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The alkyl chain length of 3-alkyl-3′,4′,5,7-tetrahydroxyflavones modulates effective inhibition of oxidative damage in biological systems: Illustration with LDL, red blood cells and human skin keratinocytes

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ABSTRACT

It is shown that the relationship between the alkyl chain length of 3-alkyl-3′,4′,5,7 tetrahydroxyflavones (FnH) bearing alkyl chains of $n = 1$, 4, 6, 10 carbons and their capacity to counter oxidative damage varies markedly with the nature of the biological system. In Cu^{2+}-induced lipid peroxidation of low-density lipoprotein (LDL), the less hydrophobic short-chain F1H and F4H are probably located in the outer layer of LDL and parallel the reference flavonoid antioxidant, quercetin (Q) as effective inhibitors of lipid peroxidation. A marked inhibition of haemolysis induced in red blood cells (RBC) suspensions by the membrane-permeant oxidant, *tert*-butylhydroperoxide (t-BuOOH), is observed with F4H and F6H present at concentration in the micromolar range. However, F10H the most hydrophobic FnH is even more effective than Q against both haemolysis and lipid peroxidation as measured by malondialdehyde (MDA) equivalents. In oxidation of RBC by H_2O_2, at least 50 times more F6H and F10H than by t-BuOOH are required to only partly inhibit haemolysis and MDA production. The F1H, F4H and Q are found rather inactive under these conditions. At concentrations in the micromolar range, a marked protection against the cytotoxic effects of the t-BuOOH-induced oxidative stress in human skin NCTC 2544 keratinocytes is also exhibited by the four FnH antioxidants and is comparable to that of Q. Thus, the four FnH species under study may be considered as potent antioxidants which manifest complementary anti-oxidative actions in biological systems of markedly different complexity.

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1. Introduction

It is well established that flavonoids present in large quantities in vegetables and fruits exhibit antioxidant properties. Their antioxidant activity has been attributed: (a) to the presence of a catechol moiety in the B-ring; (b) to the 4′-OH group conjugated with the 3-OH or 4-keto functions of the C-ring through the C2═C3 double bond; (c) to the presence of 3- and 5-hydroxyl groups in the flavone molecular structure [1,2]. The antioxidant effectiveness of hydroxyflavones has also been related to the number of hydroxyl groups in the molecule, and also to their hydrogen radical donating abilities [2]. In relation to their antioxidant properties, beneficial effects of flavones have also been reported for cancer [3], bacterial [1,4] or viral infections [5] and inflammatory diseases [4,6].

Numerous investigations have demonstrated that the flavonol quercetin (Q) [1] and the flavone luteolin [2,7] are powerful antioxidants. Recent studies have revealed that lipophilic flavones bearing methyl, isopropyl, benzyl, or isoprenyl groups enhance the binding affinity towards P-glycoprotein and diminish cancer cell chemoresistance [3]. In light of these several reports, we have synthesized a series of novel 3-alkyl-3′,4′,5,7-tetrahydroxyflavones (FnH) bearing alkyl chains of n = 1, 4, 6 or 10 carbons [8]. It is hoped that by varying the lipophilic 3-alkyl chain length of these luteolin analogues, one may promote interesting biochemical properties. For example, these compounds, which lack the hydroxyl group at C-3 position—a major site of conjugation *in vivo*—may have better pharmacokinetics in humans than other types of polyhydroxyflavones, *e.g.* Q, which are not effectively transferred from the digestive lumen into the blood stream and which additionally, are extensively metabolized [9]. Furthermore, related 3-alkyl-3′,4′-dihydroxypolymethoxyflavone derivatives are known to inhibit arachidonate 5-lipoxygenase [10].

Recently, we have demonstrated that all the FnH species synthesized are effective inhibitors of $^{\bullet}O_2^-$ radical-anions, a model reactive oxygen species (ROS) of biological significance. In addition, they can repair radical induced damage to Trp, an aromatic amino-acid essential to the structure and function of many proteins [11]. We have also shown that using FnH antioxidants of increasing alkyl chain length provides control of oxidation processes at various depths in organized microstructures such as ionic or neutral micelles. Significantly, they can act to stabilize such micro-heterogeneous structures. The observed dependence of radical kinetics on alkyl structures in these model systems is important from a biological viewpoint, and suggests a fruitful study of the effectiveness of these new antioxidants whose cellular distribution may be selectively modulated. In view of the results in micellar environments, we have investigated the capacity of these 3-alkyl-3′,4′,5,7-tetrahydroxyflavones to control oxidative processes in several well established biologically relevant but structurally different model systems. In this manner, we hope to elucidate possible structurally-dependent chain length effects on selected biological activities. The biological model systems chosen for these studies include blood LDL, RBC and human skin keratinocytes.

Here we have compared the ability of the four FnH species to inhibit the Cu^{2+}-induced lipid peroxidation of LDL. In the cellular studies, RBC haemolysis and cytotoxic effects in keratinocytes were utilized as markers of cell injuries. In measurements with RBC and keratinocytes, oxidative stress has been generated by the amphiphatic t-BuOOH, a stable organic hydroperoxide and H_2O_2. In all the cases, the protection afforded by FnH has been compared to that of Q, one of the most effective natural flavonoid antioxidant in the Cu^{2+}-induced lipid peroxidation of LDL [2,12,13] and in oxidative stress-related cytotoxic effects [14].

2. Materials and methods

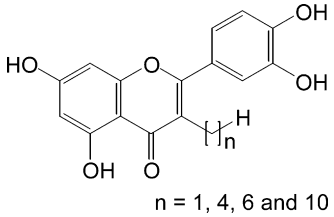
2.1. Chemicals and routine equipment

All routine chemicals were of analytical grade and were used as received from the suppliers. Quercetin dihydrate (Q), t-BuOOH and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical (Saint-Louis, MO, USA). Dimethyl sulfoxide (DMSO), H_2O_2 and absolute ethanol were supplied by Merck and were of spectroscopic grade. The phosphate buffer (pH 7.4) was prepared in pure water obtained with a reverse osmosis system from Millipore. This water exhibits a resistivity of $>18\ M\Omega\ cm^{-1}$ and a total organic content of <10 ppb. Absorption spectrophotometry was carried out with an Uvikon 922 spectrophotometer whereas fluorescence spectra were recorded with a Shimadzu RF5000 spectrofluorometer.

The 3-alkyl-3′,4′,5,7-tetrahydroxyflavones (Scheme 1) were synthesized as described in the literature [8]. They have been abbreviated as FnH in accordance with our preceding article on their redox properties [11].

2.2. Preparation and treatment of LDL

Serum samples were obtained from healthy volunteers. The LDL (d = 1.024-1.050 g/mL) was prepared by sequential ultracentrifugation according to Havel et al. [15]. Protein content was determined by the technique of Peterson [16]. The LDL samples were used within 2–3 weeks of preparation. Just before experimentation, LDL was dialyzed twice for 8 and 16 h against 1 L of 10 mM phosphate buffer (pH 7.4) to remove EDTA and then was diluted to a final concentration of 0.15 mg protein/mL (300 nM). Subsequently, 150 μL of 5 μM solutions of FnH or of Q in 10 mM phosphate buffer was added to 800 μL of the diluted LDL. Blank LDL solutions without FnH or Q were also prepared. The LDL solutions loaded with FnH or Q and the blank LDL solutions were then incubated at 37 °C for 15 min. Lipid peroxidation was initiated by adding 50 μL of 100 μM aqueous Cu^{2+} solution to produce a final concentration of 5 μM Cu^{2+} in the incubation medium.



Scheme 1

2.3. Conjugated diene determination and consumption of carotenoids

Conjugated diene formation was monitored by measuring the increase in absorbance at 234 nm and was determined periodically during incubation at 37 °C. Changes in carotenoid concentration during LDL oxidation were monitored by second derivative absorption spectroscopy (400–550 nm) through measurement of the second derivative spectrum amplitude between 489 and 516 nm and were then expressed as percent of the initial concentration [17]. In human plasma, four carotenoids, α-carotene, β-carotene, β-cryptoxanthin and lycopene are the principle contributors to the absorbance in the 400–550 nm region [18,19].

2.4. Fluorometric determination of the ApoB-100 Trp loss

The concentration of intact Trp residues in the apolipoprotein was determined in native LDL solutions (240 nM) following a previously published procedure [20]. The fluorescence of the Trp residues was excited at 292 nm, a wavelength absorbed specifically by these residues. Under these conditions, the contribution of the vitamin E fluorescence is negligible [20,21]. The fluorescence of the LDL solutions was recorded every 10 min in order to obtain accurate degradation kinetics at 37 °C.

2.5. Preparation and treatment of red blood cells

Venous blood was collected from healthy volunteers. Red blood cells were then separated from heparinized blood by centrifugation at 2000 × *g* for 10 min and used immediately after three washings with ice-cold physiological saline. Red blood cell suspensions were prepared with 10 mM phosphate buffer in 150 mM NaC1 (pH 7.4) to obtain a final concentration of 2.5% (v/v). In the case of experiments with hydrogen peroxide as the oxidant, the erythrocyte suspensions were pre-incubated with 2 mM sodium azide for 1 h at 37 °C in a shaking water bath. The percent of hemolysis was spectrophotometrically determined according to the method of Kellogg and Fridovich by measuring the absorbance change at 409 nm, 100% hemolysis being obtained by an osmotic shock with distilled water [22]. The extent of lipid peroxidation in RBC was estimated by measuring the fluorescence of thiobarbituric acid reactive substances (TBARS) as already described [23]. The TBARS levels were expressed as MDA equivalents in units of nmol/g haemoglobin [23]. The acid hydrolysis of 1,1,3,3-tetraethoxypropane which yields MDA was used for calibrating TBARS fluorescence [17]. Haemoglobin concentration was estimated spectrophotometrically by the cyanmethemoglobin method [23]. The t-BuOOH, H_2O_2, Q and FnH stock solutions in DMSO were added to diluted red cell suspensions to obtain the desired final concentrations. *tert*-Butylhydroperoxide and hydrogen peroxide were always added 15 min after the FnH.

2.6. Cell culture and treatment

The NCTC 2544 immortalized human skin keratinocyte cell line was purchased from ICN Flow (Fontenay sous Bois, France). Cultures were propagated in minimum essential medium with Earle's salts (EMEM) supplemented with 10% foetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (weekly passages, 1:10 splitting ratio). Cells from trypsinized confluent monolayers were seeded at a density of 15,000 cells/cm^2 on plates with 24 wells of about 2 cm^2 each, containing 1 mL of complete medium (EMEM supplemented with FCS). These were grown for 4 days to about 75% of confluency. After two washings with 1 mL of phosphate buffer saline (PBS), cells were incubated for 1 h at 37 °C with 250 μL of FnH in PBS at the desired concentration. Then, after addition of 5 mM t-BuOOH (from 0.125 M stock solutions in PBS) and incubation for 2 h with the oxidant, cells were washed and further incubated for 2 h at 37 °C in complete medium. After two washings with PBS, the challenged cells were submitted to the viability assay with Neutral Red (NR). The 2 h lag between treatment and assay was chosen to allow the initial damage to propagate but was short enough to avoid important proliferation of undamaged cells (population doubling time is about 1 day) which may obscure the effect of the oxidative stress.

2.7. Neutral red uptake assay

The cytotoxicity of t-BuOOH on NCTC 2544 keratinocytes, was determined by the widely used NR uptake assay [24,25]. It has been validated by the European Union for testing phototoxic chemicals and for the classification and labelling of hazardous chemicals (EU Commission Directive 2000/33/EC). The day before the assay, a saturated aqueous NR solution was diluted (1:80, v/v) with complete medium and left overnight at 37 °C. Just before use, this NR solution was centrifuged twice to eliminate excess NR precipitate. Challenged cells were washed and incubated with 250 μL of this NR solution for 2 h at 37 °C. Then, after three washings with PBS, 400 μL of a 1% SDS solution in water was added to solubilise cells and absorbance was read at 550 nm in 96 microwell plates. Data are presented as the percentage of NR uptake with respect to that of a control experiment carried out in the absence of oxidant. The data are the mean ± SD of at least three independent experiments, each performed in triplicates.

3. Results and discussion

3.1. Inhibition of conjugated diene formation and carotenoid bleaching by FnH during LDL oxidation

Low-density lipoprotein is a natural carrier of important antioxidants such as vitamin E and carotenoids. In the four LDL preparations we used for this set of experiments, the vitamin E/LDL and β-carotene/LDL concentration ratios (mol/mol) were 6.72 ± 0.72 and 0.40 ± 0.25, respectively as determined by HPLC [26]. These antioxidants inhibit LDL lipid peroxidation until they are consumed. An induction period or lag time is thus observed between the start of the oxidation by Cu^{2+} ions and the appearance of lipid peroxidation products. The duration of this induction period depends on the constitutive antioxidant content of LDL which may vary among blood donors [27]. Fig. 1A shows the time course of the conjugated diene formation after addition of 5 μM Cu^{2+} to

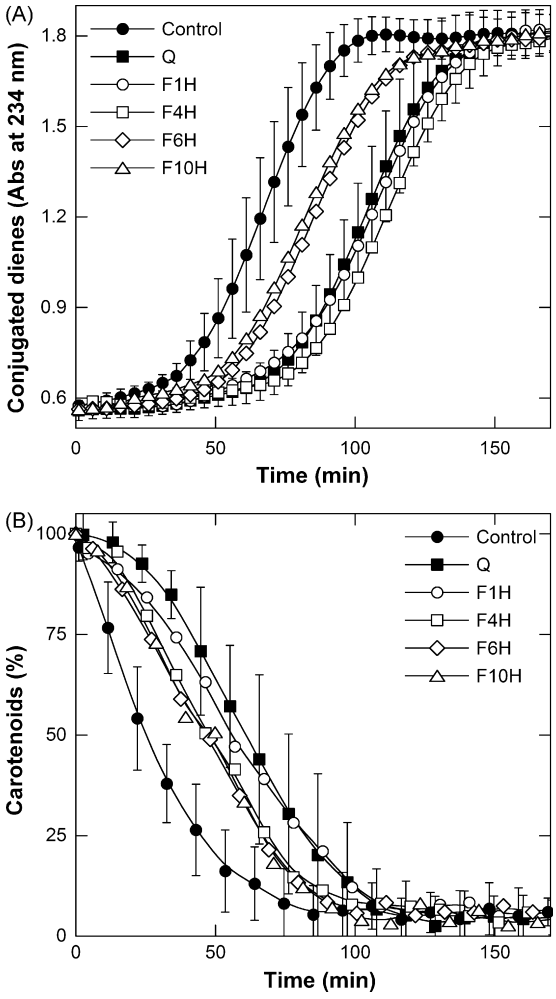


Fig. 1 – (A) Kinetics of conjugated diene formation during LDL oxidation induced by 5 μM Cu^{2+}. LDL solutions of 0.12 mg protein/mL (240 nM) final concentration in 10 mM phosphate buffer (pH 7.4) were incubated for 15 min at 37 °C with or without 0.75 μM FnH or Q before Cu^{2+} addition. Note that time zero denotes measurements made about 1 min after Cu^{2+} addition. Data are the mean ± SD of four independent experiments. For clarity, SD are only shown for control and Q-loaded LDL. (B) Kinetics of carotenoid consumption during LDL oxidation induced by 5 μM Cu^{2+}. Experimental conditions are identical to those of Fig. 1A. Data are the mean ± SD of four independent experiments. For clarity, SD are only shown for control and for Q-loaded LDL.

240 nM of native LDL occurring in the absence or in the presence of 0.75 μM FnH or Q. It may be seen that under these conditions the lipid peroxidation in the absence of FnH begins almost immediately after Cu^{2+} addition. The time lapse for producing 50% of the conjugated dienes obtained at plateau (CD50) is ~70 min. Addition of F1H, F4H or Q totally inhibits the formation of conjugated dienes during ~50 min of incubation at 37 °C and shifts the CD50 to ~2 h. By contrast, F6H and F10H only increase the CD50 by ~30% compared to that in control solution containing no antioxidants. Thus, in the Cu^{2+}-induced LDL oxidation model, F1H and F4H can be considered to be as good an antioxidant as Q, one of the most potent polyphenol antioxidant [2,28].

To further characterise the order of antioxidant effectiveness F4H > F1H = Q ≫ F6H = F10H established from Fig. 1A data, carotenoid consumption was also monitored under the same experimental conditions. Fig. 1B suggests similar inhibition of the carotenoid consumption by the FnH derivatives, F1H, again being equivalent to Q in effective protection.

These data support several structure-activity relationships regarding the antioxidant capacity of the FnH species as a function of 3-alkyl chain length. FnH is hydrophobic and readily solubilises in the LDL solution but not in buffer, suggesting that FnH must incorporate into LDL particles at some level. On the basis of results obtained with our preceding study in micellar media [11], the large variation observed here in antioxidant effectiveness may be due to differing FnH locations in LDL as a function of the alkyl chain length. Owing to their four hydroxyl groups F1H and, to a lesser extent, F4H are expected to be located in the water-rich outer layer constituted of phospholipids, cholesterol and ApoB-100 apolipoprotein which enwraps the LDL particle and contributes to its solubility in plasma. Indeed, addition of 0.75 μM of non fluorescent F1H to the 240 nM LDL solution induces a 7% quenching of Trp residue fluorescence suggesting close interaction between F1H and some Trp residues of ApoB-100 (data not shown). On the other hand, the longer alkyl tails of F6H and F10H most probably favour hydrophobic interactions with the LDL lipid core, thereby moving the polyhydroxyphenol head groups more deeply in the LDL particle. Because of the binding of Cu^{2+} ions to ApoB-100, the initial targets of the LDL oxidation by Cu^{2+} are 8–9 Trp residues of ApoB-100, and is a vitamin E-independent process [21]. The resulting formation of indolyl radicals ($Trp^{\bullet}$) triggers the radical chain reactions of peroxidation of LDL lipids most probably located in the vicinity of the oxidized Trp residues [21]. These $Trp^{\bullet}$ radicals are not repaired by vitamin E or β-carotene [26]. With the assumption that Trp residues are the initial targets of Apo-B-bound Cu^{2+} ions, F1H—the least hydrophobic FnH—is expected to be the most effective antioxidant by consequence of its proximity to these Trp residues and its ability to repair them [11] thus impeding the initiation of LDL lipid peroxidation. In this regard, Fig. 2A shows that although in the absence of Cu^{2+} ions LDL-bound Q can repair ApoB-100 radical species [29], addition of 0.75 μM Q may actually accelerate Cu^{2+} ion-induced Trp residue destruction. By contrast, the same concentration of F1H provides a level of protection. Additionally, the ability of FnH to neutralize reactive oxygen species (ROS) by hydrogen donation [11] and to form complexes with Cu^{2+} ions (Fig. 2B) must also contribute to the observed inhibition. Taking into account all these observations, it can be seen that the present experimental system defines an overall apparent antioxidant capacity of these FnH antioxidants.

3.2. *Inhibition of lysis and membrane lipid peroxidation of red blood cells by FnH*

tert-Butylhydroperoxide is a membrane-permeant oxidant extensively used to induce oxidative stress in different

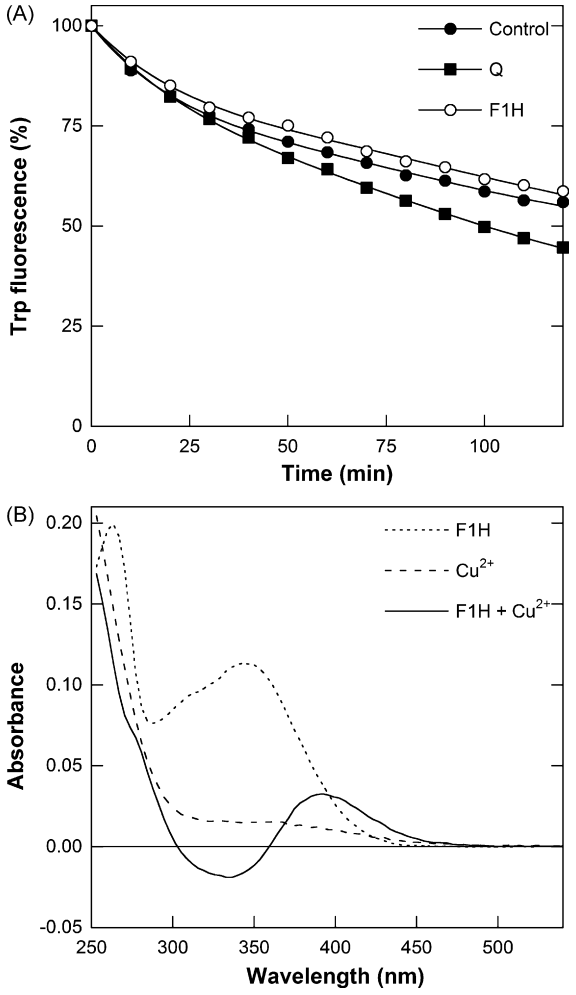


Fig. 2 – A Destruction of Trp residues during LDL oxidation induced by 5 μM Cu^{2+}. LDL solutions of 0.12 mg protein/mL (240 nM) final concentration in 10 mM phosphate buffer (pH 7.4) were incubated for 15 min at 37 °C with or without 0.75 μM F1H or Q before Cu^{2+} addition. The fluorescence of the LDL solutions (expressed in percent of the initial Trp fluorescence under excitation with 292 nm radiation) was recorded at 37 °C and measured at 10 min intervals. Note that time zero denotes measurements made immediately after Cu^{2+} addition. Data represent one experiment performed in duplicate. (B) Spectra demonstrating the complexation of Cu^{2+} ions by F1H. Absorbance spectra of 100 μM Cu^{2+} and of 10 μM F1H were measured in 10 mM phosphate buffer (pH 7.4) at 20 °C. The third spectrum corresponding to the Cu^{2+} + F1H mixture is a difference absorbance spectrum (sample cell: 10 μM F1H + 100 μM Cu^{2+}, reference cell: 10 μM F1H).

systems [30,31]. Its *tert*-butyl group confers lipid solubility. It thus easily penetrates membranes and is rapidly transferred to the cytosol. Here it is metabolized by the GSH peroxidase–reductase system with consumption of GSH and NADPH. When this enzymatic system is overwhelmed, a one-electron oxidation (or reduction) process mediated by catalytically active metal ions (for example, cytochrome P450) initially gives rise to radicals comparable to those encountered in the chain reaction of lipid peroxidation (peroxyl or alkoxyl radicals). These eventually rearrange and decompose into carbon-centered radicals such as methyl radicals. Such t-BuOOH-derived radicals are responsible for the lethal effects

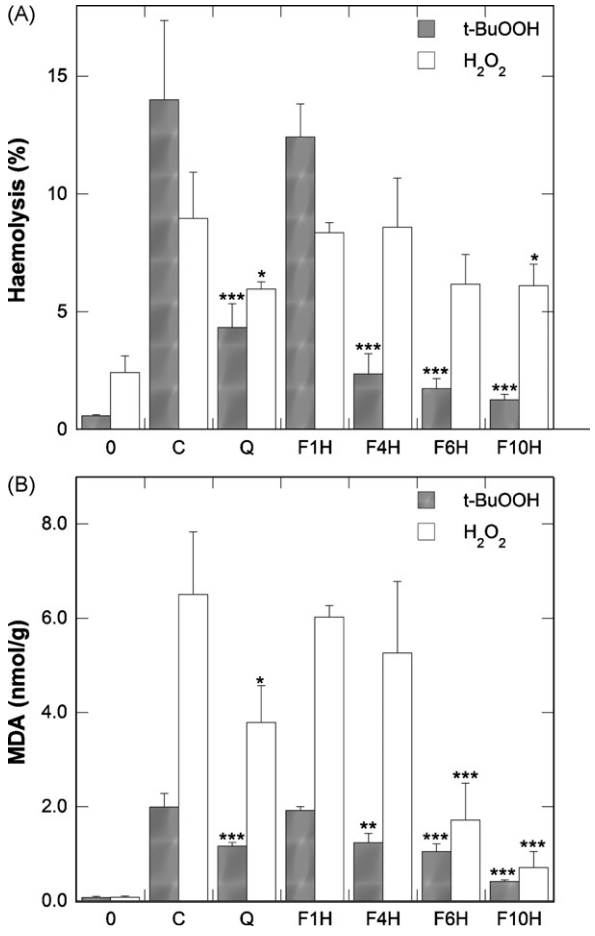


Fig. 3 – (A) Haemolysis (in percent) of RBC suspensions (2.5%, v/v) in 10 mM phosphate buffer (pH 7.4) after a 1 h incubation at 37 °C with 0.6 mM t-BuOOH or after incubation for 90 min with 10 mM H_2O_2 in the absence (C) or in the presence of FnH or Q. The concentrations of FnH or Q were 1.5 μM and 0.1 mM for oxidation by t-BuOOH and H_2O_2, respectively. Data are the mean ± SD of four independent experiments. Data are the mean ± SD of four independent experiments. Analysis of variance was performed with an unpaired Student's *t*-test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$). (0) shows the haemolysis in the suspensions in the absence of any treatment. In the control (C), DMSO was added at a concentration corresponding to the dilution of stock solutions of the antioxidants (see text). A longer incubation time, a 1 h pre-incubation with 2 mM sodium azide and a 5-fold increased in DMSO concentration explain the increased hemolysis in controls of H_2O_2 experiments as compared to those with t-BuOOH. (B) Production of MDA expressed in nmol/g of haemoglobin in the RBC suspensions. The experimental conditions are the same as in Fig. 3A. Data are the mean ± SD of four independent experiments. Analysis of variance was performed with an unpaired Student's t-test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).

observed and trigger free radical chain reactions in membranes [32–34]. Trotta et al. have demonstrated that t-BuOOH degrades haemoglobin and provokes lipid peroxidation in RBC ([34] and references therein). A concentration of 0.6 mM t-BuOOH and an incubation time of 60 min were found sufficient to induce about 15% haemolysis in human RBC suspensions and significant production of TBARS, the lipid peroxide decomposition products (Fig. 3A and B). However, significant protection against haemolysis (Fig. 3A) in the presence of only 1.5 μM F4H, F6H or F10H was observed. The diminution of RBC lysis was accompanied by a marked reduction of TBARS formation with F10H being again the most effective (Fig. 3B). It should be noted that under comparable experimental conditions, F4H, or F6H or F10H are proven to be much better inhibitors of RBC haemolysis and lipid peroxidation than 2,6-bis(1,1-dimethylethyl)-4-methylphenol (butylated hydroxytoluene), the well established lipid-soluble antioxidant [34]. By contrast, F1H was found to be essentially inactive in this system

It has long been known that H_2O_2 causes haemolysis and lipid peroxidation of RBC [35]. Hence it is of interest to compare the capacity of the various FnH species to protect RBC from the oxidative stress induced by H_2O_2 to that observed with t-BuOOH since appreciable differences in their biological action have been reported [36]. Hydrogen peroxide, a natural oxidant, diffuses across hydrophobic membranes and it is metabolized by GSH peroxidase and by catalase. Here, the latter was inhibited by addition of NaN_3, potentiating the oxidative damage. Hydrogen peroxide reacts with redox metal ions by the so-called Fenton reactions to produce $^{\bullet}OH$ radical, the strongest oxidizing species known (normal redox potential: 2.6 V *vs* NHE). As a result, $^{\bullet}OH$ radicals react unselectively at their site of formation with all biological constituents, not just those targets critical for maintaining the integrity of structure and function. A 90 min incubation of the RBC with 10 mM H_2O_2 produces haemolysis (Fig. 3A) and TBARS production (Fig. 3B) of the same order of magnitude as those obtained with t-BuOOH. However, in contrast to t-BuOOH, addition of at least 100 μM F6H or F10H is necessary to achieve the limited inhibition of RBC lysis (30%) observed. In the case of TBARS measurements, replacing F4H by F6H or F10H dramatically reduced TBARS production. At a concentration of 100 μM neither F1H nor F4H significantly modified either TBARS formation or haemolysis.

The comparison of data from RBC obtained with t-BuOOH and H_2O_2 suggests that in both cases, the FnH species with the longest alkyl chain, and hence the most hydrophobic—F10H and in a lesser extent F6H—are the most effective protectors against haemolysis and lipid peroxidation. In all instances, Fig. 3A and B suggests that they compare favourably with Q as antioxidants. By contrast, F4H can effectively protect against haemolysis but less ineffectively against lipid peroxidation only in the case of t-BuOOH-induced oxidative stress.

In the light of the above discussion it is of note that F1H and F4H were quite effective in inhibiting the Cu^{2+}-induced LDL lipid peroxidation but brought less protection to the RBC, again supporting the suggestion of site-specific oxidation reactions at the LDL–water interface where F1H and F4H are probably localized.

3.3. *Inhibition of the t-BuOOH-induced oxidative stress by FnH in NCTC 2544 keratinocytes*

As skin is the interface between the body and the environment, there are numerous studies on cell disorders induced by various agents of oxidative and photo-oxidative stress. In addition to membrane lipid peroxidation, ROS have been shown to react in all skin cell compartments and with most cell structures. For example, they are responsible for impairment of cell signalling and activation of stress-sensitive protein kinases leading to enhanced production of cytokines. They also directly react with phosphatases or redox-sensitive proteins (for a review see ref. [37]). Because lipid peroxidation is the more relevant of the processes to radical induced skin damage, t-BuOOH was preferred for this work, as it initially gives rise to oxygen radicals mimicking those encountered in lipid peroxidation. In this regard, it has been shown that t-BuOOH is metabolized by freshly isolated or cultured normal human keratinocytes with production of cytotoxic free radicals leading to severe plasma membrane damage [38]. Given the large variety of skin related targets for t-BuOOH reported in the literature, we have chosen to follow an overall response to its oxidative stress. For this purpose, NR, a lysosomotropic dye which is only retained by intact lysosomes was used to assess the viability of the NCTC 2544 keratinocytes subjected to cytotoxic effects of t-BuOOH in the absence or in the presence of the four FnH antioxidants.

Fig. 4 demonstrates the strong cytotoxicity of 5 mM t-BuOOH towards NCTC 2544 keratinocytes after 2 h incubation at 37 °C in PBS in the absence of any FnH. Thus, after the initial damage had propagated for 2 h (see materials and methods), less than 20% of cells were found to have recovered from injury. Addition of only 0.5 μM of all FnH species or of Q provided some

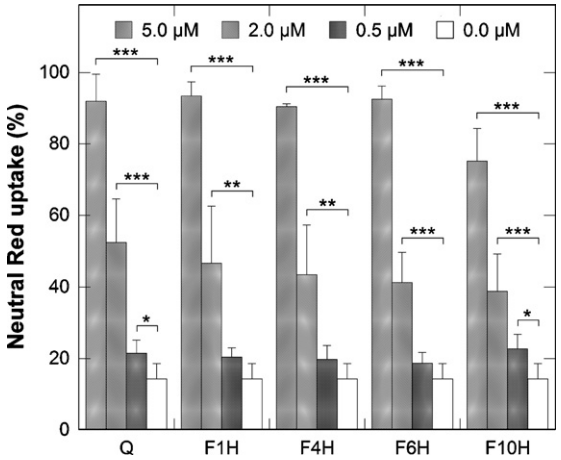


Fig. 4 – Effect of 3-alkyl-3′,4′,5,7-tetrahydroxyflavones (FnH) and of the flavonol quercetin (Q) on the cytotoxicity of 5 mM t-BuOOH towards NCTC 2544 keratinocytes. Cells were incubated in the absence or presence of several concentrations of antioxidants as indicated on the graph. The neutral red assay was performed 2 h after the incubation with t-BuOOH. Data are the mean ± SD of four independent experiments in triplicate, with untreated cells taken as 100%. Statistics were performed with an unpaired Student's t-test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).

protection to the keratinocytes. In the presence of 5 μM of all the antioxidants, most cells survived the oxidative stress. However, a trend can be noted with slightly less protection by F10H which has the longest alkyl chain length, while F1H, F4H and F6H are shown to be as effective as Q. In our previous kinetic study on the one-electron oxidation of the FnH by $^{\bullet}O_2^-$ radical-anions, we have shown that F6H and F10H can strongly increase the rigidity of hydrophobic microenvironments [11]. As a consequence, it may be hypothesized that the decyl tail on the 3′,4′,5,7-tetrahydroxyflavones may somewhat hinder the translocation of F10H from the plasma membrane to cell organelles, causing less overall protection.

4. Conclusions

As suggested by our very recent study on the high reactivity of the 3′,4′,5,7-tetrahydroxyflavones with $^{\bullet}O_2^-$ radical-anions taken as model ROS and their ability to repair $^{\bullet}$Trp radicals [11], the present work demonstrates that 3,3′,4′,5,7-tetrahydroxyflavones are potent antioxidants in the three model biological systems of increasing complexity examined here. Interestingly, the structure-activity relationships drawn from antioxidant protection provided by the various FnH antioxidants strongly depends on the model studied. Thus, significant effects are observed with F1H and F4H in the prevention of LDL oxidation by Cu^{2+} ions but have little or no effect on RBC lysis whereas the opposite situation hold for F6H and F10H. Our kinetic study on the reactivity of these FnH antioxidants shows that although there is no change in the intrinsic redox properties of the tetrahydroxyflavone ring, the apparent reactivity of FnH species are strongly modulated by their alkyl chain length. This parameter governs their partition in aqueous *vs* hydrocarbon-like environments and may be related to increase the compactness of the hydrocarbon-like core [11]. Our present work suggests that the biological activity of these compounds may follow a similar rule. Finally, it is hoped that both the physico-chemical work and the present biochemical investigation may stimulate fruitful routes for studying new antioxidants whose cellular distribution may be selectively modulated.

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