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Research Paper

Does hydrophobicity always enhance antioxidant drugs? A cut-off effect of the chain length of functionalized chlorogenate esters on ROS-overexpressing fibroblasts

Mickaël Laguerre[a], Chantal Wrutniak-Cabello[b], Béatrice Chabi[b], Luis J. López Giraldo[a], Jérôme Lecomte[a], Pierre Villeneuve[a] and Gérard Cabello[b]

[a]CIRAD, UMR Ingénierie des Agropolymères et Technologies Émergentes, and [b]INRA, UMR Différenciation Cellulaire et Croissance, Montpellier, France

Abstract

Objectives Phenolic antioxidants are currently attracting a growing interest as potential therapeutic agents to counteract diseases associated with oxidative stress. However, their high hydrophilicity results in a poor bioavailability hindering the development of efficient antioxidant strategies. A promising way to overcome this is to increase their hydrophobicity by lipophilic moiety grafting to form the newly coined 'phenolipids'. Although hydrophobicity is generally considered as advantageous regarding antioxidant properties, it is nevertheless worth investigating whether increasing hydrophobicity necessarily leads to a more efficient antioxidant drug.

Methods To answer this question, the antioxidant capacity of a homologous series of phenolics (chlorogenic acid and its methyl, butyl, octyl, dodecyl and hexadecyl esters) toward mitochondrial reactive oxygen species (ROS) generated in a ROS-overexpressing fibroblast cell line was investigated using 2′,7′-dichlorodihydrofluorescein.

Key findings Overall, the long chain esters (dodecyl and hexadecyl esters) were more active than the short ones (methyl, butyl, and octyl esters), with an optimal activity for dodecyl chlorogenate. Moreover, dodecyl chlorogenate exerted a strong antioxidant capacity, for concentration and incubation time below the cytotoxicity threshold, making it a promising candidate for further in-vivo studies. More importantly, we found that the elongation of the chain length from 12 to 16 carbons led unexpectedly to a 45% decrease of antioxidant capacity.

Conclusion The understanding of this sudden collapse of the antioxidant capacity through the cut-off theory will be discussed in this article, and may contribute towards development of a rational approach to design novel amphiphilic antioxidant drugs, especially phenolipids with medium fatty chain.

Keywords chlorogenic acid; cut-off effect; fibroblasts; lipophilization; phenolipids

Introduction

More than 50 years ago it was hypothesized that aging could be the result of the accumulation of oxidative damage from free radicals generated endogenously in cells during normal metabolism.[1] Growing interest in these oxidative phenomena has progressively emerged as the implication of oxidative stress in multifactorial diseases such as cancers[2] and cardiovascular diseases[3] has been demonstrated. It is well established that the leakage of electrons from the respiratory chain located within the inner mitochondrial membrane is the major biological process leading to oxidant species generation, especially reactive oxygen species (ROS). Because a common feature in such diseases is an increased level of mitochondrial ROS, the use of antioxidant molecules, especially phenolic compounds, has been considered as a promising way to counteract ROS-mediated damage. However, the high hydrophilicity of these antioxidant compounds results in a poor bioavailability hindering the development of efficient antioxidant strategies. A promising way to enhance their cellular uptake is to increase their hydrophobicity by grafting a lipid onto a phenolic moiety.[4,5] These functionalized molecules can be coined as 'phenolipids' and seem to be very promising as therapeutic agents to counteract oxidative stress. In Caco-2 cells, Tammela *et al*.[6] showed

Correspondence: Pierre Villeneuve, Bât. 33, 2 Place Pierre Viala, 34 060 Montpellier Cedex 02, France.
E-mail: pierre.villeneuve@cirad.fr

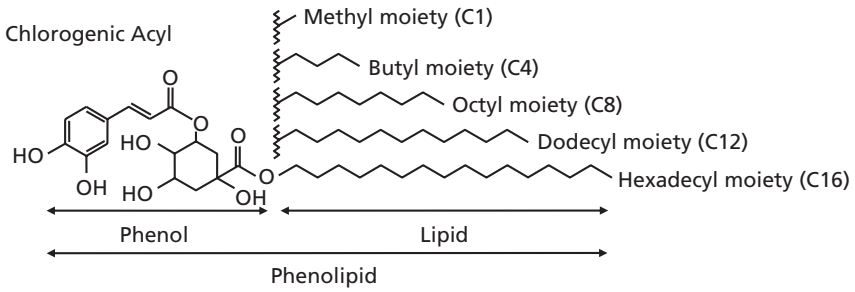

Figure 1 Chemical structure of investigated phenolic compounds.

that octyl gallate was extremely rapidly and almost totally reduced from the apical solution whereas the uptake of methyl and propyl gallate was slower. Similarly, it has been suggested in mouse B cell lymphoma Wehi 231 that dodecyl gallate exhibited a better membrane crossing than its propyl homologue.[7] Recently, Trujillo *et al*.[8] demonstrated in brain homogenates that hydroxytyrosyl linoleate is more efficient than hydroxytyrosol in protecting both lipids and proteins from oxidation induced by cumene hydroperoxides. In their review paper, Rice-Evans *et al*.[9] highlighted that the partition coefficient (or hydrophobicity) is an important parameter in predicting antioxidant activity in lipophilic media, such as liposomes and low-density lipoproteins (LDL).

Although hydrophobicity is generally considered as advantageous regarding antioxidant properties, it is worth asking whether increasing hydrophobicity necessarily leads to a more efficient antioxidant. This point is of particular interest since it may contribute to the development of a more rational approach to designing novel amphiphilic antioxidant drugs such as phenolipids.

Recently, we have shown that chlorogenic acid (5-caffeoylquinic acid, Figure 1), exhibiting antioxidant[10] and antiradical[11] properties in test tube-based assays, can be lipophilized by various fatty alcohols in a two-step lipase-catalysed transesterification strategy.[12] This reaction leads to a homologous series of alkyl chlorogenate esters (from methyl to hexadecyl, Figure 1), which constitutes an interesting phenolic model to study the impact of the hydrophobicity on the antioxidant capacity in biological samples. Nevertheless, the major challenge remains to develop analytical tools to assess these properties in a relevant and reliable manner. To date, the experimental models involve chemical assays, cultured cells, animal models of oxidative stress and clinical test in humans. Oxidative stress studies generally use exogenic inductor(s) of oxidative stress such as antimycin A (inhibition of complex III), oxidized LDLs, transient metals, diazo compounds or hydroperoxides. However, in such a system, it is difficult to distinguish the intrinsic antioxidant effect from more complex effects induced by interaction between antioxidant and exogenic inductor(s). To partially overcome these difficulties, we have developed a new cellular line of fibroblasts characterized by a strong production of oxidant species of mitochondrial origin.[13] This feature allows evaluation of antioxidant capacity without any exogenic inductor(s) of oxidation, with the equivalent analysis duration generally encountered in cellular assays. Indeed, with these cellular lines, the analysis duration is only determined by the drug incubation time sufficient to observe a significant antioxidant effect.

In this study, this strong endogenous production of oxidant species from mitochondria was advantageous to evaluate the ability of a homologous series of phenolipids to decrease the level of ROS. To monitor this level, a fluorogenic probe (2′,7-dichlorodihydrofluorescein diacetate (H_2DCFDA)), exhibiting fluorescence in visible spectrum (λ_{ex}: 492 nm /λ_{em}: 525 nm) when oxidized, was employed. The modulation of ROS level by phenolic compounds was thus estimated by means of fluorescence modulation. In this paper, we evaluated the antioxidant capacity of these novel phenolipids and gave new insight on the relationship between hydrophobicity and antioxidant properties.

Materials and Methods

Chemicals

Hoechst 33258, DNA salmon sperm, chlorogenic acid (5-caffeoylquinic acid), methanol, Amberlite IR 120 H, chloroform, *Candida antarctica* lipase B, hexane, acetonitrile, silica gel, toluene and ethyl acetate were purchased from Sigma (Saint Quentin, France). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA), Dulbecco's modified Eagle's medium (DMEM, 4.5 g/l glucose), D-PBS, gentamicin and amphotericin B were from Invitrogen (Cergy Pontoise, France).

Synthesis of chlorogenate esters

The chemo-enzymatic esterification of chlorogenic acid to obtain chlorogenate esters was carried out following the procedure described by López Giraldo *et al*.[12] Briefly, 10 mmol chlorogenic acid was dissolved in 240 ml methanol. Amberlite IR 120 H (10 g), previously dried at 110°C for 48 h, was added to the reaction mixture which was then stirred in an orbital shaker (250 rpm) for 9 h at 55°C. After cooling to room temperature, the reaction medium was filtered on a 1.6 µm glass microfiber filter (Whatman International Ltd, Maidstone, UK) and the methanol was removed under vacuum. Chloroform (150 ml) was then added, and the solution was dried over sodium sulfate, filtered on a 1.6 µm glass microfiber filter, and evaporated under vacuum at 50°C. The resulting methyl chlorogenate (5 mmol) was then added to 375 ml of desired fatty alcohol, and the mixture was then placed in sealed flasks and stirred on an orbital shaker

(250 rpm, 55°C) until complete dissolution of methyl chlorogenate. *Candida antarctica* lipase B (5% wt/wt; calculated from the total weight of both substrates) was then added to start the transesterification reaction. The suspensions were then heated at 55°C for 96 h under a nitrogen flow in order to eliminate continuously the formed methanol and favor the displacement of the reaction equilibrium towards the synthesis. The final lipophilized esters were then purified in a two step procedure. First, a liquid-liquid extraction using 250 ml of hexane and 1000 ml of a solution of acetonitrile/water (3 : 1, v/v) was realized to remove the fatty alcohol in excess. In a second step, the alcohol traces were eliminated using silica gel column chromatography (length 25 cm, i.d. 1.6 cm) using toluene/ethyl acetate (90 : 10, v/v) as eluant. All recovered esters were then characterized by mass spectrometry as described by López Giraldo *et al.* [12]

Cell culture preparation

Dark 96-well microplates with flat and transparent bottoms (Greiner, Frickenhausen, Allemagne) were seeded at a cellular density of 10 000 cells/well. Cell cultures were allowed to grow in DMEM (4.5 g/l glucose) supplemented with gentamicin (50 µg/ml), amphotericin B (50 µg/ml) and fetal calf serum (10%) at 37°C, 5% CO_2 and 100% humidity.

Cell treatment with phenolic compounds

Phenolic compounds (chlorogenic acid and its methyl, butyl, octyl, dodecyl and hexadecyl esters), being either hydrophilic, amphiphilic or hydrophobic according to their alkyl chain length, were dissolved in ethanol at the desired concentration. A 1000-fold dilution was performed in culture medium to obtain final concentrations of 0, 10, 25, 50 and 100 µM. Afterwards, 100 µl of this culture medium containing phenolic compounds was dispensed into each well of a 96-well microplate 24 h after cell seeding. The experiment involving each concentration was repeated four times ($n = 4$) in four different microplates seeded from different cell passages (each microplate comprising already triplicate wells for each concentration).

Measurement of reactive oxygen species using 2′,7′-dichlorodihydrofluorescein diacetate probe

After 24, 48 or 72 h incubation with phenolic compounds, the medium was removed. Adherent cells were washed twice with 50 µl/well Locke's buffer (140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM D-glucose and 10 mM hydroxyethylpyperazinethane acid (HEPES)), then each well was filled with 100 µl Locke's buffer. A measurement (λ_{ex}: 485 nm/λ_{em}: 530 nm) of the fluorescent background was achieved with a microplate spectrofluorimeter (Synergy 2 BioTek, Bio-tek Instruments, Inc., Winooski, USA).

Cells were incubated with 100 µl of a 10 µM solution of H_2DCFDA prepared in Locke's buffer, for 20 min at 37°C in the dark. After the removal of the H_2DCFDA solution, each well was washed twice using 50 µl Locke's buffer and finally was filled with 100 µl Locke's buffer. The microplate (covered by aluminium foil) was then incubated for 10 min at 37°C before fluorescence measurement (λ_{ex}: 485/λ_{em}: 530 nm).

Raw fluorescence was calculated for each well, subtracting the fluorescence background (without probe) from that measured in presence of probe (Equation 1).

$$Raw\ Fluo = Fluo\ (cells\ with\ probe) - Fluo\ (cells\ without\ probe) \quad (1)$$

Given that the tested substances are likely to exert an influence on the cell growth, a correction of the probe fluorescence by the DNA content (µg DNA/well, see section on DNA Quantification, below) was achieved using Equation 2.

$$Corrected\ Fluo_{H2DCFDA} = raw\ Fluo_{H2DCFDA} / DNA\ quantity\ (\text{in } \mu g)\ \text{per well} \quad (2)$$

Finally, to consider the potential effect of carrier solvent (ethanol) which can act as antioxidant in some case,[14] the corrected fluorescence was expressed as the percentage of the control (with ethanol) (Equation 3).

$$Fluo_{H2DCFDA}\ (\%\ \text{of control}) = (Corrected\ Fluo_{H2DCFDA} \times 100) / Fluo_{H2DCFDA}\ of\ control \quad (3)$$

The antioxidant capacity was thus calculated as the percentage of fluorescence inhibition in presence of the tested phenolic compared with the control (phenolic free, but containing ethanol), this value being normalized by cell DNA content.

DNA quantification by the Hoechst 33258 method

Cell DNA content was measured using the Hoechst 33258 method previously described by De Arcangelis *et al.*[15] with some modifications. Cells of each well (previously measured for the antioxidant capacity determination) were lysed with 100 µl of TE5N buffer solution (10 mM Tris-HCl pH 8, 0.1 mM EDTA and 0.5 M NaCl) containing 0.05% Triton X-100. The microplate was incubated for 10 min under stirring at room temperature. Afterwards, 100 µl/well Hoechst 33258 solution at 0.4 µg/ml in TE5N were added, then incubated for 30 min at room temperature protected from light. The fluorescence (λ_{ex}: 340/λ_{em}: 440 nm) was then read in the same spectrofluorimeter as used in the section on Measurement of Reactive Oxygen Species, above.

To calibrate the fibroblasts' DNA content, the fluorescence of a standard of salmon sperm DNA solutions (0, 0.5, 1, 2 and 4 µg/ml TE5N) incubated with 100 µl Hoechst 33258 solution in TE5N, was analysed on a separate microplate. It is worth mentioning that the DNA concentration range was prepared in the same TE5N solution as samples.

First, all fluorescence values were subtracted from the fluorescence of a 0 µg/ml sperm salmon DNA solution (Equation 4).

$$Corrected\ Fluo_{Hoechst} = Raw_{Hoechst} - Fluo_{Hoechst}(0\ \mu g / \text{ml DNA}) \quad (4)$$

Then, a linear regression was established between DNA standard concentrations and observed fluorescence (expressed as arbitrary units); the same calculation being achieved for samples. Thus, the sample DNA concentration was determined from the calibration curve slope as follows (Equation 5):

$$DNA\ concentration\ (\mu g/ml) = Corrected\ Fluo_{Hoechst}\ /\ calibration\ curve\ slope \quad (5)$$

The DNA quantity expressed in µg/well was then calculated taking into account the total volume of the solution per well (200 µl) (Equation 6).

$$DNA\ quantity\ (\mu g/well) = (DNA\ concentration\ (\mu g/ml) \times 200)/1000 \quad (6)$$

This DNA quantity was used in Equation 2 for antioxidant capacity calculation.

Cytotoxicity measurement using lactate dehydrogenase assay

The cytotoxicity was estimated using the enzymatic detection kit CytoTox-ONE G7891 (Promega, Madison, USA). After 24, 48 or 72 h of incubation with phenolic compounds, microplates were incubated at room temperature for 30 min. After dispensing 100 µl CytoTox-ONE reagent into each well, the microplate was incubated for 10 min at room temperature in the dark. Then, 50 µl/well stop solution was added and the fluorescence was immediately measured (λ_{ex}: 560/λ_{em}: 590 nm).

Because the serum used in culture medium may have contained lactate dehydrogenase (LDH), a fluorescence measurement of DMEM supplemented with 10% fetal calf serum was achieved in the absence of cells on the same microplate, thus constituting the culture medium background.

The corrected fluorescence was then calculated by subtracting the background fluorescence from the fluorescence measured in the presence of cells (Equation 7).

$$Corrected\ Fluo_{LDH} = Raw\ Fluo_{LDH}\ (\text{with cells}) - Background\ Fluo_{LDH}\ (\text{without cells}) \quad (7)$$

Taking into account that phenolics are liable to exert an influence on the cell growth, and consequently to decrease the releasable quantity of LDH, values were corrected considering the total amount of intracellular and extracellular LDH. Therefore, some wells containing cells were exposed to 2 µl total lysis solution (9% Triton X-100 in phosphate buffer) to release all LDH in the extracellular medium and finally treated identically as other wells. The absolute cytotoxicity level (percent of control) for phenolic compound was calculated by dividing the corrected fluorescence obtained without lysis solution by that obtained after total lysis (Equation 8).

$$Cytotoxicity\ (\%) = 100 \times [Corrected\ Fluo_{LDH}\ (\text{without lysis solution}) / Corrected\ Fluo_{LDH}\ (\text{with lysis solution})] \quad (8)$$

Finally, to take into account the potential influence of the carrier solvent (ethanol) on this measurement, the cytotoxicity was expressed as a percent of control (phenolic free, but containing ethanol) (Equation 9).

$$Cytotoxicity\ (\%\ \text{of control}) = Cytotoxicity\ (\%) / Cytotoxicity\ of\ control \quad (9)$$

Light microscopy observations

The microstructure of the self-assembled aggregates formed by dodecyl chlorogenate in water was assessed using phase contrast microscopy with a 60× oil immersion objective lens (Olympus optical BX60F5, Japan). Before observation, the sample was gently mixed, and a droplet was placed on a microscope slide and covered with a cover slide. The images of the sample were captured using a digital camera directly connected to the computer equipped with image processing software (Image Pro Plus, version 6; Media Cybernetics, Inc. Bethesda, USA).

Bond dissociation energy calculation

Molecular modelling was performed with Hyperchem (Autodesk, Sausalito, USA). Parameters and hydrogen bonding patterns are detailed in Table 1.

Statistical methods

Statistical analysis of the effects of increasing concentration of phenolics (10, 25, 50 and 100 µM), exposure time (24, 48 and 72 h), and chain length of phenolipids (0, 1, 4, 8, 12 and 16 carbon atoms) on antioxidant capacity and cytotoxicity using ROS-overexpressing fibroblasts was performed using the Kruskall–Wallis test. In all cases, post-hoc comparisons of the means of individual groups were performed using Dunn's test. $P < 0.001$ denoted significance in all cases.

Curve-fitting

The CurveExpert 1.3 shareware (Copyright © 1995–2001 Daniel Hyams) was used in the curve-fitting procedures using a quadratic formula for the calculation of the area under the concentration curves.

Results and Discussion

Antioxidant capacity measurement

Since hydrophobicity is generally considered as advantageous regarding antioxidant properties in cellular systems, a growing number of lipophilized phenolics (known as phenolipids) has been designed and synthesized this last decade.[16] In this context, the present study was implemented to answer the question of whether increasing hydrophobicity necessarily leads to a more efficient antioxidant. To focus the study on the influence of the hydrophobicity on the antioxidant capacity of phenolic compounds, a homologous series of alkyl esters of chlorogenic acid was used. These phenolipids were obtained by lipase-catalysed esterification of the carboxylic function of chlorogenic acid by aliphatic alcohols of various chain lengths (1, 4, 8, 12 and 16 carbons, Figure 1). Their antioxidant capacity was evaluated at various concentrations (0, 10, 25, 50

Table 1 Bond dissociation energy (BDE) calculation

Compound	Hydrogen bond	Energy ($kcal^{-1}·mol^{-1}$)	BDE[a] ($kcal^{-1}·mol^{-1}$)
Chlorogenic acid	O4′-H · · · O3′-H	–4559.84	
Chlorogenic acid radical	O4′-H · · · O3′•	–4489.59	70.25
Methyl chlorogenate	O4′-H · · · O3′-H	–4826.79	
Methyl chlorogenate radical	O4′-H · · · O3′•	–4756.61	70.18
Butyl chlorogenate	O4′-H · · · O3′-H	–5667.62	
Butyl chlorogenate radical	O4′-H · · · O3′•	–5595.73	71.89
Octyl chlorogenate	O4′-H · · · O3′-H	–6789.78	
Octyl chlorogenate radical	O4′-H · · · O3′•	–6719.53	70.25
Dodecyl chlorogenate	O4′-H · · · O3′-H	–7911.92	
Dodecyl chlorogenate radical	O4′-H · · · O3′•	–7841.67	70.25
Hexadecyl chlorogenate	O4′-H · · · O3′-H	–9034.09	
Hexadecyl chlorogenate radical	O4′-H · · · O3′•	–8961.93	72.16

[a]BDE = *E* (aryloxyl radical) – *E* (parent phenol). Energies of parent phenols and aryloxyl radicals calculated by semi-empirical quantum mechanic (parametric method 3 (PM3), unrestricted Hartree–Fock (UHF) mode, *in vacuo*, 0 K at constant temperature)

and 100 μM) and incubation durations (24, 48 and 72 h). Because phenolics were introduced in the culture medium as an ethanolic solution, which can act in some cases as an antioxidant,[14] the evaluation of ethanol influence (0.1% v/v) on DCF fluorescence was achieved, and no significant effect was observed (data not shown). Regarding the influence of the alkyl chain length on antioxidant capacity, Figure 2 shows that free chlorogenic acid along with methyl, butyl and octyl esters does not exert a significant inhibition of the probe fluorescence during 24, 48 and 72 h of incubation. In contrast, a significant inhibition ($P < 0.001$) was noticed for dodecyl and hexadecyl esters with a dose-dependent effect (Figure 2e and 2f). The dodecyl ester, which exhibited a maximal protective effect against H_2DCF oxidation, was the only one to lead to a complete inhibition of the probe fluorescence at 50 μM. Unexpectedly, increasing the alkyl chain length from 12 to 16 carbon atoms resulted in a decrease in the antioxidant capacity. The same trend was observed when microplates were seeded with 3000 cells/well (data not shown). Finally, no time-dependent relationship was found suggesting that chlorogenate esters such as dodecyl and hexadecyl exhibited a complete antioxidant action within 24 h.

To express antioxidant capacity in a quantitative way, the area under the concentration curves has been calculated: the smaller the area under the curve, the higher is the antioxidant capacity. This calculation method supposes a good fitting of experimental data by a suitable mathematic model. A quadratic model ($y = a - [(a-d) * x \wedge c]/(b \wedge c + x \wedge c)$) was empirically developed for each curve displayed in Figure 2. The first advantage of this method of calculation is that the antioxidant capacity of all substances can be quantified whatever its amplitude. Secondly, all the concentrations used are taken into account with the same weight, whatever the kinetic profile. The integration of the area under the concentration curve allowed the classification of phenolic compounds in terms of antioxidant effectiveness as follows: dodecyl chlorogenate (4.53) > hexadecyl chlorogenate (2.52) > butyl chlorogenate (1.63) ~ methyl chlorogenate (1.26) ~ octyl chlorogenate (1.14) ~ chlorogenic acid (1.12) (antioxidant capacities were expressed as 1/area calculated from all the incubation time tested (24, 48, and 72 h), then arbitrarily multiplied by 10 000) (Figure 3).

On one hand, it appeared that the short chain lengths (1, 4 and 8 carbons) did not affect antioxidant capacity, whereas a maximal activity was reached for the dodecyl chain. On the other hand, an elongation from 12 to 16 carbons led to a 45% decrease in the efficiency of the corresponding chlorogenate ester. This particular behaviour may explain why some lipophilization strategies lead sometimes to an unexpected decrease in the antioxidant effect, especially those using long aliphatic chains. To the best of our knowledge, this is the first time such a non-linear relationship between hydrophobicity and antioxidant capacity has been demonstrated in cultured cells. This finding may contribute to the development of a rational approach in the design of new phenolic-based antioxidants through lipophilization.

H-donation ability, self-assembly properties and cut-off theory

First, it is worth trying to elucidate the mechanism of action that could explain the non-linear influence of the alkyl chain, with an optimal antioxidant effect for the dodecyl chlorogenate. Interestingly, we have observed similar antioxidant behaviour for chlorogenic acid and its alkyl esters in a non-living system consisting of a stripped tung oil-in-water emulsion (without any protein).[17] Figure 4 shows the good relationship ($R^2 = 0.872$) between antioxidant capacity values measured in the present study (fibroblasts) and those previously obtained in emulsified medium. This suggests that the non-linear effect observed in fibroblasts does not necessarily result from a biological mechanism, but rather from a simple physicochemical process, which incited us to search for an explanation in the physicochemical realm.

In this context, the simplest physicochemical mechanism of action for antioxidant phenolics is the reduction of free radicals by hydrogen transfer from their phenolic hydroxyl groups, which can be theoretically estimated by their bond dissociation energy (BDE): the smaller the BDE, the higher the ability to give up a hydrogen atom.[18] To investigate the

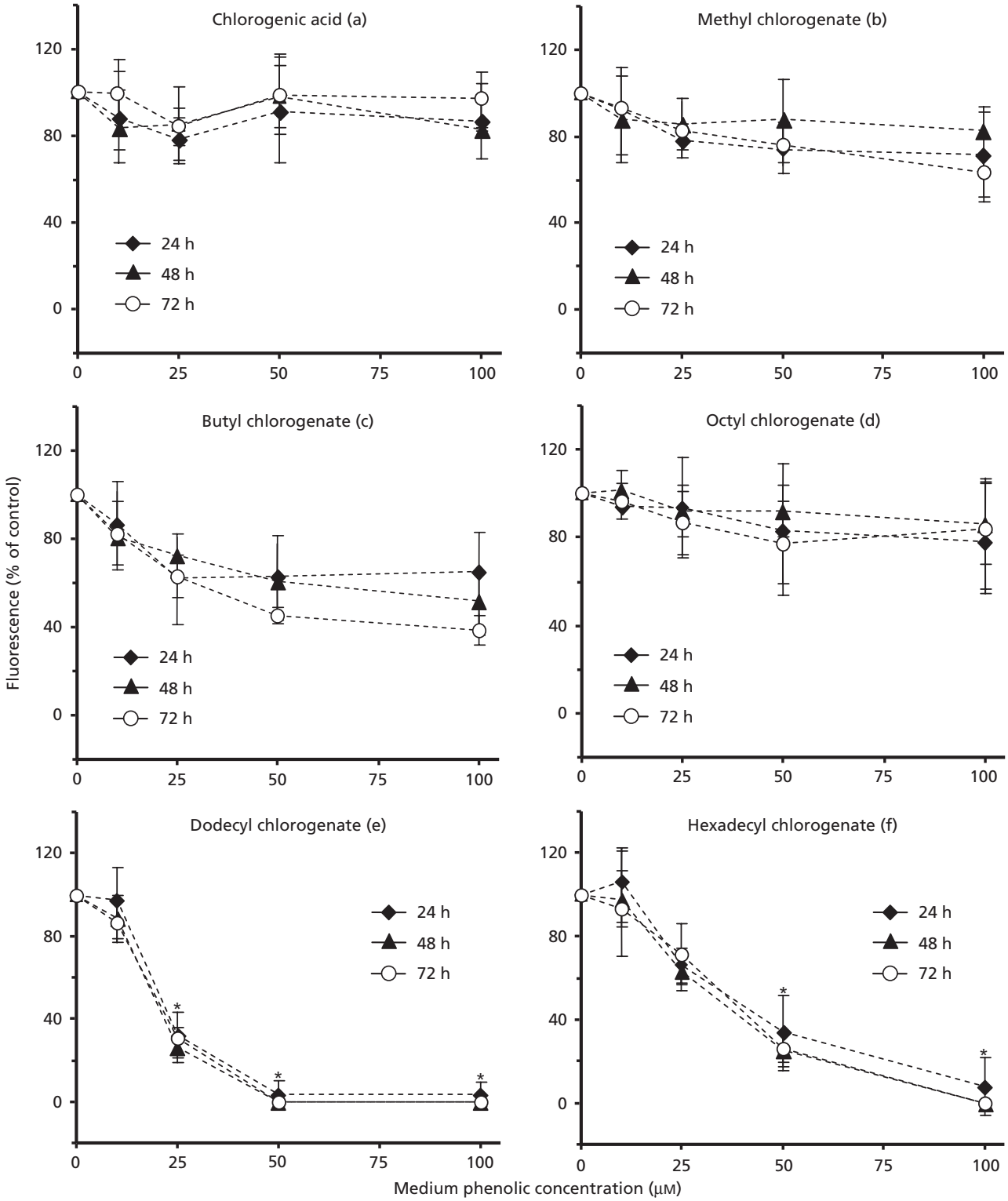

Figure 2 Influence of chlorogenic acid and its alkyl esters on 2′,7′-dichlorofluorescein formation (λ_{ex}: 485 nm/λ_{em}: 530 nm), which is an estimation of the level of reactive oxygen species. Fluorescence values on the *y*-axis were calculated according to Equation 3. Cells were cultivated with culture medium containing 10% (v/v) fetal calf serum, with 0, 10, 25, 50 and 100 μM phenolic compound and 0.1% (v/v) ethanol and then stained with 10 μM of H_2DCFDA. Results were expressed as mean ± SD of four independent measurements carried out on four separate microplates seeded with four different cell suspensions ($n = 4$). *$P < 0.001$ compared with the control without phenolic (Kruskall–Wallis followed by Dunn's multiple pairwise comparison).

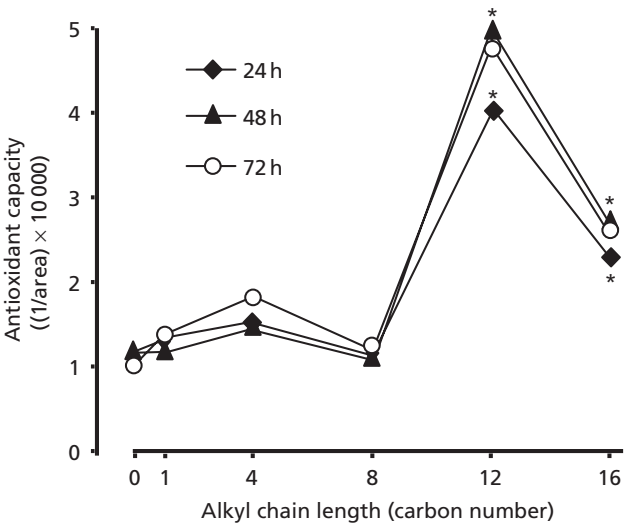

Figure 3 Influence of the alkyl chain length on the antioxidant capacity of chlorogenic acid and its alkyl esters. The antioxidant capacity was expressed in arbitrary units taking into account the reverse of the area under the concentration curves presented on Figure 2. $*P < 0.001$ compared with the control without phenolic (Kruskall–Wallis followed by Dunn's multiple pairwise comparison).

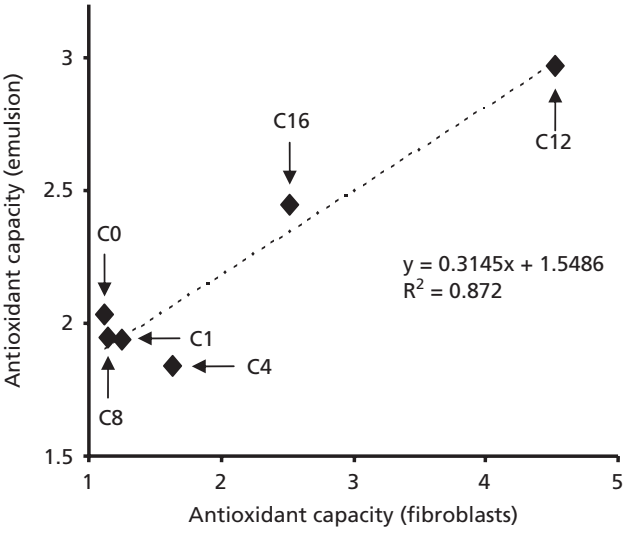

Figure 4 Correlation between antioxidant capacity values observed in this study (fibroblasts) and those obtained in emulsion for the same homologous series[17].

possible influence of the hydrophobicity on the free radical scavenging properties of chlorogenic acid and its alkyl esters, the BDE of phenolic O-H was deduced from semi-empirical quantum mechanic calculations after optimization of geometry and hydrogen bonding in both the parent phenol and the corresponding aryloxyl radical (Table 1). However, it can be observed that the alkyl chain length does not significantly influence the BDE of the corresponding O-H groups since BDE values are between 70.18 and 72.16 kcal/mol. Such a result can be explained since there is no conjugation between the carboxylic function and the aromatic ring that could affect the BDE through electron delocalisation by mesomeric effect.

In contrast, it is well documented that hydrophobicity can strongly favour the cell membrane uptake or penetration of amphiphilic molecules by giving them the so-called 'membrane-like character'.[19,20] In this way, a passive or assisted membrane transfer enabling an intracellular location of phenolics (presumably close to the oxidizing species) could strongly favour an antioxidant action and therefore could explain the strong activity of dodecyl and hexadecyl chlorogenates (Figure 2e and 2f). Besides, Serrano *et al.*[7] reported that the dodecyl ester of gallic acid presents a better membrane-crossing action than its propyl homologue.

Interestingly, we have observed by optical microscopy that dodecyl and hexadecyl chlorogenates were present (both at 50 and 100 μM) in the culture medium (DMEM + foetal calf serum + antibiotics + cells) under a self-assembled colloidal structure, which was not observed for short chain esters (1, 4 and 8 carbons) and free chlorogenic acid (data not shown). Further observations by light microscopy (60× oil immersion objective lens) of 100 μM dodecyl chlorogenate in water have shown a wide range of lipid polymorphs, most of which are characterized by a crystalline structure of an average size of 10–50 μm (Figure 5). It has also been visually observed that a temperature increase from 25 to 37°C leads to an irreversible phase transition from a gel-like structure to an aggregate. This interesting observation could explain the size and the shape of the microstructures depicted in Figure 5, which are much larger than simple micelles. Indeed, it is admitted that amphiphilic molecules self assemble spontaneously in aqueous solution to form a variety of thermodynamically stable structures known as association colloids such as micelles. It is also well known that micelles can aggregate in larger structures when the temperature is increased above the cloud point. This is due to the progressive dehydration of the hydrophilic head groups of amphiphilic molecules, which alters their molecular geometry and decreases the repulsion forces between them.[21,22]

According to McClements,[23] the properties of amphiphilic molecules dispersed as monomers are very different from those of aggregates. Indeed, ester monomers are amphiphilic and have a high surface activity whereas association colloids can have low surface activity because their surface is covered by a hydrophilic head group. Therefore, in our case, a colloidal drug delivery system could favour the transport of amphiphilic chlorogenate esters (dodecyl and hexadecyl esters) across biological membranes, compared with others tested esters, for which such a structure was not observed by optical microscopy.

Finally, if the higher efficiency could be tentatively explained for long chain esters by self-assembly properties, it is far more difficult to speculate about the possible mechanism by which an elongation from 12 to 16 carbons leads to a decrease in the antioxidant ability. Interestingly, this finding is consistent with numerous published studies on a diverse range of biological properties, such as anaesthetic,[24,25] antimicrobial,[26,27] cytotoxic[28] and spermicidal[29] activities. In these studies the dependence is quasi-parabolic, which means that the efficiency of the interaction of such compounds with biological membranes grows with an increase in their hydrophobic parts up to a certain length and then begins to diminish. Ferguson[30] in 1939 was one of the first to document this type of effect when compiling a combination of studies related to a homologous series of

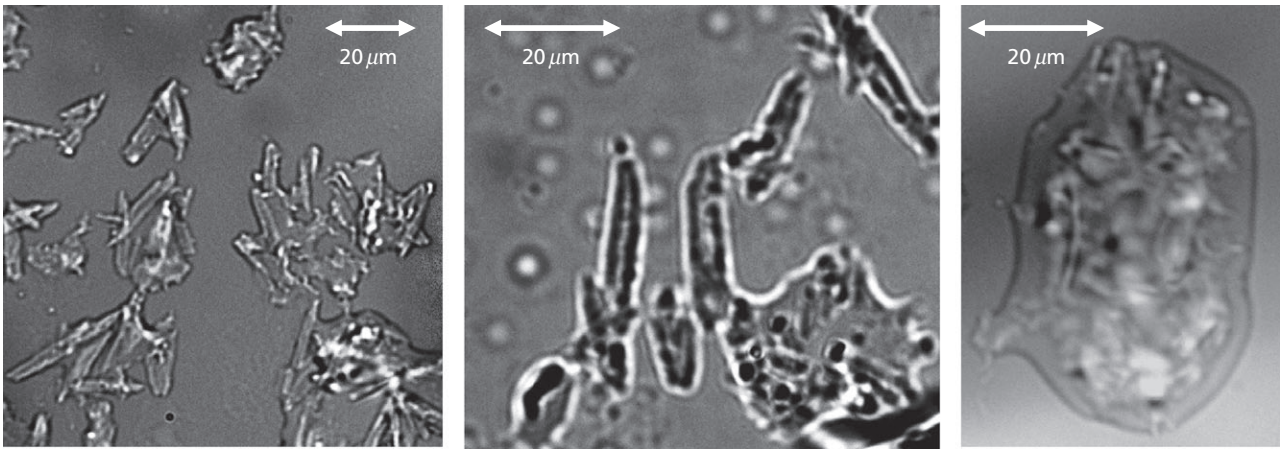

Figure 5 Representative light microscopy observations of self-assembled structures formed by dodecyl chlorogenate in water.

compounds. Sometimes named the 'parabolic case', this effect is now known under the term of 'cut-off effect'. Balgavy and Devinsky[31] reported that this effect is a general phenomenon observed in various biological and toxic actions with practically every amphiphilic homologous series tested so far. This study thus constitutes a first attempt to connect the cut-off theory to the non-linear dependency observed here between hydrophobicity and antioxidant capacity, in an effort to bridge the gap between these different fields.

In addition, as already mentioned, we have observed in emulsion on the same homologous series, a cut-off influence of the alkyl chain length, with an optimal antioxidant effect for the dodecyl chlorogenate.[17] The fact that a similar cut-off effect was observed in a non-living system (i.e. oil-in-water emulsion) without any protein, could suggest that the cut-off effect observed in the ROS-overexpressing fibroblasts results from a 'simple' physicochemical interaction between phenolics, water and lipids. This interaction, which remains unclear, could possibly take place at the membrane level. Unexpectedly, we demonstrated in an emulsified system that dodecyl chlorogenate exhibited a higher partition in the oily phase than longer esters (16, 18 and 20 carbons).[17] This suggests that in a system containing an interfacial membrane (emulsion) or a biological membrane (fibroblasts), the relationship between hydrophobicity and partitioning in the lipidic phase is not as linear as expected, and that the membrane acts as a selective barrier for some of these esters. Interestingly, Walters *et al*.[32] reported that in many biological systems, molecules show maximum membrane activity if they possess a medium chain length (~10–12 carbons). Taken together, these arguments suggest that the dodecyl chain confers to its corresponding ester the highest affinity for lipid membrane. Finally, the fact that aggregates were observed with dodecyl chlorogenate but not with short chain esters suggests that the better antioxidant capacity for dodecyl ester (which is conveyed by the phenolic hydroxyls) is largely due to its self-assembly properties.

Cytotoxicity measurement

Regardless of the mechanism of action of the chlorogenic acid and its alkyl esters, the putative application of some of these molecules (especially the dodecyl ester) to counteract oxidative stress *in vivo* supposes a lack of toxicity in the concentration and incubation time ranges used. To assess this factor, cytotoxic experiments using various concentrations (0, 10, 25, 50 and 100 μM) of chlorogenic acid and its alkyl esters were carried out during 24, 48 and 72 h of incubation using the lactate dehydrogenase (LDH) kit assay (see experimental procedure above). LDH is a cytosolic enzyme present within all mammalian cells. Briefly, the normal plasma membrane is impermeable to LDH, but damage to the membrane results in a change in its permeability and a subsequent leakage of LDH into the extracellular fluid. Release of LDH from cells provides an accurate measure of cell membrane integrity and, consequently, gives access to the cytotoxicity. The extracellular LDH level has been expressed as the percentage of the total releasable LDH after total lysis. Similarly to antioxidant capacity measurement, cytotoxicity values were expressed as the percentage of the control (phenolics free, but with ethanol).

First of all, Figure 6 shows that the LDH release is largely dependent on the nature and concentration of compounds, along with their incubation time. Indeed, only four tested compounds (chlorogenic acid and its methyl, dodecyl and hexadecyl esters) exerted a significant cytotoxicity ($P < 0.001$) in the concentration range used. In terms of concentration, it is worth noting that up to 25 μM, the dodecyl ester was the only one able to induce cytotoxicity after 72 h of incubation with cells. Thus, the concentration of the dodecyl ester necessary to exert significant cytotoxicity is less important than that of the hexadecyl ester by a factor of two. Moreover, these two compounds induce dose-dependent cytotoxicity, whereas the cytotoxicity appeared only for the highest concentration (100 μM) in the cases of free chlorogenic acid and its methyl ester. In addition, concerning the effect of incubation time, one can notice that when a strong cytotoxicity was detected (highest concentrations), the maximal effect was generally reached for an incubation time of 48 h, and even 72 h in some cases. In other words, while the antioxidant action needed less than 24 h to become apparent, the cytotoxicity needed at least 48 h of incubation.

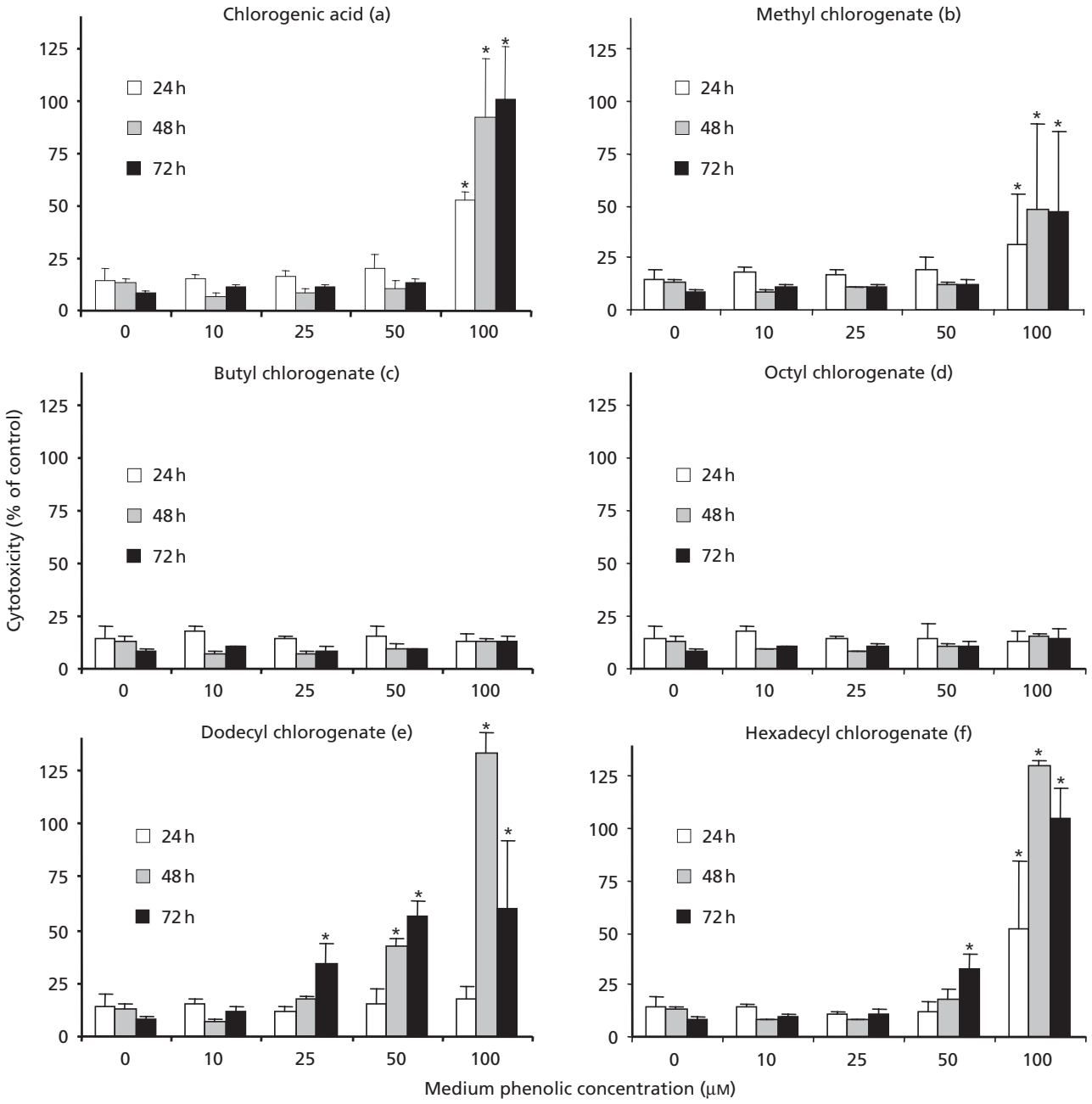

Figure 6 Cytotoxicity of chlorogenic acid and its alkyl esters estimated by lactate dehydrogenase release (λ_{ex}: 560 nm/λ_{em}: 590 nm). Cytotoxicity values on the *y*-axis were calculated according to Equation 9. Cells were cultivated with culture medium containing 10% (v/v) fetal calf serum, with 0, 10, 25, 50 and 100 μM phenolic compound and 0.1% (v/v) ethanol and lactate dehydrogenase activity was measured. Results were expressed as mean ± SD of four independent measurements carried out on four separate microplates seeded with four different cells suspensions ($n = 4$). *$P < 0.001$ compared with the control without phenolic (Kruskall–Wallis followed by Dunn's multiple pairwise comparison).

Conclusion

It can be concluded from this study that the antioxidant capacity of chlorogenate derivatives on a ROS-producing fibroblasts cell line increases as the alkyl chain is lengthened, with a threshold for the dodecyl chain, after which further chain extension leads to a drastic decrease in antioxidant capacity. This non-linear (or cut-off) trend is underlined here for the first time in a cellular system and demonstrates the interest of modulating the hydrophobicity of phenolic antioxidants through lipophilization reactions using medium chain length. This study paves the way for a systematic investigation of the effect of chain length. A hypothesis according to which medium and long alkyl chains exhibit a good affinity for lipid phase and favoured internalisation of phenolic drugs via their self-assembly properties has been put forward to explain the better antioxidant activity of dodecyl and hexadecyl esters over short chain esters. In contrast, the origin of the cut-off

phenomenon was not elucidated and requires further investigation. In addition, the self generation of ROS from the cell instead of the questionable induction of oxidation by artificial agents (diazo-compounds, transient metals) may be seen as an interesting achievement. Finally, it was shown that the dodecyl ester demonstrates a potent antioxidant capacity, for concentration and exposure time below the cytotoxicity threshold, making it a good candidate for further in-vivo study.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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