Anticancer Activity of a Quercetin-based Polymer Towards HeLa Cancer Cells

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Abstract. *Background: Quercetin is one of the most potent antioxidants showing anti-inflammatory, antiproliferative and antitumoral effects; however its short half-life in buffered solution (e.g. body fluids) has so far hampered its introduction into clinical practice. Aim: To overcome this inconvenience, quercetin was covalently conjugated into a polymethacrylic acid backbone and the conjugate was tested on HeLa cancer cells. Materials and Methods: FT-IR, UV-Vis, Gel Permeation Chromatography analyses and the Folin-Ciocalteu test were performed to characterize the conjugate. Antioxidant properties were assessed by the DPPH test and the viability experiments by trypan blue exclusion assay. Results: The conjugate showed a functionalization degree of 2.01 mg of Q per g, an* IC_{50} *of 2.62 mg* ml^{-1} *in the DPPH assay and was able to induce a 90% cell death after one day treatment, while the value for free Quercetin was 40% after three days. Conclusion: Polymer conjugation significantly increases quercetin stability, leading to a sustained activity of the flavonoid.*

In recent years, an emerging research field aimed to improve the performance of natural and synthetic polymers concerning the synthesis of antioxidant polymers by covalent insertion of antioxidant molecules into polymeric chains (1, 2). The resulting functional materials show innovative application in biomedical and pharmaceutical fields; they could be applied in pharmaceutical and cosmetic formulations in order to avoid the oxidation of their components, as preservative agents in food packaging and in biomedicine as innovative materials (*e.g.* haemodialysis membranes, contact lenses, *etc.*) (3, 4). The advantage in the use of these bioconjugates is related to the possibility of combining the advantages of both the components: the unique properties of antioxidant molecules and the high stability and slow degradation rate of macromolecular systems (5).

In the realm of biomedicine, a recent therapeutic approach is related to the discovery of the role of free radicals in cardiovascular diseases, cancer, diabetes, neurodegenerative disorders, autoimmune diseases, aging and other diseases, opening a new application field for antioxidant materials (6, 7). In particular, the first therapeutic strategies related to the use of polymer therapeutics were to improve the performance of poorly water-soluble anticancer drugs. The conjugation of such molecules with water-soluble polymers, indeed, results in enhancing the pharmacokinetics of the therapeutics by mean of increased water solubility, reduced side-effects and easier administration (8, 9).

Several different approaches have been proposed to synthesize biopolymers coupled with antioxidant compounds, indicating the need for synthetic procedures to generate multifunctional polymeric systems *via* straightforward and efficient chemistries. In this work, the adopted synthetic strategy is the coupling reaction between the antioxidant molecule and a growing polymeric chain in a single-step reaction. With this approach, it is possible to synthesize polymer antioxidant conjugates without the generation of toxic reaction by-products, while also preserving the antioxidant from the degradation processes (10). The selected antioxidant molecule was quercetin, while methacrylic acid (MAA) was the chosen co-monomer for the synthesis of a polymer-quercetin conjugate (Pol-Q). The choice of MAA is dictated by the wide use of methacrylate polymers in biomedicine and biotechnology as base materials for drug delivery systems, contact lenses, food technology, quality control systems and biosensors (11).

Quercetin (3,3',4',5,7-pentahydroxylflavone), a flavonoid ubiquitously present in fruits and vegetables, such as tea,

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Key Words: Anticancer activity, antioxidants, polymeric drugs, radical grafting, quercetin, HeLa cells.

apples, onion and berries, was selected due to its high biological benefits, including antioxidative, anti-genotoxic, anti-inflammatory, vasodilating effects, and because it has been recently proposed as a chemopreventive and anticancer agent (12). Its antitumoral effects have been attributed to various mechanisms, including the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways, and the interactions with receptors and other proteins (13). Various cellular receptors have indeed been reported to be involved in the anticancer activities of quercetin, including the androgen receptor (14), the epidermal growth factor receptor (EGFR) family (15) and the death receptor (DR) (16). In particular, quercetin has also been reported to suppress viability of HeLa cells *via* AMP-activated protein kinase (AMPK)-induced heat shock protein (HSP)70 and EGFR down-regulation (17). However, the sensitivity of quercetin to fast auto-oxidation results in its poor stability in aqueous aerobic environments (18), thus its half-life, generally ranges between 2 h (*e.g.* in McCoy's 5A cell culture medium) to 10 h [in (PBS) pH 7.2] (19), due to chemical decomposition in biologically active products, such as protocatechuic acid and phloroglucinol carboxylic acid (20).

Our hypothesis is that polymer conjugation significantly increases quercetin stability, leading to a sustained activity of the flavonoid.

Materials and Methods

Materials, cell line and culture conditions. MAA, 2,2'-azoisobutyronitrile (AIBN), quercetin, 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), Folin-Ciocalteu reagent (FCR), disodium hydrogen phosphate, sodium dihydrogen phosphate, and polymethacrylic acid (pMAA) standard samples for size-exclusion chromatography (M_n=600-500000; M_w/M_n=1.06-1.10) were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA). *N,N*-Dimethylformamide (DMF), water and ethanol were HPLC-grade and provided by Carlo Erba reagents (Milan, Italy). Human HeLa cervical adenocarcinoma cells (Interlab Cell Line Collection, ICLC, Genoa, Italy) were maintained in a humified tissue culture incubator at 5% CO_2 and 37°C and cultured in modified Eagle's medium (MEM, Sigma-Aldrich, Gillingham, Dorset, UK) supplemented with 10% fetal bovine serum (FBS), 100 IU ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, and 0.2 mM L-glutamine. HeLa cells were maintained in a humidified tissue culture incubator at 5% CO_2 and 37˚C and cultured in Modified Eagle's Medium, supplemented with 10% fetal bovine serum (FBS), 100 IU ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, and 0.2 mM L-glutamine.

Synthesis of Pol-Q conjugate. The polymerization of MAA with quercetin was carried out as follows: in a 10 ml glass tube, 0.1, 0.25, and 0.5 g of quercetin were dissolved in 8.0 ml of DMF and then 5.0 g of MAA and 100 mg of AIBN were added and the solution was magnetically stirred at 60˚C. After 24 h, the mixture was poured into acetone, and then the precipitated polymer was filtered by sintered glass filter funnel (Pyrex, Ø30 mm; porosity 3), submitted to three dissolution/precipitation cycles (methanol/acetone), and vacuum dried at room temperature.

The polymer was checked to be free of unreacted quercetin and any other compounds by HPLC analysis after each purification step.

Blank polymer (pMAA) was prepared under the same conditions without using quercetin.

Pol-Q analysis. The liquid chromatography consisted of a Jasco PU-2089 Plus liquid chromatography apparatus equipped with a Rheodyne 7725i injector (fitted with a 20 µl loop), a Jasco UV-2075 HPLC detector and Jasco-Borwin integrator (Jasco Europe s.r.l., Milan, Italy). A reverse-phase C18 Hibar column, 250 mm × 4 µm, particle size=5 µm, pore size=120Å (Merck, Darmstadt, Germany), was employed. In accordance with literature data (21), the mobile phase adopted for the detection of quercetin was a 1% (v/v) aqueous solution of formic acid-acetonitrile-2-propanol (70:22:8) at a flow rate of 0.2 ml min^{-1}. Chromatograms were recorded at 370 nm. For pMAA and Pol-Q, Mn and Mw/Mn were measured by Gel permeation chromatography (GPC) using water as eluent at 45˚C and at flow rate, 1.0 ml min-1on Waters Ultrahydrogel-1000 column connected to a Jasco PU-2089 pump and a Jasco 930-RI refractive-index detector. The columns were calibrated with standard pMAA samples (22). (FT-IR) spectra were recorded as KBr pellets on a Jasco FT-IR 4200. (UV-Vis) absorption spectra were obtained with a Jasco V-530 UV/Vis spectrometer (Jasco Europe s.r.l., Milan, Italy).

Evaluation of disposable phenolic groups by Folin-Ciocalteu procedure. The amount of total phenolic equivalents was determined using the Folin-Ciocalteu reagent procedure, according to the literature, with some modifications (23). Briefly, 20 mg of Pol-Q conjugate were dispersed in distilled water (6.0 ml) in a volumetric flask. Folin-Ciocalteu reagent (1.0 ml) was added and the contents of the flask were mixed thoroughly. After 3 min, 3 ml of Na_2CO_3 (2%, w/w) were added, and then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm against a control prepared using the blank polymer under the same reaction conditions. The amount of total phenolic groups in the polymeric material was expressed as quercetin equivalents (mg) by using the equation obtained from the calibration curve of the free antioxidant, recorded by employing five different standard solutions of quercetin; 0.5 ml of each solution were added to the Folin-Ciocalteu system to raise the final concentration to 8.0, 16.0, 24.0, 32.0, and 40.0 µM, respectively. After 2 h, the absorbance of the solutions was measured to record the calibration curve and the correlation coefficient (R^2), slope and intercept of the regression equation obtained were calculated by the method of least squares.

Determination of scavenging effect of Pol-Q on the DPPH radical. In order to evaluate the free radical scavenging properties of Pol-Q, its reactivity towards a stable free radical DPPH, was evaluated (24). For this purpose, in each of seven test tubes, 5.0, 10.0, 15.0, 20.0 mg of Pol-Q were dissolved in 6 ml of ethanol and then 4 ml of ethanol solution of the DPPH (250 µM) were added, obtaining a solution of the DPPH with a final concentration of 100 µM. The sample was incubated in a water bath at 25˚C and after 30 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The same reaction conditions were applied for the blank pMAA and standard quercetin solutions (positive control). The scavenging activity of the tested polymeric materials was measured as the decrease in the absorbance of DPPH and was expressed as percentage inhibition of the DPPH radical calculated according to the following equation (1):

$$\text{Inhibition (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \qquad \text{(Eqtn 1)}$$

where A_0 is the absorbance of a standard prepared under the same conditions, but without any polymer, and A_1 is the absorbance of polymeric samples.

Cell viability on treatment with quercetin and Pol-Q. The effect of Q and Pol-Q on cell proliferation was assessed by the trypan blue exclusion assay. HeLa cells in the exponential growth phase were plated in triplicates in 12-well plates at a density of 10^5 cells/well and grown overnight. The following day, cells were synchronized in phenol red-free serum-free media (PRF-SFM) for 24 h to avoid growth differences among cells. Following starvation, free quercetin (100 μM) or Pol-Q (corresponding to ~100 μM quercetin) were added to the cells in PRF-MEM plus 0.5% FBS. Comparable amounts of (DMSO) and polymer alone were also tested. Untreated cells were used as control. After 1, 2 or 3 days, cells were harvested by trypsinization and incubated in a 0.5% trypan blue solution for 10 min at room temperature. Cell viability was determined microscopically by counting trypan blue-negative cells in a hemacytometer (Burker, Brand, Germany).

Statistical analyses. Each measurement was carried out in five independent experiments, data are expressed as means (±SD), and analysed using one-way analysis of variance (ANOVA). For the inhibitory experiment, the IC_{50} value was determined as the concentration of antioxidant that provides 50% inhibition of DPPH.

Results and Discussion

Synthesis and Characterization of Pol-Q conjugate. The Pol-Q conjugate was synthesized by free radical coupling reaction between MAA and quercetin based on literature data which suggest that quercetin can be incorporated in a growing polymeric chain by the reaction between the radical formed in the *ortho*- and *para*- positions relative to the hydroxyl group of the flavonoid and the MAA monomer (25).

Optimization of the reaction conditions was performed in order to maximize the incorporation of quercetin in the growing polymeric chain, and thus the selected amount of quercetin in the polymerization feed represents the highest amount of quercetin which can be dissolved in the reaction medium. Pol-Q conjugates synthesized using a lower amount of quercetin, indeed, were found to be less effective antioxidants.

The synthesized polymer was then conformed to be free of un-reacted quercetin by HPLC analyses of acetone used in the precipitation-dissolution cycles which Pol-Q conjugate underwent in the purification process.

After the synthesis and purification steps, chemical characterization of Pol-Q was carried out by determination of the molecular weight distributions, FT-IR and UV-Vis analyses and the determination of the amount of quercetin incorporated into the polymeric backbone. By specific (SEC) analyses, the molecular weight distribution of Pol-Q and blank polymers were determined and corresponded to average values of 71 000 Da and 77 000 Da for Pol-Q and pMAA, respectively, with M_w/M_n <1.7.

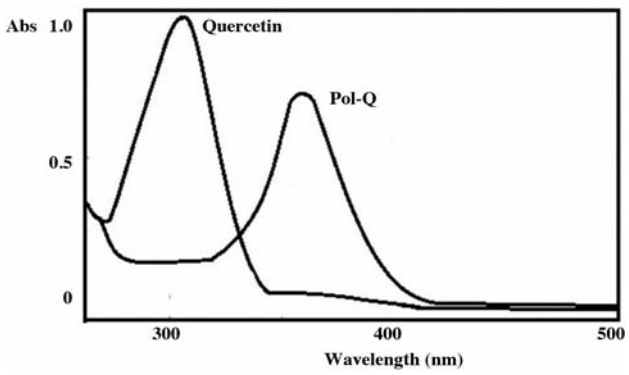

Figure 1. *UV-Vis absorption spectra of free and conjugated (Pol-Q) quercetin*.

FT-IR and UV-Vis analyses were performed to demonstrate the formation of covalent bond between quercetin and the polymeric backbone. In particular, FT-IR analysis of Pol-Q shows the presence of the carbon-to-carbon stretching band (1523 cm^{-1}) within the aromatic ring of the antioxidant which is absent from the pMAA, while the presence of a bathochromic shift of the absorption peaks of the aromatic region of quercetin from 310 nm to 367 nm after incorporation into the polymeric chain suggest the formation of a covalent linkage between Q and MAA residues (Figure 1).

A key characterization of the synthesized Pol-Q is the determination of the amount of quercetin bound into the polymeric backbone. This characterization was performed by using the Folin-Ciocalteu assay, by which it is possible to determine the efficiency of the antioxidant residues in reducing the molybdenum complex within the Folin-Ciocalteu reagent (23). Thus, a comparison between the reducing power of quercetin and Pol-Q to form the spectrophotometrically detectable blue species $(PMoW_{11}O_{40})^{4-}$ is a direct indication of the amount of quercetin incorporated into the conjugate. The assay was performed according to the literature and, as a result, it was determined that 1.0 g of Pol-Q contained 2.01±0.3 mg of quercetin.

A first functional characterization of the conjugate efficiency was performed in terms of antioxidant properties and scavenging activity in particular. In recent years, the DPPH assay has become quite popular in antioxidant studies because it is a simple and highly sensitive method to evaluate the free radical scavenging properties of a selected antioxidant (24). The antioxidant effect is proportional to the disappearance of DPPH in test samples due to the fact that DPPH accepts hydrogen from an antioxidant. Under our conditions, Pol-Q was found to be a powerful radical

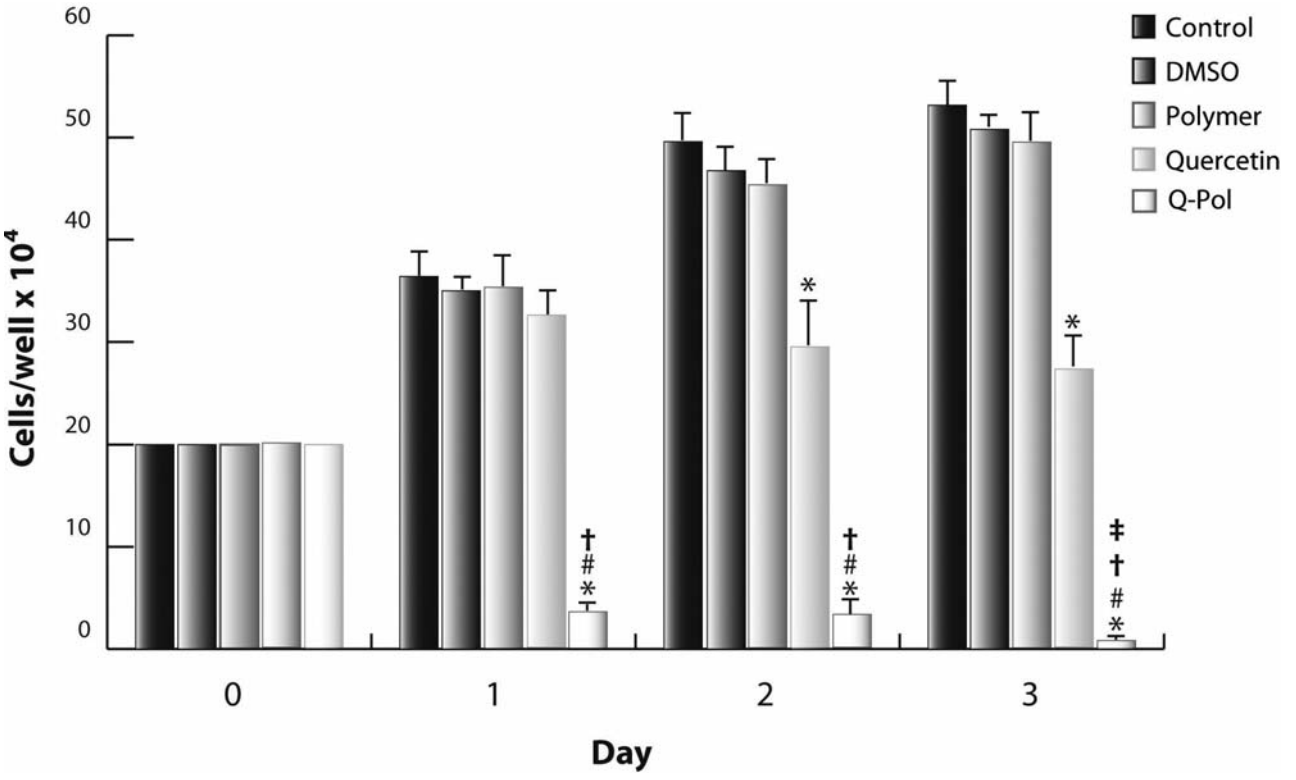

Figure 2. *Polymer conjugation accelerates and increases the efficacy of quercetin in inducing cancer cell death. Cancerous HeLa cells were treated with (DMSO), polymer alone, quercetin, polymer-conjugated quercetin (Pol-Q) or left untreated (control). Viability was determined after 1, 2 and 3 days. Values represent the mean of four triplicate independent experiments. Significance values were as follows: *p<0.01 vs. the respective control; #p<0.01 vs. the corresponding free quercetin treated samples; †p< 0.01 vs. the control at day 0; ‡p<0.01 vs. Pol-Q treated samples at day 1 and day 2. The error bars indicate SD.*

scavenger, with an IC_{50} value of 2.62 mg ml^{-1}, with pMAA exhibiting a low degree of interference with the assay. By considering the amount of quercetin per g of Pol-Q obtained by the Folin-Ciocalteu assay, this IC_{50} value corresponds to 17.7 μM of quercetin. The IC_{50} value of free quercetin (positive control) is 16.1±0.7 μM, confirming that the conjugation process did not interfere with the antioxidant properties of quercetin which are retained in the final polymer.

Anticancer activity of Pol-Q. To assess the effect of polymer conjugation on quercetin-induced tumour cell death, viability experiments were conducted on HeLa cells (Figure 2). After only two days of treatment, quercetin alone started to show a cytostatic effect (*vs.* day 0), which persisted at day 3, and caused a 40-50% growth retardation compared to the relative control at both time points. Notably, polymer conjugation dramatically increased toxicity of quercetin, inducing ~90% cell death vs control (~80% *vs.* control at day 0), as early as one day of treatment, and actually reaching 95% suppression of viability after three days. As expected, the vejicle used for free quercetin, DMSO, and polymer alone did not show any significant effect at any time point considered when compared to control samples (Figure 2).

It is worth mentioning that quercetin has recently been reported to induce a dose-dependent suppression of viability of HeLa cells (17). In agreement with our observations, the authors found a ~40% reduction of viability *vs.* the relative control after three days of quercetin treatment, but our data show that this effect is rather cytostatic, if compared to the starting cell number (day 0). On the contrary, Pol-Q is able to induce a prompt and almost complete cell kill, as early as after one day of treatment, and the cytotoxic activity persists and even increases with time. This effect cannot be ascribed to use of polymer alone, since no significant difference in HeLa cell viability was observed in polymer treated-cells, compared to controls (Figure 2).

Conclusion

A new antioxidant-polymer conjugate was synthesized and characterized in terms of anticancer activity. The quercetin flavonoid was incorporated into a methacrylate polymer by

free radical reaction and, after chemical characterization of the polymeric material, the so formed polymeric antioxidant was tested as anti-cancer agent towards HeLa cells. Our results show how polymer-bound quercetin exerts a rapid (as soon as after one day of treatment) and sustained cytotoxic effect, reaching almost 100% of cell kill within three days of exposure, if compared to free quercetin, which was only able to exert a cytostatic effect. This dramatic increase of toxicity on tumour cells observed in Pol-Q treated samples, could be most likely ascribed to a prolonged stability of the quercetin molecule following polymer conjugation. Our data support the idea that antioxidant conjugates represent a reliable and effective vehicle for anti-tumor agents, suggesting their possible implementation as co-adjuvant in the treatment of cancer disease.

Acknowledgements

This work was financially supported by MIUR (Programma di ricerca di rilevante interesse nazionale 2008) and University of Calabria funds. Financial support of Regional Operative Program (ROP) Calabria ESF 2007/2013 – IV Axis Human Capital – Operative Objective M2 - Action D.5 is gratefully acknowledged. Authors are solely responsible for the work.

References

1 Cirillo G, Kraemer K, Fuessel S, Puoci F, Curcio M, Spizzirri UG, Altimari I and Iemma F: Biological activity of a gallic acid – gelatin conjugate. Biomacromolecules *11*: 3309-3315, 2010.
2 Pasanphan W, Buettner GR and Chirachanchai S: Chitosan conjugated with deoxycholic acid and gallic acid: a novel biopolymer-based additive antioxidant for polyethylene. J Appl Polym Sci *109*: 38-46, 2008.
3 Menichetti S, Viglianisi C, Liguori F, Cogliati C, Boragno L, Stagnaro P, Losio S and Sacchi MC: Ethylene-based co-polymers with tunable content of polymerizable hindered phenols as non-releasing macromolecular additives. J Polym Sci Polym Chem *46*: 6393-6406, 2008.
4 Singh A and Kaplan DL: *In vitro* enzyme-induced vinyl polymerization. Adv Polym Sci 194: 211-222, 2006.
5 Dahe GJ, Teotia RS, Kadam SS and Bellare JR: The biocompatibility and separation performance of antioxidative polysulfone/vitamin E TPGS composite hollow fiber membranes. Biomaterials *32*: 352-365, 2011.
6 Halliwell B: Free radicals and antioxidants – Quo vadis? Trends Pharmacol Sci *32*: 125-130, 2011.
7 Nenkat Ratnam D, Ankola DD, Bhardwaj V, Sahana DK and Ravi Kumar MNV: Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective. J Control Rel *113*: 189-207, 2006.
8 Chen X, McRae S, Parelkar S and Emrick T: Polymeric phosphorylcholine-camptothecin conjugates prepared by controlled free radical polymerization and click chemistry. Bioconjugate Chem *20*: 2331-2341, 2009.
9 Ferguson EL and Duncan R: Dextrin-phospholipase A2: synthesis and evaluation as a bioresponsive anticancer conjugate. Biomacromolecules *10*: 1358-1364, 2009.
10 Puoci F, Iemma F, Curcio M, Parisi OI, Cirillo G, Spizzirri UG and Picci N: Synthesis of methacrylic – ferulic acid co-polymer with antioxidant properties by single-step free radical polymerization. J Agric Food Chem *56*: 10646-10650, 2008.
11 Herveas Perez JP, Lopez-Cabarcos E and Lopez-Ruiz B: The application of methacrylate-based polymers to enzyme biosensors. Biomol Eng *23*: 233-245, 2006.
12 Kook D, Wolf AH, Yu AL, Neubauer AS, Priglinger SG, Kampik A and Welge-Lussen UC: The protective effect of quercetin against oxidative stress in the human RPE. Invest Ophthalmol Vis Sci *49*: 1712-1720, 2008.
13 Moon YJ, Wang X and Morris ME: Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro *20*: 187-210, 2006.
14 Xing N, Chen Y, Mitchell SH and Young CY: Quercetin inhibits expression and function of the androgen receptor in LNCaP prostate cancer cells. Carcinogenesis *22*: 409-414, 2001.
15 Kim WK, Bang MH, Kim ES, Kang NE, Jung KC, Cho HJ and Park JHY: Quercetin decreases the expression of ERBB2 and ERBB3 proteins in HT-29 human colon cancer cells. J Nutr Biochem *16*: 155-162, 2005.
16 Chen W, Wang X, Zhuang J, Zhang L and Lin Y: Induction of death receptor 5 and suppression of survivin contribute to sensitization of TRAIL-induced cytotoxicity by quercetin in non-small cell lung cancer cells. Carcinogenesis *28*: 2114-2121, 2007.
17 Jung JH, Lee JO, Kim JH, Lee SK, You GY, Park SH, Park JM, Kim EK, Suh PG, An JK and Kim HS: Quercetin suppresses HeLa cell viability *via* AMPK-induced HSP70 and EGFR down-regulation. J Cell Physiol *223*: 408-414, 2010.
18 Makris DP and Rossiter JT: Heat-induced, metal-catalyzed oxidative degradation of quercetin and rutin (quercetin-3-O-rhamnosylglucoside) in aqueous model systems. J Agric Food Chem *48*: 3830-3838, 2000.
19 Van der Woude H, Gliszczyńska-Swigło A, Struijs K, Smeets A, Alink GM and Rietjens IM: Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans. Cancer Lett *200*: 41-47, 2003.
20 Boulton DW, Walle KU and Walle T: Fate of the flavonoid quercetin in human cell lines: chemical instability and metabolism. J Pharm Pharmacol *51*: 353-359, 1999.
21 Careri M, Corradini C, Elviri L, Nicoletti I and Zagnoni I: Direct HPLC analysis of quercetin and trans-resveratrol in red wine, grape, and winemaking byproducts. J Agric Food Chem *51*: 5226-5231, 2003.
22 Bai F, Huang B, Yang X and Huang W: Synthesis of monodisperse poly(methacrylic acid) microspheres by distillation–precipitation polymerization. Eur Polym J *43*: 3923-3932, 2007.
23 Pan Y, Zhu J, Wang H, Zhang X, Zhang Y, He C, Ji X and Li H: Antioxidant activity of ethanolic extract of Cortex fraxini and use in peanut oil. Food Chem *103*: 913-918, 2007.
24 Magalhaes ML, Segundo MA, Reis S and Lima JLFC: Methodological aspects about *in vitro* evaluation of antioxidant properties. Anal Chim Acta *613*: 1-19, 2009.
25 Kobayashi S and Higashimura H: Oxidative polymerization of phenols revisited. Prog Polym Sci *28*: 1015-1048, 2003.

Received January 18, 2012
Revised February 17, 2012
Accepted February 20, 2012