On the complexation of quercetin with methyl-β-cyclodextrin: photostability and antioxidant studies

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ABSTRACT

Quercetin, a plant-derived flavonoid, has been extensively investigated for a wide range of potential health benefits linked to its antioxidant properties. Unfortunately the topical administration of this molecule is restricted by its fast photodegradation. In the attempt to overcome this limitation the inclusion complex between quercetin (Q) and methyl-β-cyclodextrin (Me-β-CD) was prepared and previously investigated by a molecular modelling study, a solubility diagram and a DSC analysis. Successively the kinetics of photodegradation, the antiradical, metal chelating and anti-lipoperoxidative activities were studied by comparing the complexed with free Q. In addition the accumulation of Q in porcine skin was evaluated after *in vitro* topical application by means of vertical Franz cells. The complex formation resulted useful in enhancing the solubility of Q without significantly reducing its antioxidant ability. A modest improvement in the photostability was also observed.

Keywords Quercetin, Methyl-β-cyclodextrin, Photostability, Metal chelating, Radical scavenging Lipoperoxidation

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Introduction

Reactive oxygen species (ROS) which include oxygen radicals ($O_2^{\bullet-}$,$^{\bullet}OH$, $NO^{\bullet}$, $ROO^{\bullet}$) and non radical molecules (hydrogen peroxide, singlet oxygen) are produced by cells as result of aerobic metabolism. Radical initiating factors (UV radiation, xenobiotics, mineral dust) can increase ROS production and induce oxidative stress that causes several human diseases among which neurodegenerative disorders, atherosclerosis, diabetes, tumours etc. [1]. Flavonoids, a large group of polyphenolic compounds characterised by 2-phenyl-chroman structure, can prevent oxidative cellular damage induced by ROS generation [2-5]. Recent interest in these substances has been stimulated by the potential health benefits [6-9] and pharmacological applications [10-12] arising from their several antioxidant mechanism of action. In fact they can inhibit redox enzymes (monooxygenase, cyclooxygenase, xantine oxidase) [1,2], bind transition metals [13] and reduce both NADPH- and CCl_4-dependent microsomal lipid peroxidation [14]. Particularly they act as reducing agents of free radical [15], either with the single electron transfer mechanism in polar aqueous phase or with hydrogen atom transfer of hydroxyl hydrogens in non-polar media such as lipids [16]. Flavonoids also play an essential role as screening pigments against short wavelength-induced damage [17-21].

Nevertheless it has been shown that processing and storage conditions strongly affect biological activity of the flavonoids as quercetin, catechin, rutin [22, 23]. Moreover the therapeutic usefulness of these molecules is limited by their unfavourable physicochemical properties, particularly their poor water solubility [24] and photostability [25, 26]. In this regard inclusion complexes of some flavonols, i.e. 3-hydroxyflavone, morin and quercetin were obtained with α- and β-cyclodextrins [27, 28] which represent an extremely active area of research especially in drug photostability and phototoxicity [29-32]. In fact the ability of cyclodextrins to form inclusion complexes with different drugs is commonly used to improve their solubility and stability [33] or to reduce their photochemical reactivity [34]. Moreover in recent years, cyclodextrins complexation has been successfully used to protect flavonols from enzymatic oxidation [35]. Some authors have investigated the non-covalent complexes between three flavonoid glycosides (quercitrin hyperoside and rutin) and heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) [36]. They also proposed a binding model for the complexes and tandem mass spectrometric data of them. Other researchers assessed the interaction between quercetin and either β-cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD) or sulfobutyl ether-β-cyclodextrin (SBE-β-CD) [37]. Kim et al. evidenced the increased solubility of quercetin, galangin, kaempferol and myricetin in the presence of DM-β-CD and HP-β-CD [38].

The purpose of the work described in this report is to study if the low water solubility and the fast degradation rate of quercetin could be overcome by its complexation with Me-β-CD. Firstly, to access the structural features of the complex a molecular modelling study was performed. The kinetics of photodegradation of free and complexed quercetin were then compared to evaluate the possible stabilizing effect of the complexation phenomenon. The formation of the inclusion complex suggested by docking results was demonstrated by solubility diagrams and DSC measurements. Furthermore antiradical, metal chelating and anti-lipoperoxidative properties were tested in the free and complexed quercetin to assess whether the inclusion in Me-β-CD could modify the activity of this molecule. Finally, the accumulation of quercetin in porcine ear skin was *in vitro* investigated.

Materials

Absolute ethanol and dichlorometane were from Carlo Erba (Rodano, Italy). Sodium azide and hydrochloric acid were purchased from Fluka (Milan, Italy). Phosphoric acid (85%), thiobarbuturic acid (TBA), copper chloride, 1-butanol and sodium dodecyl sulfate (SDS) were from Merck (Milan, Italy). Octyl octanoate (Tegosoft® EE) and glycerol were from Acef (Fiorenzuola D'Arda, Italy). Potassium palmitoyl hydrolysed wheat protein-glycerin stearate-cetearyl alcohol (Phytocream® 2000) was from Sinerga (Pero, Italy). Aeroxide P 25 (Titanium dioxide, 20% rutile, 80% anastase) was from Degussa (Milan, Italy), UV-Titan M262 (Titanium dioxide, 88% rutile, 5.9% alumina, 1.2% silica) was from Kemira (Milan, Italy). Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid), quercetin (Q) (3,3',4',5,7-pentahydroxyflavone), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) and 1,1,3,3-tetraethoxypropane were from Sigma Aldrich (Milan, Italy). Methyl-β-cyclodextrin (Me-β-CD) (Kleptose®Crysmeb) was a gift from La Roquette (Lestrem, France).

Apparatus

A Modulyo freeze dryer system (Edwards, West Sussex, UK) was used to prepare the inclusion complex of Q with Me-β-CD. A RO5 multiple magnetic stirrer (IKA, Staufen, Germany), an Actinic BLT 40W UVA lamp (Philips, Milan, Italy) and a G40T10E UVB lamp (Sankyo Denki, Kanagawa, Japan) were employed for the irradiation runs. Quercetin, DPPH radical and malondialdehyde were detected by a Lambda 2 UV-Vis spectrophotometer (Perkin Elmer, Waltham, MA, USA). The thermograms were obtained by a DSC-7 power compensation (Perkin

Elmer). A DSL stirrer (Velp Scientifica, Usmate, Italy) and a SL-2 (Silverson, Bucks, England) homogenizer were employed to prepare the emulsion systems. A Micro 2001 pHmeter (Crison, Alella, Spain) was employed to control the pH. A 660/H Transsonic Sonifier (Elma, Singen, Germany) was used to disperse the TiO_2 nanoparticles and an Allegra 64R refrigerated centrifuge (Beckman Coulter, Palo Alto, CA, USA) was employed to separate TiO_2 from the samples before analysis. A RE 111 Rotavapor (Büchi, Flawil, Switzerland) was used to evaporate the organic solvent.

Methods

Molecular modelling

3D structures

The methyl-β-cyclodextrin (Kleptose Crysmeb) structure was prepared with MOE [39] as discussed in our previous paper [40]. It is a mixture of methylated β-CD which contains on average 4 methyls per native cyclodextrin molecule, i.e. a typical molar degree of substitution (M.S.) of 0.57. Quercetin structure was simply downloaded from CSD (code:FEFBEX01) and checked with standard MOE tools.

Docking

MOE-Dock methodology which consists of three steps (ligand conformational analysis, placement and scoring) was used [39]. For the initial systematic search, a random initial orientation was used, alpha triangle was the placement methodology and affinity dG scoring (a function that estimates the enthalpic contribution to the free energy of binding using a linear function) was the chosen scoring function.

One docking run was set up to generate 500 poses which were collected in a database. The resulting complexes were ranked by their scoring function (the lowest, the best). The best 50 poses out of 500 were minimized using GB-SA conditions (the newest version of the software automatically performs this operation), and ranked again by their scoring function (the best pose shown in Fig. 1 is also downloadable from OpenCDLig at https://kdd.di.unito.it/casmedchem).

Analysis of Quercetin

Diluted solutions of Q over the range 1.5×10^{-5}-15.2×10^{-5} M were spectrophotometrically determined in different media at 374 nm to obtain the calibration curves. The molar extinction coefficients (ε) were respectively 23858 M^{-1} (R^2 = 0.9956) in absolute ethanol, 23800 M^{-1} (R^2= 0.9998) in ethanol/water (15/85 v/v), 10570 M^{-1} (R^2= 0.9991) in 5% w/w SDS at pH 5.0, 9633 M^{-1} (R^2 = 0.9941) in 5% w/w SDS at pH 7.4.

Inclusion complex preparation

Freeze drying technique was used to obtain the inclusion complex of Q with Me-β-CD. A mixture of Q and Me-β-CD (1:2 molar ratio) was prepared in water and shaken for 24 hours in the dark. After equilibration the suspension was filtered and freeze dried before re-dissolution (1 mg) in 5.0 mL of absolute ethanol to assess the active loading by UV-Vis spectrophotometer. The average percentage of active loading was around 5.0% w/w with a percentage yield of 80%.

Inclusion complex characterisation

Solubility diagram and stability constant

Phase solubility studies were performed according to Higuchi-Connors method [41]. An excess of Q (2 mg in 5.0 mL of water) was added to a series of vials containing increasing amounts of Me-β-CD in water. The closed vials were shaken in the dark for 24 hours at room temperature. After equilibration, each sample was centrifuged and spectrophotometrically analysed. The experiment was performed three times and the phase diagram was drawn by plotting the molar concentration of Q found in the solution against the molar concentration of Me-β-CD. The stability constants (K_{st}) were calculated from the initial rising of the diagram according the following equation:

K_{st} = slope/S_0 (1-slope)

where the slope is obtained from the least squares linear regression of the molar concentration of Q versus the molar concentration of Me-β-CD and S_0 is the intrinsic solubility of Q in the absence of cyclodextrin.

DSC studies

The samples were placed in conventional aluminium pans and then heated under nitrogen flow at a scanning speed of 10 °C min^{-1} from 25 °C to 330 °C. The weight of free Q, and Q/Me-β-CD complex was such to have the same amount of Q in both.

Preparation of O/W emulsion

An O/W emulsion was prepared by dispersing the melted lipid phase (Phytocream 2000 and Tegosoft EE) in glycerol-containing water at 70 °C under homogenisation (Table 1). The emulsion was then cooled at room temperature under continuous stirring. Amounts of Q, free or complexed with Me-β-CD, were then dispersed under vigorous stirring to obtain 3.5 and 17.5 mM final concentration of antioxidant in the emulsion.

Table 1 Percentage compositions of O/W emulsion

Components	% w/w
Phytocream 2000	3.0
Tegosoft EE	14.0
Glycerol	5.0
Water	78.0

Photostability studies

Q photodegradation experiments were performed using an UVA lamp with a 320-400 nm wavelength range. The systems under study were a solution of Q or of Q/Me-β-CD in ethanol/water (15/85 v/v) and an aqueous solution of Q/Me-β-CD. Q concentration was 3.5×10^{-5} M in all of them. An aliquot (10 mL) of each system was introduced in Pyrex® glass containers placed at 10 cm from the light source and irradiated maintaining them under continuous stirring. In such conditions the radiation power per surface unit was around 6.0×10^{-4} W cm^{-2}. At scheduled times of 30 min, over 2 hours of total irradiation, fixed amounts (200 µL) of each sample were withdrawn and 1:10 diluted with methanol for spectrophotometrical analysis. Each sample was prepared and analysed in triplicate.

Antiradical activity

The antiradical property of Q was determined by the DPPH assay according to the method described by Brand-Williams et al. [42]. Briefly, dilutions (20 µL) of Q or of Q/Me-β-CD in the range 2.5-50 µM were treated with 3.0 mL of $DPPH^{\bullet}$-saturated ethanol-water (15/85 v/v) solution. The absorbance of each reaction medium was spectrophotometrically monitored at 515 nm after 10 minutes of incubation under magnetic stirring to reach the steady-state conditions. The $DPPH^{\bullet}$ concentration (mM) was calculated from the calibration curve determined by linear regression (R^2 0.9929):

$$\text{Absorbance} = 10800 \times [DPPH^{\bullet}] - 0.0011$$

The radical scavenging effect of Q in $DPPH^{\bullet}$ solution was calculated according Jang and Xu [43] using the following equation:

$$\text{Scavenging effect \%} = [(A_0-A_1)/A_0] \times 100$$

were A_0 was the absorbance of $DPPH^{\bullet}$ at zero time and A_1 the absorbance of $DPPH^{\bullet}$ after 10 minutes of incubation for the reaction. Each sample was prepared and analysed in triplicate and the inhibition percentage of the absorbance of $DPPH^{\bullet}$ was plotted against each dilution of Q.

Metal chelating activity

The chelation of metal ions by Q and by Q/Me-β-CD complex was estimated in a mixture of ethanol/water (15:85) since Q is insoluble in pure water. Briefly, equal aliquots (5 mL) of both free and complexed Q solutions all containing 50 µM Q concentration were spectrophotometrically analysed at 374 nm in the absence and in the presence of 100 µL of different $CuCl_2$ dilutions (0-50 µM). The mixtures were vigorously shaken in the dark at room temperature for 30 minutes. The absorbance of each reaction medium was then measured. The metal chelating capability of Q was calculated using the following equation:

$$\text{Metal chelating effect \%} = [(A_0-A_1)/A_0] \times 100$$

were A_0 was the absorbance of Q in the absence of $CuCl_2$ (control) and A_1 the absorbance in the presence of $CuCl_2$. The experiment was repeated thrice and the percentage of metal chelating effect was plotted against each dilution of $CuCl_2$.

In vitro skin penetration

A piece of excised porcine ear skin, freshly obtained from a local slaughterhouse, was mounted with the SC side facing towards the donor compartment of a Franz diffusion cell. The available area of the cell was around 1.6 cm^2. Before the experiments the skin previously frozen at -18 °C was pre-equilibrated in 0.9% w/w saline solution added with sodium azide to preserve the skin, at 25 °C for 30 min. The donor compartment was filled alternatively with Q (1.0 mM) in 5.0% w/w SDS solution (pH 5.0); Q/Me-β-CD (Q 1.0 mM) in 5.0% w/w SDS solution (pH 5.0); Q (1.0 mM) in O/W emulsion; Q/Me-β-CD (Q 1.0 mM) in O/W emulsion. The receptor compartment was filled with 5.0% w/w SDS solution at pH 7.4 and magnetically stirred at 37 °C. At appropriate intervals 200 µL-aliquots of the receptor medium were withdrawn, immediately replaced by an equal volume of fresh solution and then spectrophotometrically analysed. The amount of Q retained in the skin was determined at the end of the experiment (24 h) as follows: the application site on the skin was washed with water/methanol (50/50 v/v) mixture, cut in small pieces by scissor and extracted with 3.0 mL of methanol. After 2 h of magnetic stirring the resulting suspension was centrifuged and assayed by spectrophotometer. The skin accumulation was expressed as amount of Q on skin diffusion area (µg/cm^2).

UVB-induced linoleic acid peroxidation

The antioxidant activity of Q was determined according to the thiobarbituric acid (TBA) test [44]. During the linoleic acid oxidation malondialdehyde (MDA) is generated that form a coloured complex with TBA which absorbs at 535 nm.

Different SDS solutions (4.0% v/v) containing the same amount of linoleic acid (1.0% w/w) were added with increasing aliquots of Q (1.75, 3.5 and 17.5 mM final concentrations), free or complexed with Me-β-CD. An aliquot (10 mL) of each system was irradiated for 2 hours by an UVB lamp in closed Pyrex® cell at 10 cm distance from the light source (radiation power per surface unit around 2.3×10^{-4} W cm^{-2}). After irradiation 0.2 mL of each linoleic acid dispersion was centrifuged and then added with 0.1 mL of water, 0.2 mL of SDS (8.1% w/w), 1.5 mL of phosphoric acid (1.0% w/w) and 1.0 mL of TBA (0.6% w/w). The reaction mixtures were heated for 45 minutes in water bath at 100 °C, then cooled in ice bath and finally added with 4.0 mL of 1-buthanol to extract the TBA-MDA-TBA adduct. After centrifugation the absorbances were measured at 535 nm. The MDA concentration in the reaction medium (expressed as nmoles of

MDA per mg of linoleic acid) was calculated from the calibration curve of 1,1,3,3-tetraethoxypropane, a MDA precursor that as MDA reacts with TBA to form a chromophore:

Absorbance = 7098 × [1,1,3,3-tetraethoxypropane] + 0.0304

The experiment was repeated in the presence of TiO_2 nanoparticles (0.05% w/w) introduced through sonication in SDS solutions (4.0% v/v) of linoleic acid (1.0% w/w) containing Q (17.5 mM) and Q/ Me-β-CD (Q 17.5 mM), respectively. An uncoated (Aeroxide P 25) and a coated (UV-Titan M262) TiO_2 specimen were employed. All tests and analyses above described were run in triplicate and averaged.

Lipoperoxidation of porcine skin

To assess the UVA- and UVB-induced skin lipid peroxidation, the formation of MDA in porcine ear skin was evaluated *in vitro* through the TBA assay. Before the experiments the skin previously frozen at -18 °C was pre-equilibrated in 0.9% w/w saline solution added with sodium azide to preserve the skin, at 25 °C for 30 min. Each slice was further cut up in small pieces that were mixed to overcome a possible variability among different zones of the ear skin. The pieces (total weight around 2.4 g) were randomly allocated in Pyrex® cells, suspended in O/W emulsion added with increasing concentration of Q (3.5 and 17.5 mM) free or complexed with Me-β-CD and UVA or UVB irradiated for 2 h. Porcine skin sample without Q was used as blank. After UV irradiation the skin pieces were dried under vacuum and then incubated for 16 h in 10.0 mL dichloromethane under magnetic stirring to extract MDA. The organic solvent was then evaporated under vacuum by a Rotavapor and the residue was reconstituted with 3.0 mL of 8.1% w/w SDS. An aliquot (0.2 mL) of this dispersion was subjected to the TBA assay by means of the same procedure reported above. The experiment was repeated thrice.

Results and discussion

Molecular modelling

In previous studies we demonstrated that docking strategies are in line with experimental results about the inclusion complex of Trolox with Me-β-CD [40] and of resveratrol with HP-β-CD [45]. In these papers, the CD molecule was the receptor whereas Trolox and resveratrol were the ligands,

respectively. Here, in close analogy with the above mentioned results, Q is the ligand, being Me-β-CD the receptor. A cartoon of the most favourable pose of Q inside the Me-β-CD cavity is shown in Fig. 1 and points out that Q is deeply inserted in the CD cavity and bound to Me-β-CD with a strong hydrogen bonding (most of the final 50 poses share a similar behaviour). This finding suggests that: *(i)* the complex should guarantee the photostability of Q because of the protective action made by Me-β-CD on the central portion of the ligand; *(ii)* Q/Me-β-CD preserve the antioxidant properties of Q as the OH groups are outside the Me-β-CD cavity and thus easily accessible.

Fig. 1 Docking results: the best pose of quercetin (downloadable from OpenCDLig at https://kdd.di.unito.it/casmedchem): A) the presence of a hydrogen bond between the ligand and the Me-β-CD is shown; B) slab view

Solubility diagrams and stability constant

A quantitative investigation of the inclusion complexation of Q with Me-β-CD was performed according to Higuchi and Connors method [41]. The solubility diagram of Q at increasing concentrations of Me-β-CD is reported in Fig. 2 as example. It was observed that the solubility of Q increased as the concentration of Me-β-CD was increased, displaying an A-type phase solubility diagram. The K_{st} was calculated from the initial linear portion of the curve as early described and was 434 M^{-1}. The same order was reported by Lucas-Abellán et al. for the complex Q/β-CD [35] whereas Kim et al. observed a higher value for the complex Q/DM-β-CD which resulted to be 7024 M^{-1} [38]. These results are reflecting an enhancement of binding and solubility of Q with an increase in substitution and hydrophobicity of the CDs. In fact the degree of substitution of DM-β-CD is greater than that of Me-β-CD because there are more methyl groups in DM-β-CD (M.S. = 2) than in Me-β-CD (M.S. = 0.57).

Fig. 2 Solubility curve of quercetin in aqueous solution at increasing concentrations of Me-β-CD. Each bar represents the means ± SD obtained in three independent experiments (n=3)

DSC studies

The DSC thermograms gave further information about the interaction between Q and Me-β-CD. Firstly, no signal was observed below 300 °C; DSC of free Q displayed an endothermic peak at 322.70 °C while the thermogram of Me-β-CD did not show any peak at this temperature (Fig. 3).

The disappearance of the melting peak in the thermogram of the complex indicated the presence of an interaction between the two specimens. In fact the peak of Q was not detected since the crystalline active molecule was inside the cavity of the cyclodextrin.

Fig. 3 DSC thermograms of Q, Me-β-CD and Q/Me-β-CD

Photostability studies

Q adsorbs radiation in the UVA region with a peak at 374 nm and as assessed in the literature it can undergo photooxidation upon UV irradiation [46, 47]. The ability of cyclodextrin to protect some active substances against photodecomposition has been recently reported [48, 49]. In order to investigate the protective effect of Me-β-CD on the photodegradation of quercetin the irradiation tests were performed separately on hydroalcoholic solutions of free and complexed Q at the same concentration value (3.5×10^{-5} M) and the results were then compared (Fig. 4). Q/Me-β-CD degradation trend has also been determined in water. It can be observed that Q degradation increases with increasing irradiation time. It was also noted that the degradation of free Q is slightly faster than that of complexed Q probably as a consequence of the protective effect of Me-β-CD on the guest molecule. Our hypothesis is that when Q was enclosed in the hydrophobic cavity it experienced a more apolar environment that should partially reduce the photolytic reaction. On the other hand a small protective effect could be due to some screening phenomena of CD particles for the passage of light to reach the guest molecule. Moreover it was observed that the degradation rate of complexed Q is slightly faster in water than in ethanol/water (15/85 v/v). This finding is in agreement with our previous studies [50, 51] according to which by passing from a less polar medium to a more polar medium, the photodegradation rate of the studied molecule increases.

Fig. 4 Photodegradation kinetics of Q, free or complexed with Me-β-CD, upon UVA irradiation. Each bar represents the mean ± SD obtained in three independent experiments (n=3)

Antiradical activity

The reaction with 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) is widely used for assessing the ability of polyphenols to transfer labile atoms to radicals, a common mechanism of antioxidant protection [52]. This assay is based on the reduction of DPPH radicals which causes an absorbance decrease at 515 nm after acceptance of an electron or hydrogen radical from an antioxidant

compound. Accordingly, in this study the antioxidant activity of Q was evaluated from the $DPPH^{\bullet}$ remaining when the kinetics reached the steady state as a function of the molar concentration of the antioxidant. Fig. 5 illustrates that the scavenging effect of Q increased with an increasing Q concentration. Furthermore, comparison between free and complexed Q evidences that the inclusion phenomenon slightly reduce the antiradical activity of this compound. Anyway the scavenging ability of Q/Me-β-CD can be considered statistically significant.

Fig. 5 Antiradical activity of quercetin, free or complexed with Me-β-CD, towards $DPPH^{\bullet}$. Each bar represents the mean ± SD obtained in three independent experiments (n=3)

Metal chelating activity

Transition metals such as cuprus ion, can stimulate lipid peroxidation by generating hydroxyl radicals through Fenton reaction [53] and by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. We suggested that Q may chelate the cuprus ions with hydroxyl groups. On the other hand it was reported that the compounds with structures containing at least two functional groups (-OH, -SH, -COOH, $-PO_3H_2$, -C=O, $-NR_2$, -S and -O-) in a favourable structure-function can show metal chelating activity [54]. In this study the chelating properties of Q, either free or complexed with Me-β-CD, were compared to verify if the inclusion in the cavity of the cyclodextrin could interfere with this activity.

As shown in Fig. 6 the addition of increasing amounts of Cu^{2+} to 50 μM Q resulted in proportional increasing of the metal chelating activity. The chelating capacity of complexed Q slightly differed from that of free Q. In fact in the presence of low amounts of Cu^{2+} the chelating property of Q/Me-β-CD was lower than that of free Q whereas in the presence of higher concentration (> 0.3 μM) the situation is reversed.

Fig. 6 Metal chelating activity of quercetin, free or complexed with Me-β-CD, towards $CuCl_2$. Each bar represents the mean ± SD obtained in three independent experiments (n=3)

***In vitro* skin penetration**

An essential requirement to ensure the effectiveness of Q as topical antioxidant is its skin penetration. Accordingly, the skin accumulation of Q and its percutaneous delivery were assessed in an *in vitro* model of porcine ear skin as previously described by Diembeck et al. [55]. The amount

of Q detected in the skin section on the Franz cell is indicative of its penetration whereas the amount in the receptor phase is indicative of its percutaneous delivery. No Q was detected in the receptor phase which is an advantage because the aim is the topical (not transdermal) delivery of Q. Additionally, the data presented in Table 2 suggest that as compared to free Q, the complexation with Me-β-CD slightly reduced the cutaneous retention of the flavonoid in the skin model. However a substantial amount of Q was penetrated in the skin from the complex at 24 h post-application. Furthermore skin penetration of Q was influenced by the donor vehicle. Particularly, the reduced Q penetration from O/W emulsion relative to that presented by SDS solution suggests that the surfactant may diffuse on the skin surface and acts as enhancer increasing the partition of the molecule into the skin.

Table 2 Amounts of Q ($\mu g/cm^2$) accumulated in porcine ear skin 24 h after application

Donor phase	Q ($\mu g/cm^2$)
Q in 5.0% w/w SDS	4.32 (± 0.15)
Q/Me-β-CD in 5.0% w/w SDS	3.94 (± 0.11)
Q in O/W emulsion	1.52 (± 0.05)
Q/Me-β-CD in O/W emulsion	0.91 (± 0.03)

UVB-induced linoleic acid peroxidation

In the present study the effect of Q, free and complexed with Me-β-CD, was evaluated toward the oxidation of linoleic acid caused by UVB light. Fig. 7 reports the amount of MDA formed from the fatty acid (1.0% w/w in 4.0% w/w SDS, pH 5.0) in the presence of three different Q concentrations (1.75, 3.5, 17.5 mM) upon 2 hours of UVB irradiation. Q displayed a protective effect against the peroxidation phenomenon and this ability was dose-dependent. In fact under these conditions the yield of MDA appears to decrease with increasing Q concentration. A similar result was observed in the presence of the complex Q/Me-β-CD at the same Q concentrations above reported, even if in these systems the anti-lipoperoxidative property was less pronounced probably because the availability of complexed Q is minor than that of free Q. On the other hand conflicting results have been reported in the literature on the effect of cyclodextrin complexation on drug bioavailability [56]. Successively, this experiment was repeated in the presence of two TiO_2 samples chosen on the basis of the results previously reported by some of us [57]. The first sample, Aeroxide P 25, was

chosen for its pronounced photocatalytic activity and the other one, UV-Titan M262 for its photoprotective effect assigned to the presence of silica in its coating [58]. Both free and complexed Q were added to the linoleic acid dispersions at the higher concentration (17.5 mM) since was the most efficient to inhibit the peroxidation process. As shown in Fig. 8, in the absence of quercetin, Aeroxide P 25 displayed a higher catalytic activity compared to UV-Titan M262. This finding confirms that the presence of silica in the coating reduce the photocatalytic degradation operated by the titania nanoparticles. Moreover, Q had a pronounced protective effect toward lipid peroxidation both with linoleic acid alone and in the presence of the two specimens of TiO_2. Note that a high inhibition of lipoperoxidation was observed also with the complex Q/Me-β-CD further confirming that the complexation phenomenon does not prevent the antioxidant activity of Q neither in the presence of photocatalytic phenomena.

Fig. 7 MDA (nmol/mg) derived from 1.0% w/w linoleic acid in 4.0% w/w SDS after 2 hours of UVB irradiation in the absence and in the presence of increasing quercetin concentrations (1.75, 3.5, 17.5 mM), free or complexed with Me-β-CD. Each bar represents the mean ± SD obtained in three independent experiments (n=3)

Fig. 8 MDA (nmol/mg) derived from 1.0% w/w linoleic acid in 4.0% w/w SDS after 2 hours of UVB irradiation without or with 17.5 mM quercetin (free or complexed), in the absence and in the presence of two TiO_2 specimens (0.05% w/w). Each bar represents the mean ± SD obtained in three independent experiments (n=3)

Lipoperoxidation of porcine skin

The protective effect of Q on the UV-induced peroxidation was then evaluated on porcine ear skin, a more complex substrate than pure linoleic acid, but considered to be the closest to human skin [59]. Fig. 9 and Fig. 10 report the values of MDA formed from the skin samples suspended in O/W emulsion irradiated for 2 h under the UVA lamp or the UVB lamp, respectively. The presence of Q was found to inhibit the lipoperoxidation of the skin induced by UV irradiation under both the light sources. At the highest concentration of the antioxidant tested a high inhibition of lipoperoxidation was observed. It can be also noted that Q presents a significative anti-lipoperoxidative activity also when it is complexed with Me-β-CD.

Fig. 9 MDA (nmol/mg) derived from porcine skin samples in O/W emulsion after 2 h of UVA irradiation in the absence and in the presence of increasing quercetin concentrations (3.5, 17.5 mM), free or complexed with Me-β-CD. Each bar represents the means ± SD obtained in three independent experiments (n=3)

Fig. 10 MDA (nmol/mg) derived from porcine skin samples in O/W emulsion after 2 h of UVB irradiation in the absence and in the presence of increasing quercetin concentrations (3.5, 17.5 mM), free or complexed with Me-β-CD. Each bar represents the mean ± SD obtained in three independent experiments (n=3)

Conclusions

In this paper an inclusion complex between quercetin and Me-β-CD was investigated. Computational studies, phase diagrams and DSC thermograms suggested the formation of the inclusion complex. The present investigation underlines the possibility of increasing the solubility of quercetin by including it in Me-β-CD. Nevertheless it was found that the location of quercetin inside the cavity of the host molecule slightly reduce its photodegradation without significatively limiting its antioxidant, antiradical and metal chelating properties. Furthermore *in vitro* studies performed on Franz diffusion cells demonstrated that the complexation phenomenon does not inhibit the accumulation of quercetin in porcine skin. In conclusion the formation of Me-β-CD inclusion complex with quercetin provides an indication that it may have potential as carrier for active flavonoids.

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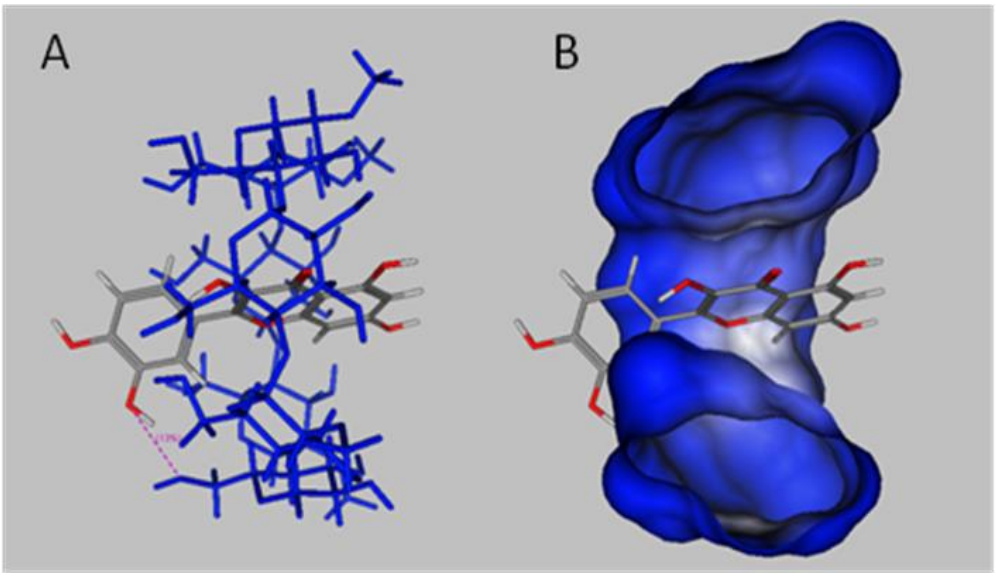

Fig. 1

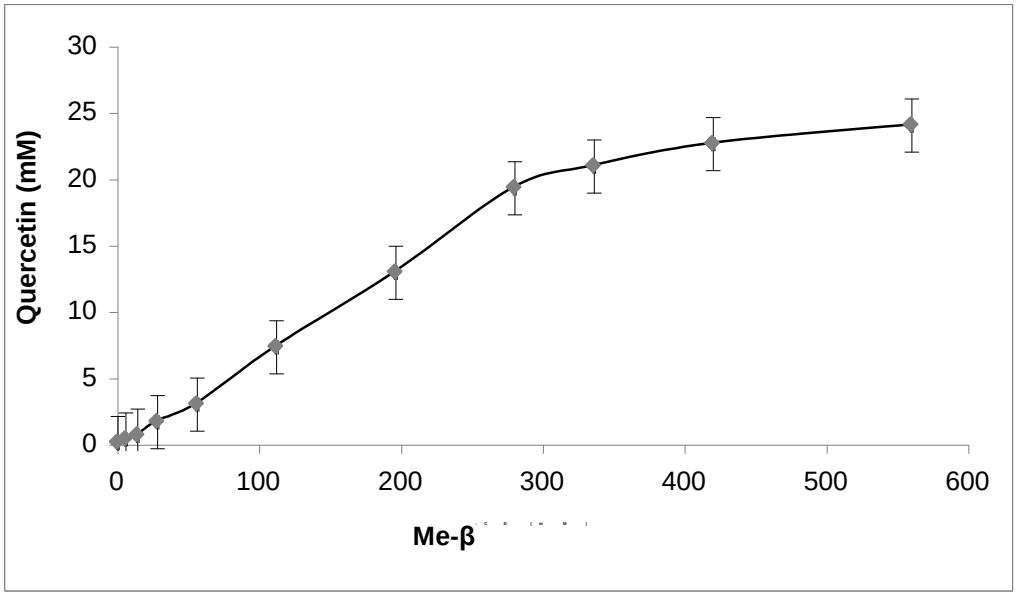

Fig. 2

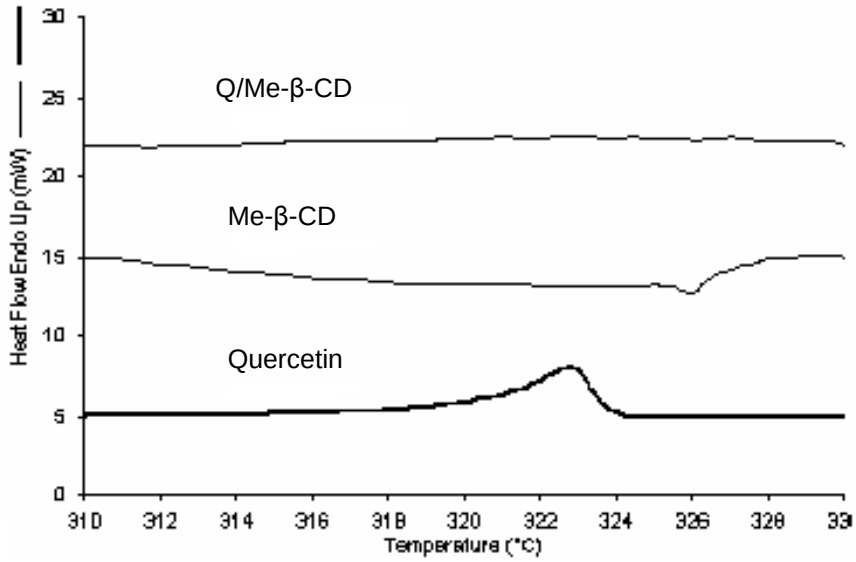
Q/Me-β-CD
Me-β-CD
Quercetin
Heat Flow Endo Up (mW)
Temperature (°C)
30
25
20
15
10
5
0
310
312
314
316
318
320
322
324
326
328

Fig. 3

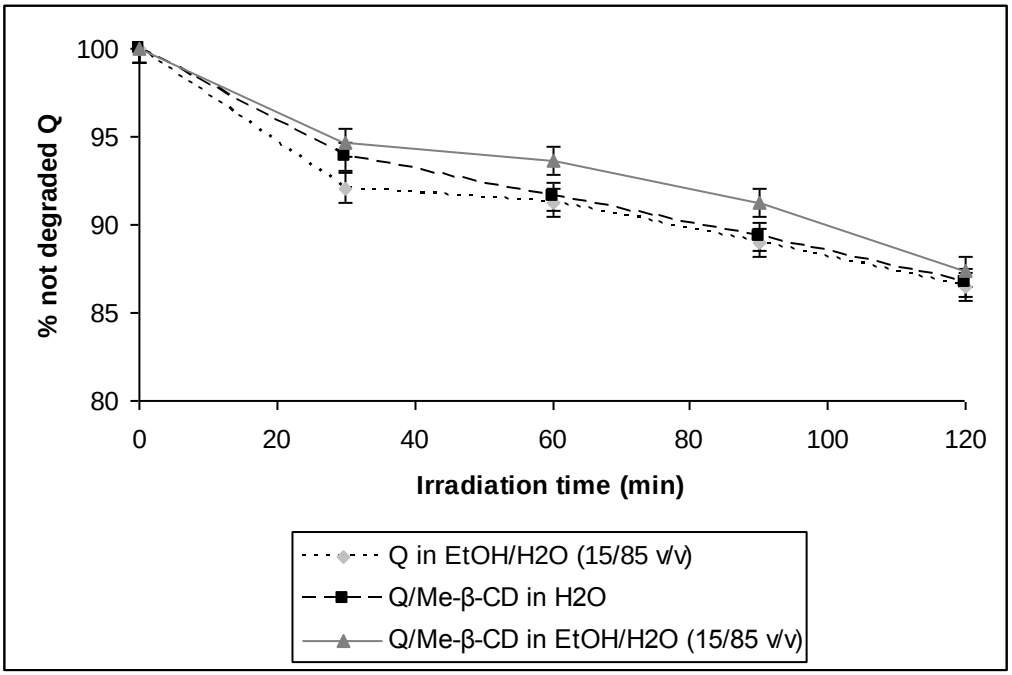
100
95
90
85
80
% not degraded Q
0
20
40
60
80
100
120
Irradiation time (min)
Q in EtOH/H2O (15/85 v/v)
Q/Me-β-CD in H2O
Q/Me-β-CD in EtOH/H2O (15/85 v/v)

Fig. 4

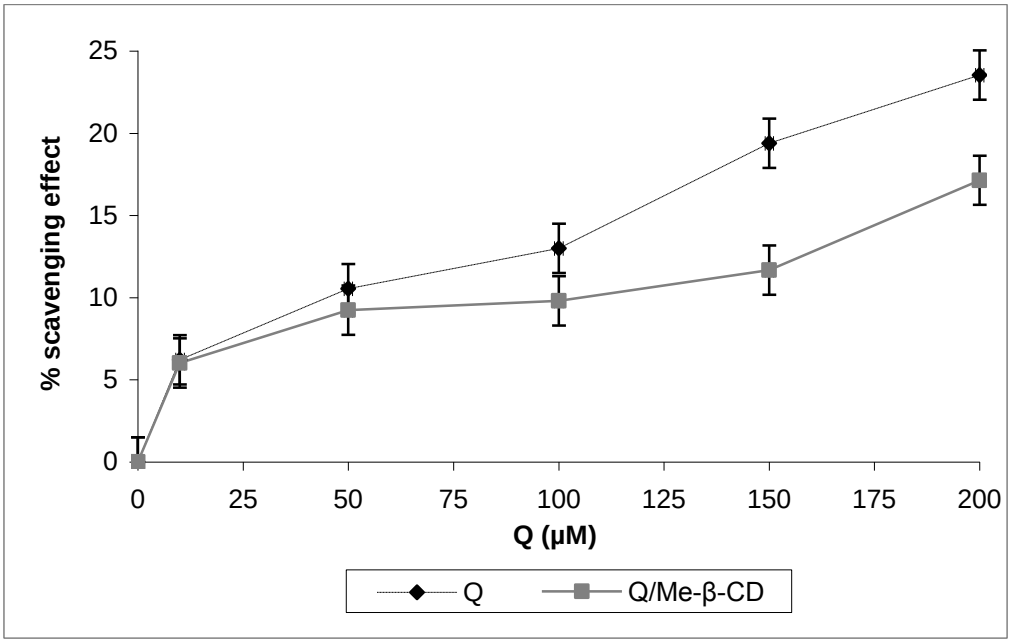
% scavenging effect
25
20
15
10
5
0
0
25
50
75
100
125
150
175
200
Q (μM)
Q
Q/Me-β-CD

Fig. 5

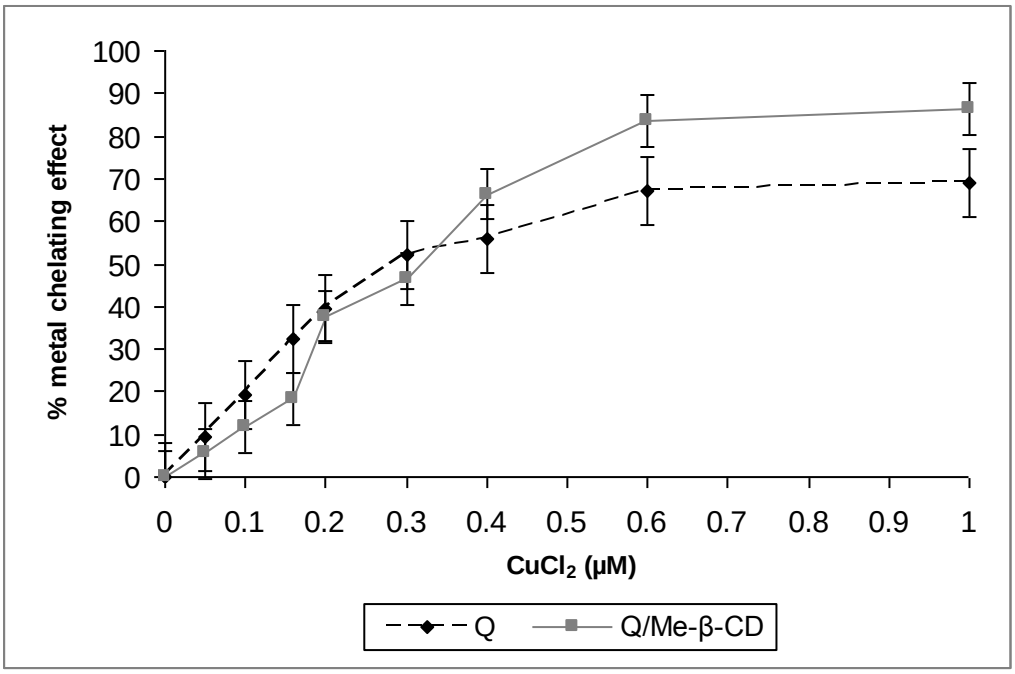
% metal chelating effect
100
90
80
70
60
50
40
30
20
10
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
CuCl2 (µM)
Q
Q/Me-β-CD

Fig. 6

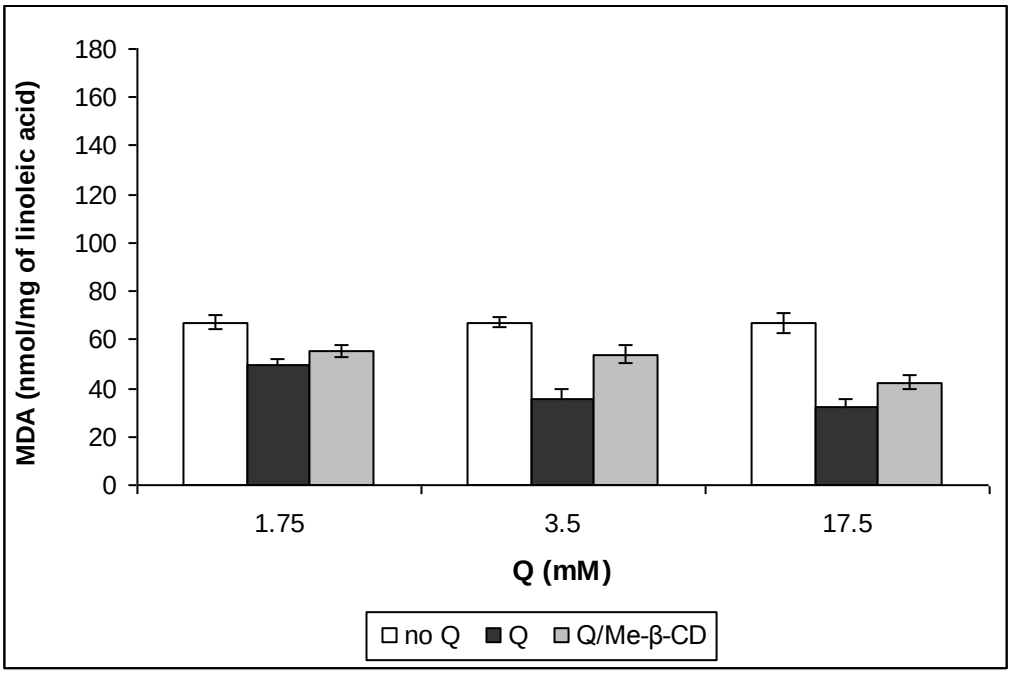
MDA (nmol/mg of linoleic acid)
180
160
140
120
100
80
60
40
20
0
1.75
3.5
17.5
Q (mM)
no Q
Q
Q/Me-β-CD

Fig. 7

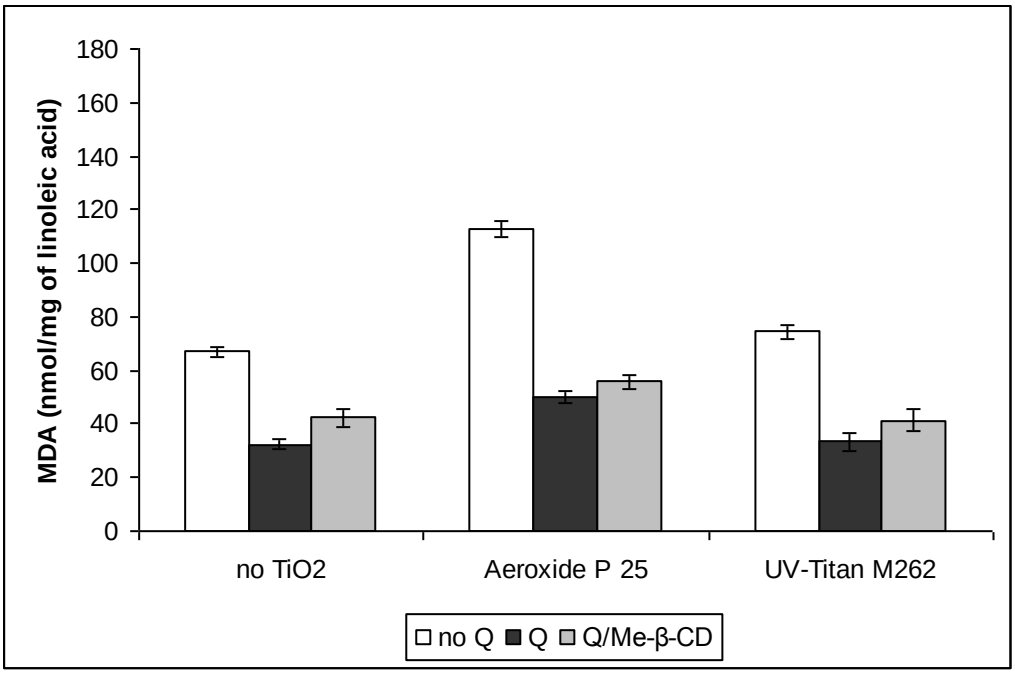
MDA (nmol/mg of linoleic acid)
180
160
140
120
100
80
60
40
20
0
no TiO2
Aeroxide P 25
UV-Titan M262
no Q
Q
Q/Me-β-CD

Fig. 8

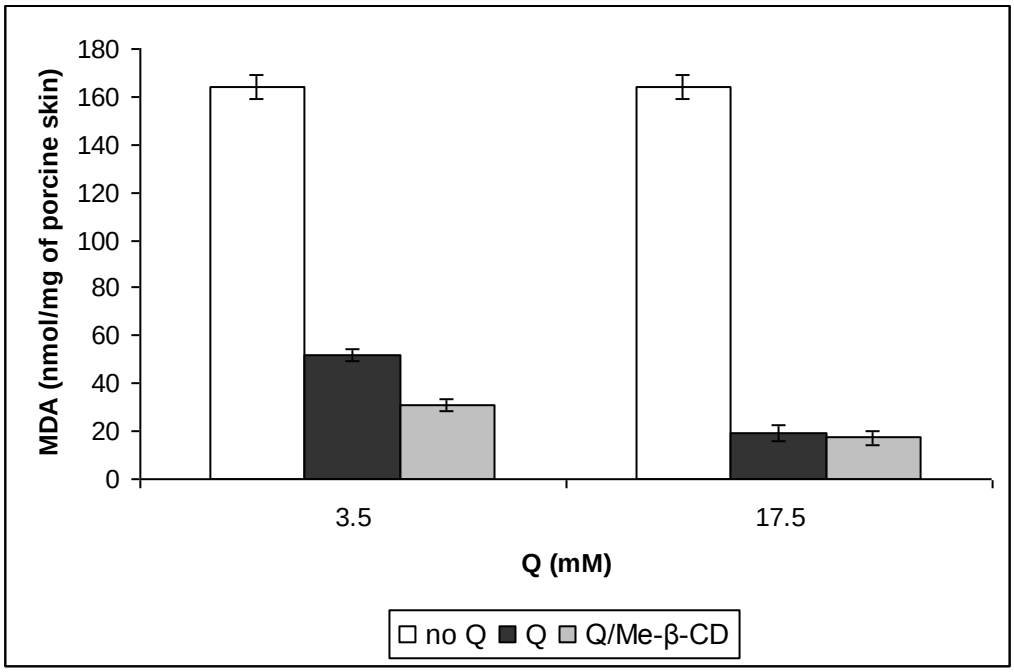
MDA (nmol/mg of porcine skin)
180
160
140
120
100
80
60
40
20
0
3.5
17.5
Q (mM)
no Q
Q
Q/Me-β-CD

Fig. 9

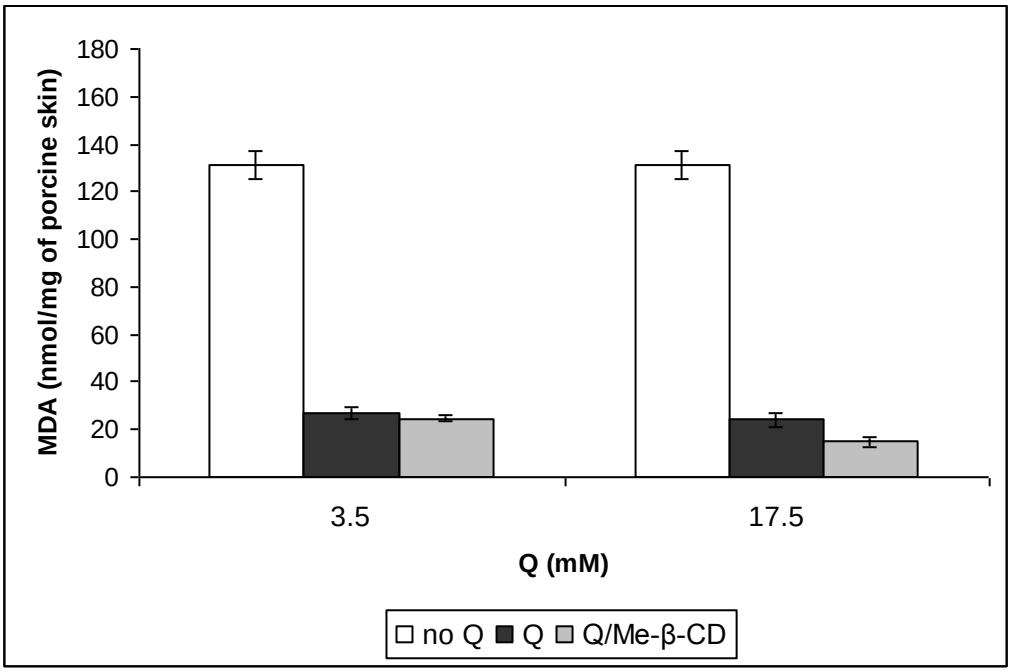
MDA (nmol/mg of porcine skin)
180
160
140
120
100
80
60
40
20
0
3.5
17.5
Q (mM)
no Q
Q
Q/Me-β-CD

Fig. 10