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**The alkyl chain length of  
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illustration with LDL, red blood cells and human skin  
keratinocytes**

Paulo Filipe, Artur M.S. Silva, Raquel S.G.R. Seixas, Diana C.G.A. Pinto,  
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## 1 ABSTRACT

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4 It is shown that the relationship between the alkyl chain length of 3-alkyl-3',4',5,7  
5 tetrahydroxyflavones (FnH) bearing alkyl chains of n = 1, 4, 6, 10 carbons and their capacity  
6 to counter oxidative damage varies markedly with the nature of the biological system. In  
7  
8 Cu<sup>2+</sup>-induced lipid peroxidation of low-density lipoprotein (LDL), the less hydrophobic short-  
9 chain F1H and F4H are probably located in the outer layer of LDL and parallel the reference  
10 flavonoid antioxidant, quercetin (Q) as effective inhibitors of lipid peroxidation. A marked  
11 inhibition of haemolysis induced in red blood cells (RBC) suspensions by the membrane-  
12 permeant oxidant, *tert*-butylhydroperoxide (t-BuOOH), is observed with F4H and F6H  
13 present at concentration in the micromolar range. However, F10H the most hydrophobic FnH  
14 is even more effective than Q against both haemolysis and lipid peroxidation as measured by  
15 malondialdehyde (MDA) equivalents. In oxidation of RBC by H<sub>2</sub>O<sub>2</sub>, at least 50 times more  
16 F6H and F10H than by t-BuOOH are required to only partly inhibit haemolysis and MDA  
17 production. The F1H, F4H and Q are found rather inactive under these conditions. At  
18 concentrations in the micromolar range, a marked protection against the cytotoxic effects of  
19 the t-BuOOH-induced oxidative stress in human skin NCTC 2544 keratinocytes is also  
20 exhibited by the four FnH antioxidants and is comparable to that of Q. Thus, the four FnH  
21 species under study may be considered as potent antioxidants which manifest complementary  
22 anti-oxidative actions in biological systems of markedly different complexity.  
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52 **Keywords:** lipid peroxidation, hydrogen peroxide, *tert*-butylhydroperoxide, flavonoid,  
53 quercetin, cytotoxicity  
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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  $\cdot\text{O}_2^-$  radical-anions, a model reactive oxygen species (ROS) of biological

1 significance. In addition, they can repair radical induced damage to Trp, an aromatic amino-  
2 acid essential to the structure and function of many proteins [11]. We have also shown that  
3 using FnH antioxidants of increasing alkyl chain length provides control of oxidation  
4 processes at various depths in organized microstructures such as ionic or neutral micelles.  
5 Significantly, they can act to stabilize such micro-heterogeneous structures. The observed  
6 dependence of radical kinetics on alkyl structures in these model systems is important from a  
7 biological viewpoint, and suggests a fruitful study of the effectiveness of these new  
8 antioxidants whose cellular distribution may be selectively modulated. In view of the results  
9 in micellar environments, we have investigated the capacity of these 3-alkyl-3',4',5,7-  
10 tetrahydroxyflavones to control oxidative processes in several well-established biologically  
11 relevant but structurally different model systems. In this manner, we hope to elucidate  
12 possible structurally-dependent chain length effects on selected biological activities. The  
13 biological model systems chosen for these studies include blood LDL, RBC and human skin  
14 keratinocytes.  
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34 Here we have compared the ability of the four FnH species to inhibit the  $\text{Cu}^{2+}$ -induced  
35 lipid peroxidation of LDL. In the cellular studies, RBC haemolysis and cytotoxic effects in  
36 keratinocytes were utilized as markers of cell injuries. In measurements with RBC and  
37 keratinocytes, oxidative stress has been generated by the amphiphatic t-BuOOH, a stable  
38 organic hydroperoxide and  $\text{H}_2\text{O}_2$ . In all the cases, the protection afforded by FnH has been  
39 compared to that of Q, one of the most effective natural flavonoid antioxidant in the  $\text{Cu}^{2+}$ -  
40 induced lipid peroxidation of LDL [2, 12, 13] and in oxidative stress-related cytotoxic effects  
41 [14].  
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## 52 **2. Materials and methods**

### 53 *2.1. Chemicals and routine equipment*

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1 All routine chemicals were of analytical grade and were used as received from the suppliers.  
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3 Quercetin dihydrate (Q), t-BuOOH and sodium dodecyl sulfate (SDS) were purchased from  
4  
5 Sigma Chemical (Saint-Louis, MO, USA). Dimethyl sulfoxide (DMSO), H<sub>2</sub>O<sub>2</sub> and absolute  
6  
7 ethanol were supplied by Merck and were of spectroscopic grade. The phosphate buffer (pH  
8  
9 7.4) was prepared in pure water obtained with a reverse osmosis system from Millipore. This  
10  
11 water exhibits a resistivity of > 18 MOhms cm<sup>-1</sup> and a total organic content of < 10 ppb.  
12  
13 Absorption spectrophotometry was carried out with an Uvikon 922 spectrophotometer  
14  
15 whereas fluorescence spectra were recorded with a Shimadzu RF5000 spectrofluorometer.  
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19 The 3-alkyl-3',4',5,7-tetrahydroxyflavones (Scheme 1) were synthesized as described in the  
20  
21 literature [8]. They have been abbreviated as FnH in accordance with our preceding article on  
22  
23 their redox properties [11].  
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## 26 2.2. Preparation and treatment of LDL

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28 Serum samples were obtained from healthy volunteers. The LDL ( $d = 1.024\text{-}1.050$  g/mL) was  
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30 prepared by sequential ultracentrifugation according to Havel *et al.* [15]. Protein content was  
31  
32 determined by the technique of Peterson [16]. The LDL samples were used within 2-3 weeks  
33  
34 of preparation. Just before experimentation, LDL was dialyzed twice for 8 and 16 h against 1  
35  
36 liter of 10 mM phosphate buffer (pH 7.4) to remove EDTA and then was diluted to a final  
37  
38 concentration of 0.15 mg protein/mL (300 nM). Subsequently, 150  $\mu\text{L}$  of 5  $\mu\text{M}$  solutions of  
39  
40 FnH or of Q in 10 mM phosphate buffer were added to 800  $\mu\text{L}$  of the diluted LDL. Blank  
41  
42 LDL solutions without FnH or Q were also prepared. The LDL solutions loaded with FnH or  
43  
44 Q and the blank LDL solutions were then incubated at 37 °C for 15 min. Lipid peroxidation  
45  
46 was initiated by adding 50  $\mu\text{L}$  of 100  $\mu\text{M}$  aqueous Cu<sup>2+</sup> solution to produce a final  
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48 concentration of 5  $\mu\text{M}$  Cu<sup>2+</sup> in the incubation medium.  
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## 51 2.3. Conjugated diene determination and consumption of carotenoids

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

#### 19 *2.4. Fluorometric determination of the ApoB-100 Trp loss*

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

#### 26 *2.5. Preparation and treatment of red blood cells*

27 Venous blood was collected from healthy volunteers. Red blood cells were then separated  
28 from heparinized blood by centrifugation at 2000 g for 10 min and used immediately after 3  
29 washings with ice-cold physiological saline. Red blood cell suspensions were prepared with  
30 10 mM phosphate buffer in 150 mM NaCl (pH 7.4) to obtain a final concentration of 2.5%  
31 (v/v). In the case of experiments with hydrogen peroxide as the oxidant, the erythrocyte  
32 suspensions were pre-incubated with 2 mM sodium azide for 1 h at 37 °C in a shaking water  
33 bath. The percent of hemolysis was spectrophotometrically determined according to the  
34 method of Kellogg and Fridovich by measuring the absorbance change at 409 nm, 100%  
35 hemolysis being obtained by an osmotic shock with distilled water [22]. The extent of lipid  
36

1 peroxidation in RBC was estimated by measuring the fluorescence of thiobarbituric acid  
2 reactive substances (TBARS) as already described [23]. The TBARS levels were expressed as  
3 MDA equivalents in units of nmol/g haemoglobin [23]. The acid hydrolysis of 1,1,3,3-  
4 tetraethoxypropane which yields MDA was used for calibrating TBARS fluorescence [17].  
5 Haemoglobin concentration was estimated spectrophotometrically by the cyanmethemoglobin  
6 method [23]. The t-BuOOH, H<sub>2</sub>O<sub>2</sub>, Q and FnH stock solutions in DMSO were added to  
7 diluted red cell suspensions to obtain the desired final concentrations. *tert*-  
8 Butylhydroperoxide and hydrogen peroxide were always added 15 min after the FnH.  
9

## 10 2.6. Cell culture and treatment

11 The NCTC 2544 immortalized human skin keratinocyte cell line was purchased from ICN  
12 Flow (Fontenay sous Bois, France). Cultures were propagated in minimum essential medium  
13 with Earle's salts (EMEM) supplemented with 10% foetal calf serum (FCS), 100 U/mL  
14 penicillin and 100 µg/mL streptomycin (weekly passages, 1:10 splitting ratio). Cells from  
15 trypsinized confluent monolayers were seeded at a density of 15,000 cells/cm<sup>2</sup> on plates with  
16 24 wells of about 2 cm<sup>2</sup> each, containing 1 mL of complete medium (EMEM supplemented  
17 with FCS). These were grown for 4 days to about 75% of confluency. After two washings  
18 with 1 mL of phosphate buffer saline (PBS), cells were incubated for 1 h at 37 °C with 250  
19 µL of FnH in PBS at the desired concentration. Then, after addition of 5 mM t-BuOOH (from  
20 0.125 M stock solutions in PBS) and incubation for 2 h with the oxidant, cells were washed  
21 and further incubated for 2 h at 37 °C in complete medium. After two washings with PBS, the  
22 challenged cells were submitted to the viability assay with Neutral Red (NR). The 2 h lag  
23 between treatment and assay was chosen to allow the initial damage to propagate but was  
24 short enough to avoid important proliferation of undamaged cells (population doubling time is  
25 about 1 day) which may obscure the effect of the oxidative stress.  
26

## 27 2.7. Neutral Red uptake assay

1 The cytotoxicity of t-BuOOH on NCTC 2544 keratinocytes, was determined by the widely  
2 used NR uptake assay [24, 25]. It has been validated by the European Union for testing  
3 phototoxic chemicals and for the classification and labelling of hazardous chemicals (EU  
4 Commission Directive 2000/33/EC). The day before the assay, a saturated aqueous NR  
5 solution was diluted (1:80 v/v) with complete medium and left overnight at 37 °C. Just before  
6 use, this NR solution was centrifuged twice to eliminate excess NR precipitate. Challenged  
7 cells were washed and incubated with 250 µL of this NR solution for 2 h at 37 °C. Then, after  
8 3 washings with PBS, 400 µL of a 1% SDS solution in water were added to solubilise cells  
9 and absorbance was read at 550 nm in 96 microwell plates. Data are presented as the  
10 percentage of NR uptake with respect to that of a control experiment carried out in the  
11 absence of oxidant. The data are the mean  $\pm$  SD of at least three independent experiments,  
12 each performed in triplicates.  
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### 29 **3. Results and discussion**

#### 30 *3.1. Inhibition of conjugated diene formation and carotenoid bleaching by FnH during LDL* 31 *oxidation*

32 Low density lipoprotein is a natural carrier of important antioxidants such as vitamin E and  
33 carotenoids. In the four LDL preparations we used for this set of experiments, the vitamin E  
34 /LDL and  $\beta$ -carotene/LDL concentration ratios (mol/mol) were  $6.72 \pm 0.72$  and  $0.40 \pm 0.25$   
35 respectively as determined by HPLC [26]. These antioxidants inhibit LDL lipid peroxidation  
36 until they are consumed. An induction period or lag time is thus observed between the start of  
37 the oxidation by  $\text{Cu}^{2+}$  ions and the appearance of lipid peroxidation products. The duration of  
38 this induction period depends on the constitutive antioxidant content of LDL which may vary  
39 among blood donors [27]. Figure 1A shows the time course of the conjugated diene formation  
40 after addition of 5 µM  $\text{Cu}^{2+}$  to 240 nM of native LDL occurring in the absence or in the  
41 presence of 0.75 µM FnH or Q. It may be seen that under these conditions the lipid  
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1 peroxidation in the absence of FnH begins almost immediately after  $\text{Cu}^{2+}$  addition. The time  
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3 lapse for producing 50% of the conjugated dienes obtained at plateau (CD50) is  $\sim 70$  min.  
4  
5 Addition of F1H, F4H or Q totally inhibits the formation of conjugated dienes during  $\sim 50$  min  
6  
7 of incubation at  $37^\circ\text{C}$  and shifts the CD50 to  $\sim 2$  h. By contrast, F6H and F10H only increase  
8  
9 the CD50 by  $\sim 30\%$  compared to that in control solution containing no antioxidants. Thus, in  
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11 the  $\text{Cu}^{2+}$ -induced LDL oxidation model, F1H and F4H can be considered to be as good an  
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13 antioxidant as Q, one of the most potent polyphenol antioxidant [2, 28].  
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17 To further characterise the order of antioxidant effectiveness  $\text{F4H} > \text{F1H} = \text{Q} \gg \text{F6H} =$   
18  
19  $\text{F10H}$  established from Fig. 1A data, carotenoid consumption was also monitored under the  
20  
21 same experimental conditions. Figure 1B suggests similar inhibition of the carotenoid  
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23 consumption by the FnH derivatives, F1H, again being equivalent to Q in effective protection.  
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25 These data support several structure-activity relationships regarding the antioxidant capacity  
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27 of the FnH species as a function of 3-alkyl chain length. FnH is hydrophobic and readily  
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29 solubilises in the LDL solution but not in buffer, suggesting that FnH must incorporate into  
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31 LDL particles at some level. On the basis of results obtained with our preceding study in  
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33 micellar media [11], the large variation observed here in antioxidant effectiveness may be due  
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35 to differing FnH locations in LDL as a function of the alkyl chain length. Owing to their four  
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37 hydroxyl groups F1H and, to a lesser extent, F4H are expected to be located in the water-rich  
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39 outer layer constituted of phospholipids, cholesterol and ApoB-100 apolipoprotein which  
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41 enwraps the LDL particle and contributes to its solubility in plasma. Indeed, addition of  $0.75$   
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43  $\mu\text{M}$  of non fluorescent F1H to the  $240$  nM LDL solution induces a 7% quenching of Trp  
44  
45 residue fluorescence suggesting close interaction between F1H and some Trp residues of  
46  
47 ApoB-100 (data not shown). On the other hand, the longer alkyl tails of F6H and F10H most  
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49 probably favour hydrophobic interactions with the LDL lipid core, thereby moving the  
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51 polyhydroxyphenol head groups more deeply in the LDL particle. Because of the binding of  
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1 Cu<sup>2+</sup> ions to ApoB-100, the initial targets of the LDL oxidation by Cu<sup>2+</sup> are 8-9 Trp residues  
2 of ApoB-100, and is a vitamin E-independent process [21]. The resulting formation of indolyl  
3 radicals (Trp<sup>•</sup>) triggers the radical chain reactions of peroxidation of LDL lipids most  
4 probably located in the vicinity of the oxidized Trp residues [21]. These Trp<sup>•</sup> radicals are not  
5 repaired by vitamin E or  $\beta$ -carotene [26]. With the assumption that Trp residues are the initial  
6 targets of Apo-B-bound Cu<sup>2+</sup> ions, F1H—the least hydrophobic FnH—is expected to be the  
7 most effective antioxidant by consequence of its proximity to these Trp residues and its ability  
8 to repair them [11] thus impeding the initiation of LDL lipid peroxidation. In this regard, Fig.  
9 2A shows that although in the absence of Cu<sup>2+</sup> ions LDL-bound Q can repair ApoB-100  
10 radical species [29], addition of 0.75  $\mu$ M Q may actually accelerate Cu<sup>2+</sup> ion-induced Trp  
11 residue destruction. By contrast, the same concentration of F1H provides a level of protection.  
12 Additionally, the ability of FnH to neutralize reactive oxygen species (ROS) by hydrogen  
13 donation [11] and to form complexes with Cu<sup>2+</sup> ions (Fig. 2B) must also contribute to the  
14 observed inhibition. Taking into account all these observations, it can be seen that the present  
15 experimental system defines an overall apparent antioxidant capacity of these FnH  
16 antioxidants.

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

41 *tert*-Butylhydroperoxide is a membrane-permeant oxidant extensively used to induce  
42 oxidative stress in different systems [30, 31]. Its *tert*-butyl group confers lipid solubility. It  
43 thus easily penetrates membranes and is rapidly transferred to the cytosol. Here it is  
44 metabolized by the GSH peroxidase-reductase system with consumption of GSH and  
45 NADPH. When this enzymatic system is overwhelmed, a one-electron oxidation (or  
46 reduction) process mediated by catalytically active metal ions (for example, cytochrome  
47 P450) initially gives rise to radicals comparable to those encountered in the chain reaction of  
48 lipid peroxidation (peroxyl or alkoxyl radicals). These eventually rearrange and decompose  
49

1 into carbon-centered radicals such as methyl radicals. Such t-BuOOH-derived radicals are  
2 responsible for the lethal effects observed and trigger free radical chain reactions in  
3 membranes [32-34]. Trotta *et al.* have demonstrated that t-BuOOH degrades haemoglobin and  
4 provokes lipid peroxidation in RBC [34 and references therein]. A concentration of 0.6 mM t-  
5 BuOOH and an incubation time of 60 min were found sufficient to induce about 15%  
6 haemolysis in human RBC suspensions and significant production of TBARS, the lipid  
7 peroxide decomposition products (Fig. 3 A,B). However, significant protection against  
8 haemolysis (Fig. 3A) in the presence of only 1.5  $\mu$ M F4H, F6H or F10H was observed. The  
9 diminution of RBC lysis was accompanied by a marked reduction of TBARS formation with  
10 F10H being again the most effective (Fig. 3B). It should be noted that under comparable  
11 experimental conditions, F4H, or F6H or F10H are proven to be much better inhibitors of  
12 RBC haemolysis and lipid peroxidation than 2,6-bis(1,1-dimethylethyl)-4-methylphenol  
13 (butylated hydroxytoluene), the well established lipid-soluble anti-oxidant [34]. By contrast,  
14 F1H was found to be essentially inactive in this system.

15 It has long been known that H<sub>2</sub>O<sub>2</sub> causes haemolysis and lipid peroxidation of RBC [35].  
16 Hence it is of interest to compare the capacity of the various FnH species to protect RBC from  
17 the oxidative stress induced by H<sub>2</sub>O<sub>2</sub> to that observed with t-BuOOH since appreciable  
18 differences in their biological action have been reported [36]. Hydrogen peroxide, a natural  
19 oxidant, diffuses across hydrophobic membranes and it is metabolized by GSH peroxidase  
20 and by catalase. Here, the latter was inhibited by addition of NaN<sub>3</sub>, potentiating the oxidative  
21 damage. Hydrogen peroxide reacts with redox metal ions by the so-called Fenton reactions to  
22 produce  $\cdot$ OH radical, the strongest oxidizing species known (normal redox potential: 2.6 V vs  
23 NHE). As a result,  $\cdot$ OH radicals react unselectively at their site of formation with all  
24 biological constituents, not just those targets critical for maintaining the integrity of structure  
25 and function. A 90 min incubation of the RBC with 10 mM H<sub>2</sub>O<sub>2</sub> produces haemolysis (Fig.

1 3A) and TBARS production (Fig. 3B) of the same order of magnitude as those obtained with  
2 t-BuOOH. However, in contrast to t-BuOOH, addition of at least 100  $\mu$ M F6H or F10H is  
3 necessary to achieve the limited inhibition of RBC lysis (30%) observed. In the case of  
4 TBARS measurements, replacing F4H by F6H or F10H dramatically reduced TBARS  
5 production. At a concentration of 100  $\mu$ M neither F1H nor F4H significantly modified either  
6 TBARS formation or haemolysis.  
7

8 The comparison of data from RBC obtained with t-BuOOH and H<sub>2</sub>O<sub>2</sub> suggests that in both  
9 cases, the FnH species with the longest alkyl chain, and hence the most hydrophobic—F10H  
10 and in a lesser extent F6H—are the most effective protectors against haemolysis and lipid  
11 peroxidation. In all instances, Figs. 3 A and B suggest that they compare favorably with Q as  
12 antioxidants. By contrast, F4H can effectively protect against haemolysis but less  
13 ineffectively against lipid peroxidation only in the case of t-BuOOH -induced oxidative stress.  
14 In the light of the above discussion it is of note that F1H and F4H were quite effective in  
15 inhibiting the Cu<sup>2+</sup>-induced LDL lipid peroxidation but brought less protection to the RBC,  
16 again supporting the suggestion of site-specific oxidation reactions at the LDL -water  
17 interface where F1H and F4H are probably localized.  
18

### 19 3.3. Inhibition of the t-BuOOH-induced oxidative stress by FnH in NCTC 2544 keratinocytes

20 As skin is the interface between the body and the environment, there are numerous studies on  
21 cell disorders induced by various agents of oxidative and photo-oxidative stress. In addition to  
22 membrane lipid peroxidation, ROS have been shown to react in all skin cell compartments  
23 and with most cell structures. For example, they are responsible for impairment of cell  
24 signalling and activation of stress-sensitive protein kinases leading to enhanced production of  
25 cytokines. They also directly react with phosphatases or redox-sensitive proteins [for a review  
26 see ref. 37]. Because lipid peroxidation is the more relevant of the processes to radical  
27 induced skin damage, t-BuOOH was preferred for this work, as it initially gives rise to  
28

1 oxygen radicals mimicking those encountered in lipid peroxidation. In this regard, it has been  
2 shown that t-BuOOH is metabolized by freshly isolated or cultured normal human  
3 keratinocytes with production of cytotoxic free radicals leading to severe plasma membrane  
4 damage [38]. Given the large variety of skin related targets for t-BuOOH reported in the  
5 literature, we have chosen to follow an overall response to its oxidative stress. For this  
6 purpose, NR, a lysosomotropic dye which is only retained by intact lysosomes was used to  
7 assess the viability of the NCTC 2544 keratinocytes subjected to cytotoxic effects of t-  
8 BuOOH in the absence or in the presence of the four FnH antioxidants.  
9

10 Figure 4 demonstrates the strong cytotoxicity of 5 mM t-BuOOH towards NCTC 2544  
11 keratinocytes after a 2 h incubation at 37 °C in PBS in the absence of any FnH. Thus, after the  
12 initial damage had propagated for 2 h (see the experimental section), less than 20% of cells  
13 were found to have recovered from injury. Addition of only 0.5 μM of all FnH species or of Q  
14 provided some protection to the keratinocytes. In the presence of 5 μM of all the antioxidants,  
15 most cells survived the oxidative stress. However, a trend can be noted with slightly less  
16 protection by F10H which has the longest alkyl chain length, while F1H, F4H and F6H are  
17 shown to be as effective as Q. In our previous kinetic study on the one-electron oxidation of  
18 the FnH by  $\cdot\text{O}_2^-$  radical-anions, we have shown that F6H and F10H can strongly increase the  
19 rigidity of hydrophobic microenvironments [11]. As a consequence, it may be hypothesized  
20 that the decyl tail on the 3',4',5,7-tetrahydroxyflavones may somewhat hinder the  
21 translocation of F10H from the plasma membrane to cell organelles, causing less overall  
22 protection.  
23

#### 24 4. Conclusions

25 As suggested by our very recent study on the high reactivity of the 3',4',5,7-  
26 tetrahydroxyflavones with  $\cdot\text{O}_2^-$  radical-anions taken as model ROS and their ability to repair  
27  $\cdot\text{Trp}$  radicals [11], the present work demonstrates that 3,3',4',5,7-tetrahydroxyflavones are  
28

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

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## Figure legends

**Figure 1A.** Kinetics of conjugated diene formation during LDL oxidation induced by 5  $\mu\text{M}$   $\text{Cu}^{2+}$ . 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  $\mu\text{M}$  FnH or Q before  $\text{Cu}^{2+}$  addition. Note that time zero denotes measurements made about 1 min after  $\text{Cu}^{2+}$  addition. Data are the mean  $\pm$  SD of four independent experiments. For clarity, SD are only shown for control and Q-loaded LDL.

**Figure 1B.** Kinetics of carotenoid consumption during LDL oxidation induced by 5  $\mu\text{M}$   $\text{Cu}^{2+}$ . Experimental conditions are identical to those of Fig. 1A. Data are the mean  $\pm$  SD of four independent experiments. For clarity, SD are only shown for control and for Q-loaded LDL.

**Figure 2A.** Destruction of Trp residues during LDL oxidation induced by 5  $\mu\text{M}$   $\text{Cu}^{2+}$ . 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  $\mu\text{M}$  F1H or Q before  $\text{Cu}^{2+}$  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  $\text{Cu}^{2+}$  addition. Data represent one experiment performed in duplicate.

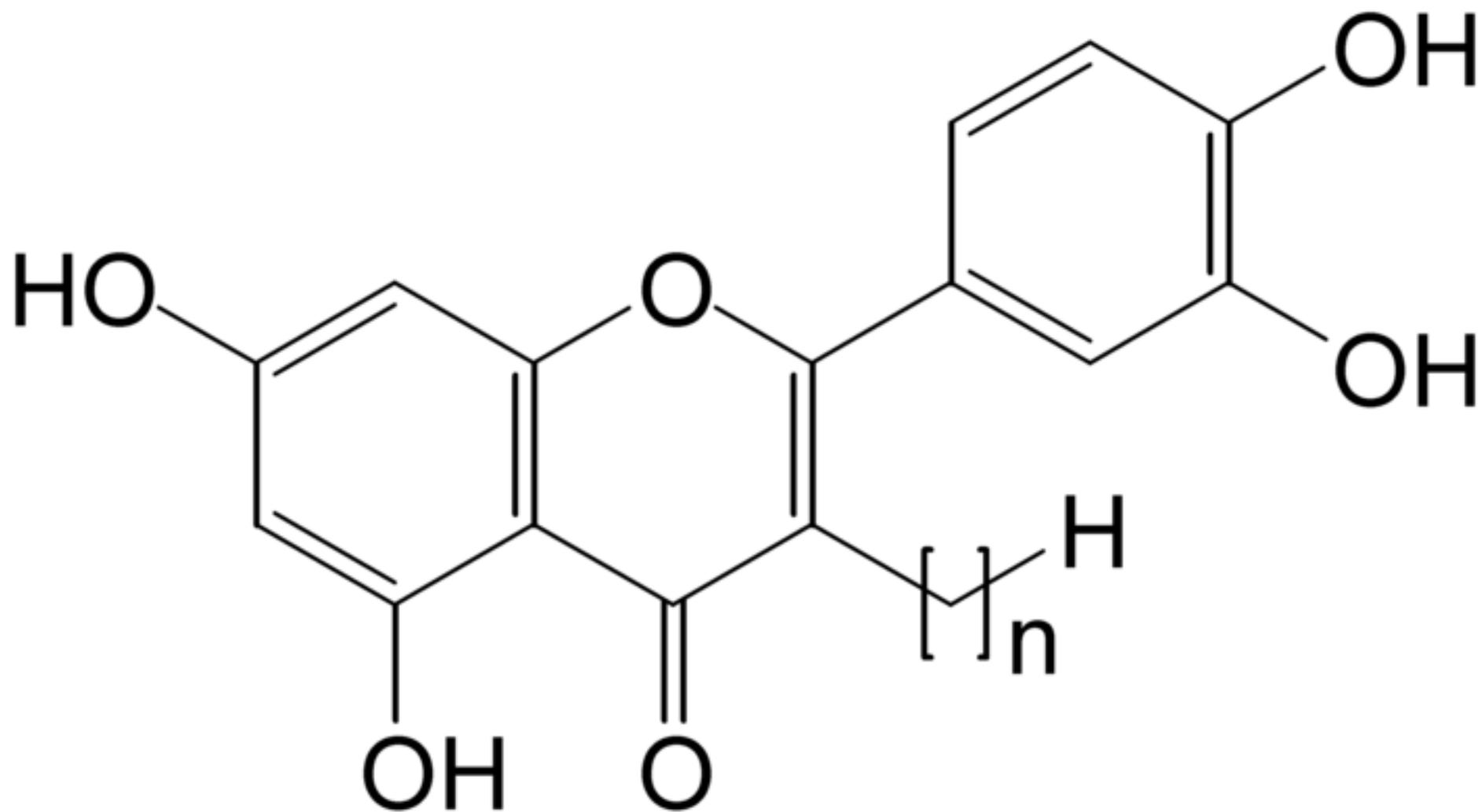
**Figure 2B.** Spectra demonstrating the complexation of  $\text{Cu}^{2+}$  ions by F1H. Absorbance spectra of 100  $\mu\text{M}$   $\text{Cu}^{2+}$  and of 10  $\mu\text{M}$  F1H were measured in 10 mM phosphate buffer (pH 7.4) at 20 °C. The third spectrum corresponding to the  $\text{Cu}^{2+}$  + F1H mixture is a difference absorbance spectrum (sample cell: 10  $\mu\text{M}$  F1H + 100  $\mu\text{M}$   $\text{Cu}^{2+}$ , reference cell: 10  $\mu\text{M}$  F1H).

**Figure 3A.** 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  $\text{H}_2\text{O}_2$  in the absence (C) or in the presence of FnH or Q. The concentrations of FnH or Q were 1.5  $\mu\text{M}$  and 0.1 mM for oxidation by t-BuOOH and  $\text{H}_2\text{O}_2$ ,

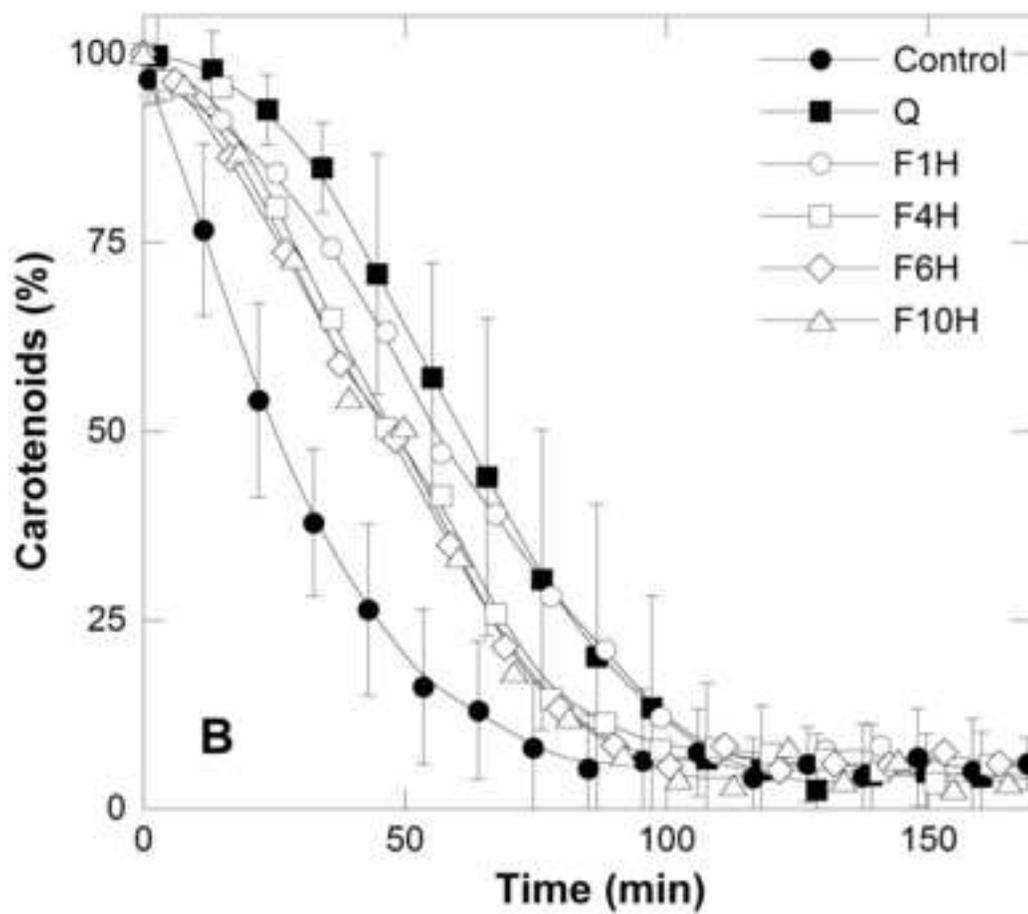
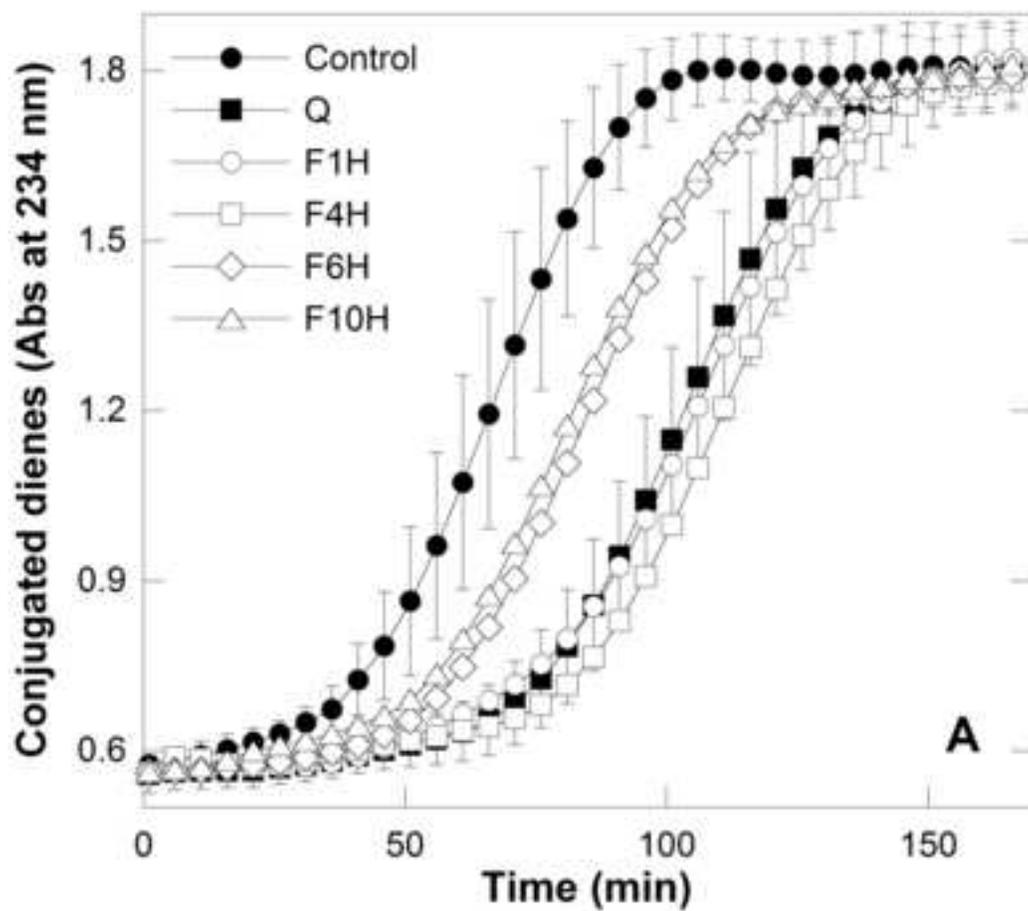
1 respectively. Data are the mean  $\pm$  SD of four independent experiments. Data are the mean  $\pm$   
2 SD of four independent experiments. Analysis of variance was performed with an unpaired  
3 Student's t-test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.005$ ). (0) shows the haemolysis in the  
4 suspensions in the absence of any treatment. In the control (C), DMSO was added at a  
5 concentration corresponding to the dilution of stock solutions of the antioxidants (see text). A  
6 longer incubation time, a 1h pre-incubation with 2 mM sodium azide and a 5-fold increased in  
7 DMSO concentration explain the increased hemolysis in controls of H<sub>2</sub>O<sub>2</sub> experiments as  
8 compared to those with t-BuOOH.  
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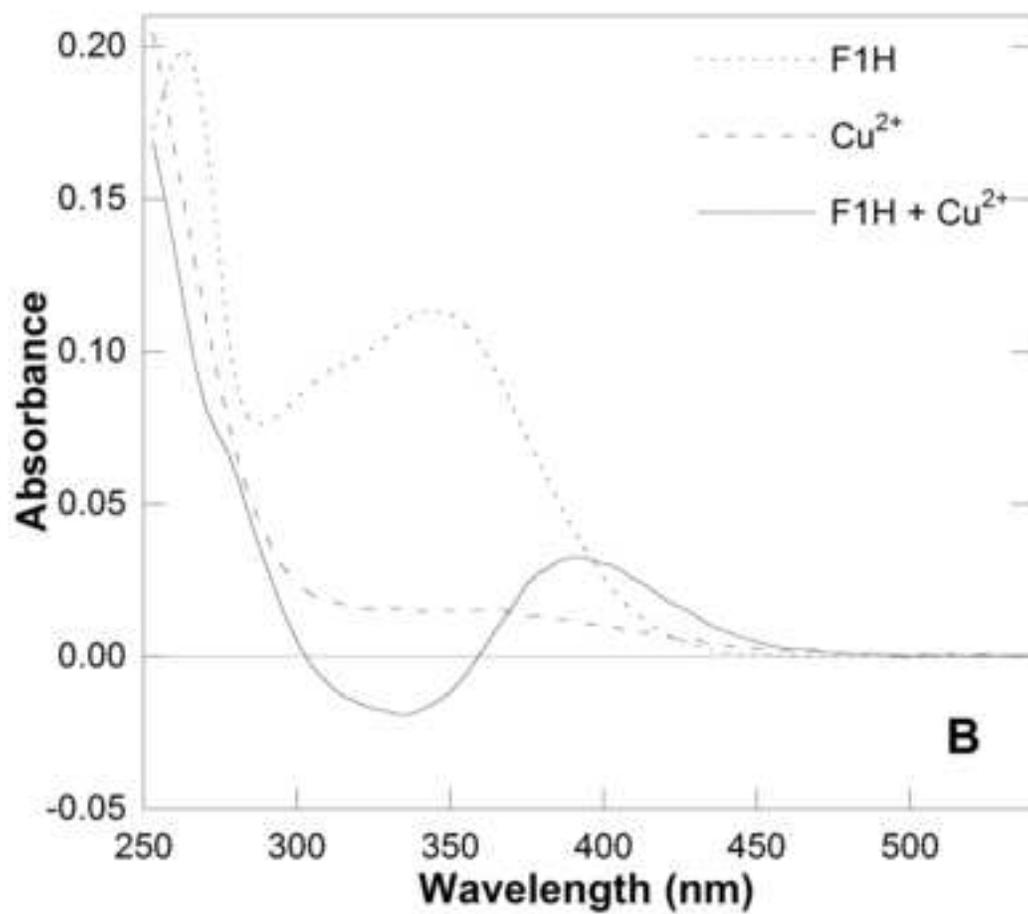
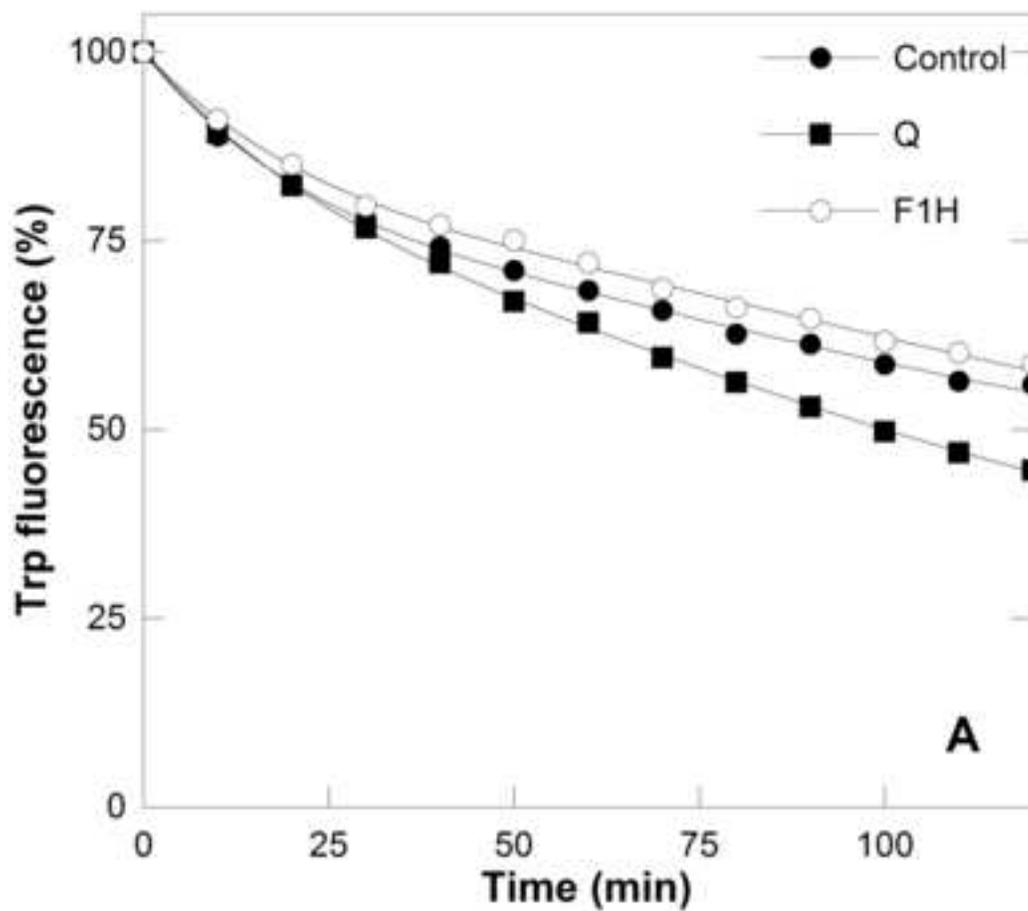
10 **Figure 3B.** Production of MDA expressed in nmol/g of haemoglobin in the RBC suspensions.  
11 The experimental conditions are the same as in Fig. 3A. Data are the mean  $\pm$  SD of four  
12 independent experiments. Analysis of variance was performed with an unpaired Student's t-  
13 test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.005$ ).  
14  
15

16 **Figure 4.** Effect of 3-alkyl-3',4',5,7-tetrahydroxyflavones (FnH) and of the flavonol quercetin  
17 (Q) on the cytotoxicity of 5mM t-BuOOH towards NCTC 2544 keratinocytes. Cells were  
18 incubated during 2h in complete medium in the absence (C) or presence of several  
19 concentrations of antioxidants as indicated on the graph. The neutral red assay was performed  
20 15 h after the incubation with t-BuOOH. Data are the mean  $\pm$  SD of four independent  
21 experiments in triplicate, with untreated cells taken as 100%. Statistics were performed with  
22 an unpaired Student's t-test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.005$ ).  
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$n = 1, 4, 6 \text{ and } 10$





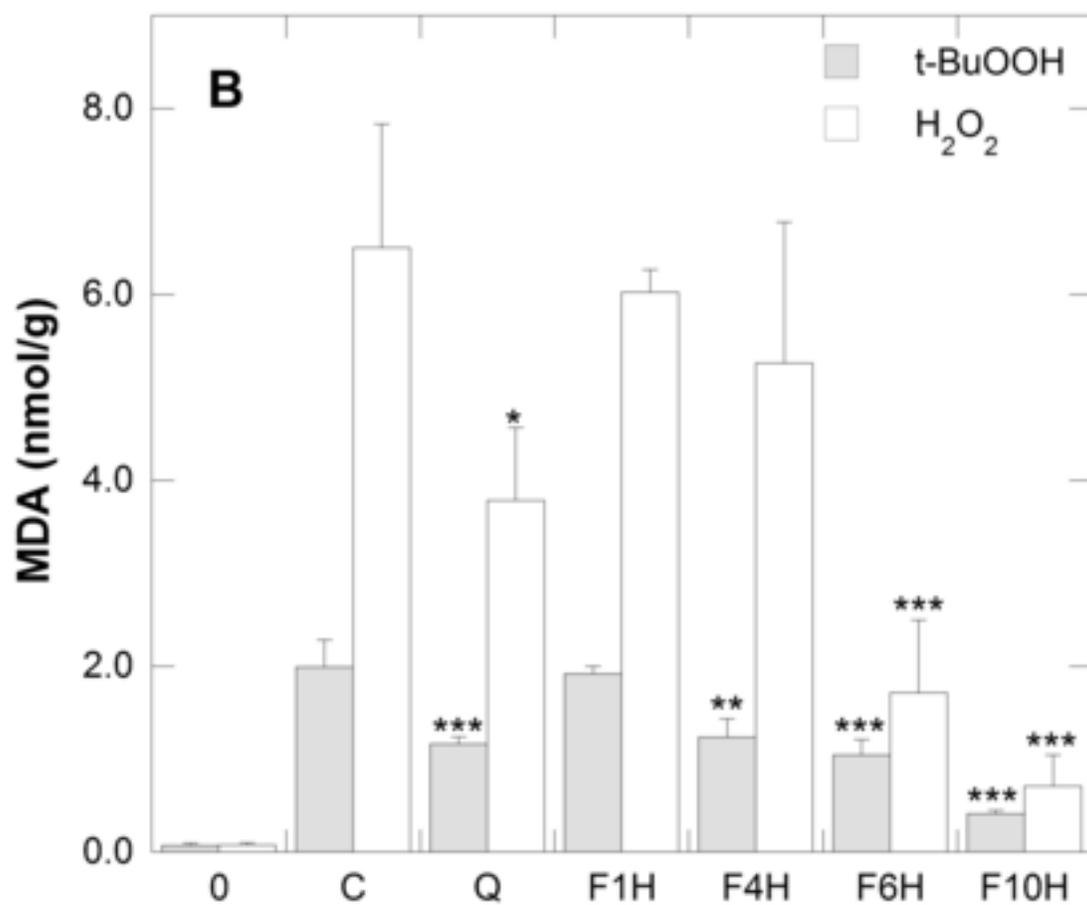
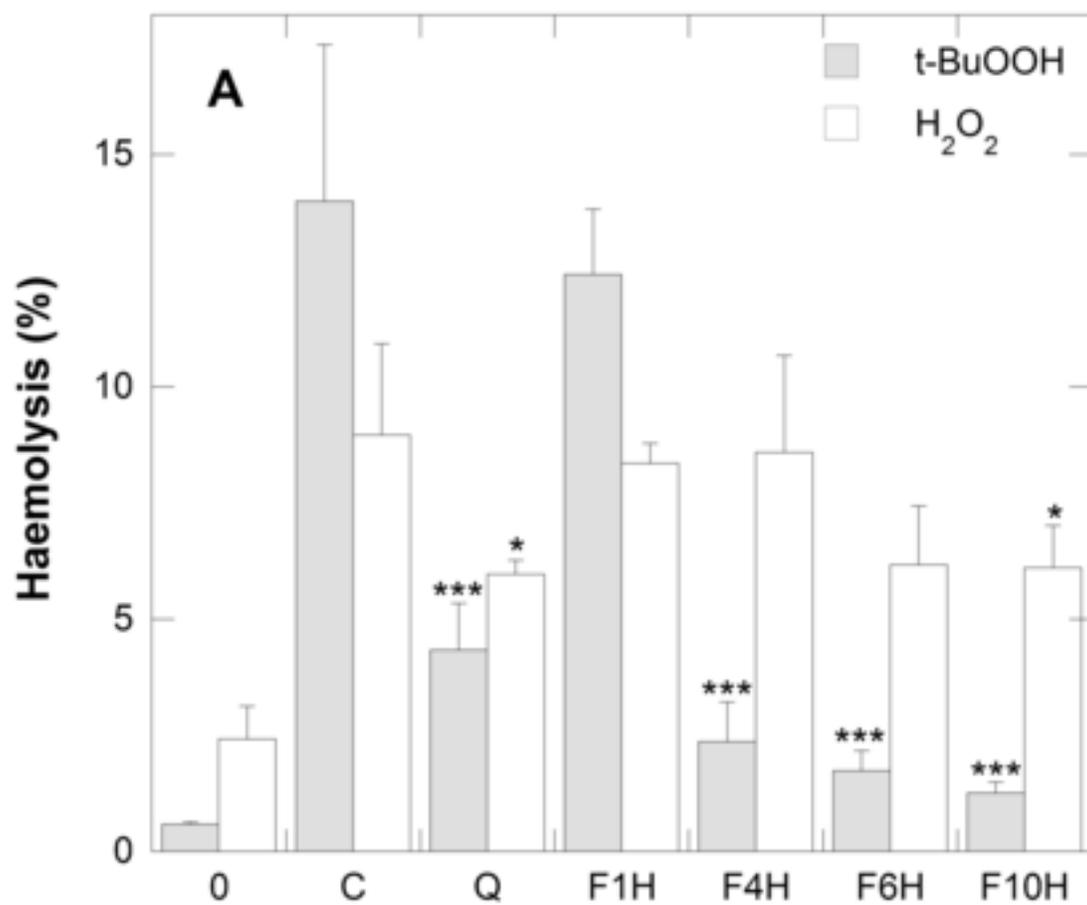


Figure4

