **13**, 1605-1614 (1996).

Smets, L.A., Programmed cell death (apoptosis) and response to anti-cancer drugs. *Anti-Cancer Drugs* **5**, 3-9 (1994).

Wong, W.S. and McLean, A.E., Effects of phenolic antioxidants and flavonoids on DNA synthesis in rat liver, spleen, and testis *in vitro*. *Toxicology* **139**, 243-253 (1999).

Yang, E.B., Zhang, K., Cheng, L.Y., and Mack, P., Butein, a specific protein tyrosine kinase inhibitor. *Biochem. Biophys. Res. Commun.* **245**, 435-438 (1998).

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