Bioorganic & Medicinal Chemistry 18 (2010) 5025–5031

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Spectroscopic characterization of the inclusion complexes of luteolin with native and derivatized β-cyclodextrin

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ARTICLE INFO

Article history:
Received 23 March 2010
Revised 25 May 2010
Accepted 31 May 2010
Available online 4 June 2010

Keywords:
Luteolin
Cyclodextrins
ROESY
DPPH
ESR
Thermodynamic study
Docking

ABSTRACT

The inclusion complexes of Luteolin (LU) with cyclodextrins (CDs) including β-cyclodextrin (βCD), hydroxypropyl-β-cyclodextrin (HPβCD) and dimethyl-β-cyclodextrin (DMβCD), Scheme 1, have been investigated using the method of steady-state fluorescence. The stoichiometric ratio of the three complexes was found to be 1:1 and the stability constants (K) were estimated from spectrofluorometric titrations, as well as the thermodynamic parameters. Maximum inclusion ability was obtained in the case of HPβCD followed by DMβCD and βCD. Moreover, ^{1}H NMR and 2D NMR were carried out, revealing that LU has different form of inclusion which is in agreement with molecular modeling studies. These models confirm that when LU–βCD and LU–DMβCD complexes are formed, the B-ring is oriented toward the primary rim; however, for LU–HPβCD complex this ring is oriented toward the secondary rim. The ESR results showed that the antioxidant activity of luteolin was the order LU–HPβCD > LU–DMβCD > LU–βCD > LU, hence the LU-complexes behave are better antioxidants than luteolin free.

1. Introduction

Flavonoids are a large and complex group of polyphenolic compounds widely distributed throughout the plant kingdom.[1] They are common dietary components of fruits, vegetables and beverages, and exhibit a multitude of biological properties, such as anti-bacteria, anti-allergy and anti-oxidation.[2,3] Due to their abundance in dietary products and their potential beneficial pharmacological and nutritional effects, the flavonoids are of considerable interest for drug development as well as health food supplement.

Luteolin (LU) (3′,4′,5,7-tetrahydroxyflavone) is a naturally occurring yellow pigment flavone found in a variety of plants, particularly vegetables such as celery, green pepper and perilla leaf. LU exhibits spasmolytic effects, anti-histaminic activities and it has antioxidant properties, which are significantly more potent than vitamin C and E. Also it has been described as an important dietary cancer preventive agent.[4] However, luteolin is sparingly soluble in water, which may be responsible for its limited absorption upon oral administration making it difficult to use as a natural food additive or in medicine.

In recent years, complexation of cyclodextrins (CD) provides a way to increase the solubility of a number of insoluble compounds, such as drugs, vitamins and food colorants.[5–8] CDs are a group of naturally occurring cyclic oligosaccharides linked by α(1→4) glycosidic bonds in a cylinder-shaped structure with hydrophobic interior and hydrophilic exterior. CDs have the ability to include, entirely or partially into their hydrophobic cavity, a variety of molecules with appropriate molecular size and polarity.[9,10] The inclusion interaction has been often applied in the improvement of solubility, dissolution rate, bioavailability and chemical stability from oxidation, hydrolysis and photodegradation reaction of drugs.[11–14] β-Cyclodextrin has been extensively studied despite its low aqueous solubility. With the aim of the functionalities such as better solubility or larger inclusion ability of natural CDs, various CD derivatives such as hydrophilic, hydrophobic, and ionic derivatives have been synthesized. Methylated and hydroxypropylated derivatives resulted from expediently esterification at the hydroxyl groups of the glucose residues. These chemically modified CDs have gained importance because of their relatively flexible cavity sizes, greater water solubility or less toxicity.[13,15]

As a group, cyclodextrin complexes have been formed with several flavonoids (quercetin,[11] morin,[16] galangin[17]) improving their solubility and their antioxidant properties. Also, we have determined the thermodynamic parameters which explain the difference in the stability constant (K) obtained. Using NMR techniques and molecular modeling studies we have characterized the inclusion geometries for these complexes.

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doi:10.1016/j.bmc.2010.05.079

The present work was designed to study the complexation of luteolin utilizing three different cyclodextrins (HPβCD, DMβCD and βCD) to improve its solubility (Scheme 1). Thermodynamic parameters, from van't Hoff plots, were analyzed in order to gain information about the association mechanism. Nuclear magnetic resonance (NMR) spectroscopy was used to obtain detailed information about the structure of the inclusion complexes in aqueous solution which was rationalized with docking studies. The scavenging ability of luteolin was evaluated by ESR using DPPH· radical in the absence and presence of CDs.

2. Results and discussion

Addition of various amounts of CDs in aqueous solution of LU leads to a weak modification of the absorption which indicates the formation of inclusion complexes between luteolin and the different cyclodextrins. Although absorbance changes on inclusion of guest molecule in cyclodextrins have been employed to determine the corresponding binding constant, the observed changes in the absorption spectra are too small to allow for the determination of the binding constant. However, the effect of CDs on the fluorescence spectra of LU is more pronounced than the corresponding effect on the absorption spectra. Figure 1 shows that adding CDs to LU solution results in a significant increase in the fluorescence signal. The CD cavity provided an apolar environment for Luteolin molecule and the motion of the molecule in the cavity was largely confined. Thus, the enhanced rigidity of luteolin resulted in an increase in its fluorescence quantum yield.[18] The data in Figure 1 can be treated using the modified Benesi–Hildebrand equation

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{\max}} + \left(\frac{1}{K[\mathrm{CD}]^n}\right)\left(\frac{1}{\Delta F_{\max}}\right) \qquad (1)$$

where $\Delta F = F_x - F_o$, F_x and F_o represent the fluorescence intensities of luteolin in the presence and absence of total added cyclodextrins concentration, respectively. $\Delta F_{\max}$ is the maximum change in fluorescence intensity, K is the binding constant for luteolin–cyclodextrin complex, and n represents the stoichiometry of the complex formed. Typical double reciprocal plot for $n = 1$ is shown in Figure 2 for luteolin complexed with βCD, DMβCD and HPβCD at 30 °C. For the three cyclodextrins the $n = 1$ plots exhibit good linearity whereas the $n = 2$ plots (data not shown) deviate from linearity. From these results we can infer that luteolin forms 1:1 inclusion complexes with both substituted cyclodextrins and the native cyclodextrin.

According to the continuous variation method, a physical parameter directly related to the concentration of the complex can be measured for a set of samples with continuously varying molar fraction of its components. The maximum concentration of the complex will be present in the sample where the molar ratio R corresponds to the complexation stoichiometry. In Figure 3, the maximum absorbance variation for LU in the cyclodextrins was observed for $R = 0.5$, which might indicate that the main stoichiometry is 1:1, in agreement with the stoichiometry suggested from the double reciprocal plot.

Fluorescence anisotropy (r) measurements were performed since this parameter serves as a useful indicator of the rigidity of

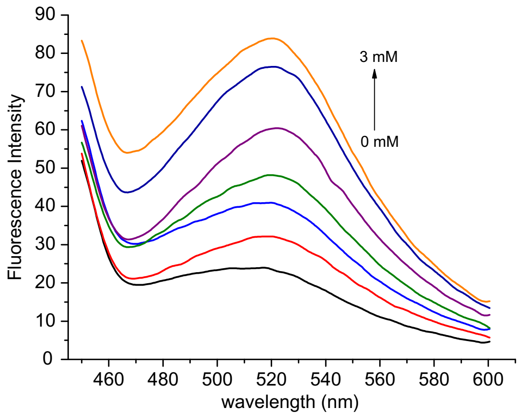


Figure 1. Fluorescence emission spectra of luteolin in presence of various concentrations of DMβCD obtained at $\lambda_{exc} = 374$ nm.

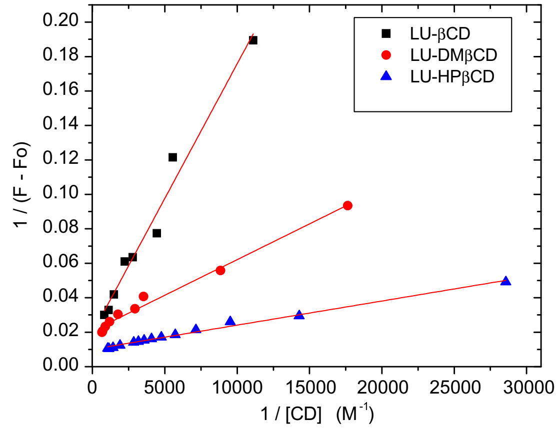
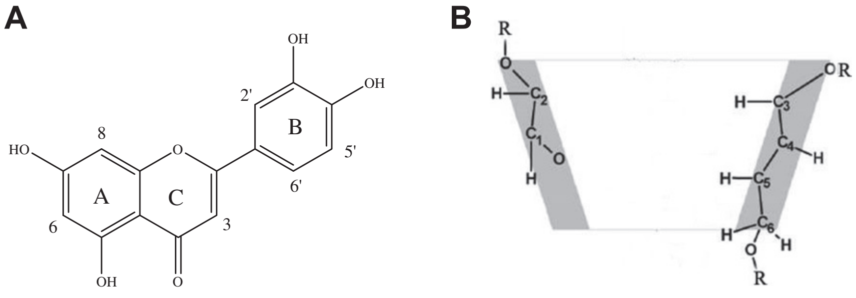


Figure 2. Double reciprocal plot 1/ΔF versus 1/[CD] obtained from the fluorescence intensities recorded for the LU–CDs complexes. λ_{exc} 374 nm, λ_{em} 524 nm. Excitation and emission bandwidths set at 15 nm.



β-cyclodextrin, R = H
2-Hydroxypropyl β-cyclodextrin, R = $CH_2CHOHCH_3$ or H
Heptakis (2,6 *O* di methyl) β-cyclodextrin, $R_{2,6} = CH_3$ $R_3 = H$

Scheme 1. (A) Molecular structure of luteolin. (B) Schematic representation of β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, and Heptakis-2,6-*O*-dimethyl-β-cyclodextrin.

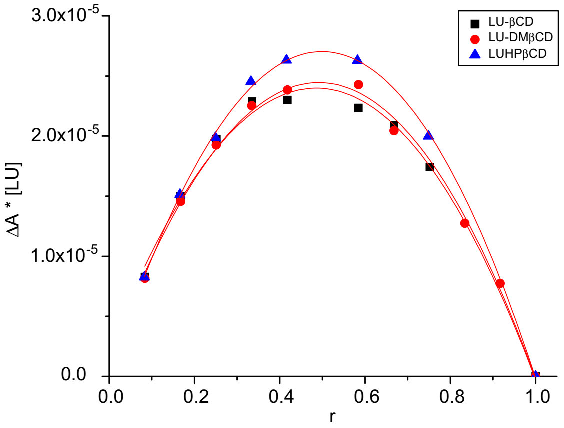


Figure 3. Continuous variation plot for LU–βCD, LU–HPβCD and LU–DMβCD system from absorbance measurements.

the local environment of the fluorophore and also for monitoring ligand binding to macromolecular systems.[18] The anisotropy values are low in fluid solutions where the fluorophore molecules can freely rotate and increase in motionally constrained environments.[19] Upon complexation with cyclodextrins the anisotropy values become considerably higher than that in fluid solutions (Table 1). This indicates that the flavone molecules are incorporated in the motionally constrained central cavities of the cyclodextrins where rotational diffusion is restricted. Fluorescence anisotropy can also give estimates of the microviscosity of the environment of the fluorophore. Microviscosity, at a defined temperature, is often estimated by comparing the fluorescence anisotropy of a fluorophore in a specific environment with those of the probe in solvents of known viscosity. To have the relative measure of microviscosity in CDs environments, fluorescence anisotropies of luteolin in the CDs solutions under saturating conditions were compared with the values in glycerol–water mixtures of different compositions[20] at 298 K. The anisotropy value suggests the average environment around the probe molecules upon incorporation into βCD, DMβCD and HPβCD corresponds to 91.5%, 92% and 95% glycerol–water mixture (mass percentage), respectively (data not shown). By using the calibration curve based on available data,[21] the effective microviscosity values in these environments were estimated as 204 cP, 222 cP and 366 cP.

On the other hand, the stability constants K, of the complexes at different temperatures (25, 30 and 35 °C) were calculated from the double reciprocal plot, and results are summarized in Table 1. The stability constant obtained indicated the following trend upon complex formation HPβCD > DMβCD > βCD. These results have the same trend obtained by Kim et al.[22] by phase solubility method. As shown in Table 1, we can note, that the stability constant for LU–HPβCD and LU–DMβCD complexes decrease with increasing temperature as expected for an exothermic process, which might be interpreted as a lower degree of interaction at higher temperatures, possibly due to the fact that hydrogen bonds are usually weakened by heating.[23] However, for LU–βCD, stability constant increase as temperature rises, as expected for an endothermic process. These temperature effects on the association constant were also found for galangin–cyclodextrin complexes.[24]

Thermodynamic parameters were calculated based on the temperature dependence of the binding constant for LU–CDs binding. The thermodynamic parameters enthalpy changes (ΔH) and entropy changes (ΔS) of binding reaction are important to confirm the driving force of interactions of luteolin with cyclodextrins. It has been generally accepted that the main driving forces for complex formation are hydrogen binding between the hydroxyl groups of the CD and the guest, van der Waals force interactions between host and guest molecules, hydrophobic interaction, and the release of 'high energy water' molecules from the cavities of CD to bulk water. Hydrophobic interaction essentially involves favorable positive entropy together with a slightly positive enthalpy change, while the other forces involve negative ΔH and ΔS. The thermodynamic parameters (ΔG, ΔH and ΔS) for the formation of inclusion complex were determined from temperature dependence of apparent formation constants, by using classical van't Hoff equation and plotting $\ln K$ versus $1/T$.

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{2}$$

where K is the stability constants corresponding to various temperatures, and R is the gas constant (8.314 J/mol K). The enthalpy change (ΔH) can be calculated from the slope of the van't Hoff relationship and the free energy change (ΔG) can be estimated from the following relationship:

$$\Delta G = \Delta H - T\Delta S \tag{3}$$

The van't Hoff plots for the complexes are linear, Figure 4, and the thermodynamic parameters of the host–guest inclusion compounds are listed in Table 1. The negative value for free energy (ΔG) of the three complexes means that the binding process is a spontaneous process and thermodynamically favored. The entropic terms accompanying the complexation are favorable for LU–βCD and LU–DMβCD complexes. However for LU–HPβCD is accompanied by a small negative entropy change. ΔS <0 values are typically obtained for guest that partially penetrate inside the CD cavity and/or whose movements are hindered by strong intermolecular guest–CD interactions[25] which agrees with our anisotropy results described above.

By inspection of Table 1, ΔH and ΔS for LU–HPβCD are negative in the experimental temperature range and for LU–DMβCD ΔH is negative and ΔS is positive, which indicates that for both complexes the inclusion process is an exothermic and an enthalpically controlled process. The different behavior exhibited by LU–βCD complex, where the complexation gave positive enthalpic changes and positive entropic term, indicating that this inclusion is mainly entropically driven. Apparently, when luteolin is free in solution, it seems to have a strong interaction with the solvent shell. Upon binding, this solvent shell is broken up, leading to the partly unfavorable enthalpic change. The same effect was observed by us, on the complexation of morin[16] and galangin[24] with βCD.

The scavenging ability of luteolin toward 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl radical (DPPH$^\cdot$) in the absence and

Table 1
Anisotropy values (*r*), microviscosity (η) of the environment of LU in the CDs cavity and thermodynamic parameters of inclusion complexes between LU and CDs

	r	η (cP)	*K* (M^{-1}) 25 °C	*K* (M^{-1}) 30 °C	*K* (M^{-1}) 35 °C	ΔH (kJ/mol)	ΔS (J/mol K)	ΔG (kJ/mol)
LU	0.184	0.89						
LU–βCD	0.270	204	602 ± 24	697 ± 72	778 ± 22	19.58	119	−15.88
LU–DMβCD	0.280	222	1617 ± 33	1554 ± 105	1387 ± 36	−11.62	22.53	−18.33
LU–HPβCD	0.289	366	5064 ± 50	4554 ± 63	3669 ± 67	−24.54	−11.28	−21.18

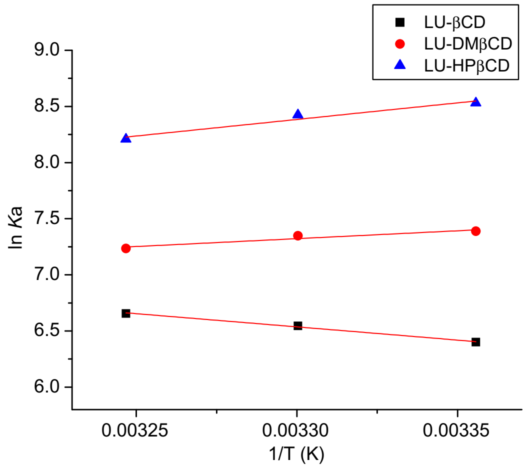


Figure 4. van't Hoff plots (ln K vs 1/T) for luteolin–cyclodextrins association.

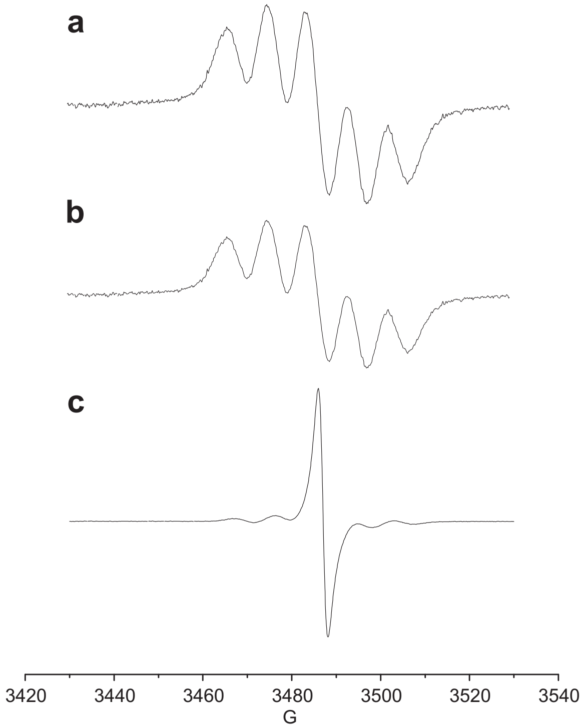


Figure 6. The scavenging effects of LU and LU–DMβCD on DPPH radical. (a) DPPH control, (b) LU free and (c) LU–DMβCD.

presence of CDs was also investigated. No decay was observed when cyclodextrin alone was mixture with DPPH$^{\cdot}$. By measuring the activity of luteolin and its complexes by ESR, the results clearly showed their antioxidant activity against this radical. The DPPH$^{\cdot}$ peak area was reduced in the presence of the complexes in comparison with free luteolin. The antioxidant activity results were expressed as %DPPH$^{\cdot}$ remaining, Figure 5. These results showed that the antioxidant activity of the complexes was the order LU–HPβCD > LU–DMβCD > LU–βCD, hence LU-complexes behave as better antioxidants than luteolin alone. This enhancement of the antioxidant activity could be due to a stabilization of the radical in the cyclodextrin cavity. Figure 6 shows the decay of DPPH$^{\cdot}$ signal due to the antioxidant activity of LU in comparison with the control, Figure 6a and b. Figure 6c shows the ESR spectrum of DPPH$^{\cdot}$ in presence of 5 μM of LU–βCD complex. This ESR spectrum corresponds to a mixture of remaining DPPH$^{\cdot}$ and LU radical specie. The same behavior was observed for LU–HPβCD and LU–DMβCD complexes. ESR spectra provide evidence that in presence of CDs, Lu radical is more stable, probably due to the apolar cavity of the CD.

Further support for the inclusion of luteolin inside the CDs cavity can be obtained using proton nuclear magnetic resonance spectroscopy (^{1}H NMR), which has proved to be useful tool in the study of cyclodextrin inclusion complex. The information gained from NMR spectroscopy relies on the observation of selective line broadening and/or chemical shift displacement of ^{1}H NMR spectral signal of the guest and host protons. The formation of inclusion complexes can be proved from the changes of chemical shifts of LU or CDs in ^{1}H NMR spectra. Figure 7 illustrates that most of the aromatic protons of LU are influenced owing to the presence of CDs. Table 2 lists the detailed variation of the aromatic chemical shifts of LU before and after forming inclusion complexes with CDs. The induced shift Δδ is defined as the difference in chemical shift in the absence and presence of the other reactants. In the present case, the induced shifts were calculated by the following equation: $\Delta\delta = \delta_{(\text{complex})} - \delta_{(\text{free})}$. In this convention, negative and positive signs show high and low frequency shifts, respectively. Downfield shifts are observed for most of the aromatic protons of the A- and B-rings. However, the major induced deshielding is observed for all the A-ring protons of LU–HPβCD. In contrast LU–DMβCD complex experiences minor changes in chemical shift and for LU–βCD complex the difference in chemical shift decreased even more. For the three luteolin complexes the induced chemical shift (Δδ) of H-5′ from B-ring is almost similar. The broadening of the proton signals of LU in the presence of CD suggests that the B-ring, the C-ring, and the A-ring are included in the cavity and hence the motions of these protons are restricted indicating that LU molecule fit tightly.[26]

Further information about the inclusion mode of LU in the cyclodextrin cavity can be derived from the evidence of spatial proximities between protons of CD and LU. Two-dimensional NMR is a powerful tool for investigating inter- and intra-molecular interaction. The presence of NOE cross-peaks between protons from two species indicates spatial contacts within 5 Å. To gain more conformational information, we used 2D Rotating-frame Overhauser Effect SpectroscopY (2D ROESY) to study the inclusion complexes and the effects were only qualitatively used. These studies were carried out only for LU–DMβCD and LU–HPβCD due to the low solubility of the LU–βCD complex.

Figure 8 shows a partial contour plot of 2D ROESY spectra of the inclusion complex of LU and DMβCD. The inspection of this ROESY map allows us to establish a spatial proximity between the guest

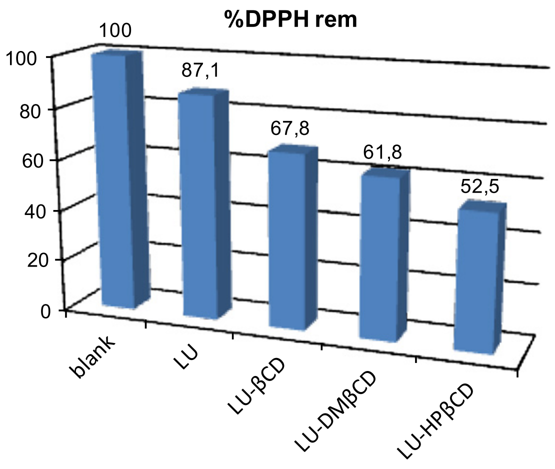


Figure 5. %DPPH$^{\cdot}$ remaining for free luteolin and in the presence of βCD, DMβCD and HPβCD.

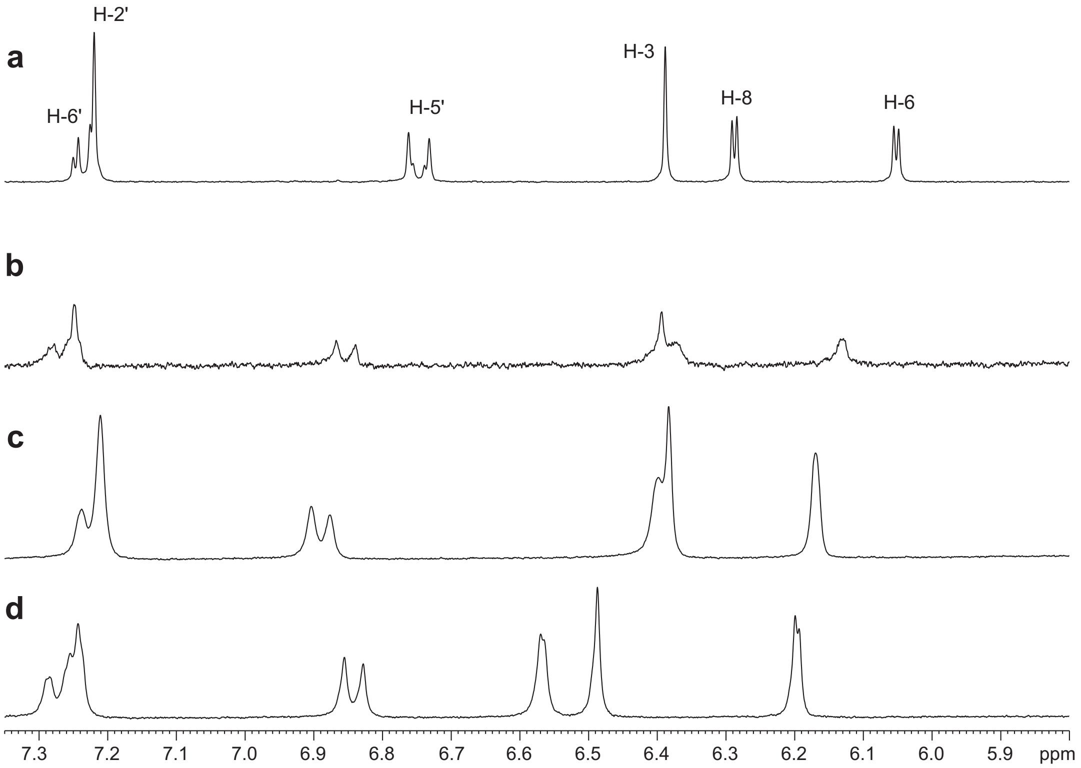


Figure 7. Chemical shifts of aromatic protons of LU in the absence and presence of CDs in 10% v/v MeOD in D_2O. (a) LU, (b) LU–βCD, (c) LU–DMβCD and (d) LU–HPβCD.

Table 2
Change of ^{1}H-chemical shift ($\Delta\delta$) of LU–CDs complexes at 300 K

Luteolin proton	$\Delta\delta$ (ppm) LU–βCD	$\Delta\delta$ (ppm) LU–DMβCD	$\Delta\delta$ (ppm) LU–HPβCD
H-8	0.09	0.11	0.28
H-6	0.08	0.12	0.15
H-3	0.00	0.01	0.10
H-6′	0.04	0.00	0.04
H-5′	0.10	0.14	0.09
H-2′	0.03	0.01	0.03
CD proton			
H-3	−0.11	−0.12	−0.09
H-5	−0.13	−0.14	−0.1
H-6a	−0.07	−0.08	0.02
H-6b	−0.07	−0.08	−0.08

Complexation shifts $\Delta\delta = (\delta_{complex} - \delta_{free})$. (0.5 mM LU, 10 mM of CDs).

protons and the inner protons of CDs. There are intermolecular cross-peaks between H-2′ and H-3 of LU with H-3 cyclodextrins proton and then to a lesser extent through the proton H-5 of DMβCD, demonstrating the inclusion in the hydrophobic cavity. This result suggests geometry of complexation where the chromene is inserted in the cyclodextrin cavity by the wide side and the B-ring is protruding from the primary rim.

To attribute unambiguously the protons H-3, H-5, and H-6 of the cyclodextrin region, a Heteronuclear Single Quantum Coherence (HSQC) spectrum of LU–HPβCD system was performed in the same conditions as those used for the ROESY spectrum (data not shown). The ROESY spectrum of the LU–HPβCD complex, Figure 9, shows correlations between H-2′ and H-8 of LU with H-5 and/or H-6 of HPβCD this means that luteolin is inserted in the cyclodextrin cavity with the B-ring protruding by the wide side. This indicates that we have different form of inclusion for LU–DMβCD and LU–HPβCD which means that both cyclodextrins offers different bindings environments for luteolin.

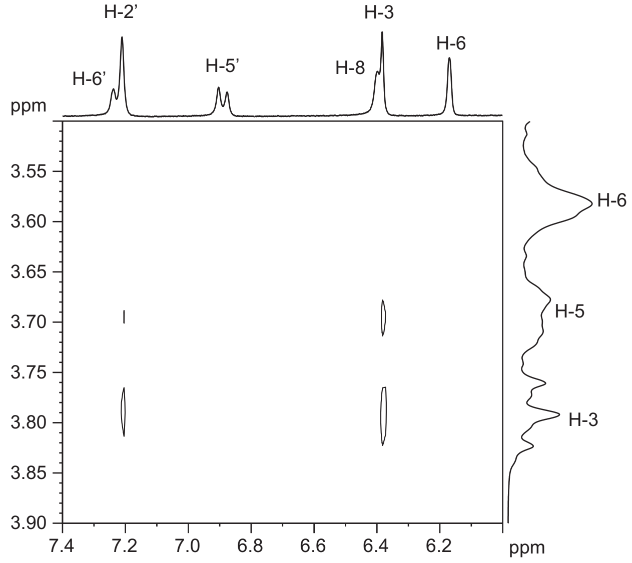


Figure 8. Partial contour plot of the two-dimensional ROESY spectrum of luteolin in the presence of DMβCD in D_2O.

In order to rationalize the NMR experimental results described above, we carried out molecular modeling studies of the complexes. These studies revealed that a preferred final relative orientation for all the complexes study occurs in spite of the different initial configurations arbitrarily imposed. The best docking poses obtained for the three CDs are shown in Figure 10. All these complexes were further refined using a semiempirical methodology PM3. In the case of LU–βCD complex, Figure 10a, the conformation obtained has the B-ring of luteolin oriented toward the primary rim, while A-ring remains exposed to the external surface by the secondary rim. The theoretical results obtained for LU–DMβCD

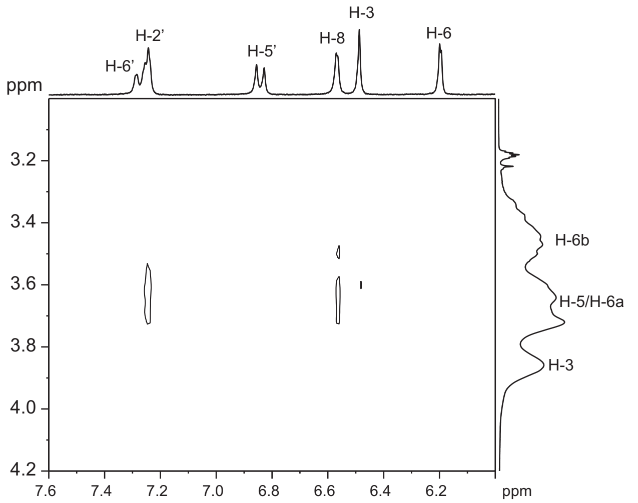


Figure 9. Partial contour plot of the two-dimensional ROESY spectrum of luteolin in the presence of HPβCD in D_2O.

complex, Figure 10b, indicated that the inclusion occurs in a similar fashion to LU–βCD complex. In the latter, both the A- and C-rings are almost completely inserted in the interior of the DMβCD, yielding the OH groups more buried than in the LU–βCD complex. In the case of LU–HPβCD complex, Figure 10c, the flavonoid is included completely in the cyclodextrin cavity. The results indicated that the B-ring of luteolin is oriented to the secondary rim, resulting in a complex where LU is located in the opposite direction with respect to the previous complexes.

These results are in agreement with our geometry obtained by NMR experiment and with the ESR results, where the radical formed ($LU^{\cdot}$) is stabilized, due to the inclusion in the apolar cavity of cyclodextrins.

3. Conclusions

The effect of βCD, HPβCD and DMβCD on the fluorescence spectrum of luteolin was studied. Adding CDs to LU solution resulted in a significant enhancement of the fluorescence signal. Also, the anisotropy value increase upon complexation indicating that luteolin fit tightly into the cyclodextrin cavity. By the double reciprocal plot we obtain the association constant for each complex at three different temperatures. For LU–DMβCD and LU–HPβCD inclusion is an exothermic and enthalpically controlled process while for LU–βCD the inclusion is an entropically driven process.

By means of experimental and theoretical methods, the present work unambiguously determined the geometrical inclusion parameters of LU on both derivatized cyclodextrins. Besides the ROESY experiments showed that the inclusion of LU in DMβCD has a different trend when it is compared with HPβCD. These results were corroborated by molecular modeling studies. The ESR results showed that the LU-complexes behave are better antioxidant than luteolin alone and that the apolar cavity stabilized the luteolin radical specie.

4. Materials and methods

4.1. Materials

Luteolin (3′,4′,5,7,-tetrahydroxyflavone), β-cyclodextrin (βCD), Heptakis-2,6-*O*-dimethyl-β-cyclodextrin (DMβCD), 2 hydroxypropyl-β-cyclodextrin (HPβCD) [D.S. = 7], DPPH (2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl) were purchased from Sigma–Aldrich, Inc., St. Louis, MO. They were employed without further purification. All other materials were analytical grade, and all water used double-distilled and deionized.

4.2. Method

4.2.1. Fluorescence spectroscopy

Fluorescence spectra were recorded with a LS 55 Perkin–Elmer spectrofluorometer equipped with a xenon lamp source, polarized filters and thermostated bath. Luteolin (300 μL, stock solution 1.0×10^{-4} M in methanol) was added to increased buffered solutions of CDs (1–3 mM). The final volume of the system was kept constant to 5 mL buffer Britton–Robinson 0.1 M pH 7.4. The resulting mixture was equilibrated in a Julabo thermostatic shaking water bath for 24 h at variable temperature (25–35 °C) after which the equilibrium was reached.

The fluorescence anisotropy (r) values were obtained using the expression

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \tag{4}$$

where I_{VV} and I_{VH} are the vertically and horizontally polarized components of probe emission with excitation by vertically polarized light at the respective wavelength and G is the sensitivity factor of the detection system.[19]

4.2.2. ESR spectroscopy

DPPH radical scavenging capacities of the samples (luteolin free and complexed) were determined by electron spin resonance spectrometry method. ESR analyses were conducted using a Bruker ECS 106 X-Band ESR spectrometer at room temperature. Each sample was mixed with 10 mM $DDPH^{\cdot}$ stock solution to initiate the antioxidant-radical reaction. The final concentration was 1 mM for $DDPH^{\cdot}$ in all reaction mixtures with 100 μL of the sample (1×10^{-2} M). ESR signals were recorded at 1 min intervals following the start of the reaction using the following conditions on the spectrometer: the frequency used was 9.79 GHz, the receiver gain was adjusted to 1×10^5, modulation amplitude was 0.9 G, modulation frequency 50.0 KHz using a rectangular cavity, the time constant was

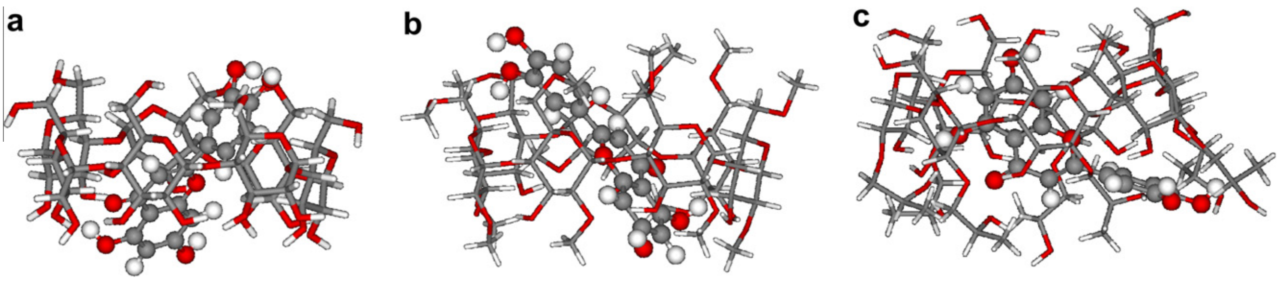


Figure 10. Relative host–guest geometry corresponding to the minimum of the energy of the formation of (a) LU–βCD complex (b) LU–DMβCD complex and (c) LU–HPβCD complex.

81.92 ms and finally the conversion time used was 40.96 ms. The intensities were determined using the double integration of the respective lines using WINEPR SimFonia v1.25.

The scavenging activity of each sample was estimated by comparison with DPPH· signal in the control reaction and was expressed as %DPPH· remaining.

$$\%\text{DPPH}^{\cdot}\ \text{rem} = \left[\frac{A_{A(t)}}{A_{C(0)}}\right] \times 100 \qquad (5)$$

$A_{C(0)}$: initial absorbance of DPPH at $t = 0$
$A_{A(t)}$: final absorbance of DPPH at $t = t$

4.2.3. NMR spectroscopy

One-dimensional ^{1}H NMR spectra were recorded at 300 K on a Bruker Avance DRX operating at a proton NMR frequency of 300.13 MHz in unbuffered D_2O solutions. Acquisition parameters consisted of a spectral width of 3000 Hz, an acquisition time of 2.67 s and a relaxation delay of 1 s. 128 scans were recorded. FIDs were Fourier transformed with LB = 0.3 Hz and GB = 0. The resonance at 4.7 ppm due to partially deuterated water (HDO) was used as internal reference.

Rotating-frame Overhauser Effect SpectroscopY (ROESY) spectra were acquired in the phase sensitive mode using the same spectrometer and Bruker standard parameters (pulse program roesygpph19). Each spectrum consisted of a matrix of 16 K (F2) by 8 K (F1) points covering a spectral width of 3000 Hz. Spectra were obtained from the samples solutions prepared for the ^{1}H NMR studies, using a spin-lock mixing time of 400 ms, relaxation delay 2 s, and 32 scans were recorded.

4.2.4. Molecular modeling

In silico built-up of the βCD and its derivatives forms were carried out using the *Builder* module of the INSIGHTII program[27] by adding to βCD 14 methyl in position 2 and 6 (DMβCD) and 7 hydroxypropyl (MS 1) groups (HPβCD). The obtained models were subjected to optimization using a protocol of 300 steps of conjugate gradients to avoid steric hindrance and clashes that can appear in the building process. The luteolin was build using GAUSSVIEW and then it was optimized using PM3 as implemented in GAUSSIAN98 package of programs.[28]

AUTODOCK3.0.5[29] with Lamarkian Genetic Algorithm (LGA) was used to generate the starting complexes. The parameters used for the global search was an initial population of 50 individuals, with a maximal number of energy evaluations of 1.5×10^6 and a maximal number of generations of 5.0×10^4 as an end criterion. An elitism value of 1 was used, and a probability of mutation and crossing-over of 0.02 and 0.08 was used, respectively. From the best solutions obtained according to these parameters, some of them defined by the user as the best probabilities in our case 0.06 were further refined by the local search method pseudo Solis and Wets 'PSW'. AUTODOCK defines the conformational space implementing grids all over the space of the possible solutions. With the aim of testing the ability of AUTODOCK to converge into solutions that are inside of the βCD, a grid of 80 Å by side and 0.3 Å spacing between each point was setup in such a way that it covered both the external surface and the internal cavity of the βCD. The following procedure was employed on the βCD docking simulations: 250 runs were done for each βCD. At the end of each run, the solutions were separated into clusters according to their lowest RMSD and the best score value based on a free empiric energy function. Cluster solutions whose average score was not over 1 kcal mol^{-1} respect to the best energy obtained in the respective run were selected. Then, the solution that represents most of the complexes obtained in the run was compared with the NMR experimental data, assuring that this solution is able to represent it accurately. The selected final complexes were optimized using the semiempirical PM3 method as a refining procedure with GAUSSIAN98.

Acknowledgement

Our thanks are to Fondecyt 11080038.

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