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# Studies on coumestrol/β-cyclodextrin association: Inclusion complex characterization

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ABSTRACT

Coumestrol is an estrogenic and antioxidant agent, characterized by its low solubility in aqueous and lipophilic media, once in the aglicone form. In order to improve its solubility in water, coumestrol was associated with β-cyclodextrin in aqueous media followed by freeze-drying and characterized by SEM, $^1$H NMR and molecular modeling. The analysis proved the existence of an inclusion complex, with higher probability of inclusion of the coumestrol B-ring into the wider rim of the β-cyclodextrin molecule.

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## 1. Introduction

Coumestrol belongs to the group of coumestans, included in the Leguminosae family. It is structurally similar to isoflavones and constitutes a fully oxidized version of pterocarpans (Al-Maharik and Botting, 2004; Ganry, 2005). Its molecular structure is presented in Fig. 1.

This compound is found in clovers and alfalfa sprouts (Al-Maharik and Botting, 2004; Ganry, 2005) and has been receiving attention due to its estrogenic and antioxidant properties. Coumestrol acts on ERα and ERβ, estrogen receptors, having seven times more affinity for ERβ than for ERα (Benassayag et al., 2002; Garey et al., 2001). These receptors are present in the epidermis (queratinocites, Langerhans cells and melanocites), blood vessels and dermis (fibroblasts), among other places of the human body (Birt et al., 2001; Krazeisen et al., 2001; Pocock et al., 2002; Lapcik et al., 2003; Thornton et al., 2003; Sator et al., 2004). Coumestrol is the most potent phytoestrogen and competes with zearalenol and genistein for the 17β-estradiol receptors binding, depending on which receptor it acts (ERα or ERβ) (Benassayag et al., 2002).

Besides its estrogenic activity, coumestrol acts as an antioxidant due to its capacity of donating electrons from the hydroxyl groups present in the A and B-rings; removing free radical and preventing oxidative damages (Mitchell et al., 1998). Mitchell et al. (1998) reported that coumestans can inhibit peroxidation reactions sixteen times more than α-tocoferol, a natural antioxidant present in the membranes. More recently, Georgetti et al. (2003) and Lee et al. (2006) demonstrated that the red clover extract (*Trifolium pratense* L.), which contains coumestrol, presents a high antioxidant activity, and that the pterocarpans from roots of *Glycine max* L. have potent low-density lipoprotein (LDL) oxidation inhibitory activity, showing that coumestrol is 20 times more antioxidant than genistein and daidzein.

Taken together, coumestrol seems to be a promising agent for skin aging prevention, especially for post-menopausal women. However, its activities are conditioned to the aglicone form, which presents reduced solubility in organic solvents and in hydrophilic vehicles (Budavari, 2001; Silva et al., 2001; Havsteen, 2002). This fact impairs the development of a topical pharmaceutical dosage form. In order to improve the solubility of the aglicone form in water, and therefore to facilitate its delivery to the skin as well as its incorporation into a hydrophilic vehicle, the association of coumestrol with cyclodextrins seems to be a promising strategy. In fact, the association of coumestrol with a cyclodextrin was for the first time investigated by Cannavà et al. (2008), who performed phase-solubility studies and employed FTIR-ATR analysis to point out the implication of particular functional groups of coumestrol in the inclusion complexes with β-cyclodextrin and hydroxypropyl-

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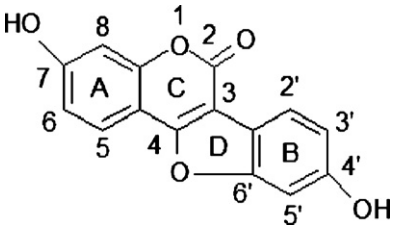


**Fig. 1.** Chemical structure of coumestrol.

β-cyclodextrin prepared by the co-precipitation method. In the present study, a solid coumestrol:β-cyclodextrin complex was prepared by freeze-drying method. The morphology of the complex was characterized by scanning electron microscopy (SEM) and the spatial configuration of this association was firstly proposed by means of $^1$H Nuclear Magnetic Resonance ($^1$H NMR) and molecular mechanics calculation. β-Cyclodextrin was chosen due to its adequate cavity size, which enables the formation of inclusion complexes with many substances, as well as to its accessible cost and related advantages concerning the industrial feasibility of a topical dosage form containing coumestrol (Loftsson and Brewster, 1996; Singh et al., 2002; Del Valle, 2004; Yan et al., 2006).

## 2. Materials and methods

### 2.1. Materials

Coumestrol, code 27885 (95% of purity), was purchased from Sigma–Aldrich (São Paulo, Brazil) and β-cyclodextrin was kindly donated by Roquette et Frères (France). Dimethyl sulfoxide $d_6$ was purchased from Tedia (Rio de Janeiro, Brazil) and potassium bromide from Synth (Porto Alegre, Brazil). All other reagents and solvents used were of analytical grade.

### 2.2. Preparation of coumestrol associations

The complex preparation followed the procedure reported by Higuchi and Connors (1965). An excess amount of coumestrol (1.5 mg) was added to a vial containing 2.5 ml of either water or a β-cyclodextrin solution (considering a 1:1 molar ratio). These dispersions were stirred in a water bath (IKA®-Werke EH4 Basic) at 37 °C, during 48 h. After this period, the dispersions were cooled down to room temperature, filtered through a 0.45 μm pore diameter membrane to volumetric flasks of 5 ml, and the volume was made up with water. An aliquot (1 ml) of this solution was transferred to other 5 ml volumetric flasks and diluted with methanol for drug assay by ultraviolet spectrophotometry at 343 nm (Hewlett Packard 8452A—Diode Array Spectrophotometer) over the concentration range of 1–5 μg/ml of coumestrol ($R^2 > 0.999$), following a previously validated method based on ICH guidelines (2005). The solution (with theoretical 1:1 drug:cyclodextrin content), was freeze-dried (Edwards Modulyo 4K, −60 °C, under light protection) and stored for further analysis and drug content evaluation.

### 2.3. Characterization of coumestrol associations

The scanning electron microscopy (SEM) was performed to the raw materials and to coumestrol: β-cyclodextrin freeze-dried product at three magnifications (2000×, 1000× and 500×), using the Jeol 6060 apparatus, after samples had been gold sputtered. The parameters used were: SEI mode, samples height less than 5 mm; voltage of 20 kV; WD of 11 mm, spotsize of 40 and LC of 60 μA.

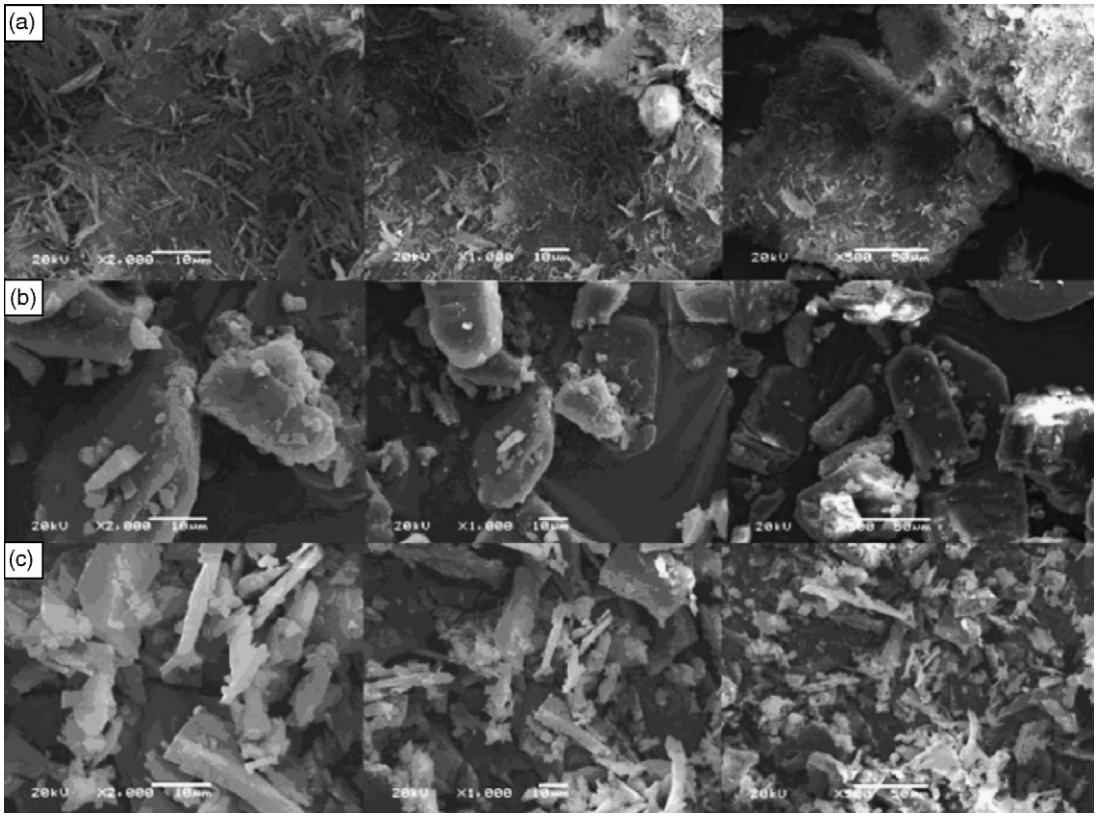


**Fig. 2.** SEM of (a) coumestrol, (b) β-cyclodextrin and (c) coumestrol:β-cyclodextrin complex (magnifications of 2000×, 1000× and 500×, respectively).

Specific adjustments of magnitude, focus, brightness, contrast and astigmatism were performed.

NMR spectra were recorded in a Bruker DRX400—*AVANCE* spectrometer operating at 400 MHz to the hydrogen nucleus, equipped with direct detection 5 mm $^{1}H/^{13}C$ dual probe and inverse detection 5 mm probe with z-gradient coil, having dimethyl sulfoxide (DMSO-$d_6$) as solvent and tetramethilsilane (TMS) as internal standard ($\delta$ 0.0). The $^{1}H$/NMR analyses were carried out at 27 °C (300 K) for coumestrol, β-cyclodextrin and the (1:1) coumestrol/β-cyclodextrin complex.

The molecular modeling was obtained with the Software ChemBio Office ChemBio 3D/ChemBio Draw Ultra, v. 9.0. Cambridge Soft (2005), using the molecular mechanics method (MM2).

## 3. Results and discussions

The mean aqueous solubility of coumestrol with and without β-cyclodextrin was found to be 12.90 (RSD < 1.1%) and 3.23 μg/ml (RSD < 1.8%), respectively. In this way, coumestrol solubility in water was enhanced up to ~4 times at the presence of β-cyclodextrin in a 1:1 molar ratio. After freeze-drying, the solid product (complex) was analyzed with respect to coumestrol content and the actual stoichiometry ratio of the complex, after all steps, was ~1:1.3 coumestrol: βCD.

The SEM images presented in Fig. 2 showed that coumestrol particles (Fig. 2a) present acicular form, irregular surface, a little wrinkled and no porous while β-cyclodextrin particles (Fig. 2b) had parallelogram form, smooth surface, with some irregularities. The complex was found to be a slightly homogeneous crystal mass with smooth surface, little wrinkled and no porous. Particles of around 20 μm prevail in this new arrangement (Fig. 2c), not being possible to infer, by this technique, about higher interaction.

**Table 1**
$^{1}H$/NMR attributions to coumestrol and β-cyclodextrin.

| Coumestrol atoms | $\delta$ $^{1}H$ (ppm) | β-Cyclodextrin atoms | $\delta$ $^{1}H$ (ppm) |
|---|---|---|---|
| H5 | 7.84 | H1 | 4.83 |
| H6 | 6.93 | H2 | 3.28–3.30 |
| H8 | 6.90 | H3 | 3.61–3.67 |
| H2′ | 7.69 | H4 | 3.32–3.38 |
| H3′ | 6.95 | H5 | 3.55–3.61 |
| H5′ | 7.16 | H6 | 3.61–3.67 |

Modern NMR techniques based on gradient-pulsed field were used in this study in order to make the assignment and the determination of the coumestrol and its inclusion compound structures (Derome, 1987; Claridge, 1999). $^{1}H$ NMR resonance assignments of the coumestrol molecule were carried out by 2D shift-correlated NMR techniques. The chemical shifts of hydrogens atoms are summarized in Table 1.

The attributions were made by comparison with the studies of Moravcová and Kleinová (2001), Schneider et al. (1998) and (2D) NMR analysis. $^{1}H$ NMR spectra for coumestrol and its freeze-dried product are presented in Fig. 3, (the β-cyclodextrin $^{1}H$ NMR spectra is also presented only for comparison, Fig. 3a). The comparison between $^{1}H$ NMR spectra for coumestrol and its freeze-dried product shows that the preparation of the inclusion compound did not

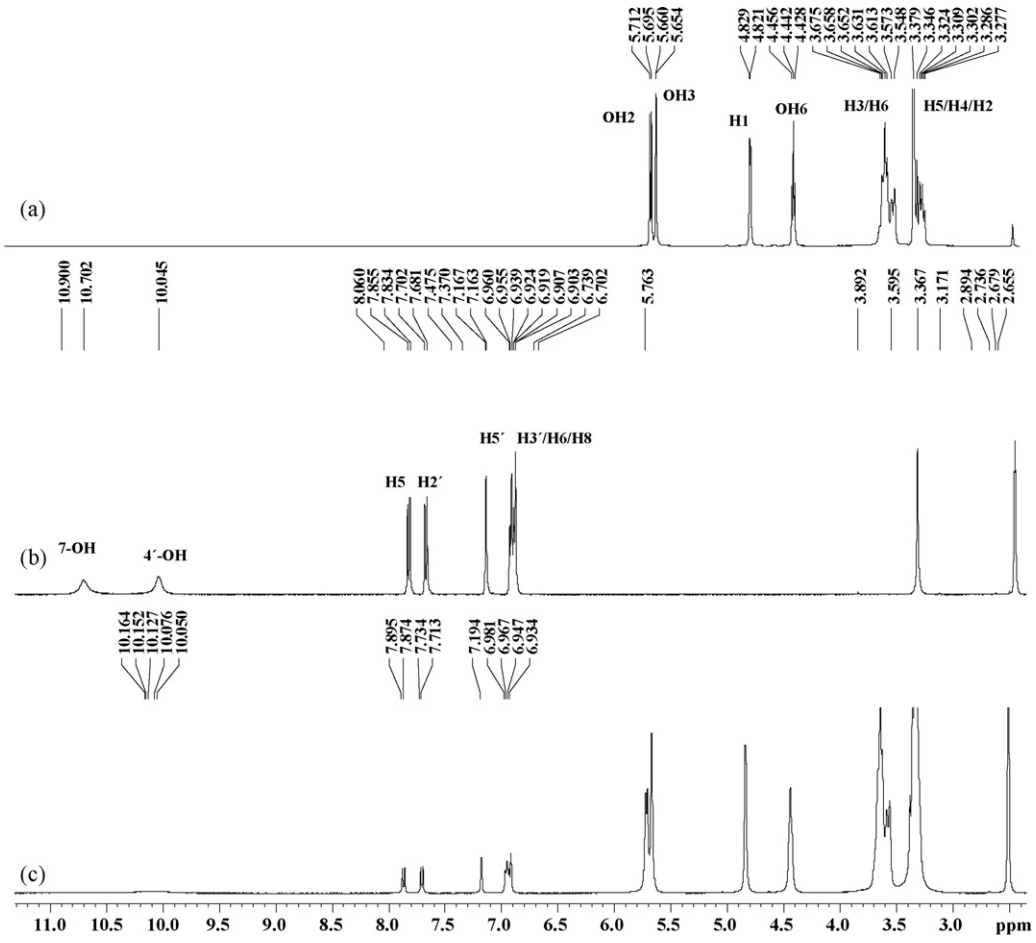


**Fig. 3.** $^{1}H$/NMR analysis of (a) β-cyclodextrin, (b) coumestrol and (c) coumestrol:β-cyclodextrin complex.

degrade the coumestrol molecule. Also, the comparison between $^1$H NMR spectrum of coumestrol (Fig. 3b), and $^1$H NMR spectrum of the coumestrol/β-cyclodextrin freeze-dried product in DMSO-$d_6$ solution (Fig. 3c) reveals some loss of resolution in the spectral lines of the NMR spectra and the coalescence of the OH signals of the coumestrol, due to the complexation effects with the host molecule.

In order to prove the host:guest interaction and to get information about the cyclodextrin geometry complex, Nuclear Overhauser Effect measurements (NOESY spectrum) of the coumestrol/β-cyclodextrin freeze-dried product in DMSO-$d_6$ solution were obtained (Fig. 4).

The NOEs observed between the hydrogens H5′ ($\delta_H$ 7.16), H3′ ($\delta_H$ 6.95), H6 ($\delta_H$ 6.93) and H8 ($\delta_H$ 6.90) of coumestrol molecule and the hydrogens H-3 ($\delta_H$ 3.89), H-5 ($\delta_H$ 3.69–3.80) and OH2 ($\delta_H$ 5.70), OH3 ($\delta_H$ 5.66) and OH6 ($\delta_H$ 4.44) of β-cyclodextrin, as detected in the 2D-NOESY experiments, could only arise if a coumestrol/β-cyclodextrin complex has been formed (Fig. 4).

The data suggest that the coumestrol A-ring is situated in the torus cavity of the cyclodextrin due to the interaction between H6 and H8 of coumestrol with H-3 of β-cyclodextrin, being this hydrogen (H3) situated inside the cyclodextrin cavity.

In addition, the cross peak correlation between the coumestrol hydrogens with the β-cyclodextrin OH hydrogens (OH2, OH3 and OH6) might be explained by the formation of a large supramolecular structure, with a high molecular weight, as the self-assembly of the supramolecular complex besides of an interaction between the coumestrol molecule and the outer face of β-cyclodextrin in a projection of the A and B-rings of coumestrol onto the OH2/OH3/OH6 face of β-cyclodextrin (Sousa et al., 2008).

The NOESY spectrum also indicated the insertion of the coumestrol B-ring into the cyclodextrin cavity by the interaction between H3′ from coumestrol molecule and H3 from the β-cyclodextrin.

The chemical shift of H3 of β-cyclodextrin was confirmed by the molecular modeling. These chemicals shifts may indicate the formation of four types of complexation: (i) insertion of A-ring of coumestrol in the β-cyclodextrin cavity; (ii) insertion of B-ring of coumestrol in the β-cyclodextrin cavity; (iii) insertion of two molecules of coumestrol in both sides of a β-cyclodextrin cavity or (iv) the insertion of one molecule of coumestrol in two molecules of β-cyclodextrins, by both sides.

The simulations were performed by manual insertion of the coumestrol molecule in the vertical position into the cavity in a perpendicular way of its diameter. The dynamic calculations were performed at 300 K by molecular mechanics method (MM2). The inclusion complex models and their total conformational energies are presented in Figs. 5 and 6. The carbon atoms are represented by gray spheres, the oxygen atoms by red spheres and hydrogen atoms by the white ones.

By these preliminary tests, it is possible to infer that all simulated inclusion complex models, which were energetically favorable, could be occurring in solution since this can be considered a dynamic process in which different parts of the molecule could be alternatively included in the CD (Loftsson and Brewster, 1996; Piel et al., 2001; Del Valle, 2004).

The total energy values obtained were very close to each other when the complexation occurred with one molecule of coumestrol and one of β-cyclodextrin, no matter which ring was introduced or which side of the cyclodextrin was tested. The low difference in the energy values is dependent on the coordinates and was considered acceptable for comparison in other models cited in the literature,

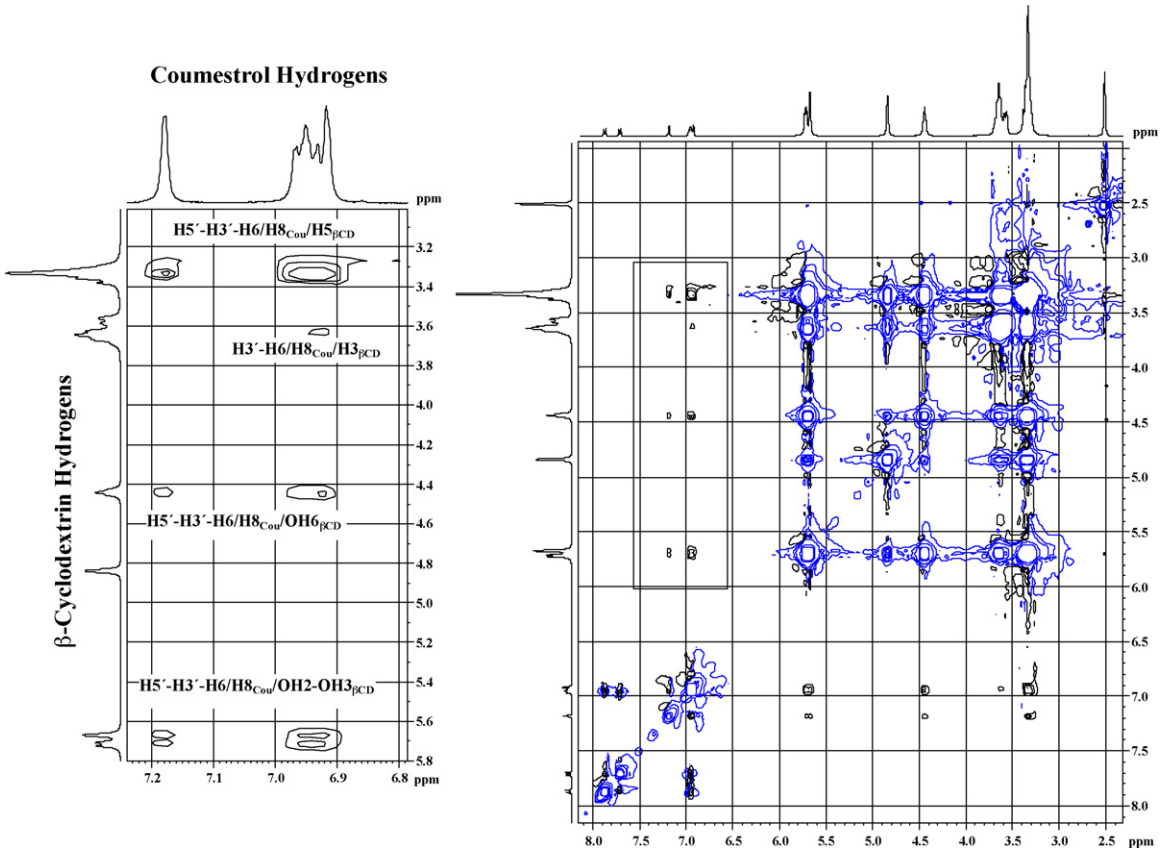


**Fig. 4.** NOESY contours map ($^1$H/$^1$H) of the coumestrol:β-cyclodextrin complex and expansion region of the aromatic resonance hydrogen (DMSO-$d_6$, 400 MHz).

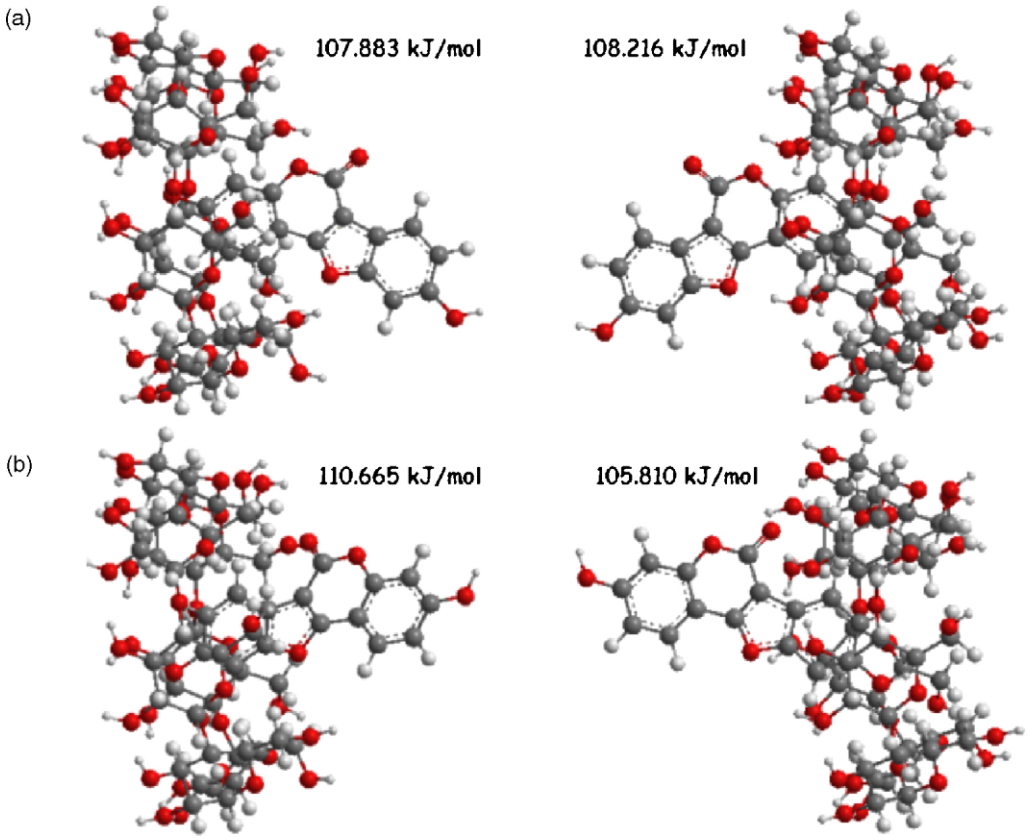


**Fig. 5.** Complexation models of the insertion of (a) A and (b) B rings of one molecule of coumestrol by the narrower and wider rims of one molecule of β-cyclodextrin, with its total energy value calculated by MM2.

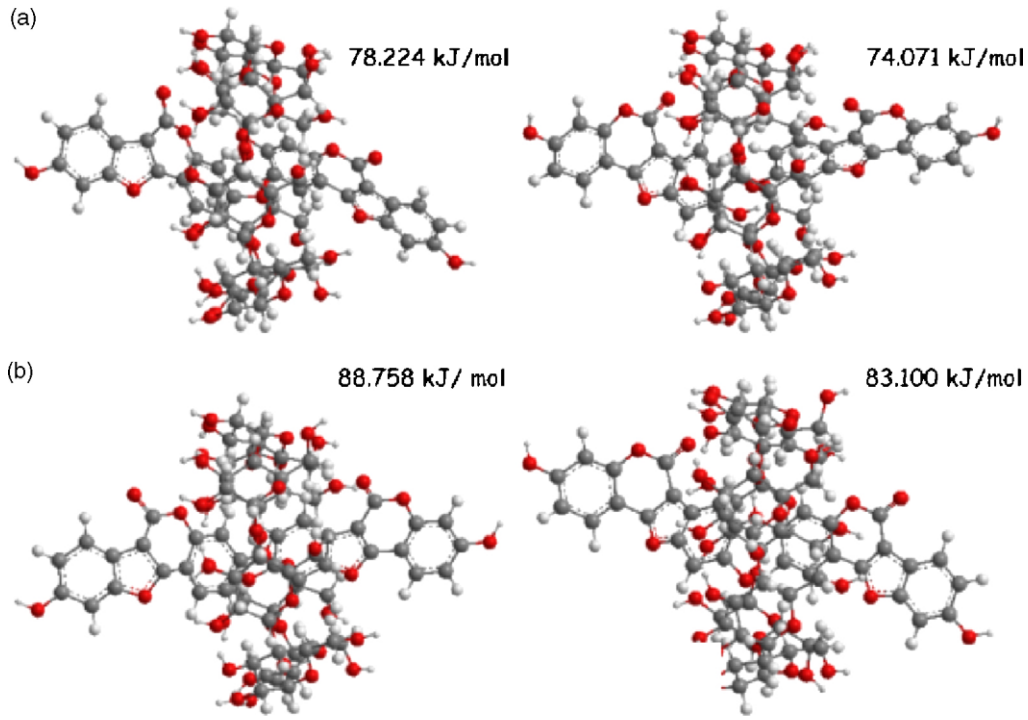


**Fig. 6.** Complexation models of the insertion of (a) A and (b) B rings of two molecules of coumestrol in the wider and narrower rims of one molecule of β-cyclodextrin, with its total energy value calculated by MM2.

e.g. for rutin (difference of 2.89 kJ/mol) (Haiyun et al., 2003), 3-hydroxyflavone and fisetin (differences of 7.77 and 5.60 kcal/mol, respectively) (Banerjee and Sengupta, 2006) and robinetin (difference of 2.3 kcal/mol) (Banerjee et al., 2007).

The coumestrol B-ring inserted by the wider rim of β-cyclodextrin demonstrated the lower energy (105.810 kJ/mol) in the 1:1 simulations. This can be confirmed by the NOESY spectrum where it is possible to observe the interaction between H3′ of the coumestrol molecule and the H3 from the cyclodextrin, which are positioned inside the cavity.

The complexation of two molecules of coumestrol with one of β-cyclodextrin was energetically more favorable than the complexation of one molecule of each. However, the complex model that obtained the smaller total energy (74.071 kJ/mol) was that obtained by the insertion of the B-rings of two molecules of coumestrol in both sides of the β-cyclodextrin molecule, being this the most probable complexation pathway according to the molecular modeling (Fig. 6a).

A test was also performed with two molecules of β-cyclodextrin and one molecule of coumestrol, and the four other inclusion models developed presented higher energies compared with the models of one molecule of each compound (data not shown).

The inclusion complexes of other flavonoids with β-cyclodextrin previously discussed in the literature may help to understand coumestrol interactions with β-cyclodextrin.

Zheng et al. (2005) developed a molecular modeling study of quercetin:β-cyclodextrin at a 1:1 molar ratio, showing the projection of the B-ring onto the OH2/OH3 face of β-cyclodextrin and the projection of the A-ring from the OH6 face, such relation was confirmed by NMR results for the coumestrol molecule suggesting the same way of interaction as observed in the quercetin complex. In the quercetin complex orientation, four quercetin hydroxyl groups were outside the hydrophobic β-cyclodextrin cavity. The conformational arrangement of the quercetin:β-cyclodextrin complex with the aromatic B-ring positioned inside the cavity and the formation of one hydrogen bound between OH7 of the quercetin A-ring with OH6 of β-cyclodextrin molecule helps in the protection from the molecule degradation (Yan et al., 2006).

The same orientation (B-ring positioned in the wider side of the cyclodextrin) was reported to naringin/β-cyclodextrin and 3-*O*-methylquercetin/β-cyclodextrin complexes by Fronza et al. (2002) and Schwingel et al. (2008), respectively.

Bergonzi et al. (2007) confirmed that the inclusion of the B-ring of flavonoids galangin, kaempferol and quercetin in the wider rim of β-cyclodextrin was the most probable way of interaction among the molecules. In addition, freeze-drying was considered the best method to prepare inclusion complexes.

Cannavà et al. (2008) suggested that a single coumestrol molecule could be inserted more or less deeply in the hydrophobic cavity of the β-cyclodextrin and that new hydrogen bonds are supposed to be formed, turning weaker the C–O–C and C–O bonds during the inclusion phenomena.

Recently, Daruházi et al. (2008) evaluated the inclusion complex of genistein/β-cyclodextrin at a 1:2 molar ratio prepared by kneading method. The $^{1}$H NMR was performed at 600 MHz/30 °C with ROESY analysis and molecular modeling was made by the PM3 semi-empirical method. It is worth emphasizing that both genistein and coumestrol molecules present very similar chemical structures. This research confirmed the inclusion of the genistein molecule through the insertion of the B-ring in the wider rim of the β-cyclodextrin. This interaction was detected between the OH4 of the genistein with H3 and, specially, with H5 of the cyclodextrin.

The formation of an inclusion complex between the coumestrol and β-cyclodextrin is postulated, as demonstrated by NOESY and molecular modeling experiments. This inclusion may occur by means of the insertion of coumestrol A or B-rings in the cyclodextrin cavity, which will interact with the H3 of the β-cyclodextrin, as demonstrated by NOESY. According to our results, this inclusion is likely to occur by means of the insertion of coumestrol B-ring in the wider side of the cyclodextrin, considering the interaction with one molecule of each substance. However, it was also demonstrated that a more energetically favorable interaction occurs by the insertion of two molecules of coumestrol (B-rings) into both sides of one molecule of β-cyclodextrin.

## 4. Conclusions

Coumestrol association with β-cyclodextrin improved its solubility in water up to 4 times at the 1:1 molar ratio. According to the NMR data and molecular modeling, the formation of an inclusion complex is possible through the insertion of the A or B-rings of coumestrol into the β-cyclodextrin cavity. However, the insertion of the B-ring of one molecule of coumestrol by the wider side of the β-cyclodextrin is supposed to be the most probable complexation pathway in a complex 1:1.

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