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Effect of acyl donor chain length on isoquercitrin acylation and biological activities of corresponding esters

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ABSTRACT

The enzymatic acylation of isoquercitrin with fatty acid esters of various carbon chain lengths was carried out in 2-methyl-2-butanol using Novozym 435®. The conversion yield and the initial rate decreased from 66% to 38% and from 17.7 to 10.1 μmol/h respectively, as the carbon chain of the acyl donor increased from C4 to C18. Isoquercitrin acylated derivatives showed higher xanthine oxidase inhibition activities than isoquercitrin. This property increased with the lipophilicity of the derivative esters. The scavenging activity of isoquercitrin esters against ABTS and DPPH radicals decreased with the acyl chain length. Conversely, for esters from C6 to C18, a linear growing relationship can be established between the chain length and the superoxide radical scavenging activity. Furthermore, an improved antiproliferative effect on Caco2 cancer cells was induced by addition of isoquercitrin esters comparing with isoquercitrin.

1. Introduction

Free radicals like reactive oxygen species (ROS) which are implicated in many human degenerative diseases can cause DNA lesions [1,2], loss of enzymatic activities [3,4], increase of cell permeability [5,6] and eventually necrotic cell death. Damages induced by these species are often suggested to play a role in the patho-physiology of various diseases, including diabetes [7], cancer [8] and lung diseases [9–12]. Search for new efficient radical inhibitors from natural sources constitutes an expanding field to prevent the risks and effects of acute and chronic free radical induced pathologies.

In this context, antioxidants in human diets are of great interest as possible protective agents to reduce oxidative damages. Many natural antioxidants have already been isolated from different plant materials such as seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs [13,14]. Among them, flavonoids present various beneficial effects towards human health and have been studied to elucidate their mechanism of action. Flavonoid antioxidant properties include the chelation of trace elements (free iron or copper) which are potential enhancers of free radicals formation, the stabilisation of free radicals involved in oxidative processes by complexing with them [15] and the inhibition of enzymes which lead to the formation of reactive oxygen species [16]. In addition, some flavonoids have been found to exert specific cytotoxic activities towards cancer cells which has generated an expanding interest in developing flavonoid-based cytostatics for anti-cancer therapy [17]. In order to predict the cytotoxic and/or antioxidant potential of a given flavonoid, structure–activity relationships have to be established.

However, flavonoids can exhibit very low solubility and stability in lipophilic and/or hydrophobic media [18–21], thus limiting their use in oil based foods and cosmetics.

To improve these properties several authors have studied the modification of flavonoid structures by chemical, enzymatic or chemo-enzymatic reactions. The acylation and the glycosylation have received particular attention. Glycosylation allowed reinforcing the hydrophilic character of some flavonoids by adding sugars, while their hydrophobicity can be enhanced by chemical or enzymatic acylation with fatty acids.

Chemical and enzymatic acylation of phenolic compounds with various acyl donors has been already reported. However, chemical processes are not regioselective: they lead to the functionalization of phenolic groups, and thus possibly decrease their antioxidant activity [22,23,16]. The enzymatic acylation was shown to be more regioselective and to enhance not only flavonoids solubility in various media, but also their stability and their antioxidant activity [24–26]. In addition, flavonoid derivatives are expected to exhibit a

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higher affinity for phospholipidic membranes and could be useful to establish structure–activity relationships relating their amphiphilic and lipophilic properties to their ability to be transferred into cells. For instance, studies showed that acylation of mono- and diglycosylated chrysoeriol enhanced their protective effect against human LDL and serum oxidation as well as their antioxidant activity in oils and their xanthine oxidase inhibition property [27,28]. In food and cosmetic preparations, various phenolic esters are used, such as esters of gallic acid [propyl (E310), octyl (E311), and dodecyl (E312) gallates] as food antioxidant additives [29–31], octyl gallate for its broad antimicrobial spectrum and its antifungal efficiency [32,33], or flavanols esters as cosmetic additives [34]. Some authors also showed that the lipophilicity of acylated derivatives may increase their ability to interact with the cell membrane and to transfer through it [27,35,36].

Various enzymes have been tested to catalyze the acylation of flavonoids. The *Candida antarctica* lipase B (CALB) showed a strong selectivity for flavonoids glycosides presenting a primary or a secondary aliphatic hydroxyl group on their sugar moiety [37,38]. However, studies about the alcohol donor effect showed that substrates with a primary hydroxyl group were easier to acylate than those exhibiting secondary hydroxyl groups [39–41].

Among flavonoids, the widely spread isoquercitrin has been reported to present many biological activities, such as ABTS, DPPH and superoxide radicals scavenging [42–44]. This molecule is a good substrate for the CALB which exhibits regioselectivity in favour of its primary 6″-OH group [40,45,46]. However, in some cases, the formation of di- and tri-acylated isoquercitrin has been reported [47]. Ishihara and Nakajima [25] showed that the enzymatic synthesis of acylated isoquercitrin was accomplished by a lipase-catalyzed transesterification with carboxylic acid vinyl esters as acyl donors in acetone or acetonitrile as solvent. These authors found that acylation occurred on the primary OH of the sugar moiety and indicated that the introduction of an acyl group onto isoquercitrin structure improved its thermostability and light-stability.

In the present work, the enzymatic synthesis of fatty acid derivatives of isoquercitrin was studied. Then, a systematic study concerning the effect of the structure of isoquercitrin derivatives on their antioxidant properties and antiproliferative activity towards Caco2 cancer cells was investigated in vitro. Particularly, the influence of the acyl donor carbon chain length on the performances of isoquercitrin acylation and derivative properties was evaluated.

2. Materials and methods

2.1. Chemicals

Ethyl decanoate (>99%), ethyl caprylate (>98%), ethyl caproate (>99%) and ethyl butyrate (>98%) were purchased from Fluka (Switzerland). Ethyl laurate (99%), ethyl stearate (99%) and ethyl palmitate (99%) were from Sigma (Germany) and ethyl oleate (98%) was purchased from Sigma–Aldrich (Germany). 2-Methyl-2-butanol, hexane, acetic acid, methanol, chloroform and trifluoroacetic acid (TFA) with 99% of purity were acquired from Carlo Erba (Spain). Ethyl acetate was from Fisher scientific (UK).

2.2. Synthesis of isoquercitrin esters

The enzymatic synthesis of isoquercitrin esters was performed in the glass device of a rotary evaporator equipped with a vacuum controller. The reaction medium was maintained at 65 °C and stirred at 150 rpm under vacuum (700 mbar). Each reaction was performed using isoquercitrin (10 mM) (Extrasynthèse, France) in 10 mL of 2-methyl-2-butanol previously dried on 4 Å molecular sieves and 100 mM acyl donor: ethyl oleate, ethyl stearate, ethyl palmitate, ethyl laurate, ethyl decanoate, ethyl caprylate, ethyl caproate or ethyl butyrate. After complete dissolution of the substrates (65 °C, under stirring overnight), the esterification reaction was started by adding 30 g/L of Novozym 435®, lipase B from *C. antarctica* (CALB lipase) immobilized on an acrylic resin. This enzyme presents a propyl laurate synthesis activity of 7000 propyl laurate units (PLU) g^{-1} and a protein grade of [1–10%], (Novo Nordisk A/S, Denmark). To evaluate the evaporation of the solvent during reactions, an internal inert standard was used (2,6-dimethylphenol at 0.2 g/L) [48]. The reaction was stopped after 72 h by filtration to remove the enzyme.

2.3. Thin layer chromatography

Qualitative analyses of reaction mixtures were performed by TLC on silica gel 60 F254 plates (Merck, Germany) using a solvent mixture system: ethyl acetate/methanol/water (100/8/10, v/v/v). The products were detected by spraying a methanol solution of 2-aminoethyldiphenylborinate and revealed under UV light (254 nm).

2.4. High-performance liquid chromatography (HPLC) analysis

The time course of each reaction was monitored at 214 nm using HPLC (LC 10 AD-VP, Shimadzu, France) equipped with an UV detector and a light-scattering low temperature evaporative detector (Shimadzu, France). The column was a C18 amide 2.1–125 mm (Altima®, Altech, France) maintained at 25 °C. The mobile phases (0.2 mL/min flow rate) consisted of water/methanol/TFA (60/40/0.1 v/v/v) (phase A) and methanol/TFA (100/0.1 v/v) (phase B). The gradient applied was: 0–1 min: 100% A; 1–16 min: 0–100% B; 16–24 min: 100% B; 24–25 min: 0–100% A; 25–34 min: 100% A. Calibrations were performed using standard substrates and purified products. The substrate conversion yield at the thermodynamic equilibrium was determined applying the following equation:

$$Y\,(\%) = \left(1 - \frac{[\text{substrate}]_{\text{equilibrium}}}{[\text{substrate}]_{\text{initial}}}\right) \times 100 \quad (1)$$

Initial specific reaction rates were estimated as the slope of the linear approximation during the first 2 h of reaction. The variation coefficient of reproducibility for both substrate conversion rates and initial reaction rates was found to be inferior to 10%.

2.5. Purification of acylated products

After filtration of the reaction medium to remove the enzyme and partial evaporation of the solvent, the residue was applied to a silica gel column (Silica Gel 60, 230–400 mesh, Merck, Germany) and eluted with ethyl acetate/methanol/water (100/8/10, v/v/v). Fractions were collected then analyzed. The fractions containing the product were pooled together and the solvent was evaporated under vacuum.

2.6. LC–MS characterization of isoquercitrin esters

After dilution of the reaction medium in the mobile phase and filtration, samples were analyzed using a HPLC system (Thermo Fisher Scientific, France) equipped with a UV detector (214 nm) coupled to a mass spectrometer with electron spray (ES) ionization source (LTQ, Thermo electron corporation®, USA). The HPLC method was the one described above. MS measurements were carried out with helium as the collision gas in the ion trap and nitrogen as sheath (50), sweep (10) and auxiliary (10) gas in the source. MS parameters were tuned as follows: electrospray positive ionization mode, capillary temperature of 300 °C and source voltage at 5.0 kV.

2.7. Nuclear magnetic resonance

The chemical structure of the acylated products was determined by ^{13}C NMR and ^{1}H NMR spectroscopic analysis in $CDCl_3$ on a Brücker Avance 300 spectrometer (Germany). The following notations were used: s: singlet, t: triplet, m: multiplet, br: broad; bold data: data used for the structural elucidation of isoquercitrin esters focusing on the acyl group position.

Isoquercitrin: ^{1}H NMR (DMSO-d_6, 300 Hz) δ: 12.63 (s, 1H, OH5), 7.58 (m, 2H, H6′, H2′), 6.84 (d, J = 9.0 Hz, 1H, H5′), 6.40 (d, J = 2.0 Hz, 1H, H8), 6.20 (d, J = 2.0 Hz, 1H, H6), 5.45 (d, J = 7.4 Hz, 1H, H1″), **3.58, 3.34 (ABX, J_{AB} = 11.4 Hz, J_{AX} = 1.4 Hz, J_{BX} = 5.4 Hz, 2 H, H6″)**, 3.24 (m, 2H, H2″, H4″), 3.09 (m, 2H, H3″, H5″).

^{13}C NMR (DMSO-d_6): δ: 177.43 (C4), 164.08 (C7), 161.23 (C9), 156.30 (C5), 156.16 (C2), 148.43 (C4′), 144.78 (C3′), 133.432 (C3), 121.58 (C1′), 121.16 (C6′), 116.19 (C5′), 115.19 (C2′), 103.97 (C10), 100.87 (C1″), 98.63 (C6), 93.47 (C8), 77.54 (C5″), 76.50 (C3″), 74.08 (C2″), 69.93 (C4″), **60.97 (C6″)**.

Isoquercitrin butyrate: ^{1}H NMR (DMSO-d_6, 300 Hz) δ: 12.61 (s, 1H, OH5), 7.53 (ps, 2H, H6′, H2′), 6.82 (d, J = 9.0 Hz, 1H, H5′), 6.38 (d, J = 1.8 Hz, 1H, H8), 6.18 (d, J = 1.8 Hz, 1H, H6), 5.43 (d, J = 7.1 Hz, 1H, H1″), **4.15, 3.95 (ABX, J_{AB} = 10.9 Hz, J_{AX} = 4.4 Hz, J_{BX} = 5.9 Hz, 2 H, H6″)**, 3.67 (m, 2H, H2″, H4″), 3.28 (m, 2H, H3″, H5″), 1.96 (m, 2H, Hb), 1.25 (m, 2H, Hc) 0.65 (t, J = 4.3 Hz, 3 h, Hd).

^{13}C NMR (DMSO-d_6): δ: 177.28 (C4), 172.18 (Ca), 164.09 (C7), 161.19 (C9), 156.25 (C5, C2), 148.42 (C4′), 144.71 (C3′), 132.90 (C3), 121.41 (C1′), 120.99 (C6′), 116.01 (C5′), 115.05 (C2′), 103.79 (C10), 100.52 (C1″), 98.58 (C6), 93.40 (C8), 76.22 (C5″), 74.53 (C3″), 74.42 (C2″), 70.01 (C4″), **62.84 (C6″)**, 35.14 (Cb), 17.71 (Cc), 13.09 (Cd).

2.8. Log P evaluation

Theoretical log P of isoquercitrin and its acylated derivatives were determined using the Molinspiration program [49]. The efficiency of this program was tested by calculating the log P of some flavonoids already experimentally determined [50].

2.9. Xanthine oxidase inhibition assay

Xanthine oxidase inhibition activity was evaluated by the slightly modified method of Cos et al. [51], which consists in the spectrophotometric monitoring of the formation of uric acid from xanthine. Isoquercitrin and its acylated derivatives were first dissolved in a small amount of DMSO (5%). 2 mL reaction mixture containing 200 mM phosphate buffer pH 7.8, 0.2 mM hydroxylamine, HCl, 50 μM xanthine as the substrate, 0.1 mM EDTA (pH 7.0), isoquercitrin or its esters (0–200 μM) were prepared. The assay was initiated by adding the enzyme (2.5 mU/mL) to the reaction mixture. A negative control containing all reagents except the test sample was used. After incubation at 37 °C during 30 min, the reaction was stopped by adding 200 μL of 0.58 M HCl. The enzyme activity was evaluated by measuring uric acid formation at 290 nm. For each compound concentration tested, the percentage of xanthine oxidase inhibition (XOI) was calculated using the following equation:

$$\text{XOI}\,(\%) = \left(1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100 \quad (2)$$

with $\text{Abs}_{\text{sample}}$ and $\text{Abs}_{\text{control}}$ be the absorbance values at 290 nm of sample and control respectively.

The extent of inhibition was expressed as the chemical concentration required to inhibit 50% of the enzyme activity (IC50) using a second order polynomial model. Allopurinol was used as the reference compound. All tests were carried out in triplicate. Results were expressed as mean values with standard deviations (±SD).

2.10. Superoxide scavenging activity

To detect superoxide, the colouring reagent (300 μg/mL sulfanilic acid, 5 μg/mL of N-(1-naphthyl)-ethylenediamine dihydrochloride, and 16.7% (v/v) acetic acid) was added to the reaction medium of the xanthine oxidase inhibition test. The mixture stood in the dark at room temperature for 30 min, then the absorbance at 550 nm was measured. A solution without any tested compound was used as control. Antioxidant activity (AA) was expressed as an inhibition percentage of superoxide radical, and calculated using Eq. (2). For each compound, the half-maximal scavenging concentration (SC50) was calculated by linear regression analysis [51]. All analyses were carried out in triplicate and results represented the mean values with standard deviation (±SD).

2.11. DPPH test

Determination of the antioxidant activity with the stable radical 2,2-diphenyl-l-picrylhydrazyl ($\text{DPPH}^{\bullet-}$) (Sigma, Germany) radical scavenging method was performed. The ability to scavenge the $\text{DPPH}^{\bullet-}$ free radical was determined according to the method of Atoui et al. [52]. A methanolic solution (50 μL) of the compound to be tested was prepared at four different concentrations (between 0 and 40 μM) and added to 1.95 mL of DPPH solution (6×10^{-5} M in methanol). The mixture was vigorously shaken with a vortex mixer and incubated for 30 min in the dark, then the decrease in the absorbance corresponding to the remaining $\text{DPPH}^{\bullet-}$ was measured at 517 nm. Methanol was used as a blank solution and DPPH solution in methanol without any tested compound was used as control. The ability to scavenge DPPH radical was calculated using Eq. (2). The antioxidant activities of compounds were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) values. TEAC value is defined as the concentration of standard trolox (Fluka, Germany), a water-soluble vitamin E analogue that exhibited the same antioxidant capacity as a 1 mM solution of the antioxidant compound under investigation. All analyses were carried out in triplicate and results represented the mean values with standard deviation.

2.12. ABTS radicals scavenging activity

The evaluation of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($\text{ABTS}^{\bullet+}$) radical scavenging activity is based on the ability of antioxidants to inhibit the long-life ABTS radical cation (Sigma, Germany), a blue/green chromophore with characteristic absorption at 734 nm, in comparison with that of trolox. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark, at room temperature, for 12–16 h before use.

For the study of the antiradical activity of isoquercitrin and its acylated derivatives, the $\text{ABTS}^{\bullet+}$ solution was diluted with ethanol at 30 °C, in order to obtain an absorbance of 0.70 (±0.02) at 734 nm. After addition of 1.0 mL of diluted $\text{ABTS}^{\bullet+}$ solution to 10 μL of sample or trolox standard in ethanol (concentration between 0 and 16 μM), the absorbance was measured at 30 °C exactly 6 min after initial mixing. Appropriate solvent blanks were run in each assay. All experiments were performed in triplicate. The extent of decolourization is calculated as the percentage reduction of ABTS absorbance. The antioxidant activities of compounds were expressed as TEAC values. TEAC value is defined as the concentration of standard trolox with the same antioxidant capacity as a 1 mM concentration or 1 mg/mL of the antioxidant compound under investigation [53].

2.13. Cell culture

Caco2 cells were kindly provided by the laboratory URAFPA (Nancy, France). These cells were used between passages 30 and 50 and were cultivated in Dulbecco's modified eagle medium (DMEM) with high glucose (4.5 g/L), (Sigma, Germany) and supplemented with 10% fetal calf serum (FCS), (EuroBio, France), 2 mM L-glutamine and 1% nonessential amino acids (GIBCO, USA). The cells were usually split when reaching confluence (5–7 days). They were first rinsed with Dulbecco's phosphate-buffered saline without calcium (DPBS) (Sigma, Germany) and then trypsinized with a solution containing 0.25% trypsin and 1 mM EDTA (GIBCO, USA). For maintenance of the cell line, cells were seeded at 2×10^4 cells/cm^2 in flasks.

2.14. Antiproliferative activity

Caco2 cells were seeded into 96-well microplates at 4×10^4 cells per well in 200 μL of DMEM medium supplemented with 10% FCS, 2 mM glutamine and 1% non essential amino acids. After 24 h, cells were exposed to various concentrations of the compounds solubilized in DMSO and incubated for 48 h at 37 °C, under 5% CO_2 atmosphere. The cytotoxicity of isoquercitrin and its esters was determined using the colorimetric methylthiazoletetrazolium method based on the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-y)-2,5 diphenyltetrazolium bromide (MTT) (Sigma, Germany) into a crystalline blue formazan product by the mitochondrial oxidoreductases of viable cells [54]. 50 μL of MTT solution (2 g/L) was added to each well. After incubation for 4 h at 37 °C, the formazan crystals produced by active reductases were dissolved in 150 μL isopropanol. The product was quantified spectrophotometrically by absorbance measurement at 540 nm using a microplate reader. Each test was carried out in quadruplicate, and each experiment was repeated twice. The relative cell viability was calculated according to the following equation:

$$\text{Relative cell viability}\,(\%) = \left(1 - \frac{\text{Abs}_{\text{treated cells}}}{\text{Abs}_{\text{control}}}\right) \times 100$$

with $\text{Abs}_{\text{treated cells}}$ and $\text{Abs}_{\text{control}}$ be the absorbance values at 540 nm of sample with treated cells and control respectively.

Results were expressed as mean values with the standard deviations.

3. Results and discussion

3.1. Enzymatic acylation of isoquercitrin

Isoquercitrin was acylated by fatty acid ethyl esters using the lipase B of *C. antarctica* (Fig. 1) in 2-methyl-2-butanol which allowed the complete solubilization of both substrates at the concentrations used in the present study. A molar ratio of 1/10 (flavonoid/acyl donor) was chosen as it was previously demonstrated to be optimal in terms of acylation rate [41].

The temperature was kept at 65 °C and a pressure of 700 mbar was applied to favour the alcohol by-product evaporation. No product was detected when fatty acid esters and isoquercitrin were incubated in the absence of enzyme. For all the acyl donors, only one major product was identified by TLC, HPLC and LC–MS analyses, which indicated that the reaction was regioselective. The regioselectivity was confirmed by LC–MS and ^{1}H NMR analyses of the purified isoquercitrin esters. LC–MS results showed that only monoacylated esters were synthesized (Fig. 1): isoquercitrin butyrate (C4:0) ($M+H^+$ = 535 g/mol), isoquercitrin caproate (C6:0) ($M+H^+$ = 563 g/mol), isoquercitrin caprylate (C8:0) ($M+H^+$ = 591 g/mol), isoquercitrin decanoate (C10:0) ($M+H^+$ = 619 g/mol), isoquercitrin laurate (C12:0) ($M+H^+$ = 647 g/mol), isoquercitrin palmitate (C16:0) ($M+H^+$ = 703 g/mol), isoquercitrin stearate (C18: 0) ($M+H^+$ = 731 g/mol) and isoquercitrin oleate (C18:1) ($M+H^+$ = 729 g/mol).

To confirm the acylation site, ^{1}H and ^{13}C NMR spectra of isoquercitrin and isoquercitrin butyrate were recorded in DMSO-d_6. Comparison of these spectra showed that acylation took place at the 6″-OH of the sugar moiety.

The acylation of isoquercitrin affected only the two hydrogens H6″ (in bold in the data). For the "free" isoquercitrin, the ABX system presented two signals at 3.58 and 3.34 ppm, whereas these two H6″ atoms assumed higher values at 4.15 and 3.95 ppm for the ester, indicating the deshielding effect of the butyrate moiety on the chemical shifts of these two protons.

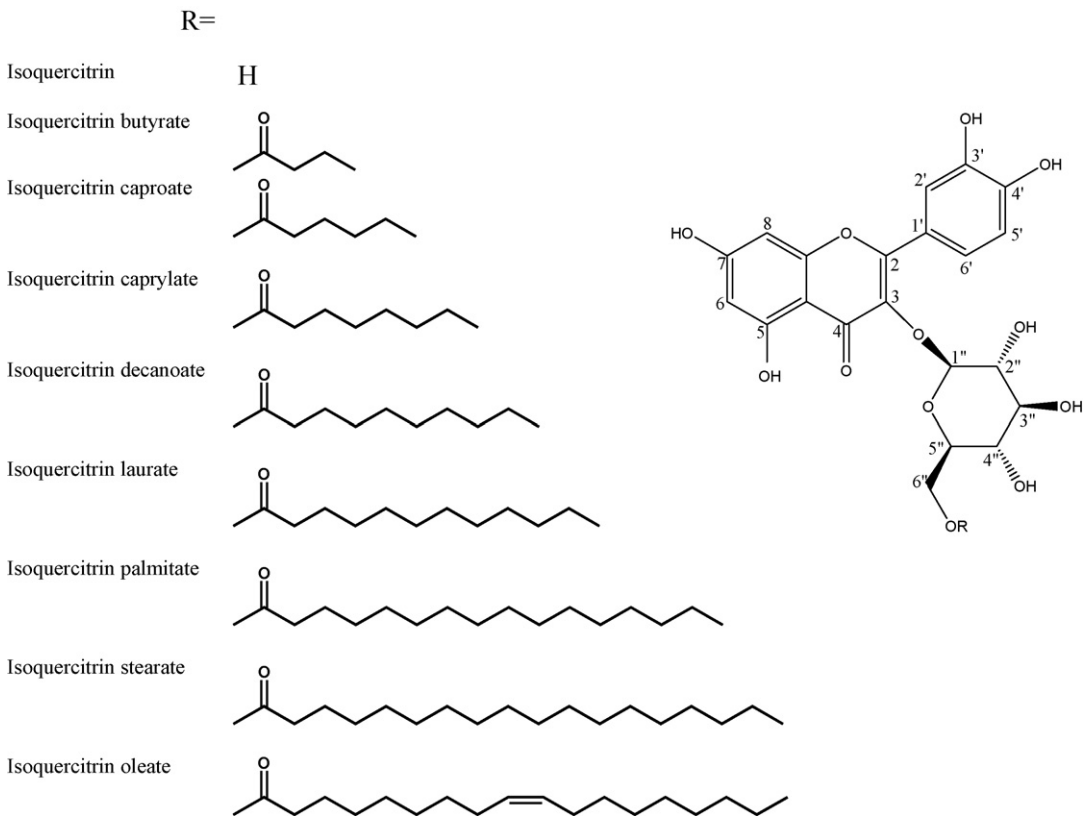

Fig. 1. Chemical structure of isoquercitrin and its esters.

Similarly, the chemical shift of C6″ was 60.97 ppm for the "free" isoquercitrin and slightly downfield for the ester (62.84 ppm), all the other values being rather similar. The same approach that has been described by Yoshimoto et al. [55] was used for the elucidation of lauroyl isoquercitrin structure and the same trend of chemical shifts was observed, showing that the regioselectivity of the acylation reaction did not differ depending on the acyl chain length.

This result is in accordance with other studies concerning the ability of CALB to catalyze the esterification of isoquercitrin. Nakajima et al. [45] and Stevenson et al. [37] indicated that the acylation of isoquercitrin with vinyl cinnamate or 2-hydroxy-phenylpropionic acid led to the synthesis of the sole isoquercitrin 6″-ester. However, Chebil et al. [47] indicated that the acetylation of isoquercitrin in acetone at 50 °C with CALB led to two products, isoquercitrin 3″,6″-diacetate ester and isoquercitrin 2″,3″,6″-triacetate, when vinyl acetate was used as acyl donor. Danieli et al. [40] obtained only the diacetylated product, isoquercitrin 3″,6″-diacetate after isoquercitrin acetylation with vinyl acetate catalyzed by CALB using a mixture of acetone/pyridin as solvent, at 45 °C. In our case, neither diester nor triester was obtained.

3.2. Effect of the acyl donor structure on acylation reaction efficiency

The effect of the carbon chain length of the acyl donor on the reaction efficiency was studied using eight fatty acids with carbon chain length varying from 4 to 18. Both the conversion yields of isoquercitrin and the initial rates of reaction for the eight fatty acids are given in Fig. 2A and B. All reactions reached their thermodynamic equilibrium between 48 and 72 h. The total conversion yield of isoquercitrin depended on the carbon chain length of the fatty acyl donor used. It decreased from 66% for ethyl butyrate to 38% for ethyl stearate. Katsoura et al. [56] reported a similar trend during the acylation of naringin and rutin with CALB lipase using free fatty acids and their vinyl esters in ionic liquids. In their study, the higher conversion yield, about 65%, was observed

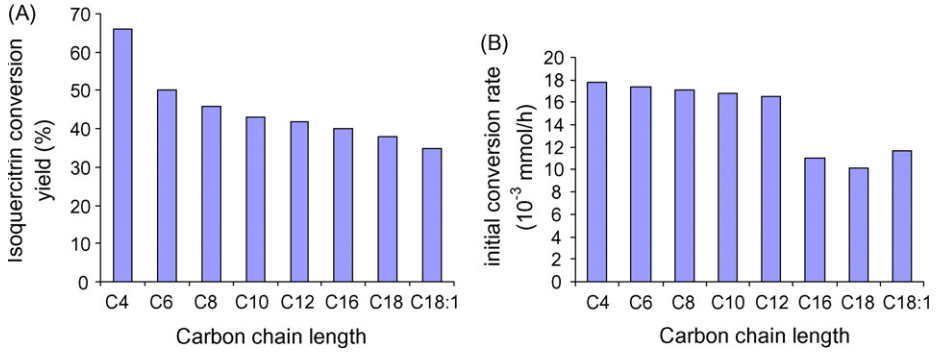

Fig. 2. Effect of acyl donor structure on the conversion yields after 72 h of reaction (A), initial rates of isoquercitrin acylation reactions (B) with different ethyl ester fatty acids in 2-methyl-2-butanol catalyzed by the lipase B of *Candida antarctica*. The variation coefficient was found to be inferior to 10% for all results.

for short chain length acyl donors. Kontogianni et al. [57] showed that no relationship can be established between the acyl donor chain length and the conversion yield of esterification of rutin and naringin by fatty acids (C8, C10 and C12), catalyzed by the CALB lipase in various solvent systems. Another trend was reported by Ardhaoui et al. [58], who studied rutin acylation reactions with CALB using free fatty acids with carbon chain length varying from 6 to 18 as acyl donors. They showed that for carbon chain lengths between C6 and C12, the performance of the reaction increased with the fatty acid chain length, whereas for higher carbon chain length, no significant effect was observed.

The influence of the acyl donor chain length on the kinetics of isoquercitrin acylation was also studied. For chain lengths from C4 to C12, similar initial rates of reaction were observed (around 17×10^{-3} mmol/h) independently of the fatty acid esters used. On the contrary, Pedersen et al. [59] reported that initial reaction rates decreased with increasing fatty acid chain length from C4 to C12 during CALB catalyzed esterification of carbohydrates. For esters longer than C16 the initial rate stood around 10×10^{-3} mmol/h. This latter result is similar to that reported by Mellou et al. [27] who showed that the carbon chain length of the acyl donor did not affect the reaction rate of naringin acylation by fatty acids or their vinyl esters.

Both results about initial reaction rates and conversion yields may be related to the structure of CALB. Indeed, it exhibits a funnel-like scissile fatty acid binding site, inside which the longest fatty acid that completely binds is a 13 carbons chain length one [60].

In the present study, conversion yields of stearic and oleic acid ethyl esters were 38% and 35% respectively, and similar reaction rates were obtained showing that the presence of an unsaturation did not influence the kinetic of the reaction. This result is in accordance with those reported by other authors about rutin acylation with oleic, linoleic and γ-linolenic acids and with stearic and oleic ethyl esters [35,58].

All isoquercitrin esters were purified in order to study their antioxidant and antiproliferative activities and to compare them with isoquercitrin activities.

3.3. Xanthine oxidase inhibition

The XO catalyzes the oxidation of hypoxanthine and xanthine producing uric acid, superoxide radical and hydrogen peroxide. Consequently, XO is considered as an important biological source of superoxide radicals.

In the present study, inhibition of XO by isoquercitrin and its acylated derivatives was evaluated (Fig. 3). Allopurinol, routinely used as XO inhibitor, constituted the reference molecule with an IC50 value of 2.4 μM. Isoquercitrin did not display an important xanthine oxidase inhibitory activity with an IC50 value of 183 μM. The acylation of this molecule significantly enhanced its xanthine oxidase inhibitory potential. Indeed, all isoquercitrin esters showed a higher XO inhibition activity than isoquercitrin. For saturated esters, IC50 values increased when decreasing the carbon chain length, from 61 to 144 μM for isoquercitrin stearate and isoquercitrin butyrate respectively. As shown in Fig. 3, a linear relationship could be established between the IC50 value and the acyl chain length. Isoquercitrin oleate (C18:1) which presents an unsaturation in the acyl group exhibited a higher activity (IC50 value of 27 μM) than that of its saturated analogue (IC50 value of 61 μM). These results are in accordance with those described by Rao et al. [28] who found a linear relationship between the log IC50 of mesquitol esters and their acyl chain length. This result can be explained by the improvement of the lipophilicity of the molecule allowing a better accessibility to the active site of XO. In fact, Iio et al. [61] reported that the XO inhibition activity of flavonoids may be due to their surface properties, especially their amphiphilic character.

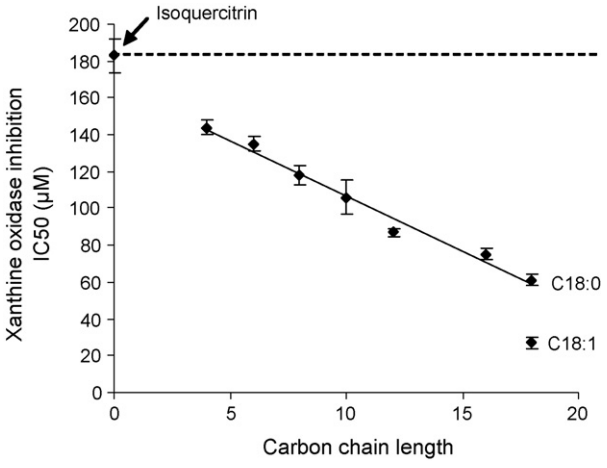

Fig. 3. Xanthine oxidase inhibitory potential of isoquercitrin and its esters depending on acyl chain length, expressed as IC50 values (μM).

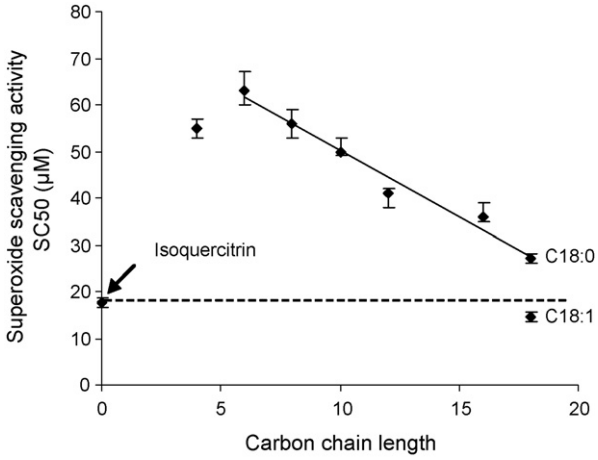

Fig. 4. Superoxide scavenging potential of isoquercitrin and its esters depending on acyl chain length, expressed as SC50 values (μM).

3.4. Superoxide radical scavenging activity

A molecule is considered as a superoxide scavenger when its SC50 value for reduction of the superoxide radical is lower than that found for XO inhibition [51]. Isoquercitrin and its acylated derivatives respected this condition as isoquercitrin saturated esters presented SC50 values from 63 μM for isoquercitrin caproate to 27 μM for isoquercitrin stearate. These esters were then less active than isoquercitrin (SC50 value = 17 μM). Isoquercitrin oleate exhibited a scavenging activity (SC50 value = 14 μM) similar to that of isoquercitrin.

Except for isoquercitrin butyrate (C4) that showed a higher activity than isoquercitrin caproate (C6), a linear relationship between SC50 values and the esters carbon chain length was established (Fig. 4).

3.5. DPPH radical scavenging activity

The DPPH radical scavenging ability of isoquercitrin and its derivatives was assessed and trolox was used as reference molecule. This activity was expressed as Trolox Equivalent Antioxidant Capacity (TEAC). Esters were found to exhibit a lower radical scavenging activity than isoquercitrin (TEAC value of 2.0) (Fig. 5A). For esters from C4 to C10, the antiradical activity

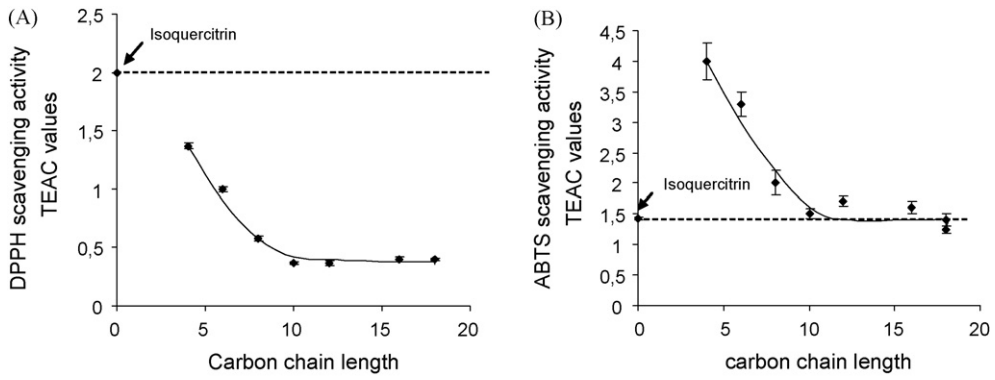

Fig. 5. Antioxidant activities of isoquercitrin esters of various chain lengths against $DPPH^{\bullet -}$ (A) and $ABTS^{\bullet +}$ (B) radicals, expressed as TEAC values.

decreased when increasing carbon chain length. Isoquercitrin butyrate (C4) exhibited the highest antiradical activity (TEAC value of 1.37). TEAC values decreased with the carbon chain length to reach 0.37 for isoquercitrin decanoate (C10). Rather similar antiradical activities were observed for esters with chain lengths higher than 10 carbon atoms (TEAC value around 0.37).

Some authors studied the DPPH antiradical activity of phenolic esters and showed that this activity was independent of the acyl chain length [62]. Takahashi et al. [63] showed similar results in the case of alkylaminophenols of various alkyl chain lengths.

3.6. ABTS radical scavenging activity

The ABTS radical scavenging activity method is based on the ability of molecules to quench the ABTS radical cation, in comparison with that of trolox. The ABTS radical scavenging activities of isoquercitrin and its esters, expressed as TEAC, are given in Fig. 5B.

All isoquercitrin esters were shown to be effective antiradical agents compared to the trolox (TEAC values higher than 1). They displayed similar or higher free radical scavenging activities than isoquercitrin (TEAC = 1.4). In fact, TEAC values decreased from 4 to 1.5 when the acyl chain length increased from C4 (isoquercitrin butyrate) to C10 (isoquercitrin decanoate). Esters with longer acyl chain (C10–C18) showed antiradical activities similar to that of isoquercitrin with TEAC values of 1.4 for isoquercitrin stearate and 1.7 for isoquercitrin laurate. These results are in accordance with those of Torres de Pinedo et al. [64], who showed that the acyl chain length affected the radical scavenging activity. They reported that palmitoyl esters of phenolic acids were more effective ABTS radical scavengers than stearoyl or oleyl esters.

The variation of ABTS antiradical activity versus the acyl chain length followed a similar trend to that of the DPPH scavenging activity (Fig. 5A and B).

3.7. Antiproliferative activity

Several studies reported that flavonoids may exhibit cytotoxic activities towards cancer cells [65–70]. In the present work, the effect of isoquercitrin and its fatty acid esters on tumoral Caco2 cells growth was investigated. At a concentration of 200 μM, isoquercitrin led to an antiproliferative activity of 42% referring to the growth of Caco2 cells without isoquercitrin. All isoquercitrin esters exhibited a dose dependent antiproliferative activity on Caco2 cells and were shown to be more active than isoquercitrin. Esters with acyl chain lengths from C8 to C16 showed the highest activities with IC50 values comprised between 51 and 66 μM. C4, C6 and C18 esters were less effective with IC50 values over 100 μM (Fig. 6).

The enzymatic acylation of flavonoids was expected to increase their lipophilicity and consequently their ability to interact with the cell membrane and their transfer through it [27,35,71]. However, no relationship between the acyl chain length and cytotoxic activity on Caco2 cells could be established. The activity of a compound in a biological system does not only depend on its

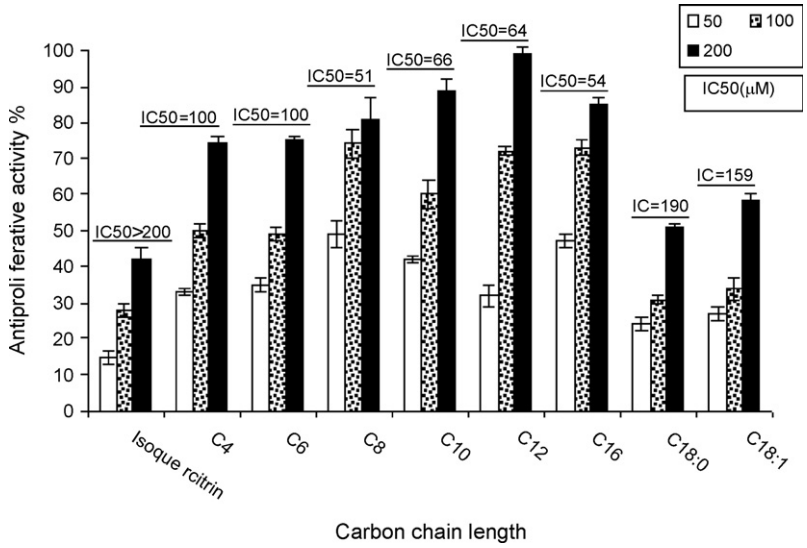

Fig. 6. Antiproliferative effect of isoquercitrin esters of various chain lengths against Caco2 cancer cells at 50, 100 and 200 μM and their IC50.

interaction with cell membranes due to its lipophilicity but also on its affinity for specific cell receptors. For instance, Dasgupta et al. [72] showed that in human breast cancer cells, the receptor affinity of somatostatin analogue was not affected by its acylation with palmitic acid, whereas this affinity was reduced when the peptide was acylated with stearic or butyric acids.

4. Conclusion

The present work focused on a systematic study concerning the influence of the chain length of acyl donors on the efficiency of isoquercitrin enzymatic acylation. Then, the influence of the structure of corresponding esters on their antioxidant and cytotoxic activities on cancer cells was evaluated.

The results indicated that the efficiency of the synthesis depended on the length of the acyl donor. *C. antarctica* lipase B exhibited a high activity towards short and medium chain length acyl donors and was less active towards long chain substrates.

For the first time, the effect of acyl donor chain length on isoquercitrin esters antioxidant properties and antiproliferative activity towards tumor cells was systematically studied. Isoquercitrin esters were shown to exhibit an antioxidant activity that depended on the acyl chain length. First, the XO inhibition property of isoquercitrin esters was shown to increase with their lipophilicity. The acylation reaction enhanced the scavenging activity of isoquercitrin against ABTS radicals but decreased it against DPPH and superoxide radicals. A structure–activity relationship could be established between isoquercitrin esters activities and corresponding acyl chain length. Although acylated derivatives of isoquercitrin presented a lower superoxide and DDPH antiradical activity than isoquercitrin, their improved solubility in lipidic phases can lead to a good compromise for their use in food or cosmetic formulations.

The antiproliferative activity of isoquercitrin esters seemed to depend not only on their lipophilicity but also on their chemical structure that may influence their affinity for membrane receptors. In the present study, evidence was pointed out that although the parental molecule isoquercitrin was rather inactive towards Caco2 human colon cancer cells, its synthesized esters exhibited higher antiproliferative activities, indicating that these novel compounds might possess improved anti-tumor properties. Esters from C8 to C16 showed the highest antiproliferative activity. To explain these results, studies are in progress to evaluate interaction of these derivatives with cell membrane mimetic systems.

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