

Influence of Extracellular Protein on the Cytoprotective Effects of Two Model Phytochemicals

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Abstract

Consumption of antioxidant compounds in the diet may provide cytoprotection against oxidative damage associated with diseases or exposure to toxic agents. In the present study, we have investigated the influence of extracellular protein-binding on the cytoprotective properties of two free-radical-scavenging natural products: quercetin (Q) and epigallocatechin-3-gallate (EGCG). Cytoprotection was determined by the ability of Q/EGCG to reduce the toxicity elicited by the oxidant *tert*-butyl hydroperoxide (t-BHP) in human hepatoma HepG2 cells, performed in serum-free medium or medium containing low [2%(v/v)] or high [10%(v/v)] levels of foetal bovine serum. Initial studies confirmed that the presence of serum (up to 10% v/v) was without effect on HepG2 viability. Furthermore, Q and EGCG were not toxic (up to a concentration of 100 µg/mL) under any of the culture conditions. Cytoprotection elicited by Q was significantly greater in serum-free and low-serum conditions compared to high-serum conditions. Similar results were obtained with EGCG, with additional evidence of a significant difference between serum-free and low-serum conditions. In conclusion, cytoprotective effects of Q and EGCG are modified by the presence of extracellular protein

Keywords: Cytoprotection; Quercetin; Epigallocatechin-3-gallate; Protein-binding

Introduction

Cell metabolism constantly produces reactive oxygen species (ROS) as a natural by-product of the normal metabolism of oxygen [1]. This situation is not damaging because cells are able to compensate and maintain an adequate homeostasis between ROS production and its removal *via* enzymatic or non-enzymatic pathways [2-5]. On the other hand, if this balance is disturbed by an excessive accumulation of ROS [6] due to some reason related with environment, life style and pathologic factors these result in a situation called oxidative stress (OS). In consequence, this accumulation of ROS above the protection system of cells may damage the integrity and function of critical molecules, ultimately leading to cell death [3,7]. There is increasing evidence that oxidative stress contributes to disease conditions such as cancer, chronic obstructive pulmonary disease, asthma, cardiovascular dysfunction and immunosuppression [8,9].

Quercetin (Q) (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one; shown in Figure 1) is considered as one of the most abundant flavonoids and represents an integral part of human diet. High amounts of quercetin are found in numerous vegetables, fruits, nuts, tea, seeds and wine [10]. It exhibits a wide range of biological activities, including anti-inflammatory [11,12], anti-carcinogenic [13] and antiviral actions [14]. The chemoprevention elicited by polyphenols such as Q is mediated by apoptosis in tumor cells [15,16] through direct activation of caspase cascade (mitochondrial pathway) such as caspase 3 and 9 as well as Bcl-2 family members [17,18] but is without effect on their normal cell counterparts [19]. Moreover, it exerts a notable cytoprotection activity against t-BHP-induced oxidative stress in HepG2 cells [20].

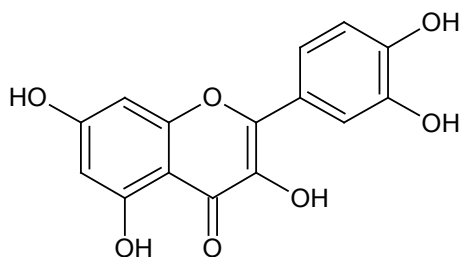


Figure 1: Structure of quercetin.

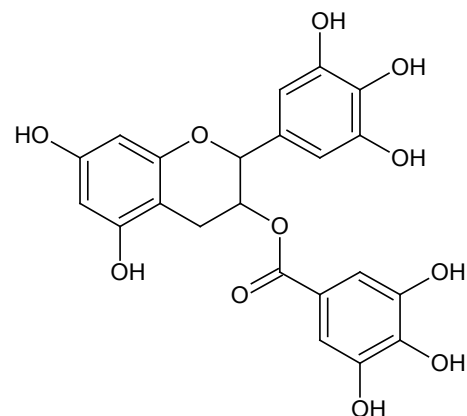


Figure 2: Structure of epigallocatechin-3-gallate.

Epigallocatechin-3-gallate (EGCG; shown in Figure 2) is found in high amounts in green tea (*Camellia sinensis*), a popular drink throughout the world. EGCG is a potent chemopreventive agent, protecting against many types of cancer [21] such as those induced by chemicals or radiation [22,23], hepatic injury [24], as well as protecting cells from damage induced by free radicals [25]. The protective role of EGCG is selective, its effect being achieved in cancer cells but not in normal cells [26,27]. These differences in EGCG actions may be attributed to the differences between antioxidant defence mechanism in normal cells and mechanism of oxidative stress in cancer cells [21].

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Q and EGCG (and, indeed, many other naturally occurring polyphenolic antioxidants) are known to bind extensively to serum/plasma proteins [28,29], and a number of studies have demonstrated that the biological effects of chemicals to cells in culture is influenced by the presence of serum in the culture medium, this ascribed to the binding of the chemicals to the serum/plasma proteins [30-32]. Accordingly, in this study the influence of serum addition to culture medium on the antioxidant effects of Q and EGCG have been determined in an *in vitro* cytoprotection assay.

Materials and Methods

Chemicals

All chemicals used in this study were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK, unless otherwise noted. Stock solutions of Q and EGCG were prepared in DMSO at $\mu\text{g}/\text{mL}$ concentration units and stored at 4°C until use. Q and EGCG were of >95% purity, as specified by the supplier.

Culture of HepG2 cells

Human hepatoma HepG2 cells obtained from ECACC (Salisbury, UK), were cultured in 175 cm² Nunclon culture flasks, in 5% CO₂ in air atmosphere at 37°C in humidified incubator prior to use, using 50 mL of minimum Essential Eagles MEM medium supplemented with 10% (v/v) foetal calf serum, 2 $\mu\text{g}/\text{mL}$ fungizone, 0.05 mg/mL gentamicin, 1% (v/v) non-essential amino acid solution, 2 mM L-glutamine (standard culture medium). The cell stocks were sub-cultured by trypsinisation in a 1:8 split ratio at 80% confluence, once a week, with medium changes every 72 hrs. For experiments including treatments, cells were sub-cultured at high density in wells of a 24-well plate in 1 mL standard culture medium; under these conditions confluence was achieved within 24 hrs.

Cytoprotection assay

The ability of plant-derived antioxidants to protect against the cytotoxicity elicited by the organic oxidant stressor t-BHP is a widely used model of cellular antioxidant activity [3,20,30], and this model was used in the present study.

For this study, we chose liver as a model for toxicity due to its susceptibility to toxins and oxidative insults [33], and HepG2 cells were used to mimic non-dividing hepatic cells by culturing the cells in a high-density confluent monolayer. Accordingly, HepG2 cells were seeded in wells of a 24-wells plate in 1 mL standard culture medium. After 24 hrs, cultures were exposed to different series of concentrations of Q or EGCG (0-100 $\mu\text{g}/\text{mL}$) with or without 0.8 mM t-BHP simultaneously for 5 hrs in serum-free medium or containing 2% (v/v) or 10% (v/v) serum, after which cell damage mediated by oxidant stress was assessed by neutral red uptake.

Neutral red assay

Cell viability determined by neutral red uptake is based on the incorporation and binding of neutral red into the lysosomes of viable cells [34]. The assay was performed as described by Adomaku-Bonsu et al. [35].

Evidence for changes in cell viability assessed by this assay were confirmed by repeated microscopic visualization of the cultures (data not shown).

Statistical analysis

Experimental data were entered into GraphPad Prism and analysed

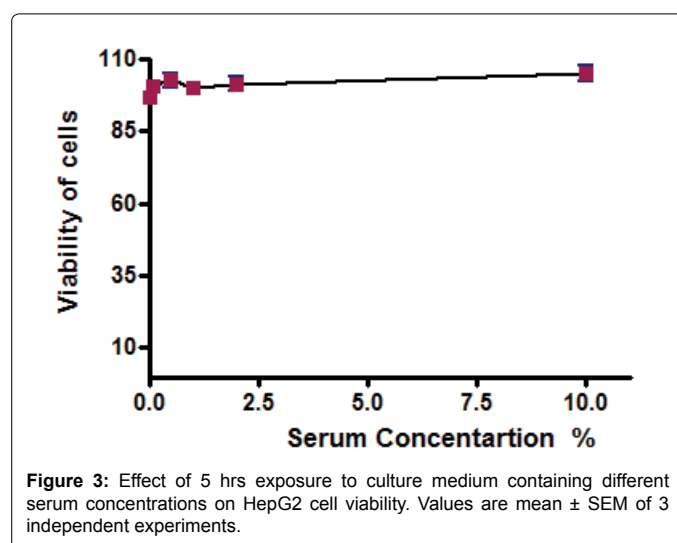


Figure 3: Effect of 5 hrs exposure to culture medium containing different serum concentrations on HepG2 cell viability. Values are mean \pm SEM of 3 independent experiments.

to identify the model that best fit the concentration-response curve, from which mean \pm S.D. EC₅₀ values were determined for Q and EGCG. Values were derived from at least 3 independent experiments. One-way ANOVA followed by selected comparisons by the Bonferroni method was used to compare the EC₅₀ values obtained under different culture conditions.

Results

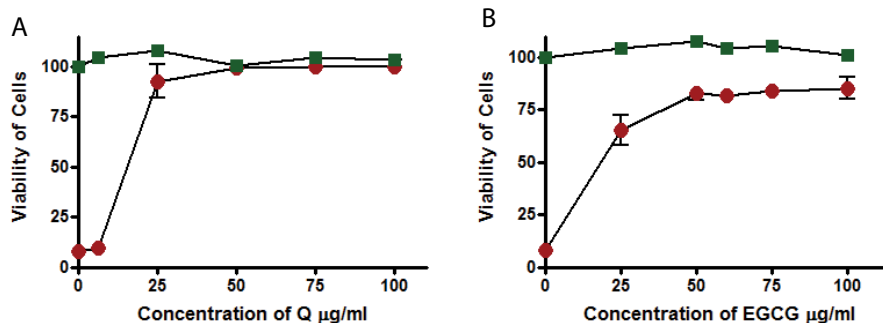
Effect of serum

Cell viability was not altered when cells were cultured for five hours in different serum conditions as shown in Figure 3. This allowed a further study of the cytoprotection provided by Q and EGCG, this time under standard (10%) and reduced serum (2%) and serum-free conditions.

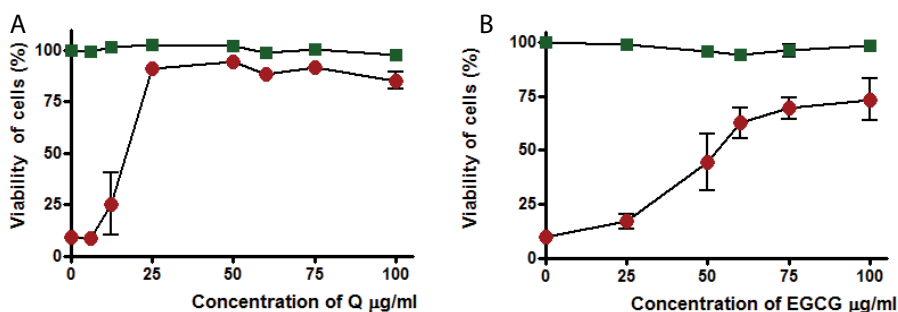
Cytoprotection activity of phytochemicals: Effect of serum

Serum-free medium: When tested in serum-free medium, Q and EGCG were not toxic to the cells during incubation individually with HepG2 cells as shown in Figures 4a and b. When cells were incubated with Q and t-BHP simultaneously for 5 hrs, Q protected against t-BHP toxicity in a concentration-dependent manner, cell viability reaching 80-100% at concentrations of 25-100 $\mu\text{g}/\text{mL}$ and EC₅₀ was 17.9 \pm 18 $\mu\text{g}/\text{mL}$. Similarly, EGCG protected the cells against oxidative stress induced by t-BHP in a concentration-dependent manner, and increased the viability of the cells to about 55-80% at concentrations of 25-100 $\mu\text{g}/\text{mL}$; EC₅₀ was determined as 23.4 \pm 7.2 $\mu\text{g}/\text{mL}$.

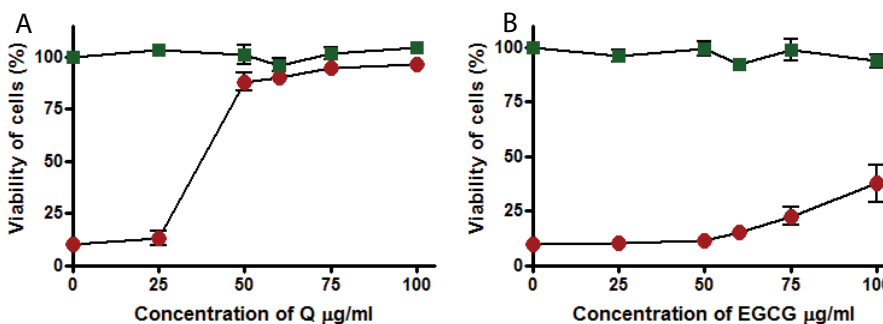
Medium 2% serum: Results displayed no loss in cell viability when HepG2 cells were incubated with different concentrations of Q (0-100 $\mu\text{g}/\text{mL}$) in the presence of 2% serum. On the other hand when cells were exposed to t-BHP for 5 hrs in the presence of Q, cells were protected against toxicity by Q and protection was very notable as concentration of Q increased, to reach to about 90-95% at 100 $\mu\text{g}/\text{mL}$ of Q and EC₅₀ was recorded 16.1 \pm 2.4 $\mu\text{g}/\text{mL}$ as shown in Figure 5a. Under similar exposure conditions, viability of cells was maintained 100% during incubation with EGCG and EGCG was not toxic at any concentration used. An enhancement in protection was demonstrated when HepG2 cells were exposed to t-BHP in the presence of EGCG; cell viability gradually increased to 75% at 100 $\mu\text{g}/\text{mL}$ EGCG and EC₅₀ was 51.2 \pm 8.3 $\mu\text{g}/\text{mL}$ as shown in Figure 5b.



Figures 4a and b: Effect of 5 hrs exposure to (a) Q and (b) EGCG in serum-free medium on the viability of HepG2 cells, in the absence (■) or presence (●) of 0.8 mM t-BHP. EC₅₀ values were 17.9 ± 1.8 and 23.4 ± 7.2 µg/mL respectively. Values are mean ± SEM of 3-7 independent experiments.



Figures 5a and b: Effect of 5 hrs exposure to (a) Q and (b) EGCG in medium containing 2% serum on the viability of HepG2 cells, in the absence (■) or presence (●) of 0.8 mM t-BHP. EC₅₀ values were 16.1 ± 2.4 and 51.2 ± 8.3 µg/mL respectively. Values are mean ± SEM of 3-7 independent experiments.



Figures 6a and b. Effect of 5 hrs exposure to (a) Q and (b) EGCG in medium containing 10% serum on the viability of HepG2 cells, in the absence (■) or presence (●) of 0.8 mM t-BHP. EC₅₀ (µg/mL) for Q and EGCG was 39 ± 4.5 and 84.7 ± 7.4 µg/mL respectively. Values are mean ± SEM of 3-7 independent experiments.

Medium 10% serum: When cells were cultured in medium containing 10% serum, cell viability was not affected by the incubation with Q for 5 hrs. Moreover, Q was able to protect HepG2 cells against oxidative stress induced by t-BHP and cell viability reached to about 85-90% at concentration of 100 µg/mL of Q and EC₅₀ was 39 ± 4.5 µg/mL as shown in Figure 6a. A notable positive relationship was reported for EGCG and cell viability for 5 hrs, in that cytoprotection was increased on increase in the concentration of EGCG, and EC₅₀ was 84.7 ± 7.4 µg/mL. Furthermore, EGCG was not toxic to HepG2 cell during the period of incubation as shown in Figure 6b. In addition, Q was significantly more active than EGCG (lower EC₅₀, p ≤ 0.001) (Table 1).

Antioxidant	Cytoprotection (expressed as EC ₅₀ in µg/mL)		
	Serum-free medium	Medium + 2% serum	Medium + 10% serum
Q	17.9 ± 1.8*	16.1 ± 2.4*	39.9 ± 4.5
EGCG	23.4 ± 7.2 ^o	51.2 ± 8.3*	84.7 ± 7.4

Note: EC₅₀ = the effective concentration of phytochemical providing 50% protection. Values are reported as mean ± S.D of 3-7 separate experiments. Data analysis was carried out as described in 'Materials and Methods'. Where indicated by *, values are significantly different (P<0.001) to that for medium + 10% serum. Where indicated by ^o, value is significantly different to that in medium + 2% serum (P<0.001).

Table 1: Influence of medium conditions on the cytoprotection exerted by Q and EGCG against oxidative stress toxicity elicited by t-BHP.

Discussion

The data presented in this report demonstrate that the presence of serum, and by implication, its protein component, modifies the cytoprotection action of Q and EGCG in human hepatoma HepG2 cells exposed to an oxidative stress elicited by t-BHP. This is most clearly demonstrated in the data in Table 1, which is a summary of the individual data presented in Figures 4-6. These data add to the body of literature illustrating the role of serum in the biological actions of organic compounds on cells in culture (see Introduction), and this report is one of the few that specifically does so in the context of cytoprotection against oxidative stress, is consistent with previous reports on the role of protein binding on antioxidant behavior of phenolic compounds in a non-cellular environment [36-38].

The modulatory effect of serum on biological actions in cell culture models is ascribed to the binding of organic compounds to proteins, predominantly albumin, present in serum, so reducing the "free" biologically active fraction in the culture medium. This interpretation is consistent with the extensive binding of Q, EGCG and related compounds that has been reported by others (see Introduction).

An important determinant in the binding affinity of Q, EGCG and related compounds to albumin appears to be the presence of multiple free hydroxyl groups in their structure (Figures 1 and 2) [29,39,40]. The effectiveness of Q, EGCG and related compounds as antioxidants and, hence, cytoprotectants, is similarly strongly dependent on the presence of multiple free hydroxyl groups [41-43]. This consideration is in keeping with the study reported by Wang and Goodman [38], who demonstrated a significant positive correlation of protein binding of dietary phenols with their antioxidant properties. Consequently, attempts at chemical modification of the structure of Q, EGCG and related compounds to minimize their protein binding affinity is likely to also reduce their antioxidant and cytoprotection properties.

Having established that the presence of serum in culture medium reduces the cytoprotection effectiveness of Q and EGCG, the question arises as to which cell culture conditions might most mimic the *in vivo* situation. The answer to this is not straightforward.

The albumin content of foetal bovine serum, the serum used in this study, is in the range 15-25 g/L [30,44], so the albumin content of media containing 2% and 10% foetal bovine serum would be in the order of 0.4 and 2 g/L respectively. Whilst there are appreciable technical difficulties in the sampling of interstitial fluid (ISF), it is generally accepted that the albumin content of ISF is of the order of 15-18 g/L [45,46], although a more recent study suggests a tissue level of approximately 1-2 g/L [47]. However, in human cerebrospinal fluid (CSF) the albumin content is less than 0.3 g/L [48,49]. In light of these observations then, cytoprotection assays performed in medium containing the higher levels of serum may reflect the *in vivo* situation in ISF, whereas assays performed in medium containing low levels of serum or in serum-free medium may reflect the *in vivo* situation in CSF.

Conclusion

In the present study we have demonstrated that the presence of serum in the culture medium modifies the cytoprotective effects of Q and EGCG, an effect which we ascribe to the well-characterized protein-binding effects of both compounds. Comparison of the protein concentrations in the various culture models used in this study with those measured in various body fluid compartments points to both low-serum and high-serum cell culture models being of relevance to prediction of likely *in vivo* antioxidant behavior of these naturally occurring polyphenolic compounds.

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