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INFLUENCE OF HYDROXYPROPYL-BETA-CYCLODEXTRIN ON THE PHYSICOCHEMICAL AND BIOLOGICAL CHARACTERISTICS OF A FLAVONE WITH IMPORTANT PHARMACOLOGICAL PROPERTIES

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Abstract

The purpose of this study was to investigate the influence of hydroxypropyl-β-cyclodextrin on diosmin, substance from the flavones category with important pharmacological properties. We carried out solubility studies, calculating the solubility constant of the complex formed between diosmin and hydroxypropyl-cyclodextrin at various temperatures (20, 25 and 37 ^{0}C) and in the presence of increasing cyclodextrin concentrations. A_L type curves were obtained which suggest the formation of an inclusion compound on a 1:1 molar ratio and the solubility constants had values between 370 and 453 M^{-1}. Knowing that between diosmin, hydroxypropyl-cyclodextrin and the used solvent, thermodynamic interactions occur, we investigated the influence of these interactions on several thermodynamic parameters such as Gibbs free energy change, free energy change, enthalpy change and entropy change. The calculated values showed that the reaction is positively influenced by the cyclodextrin's concentration increment and the temperature, the process being spontaneously. Next, inclusion compounds were obtained by co-evaporation and co-precipitation, and their structures were confirmed by FTIR and MS analysis. The complexes were tested *in vitro* compared with the parent substances, the results indicating improved antioxidant and antimicrobial activities. Moreover, the dissolution of diosmin increased in mediums similar to physiological conditions (simulated gastric and intestinal).

Key words: antimicrobial, antioxidant, beta-cyclodextrin, diosmin, dissolution

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1. Introduction

Diosmin (D) is a hesperidin semisynthetic derivative that has vasoprotective (Tong et al., 2013), antioxidant and anti-inflammatory properties (Sezer et al., 2011), antiproliferative and anti-cancer activities (Alvarez et al., 2009). Diosmin exhibits a neuroprotective effect and might have potential in the

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treatment of neurodegenerative diseases (Abdel-Salam et al., 2012). Also, diosmin manifests antihyperglycemic and hepatoprotective properties (Leelavinothan and Subramani, 2010; Tahir et al., 2013). However, its poor solubility in water increases the difficulty of formulation. In order to overcome this disadvantage, we used the complexation method with "host" substances of cyclodextrins type (Higuchi and Connors, 1965).

Cyclodextrins (CD) are cyclic oligosaccharides which are able to form inclusion compounds in the form of a truncated cone, with the secondary hydroxyl groups present on the lower base and the primary hydroxyl groups on the upper base, resulting in increased water solubility of the cyclodextrins, while the interior cavity is hydrophobic (Davis and Brewster, 2004; Martin Del Valle, 2004). Furthermore, for the improvement of solubility, stability to light and oxygen, and a better control of the chemical reactivity of the guest substance, other cyclodextrin derivatives obtained by esterification and etherification of the primary and secondary hydroxyl groups may be used (Higuchi and Connors, 1965; Liu et al., 2005).

Due to the important pharmacological properties of diosmin, the objective of this study was to investigate the complexation between diosmin and hydroxypropyl-β-cyclodextrin and the influence of this process on some physicochemical and biological characteristics. All determinations were carried out by comparison between the prepared inclusion compounds and parent substances.

2. Experimental

2.1. Materials

The substances used in this study were purchased from Sigma Aldrich (USA), having the following characteristics: diosmin (D) (5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)- 7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy -6-[[(2*R*,3*R*,4*R*,5*R*,6*S*) -3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one), CAS number 520-27-4, molar mass 608.545 g mol^{-1}, molecular formula $C_{28}H_{32}O_{15}$, β-cyclodextrin (β-CD) (CAS Number 7585-39-9, empirical formula $C_{42}H_{70}O_{35}$, molecular weight 1135 g mol^{-1}), hydroxypropyl-β-cyclodextrin (HP-β-CD) (CAS Number 128446-35-5, 0.8 molar substitution, molecular weight 1460 g mol^{-1}), and sulfated-β-cyclodextrin (sulfated-β-CD) (CAS Number 37191-69-8, 7mol per mol β-CD, molecular weight 3277 g mol^{-1}).

2.2. Methods

2.2.1. Phase solubility studies

Solutions with different concentrations of cyclodextrin were prepared (on the range of 0.969 - 16.29 x 10^{-3} M for β-CD, 0.684-13.69 x 10^{-3} M for HP-β-CD and 0.305 - 6.10 x 10^{-3} M for sulfated-β-CD), then an excess of diosmin was added (Domańska et al., 2011; Higuchi and Connors, 1965). The mixtures were stirred for 24 hours at various temperatures: 20, 25 and 37 ± 1 ^{0}C, then the unreacted diosmin was removed through filtering. The concentration of diosmin was determined spectrophotometrically, using a double beam Jasco V 530 spectrophotometer, by measuring the absorbance of samples against a blank containing the same concentration of cyclodextrin as the sample, at 257 nm.

2.2.2. Preparation of inclusion compounds

The inclusion compounds were prepared by co-evaporation and co-precipitation, using a molar ratio of diosmin: cyclodextrin of 1:1. Co-evaporation (CV): to a saturated solution of cyclodextrin was added a solution containing hesperidin in equimolar amount, under continuous stirring. The mixture was stirred at 30 ºC for 72 hours, then at room temperature until the solvent evaporated.

Co-precipitation (CP): cyclodextrin was dissolved in a given volume of water to the limit of solubility and solid diosmin was added, with vigorous stirring. The mixture was stirred continuously until a white precipitate was formed. The precipitate was filtered and dried to constant weight.

2.2.3. Physicochemical characterization

- **FTIR spectroscopic analysis**: was performed using a Tensor 27 Optics FT-IR spectrophotometer from Bruker, Germany, with a spectral range of 7500 – 370 cm^{-1}.

- **Mass spectroscopic analysis**: was performed using a triple quadrupole - TSQ Quantum Access Max mass spectrometer. Analysis conditions were: spray discharge voltage 3 kV, vaporizer temperature 400 ^{0}C, capillary temperature 375 ^{0}C, auxiliar gas pressure 40 mtorr.

2.2.4. Biological characterization

- **Antioxidant activity**: was tested by determining the ability of diosmin to inhibit lipoxygenase activity (Malterud and Rydland, 2000), an enzyme in the oxidoreductases class involved in the metabolism of arachidonic acid and linoleic acid.

The assay was carried out in borate buffer (pH - 9), after the addition of lipoxygenase solution and of the test compounds prepared in DMSO and using linoleic acid as substrate (0.16 mM). The absorbance of the solution was measured at 234 nm in the time interval of 0-120 seconds. The ability to inhibit lipoxygenase was calculated according to Eq. (1) and for each sample IC_{50} was calculated (mM diosmin in the final solution).

$$\% \ activity = \frac{(A_E - A_{ES})}{A_E} x\,100 \qquad (1)$$

where: A_E - the difference between the absorbance of the enzyme without sample at second 90 and the absorbance of the same solution at second 30; A_{ES} -

the difference between the absorbance of the enzyme with sample at second 90 and the absorbance of the same solution at second 30.

- **Antimicrobial activity** of inclusion compounds compared to the parent substances was evaluated by the agar diffusion method (Atilano et al., 2011; LaJean Chaffin, 2008). On the surface of Petri plates, with agar medium Mueller-Hinton for bacteria and agar medium Sabourand for fungi, inoculated with suspension of the test microorganisms, were placed stainless steel cylinders with an inner diameter of 6 mm in which was deposited 100 µl of solutions of the analyzed compounds. The test microorganisms used were: Gram positive bacteria - *Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Bacillus cereus* ATCC 14579, and fungi - *Candida albicans* ATCC 10231, *Candida glabrata* ATCC MYA 2950, *Candida parapsilosis* ATCC 22019. After incubation, for 24 hours at 37 ^{0}C, diameters of inhibition zones of microbial growth were registered. Results represent the mean diameters registered on three plates. The antibacterial activity of the investigated compounds was compared with the inhibition zone obtained with a 25 mg ampicillin disc and 30 mg chloramphenicol disc, placed on plates at the same time as the samples. Antifungal activity was compared to a 100 mg nystatin disc.

2.2.5. In vitro dissolution studies

The dissolution rate of diosmin and inclusion compounds was measured using the USP paddle apparatus, at a stirrer speed of 100 rpm, and temperature of 37 ± 0.5 ^{0}C. At intervals of 10, 20, 30, 40, 60, 90 and 120 minutes samples were collected, and replaced with the used medium. Samples absorbance was read at 257 nm. The dissolution medium replicated the physiological conditions: 0.1 N hydrochloric acid solutions simulated gastric fluid pH 1.2 and phosphate buffer simulated intestinal fluid pH 6.8 (García et al., 2009; Patil et al., 2008).

3. Results and discussion

3.1. Phase solubility studies

The phase solubility diagram was drawn by plotting the concentration of diosmin to increasing concentrations of cyclodextrin, and it was of A_L type. From the linear portion of the phase diagram Higuchi & Connors (Domańska et al., 2011; Higuchi and Connors, 1965), the stability constants (K_S) were calculated, at each temperature, according to Eq. (2).

$$Ks = \frac{slope}{S_0\,(1 - slope)} \tag{2}$$

where: *slope* – was calculated from the graph, S_0 – was intrinsic solubility of diosmin in the absence of cyclodextrins.

Fig. 1 shows the solubility diagrams of diosmin, in aqueous solutions of the three cyclodextrins, at increasing temperature. As can be seen, the solubility of diosmin in water increases linearly with cyclodextrin concentration and with temperature. The highest concentrations are obtained in the presence of HP-β-CD, followed by β-CD and sulfated-β-CD, regardless of temperature. Ks values were between 112-345 M^{-1} for β-CD, 370-453 M^{-1} for HP-β-CD and 112 – 333 M^{-1} for sulfated-β-CD. The slopes obtained from the phase diagrams are less than 1, indicating the formation of inclusion compounds in a 1:1 molar ratio between diosmin and cyclodextrins. The stability of the complex decreases in the following order: HP-beta-CD> beta-CD> sulfated-beta-CD. For this reason, further in the research was used HP-β-CD.

3.1.1. Determination of thermodynamic parameters

Knowing that between diosmin, hydroxypropyl-cyclodextrin and the used solvent, thermodynamic interactions occur, we investigated the influence of these interactions on several thermodynamic parameters such as Gibbs free energy change (ΔG^0_{tr}), free energy change (ΔG^0), enthalpy change (ΔH^0) and entropy change (ΔS^0). The thermodynamic parameters of the reaction depending on the temperature and CD concentration were calculated using the Eqs. (3-7) (Chadha et al., 2012; Domańska et al., 2011).

$$\Delta G^0_{tr} = -\,RT \log \frac{S}{S_0} \tag{3}$$

where: R = gas constant, T = absolute temperature of the reaction, S/S_0 = the ratio between the solubility of diosmin in cyclodextrin solution and the solubility of diosmin in water.

$$\Delta G^0 = -\,2.303\,RT \log K_s \tag{4}$$

where: Ks = equilibrium constant of the complex formed with 1:1 stoichiometry

$$\log Ks = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \tag{5}$$

It was graphically represented *log Ks* versus *1/T*, and the slope of the line obtained gives the value of enthalpy change (ΔH^0) from Eq. (6):

$$Slope = \frac{\Delta H^0}{2.303R} \tag{6}$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{7}$$

From calculation of thermodynamic parameters, we can assert the following:

- ΔG^0_{tr} has increasing values, depending on the concentration of the cyclodextrin (mM) and the temperature, with values between - 0733 and - 1420 $kJmol^{-1}$.

- ΔG^0 ranged between - 14 412 -15. 764 kJmol^{-1}
- ΔS^0 ranged between 52.03 and 53.53 Jmol^{-1}K^{-1}
- the calculated ΔH^0 was 0.833 kJmol^{-1}.

Results indicate a favorable solubilization process of diosmin in the presence of HP-β-CD, influenced by increasing concentrations of cyclodextrin. The formation of inclusion compounds diosmin - HP-β-CD is done through an endothermic process and between the two parent substances hydrophobic interactions are established.

3.2. Physico-chemical characterization

3.2.1. FTIR spectroscopic analysis

One of the methods used to confirm the formation of inclusion compounds is FTIR spectroscopic analysis (Gajare et al., 2009; García et al., 2014; Liu et al., 2005; Patil et al., 2008). The characteristic spectrum of diosmin (Fig. 2a.) is defined by the following absorption maxima - 3409.43, 1661, 1610 1501, 1448, 1319, 1261, 1182, 1069, 854 and 821 cm^{-1}. The bands specific to free OH groups are wide bands, in the 3200 - 3600 cm^{-1} domain.

The band at 3409 cm^{-1} indicates the presence of these groups in the diosmin molecule. The bands characteristic of C-H bonds deflection appear in the domain 1275 - 1000 cm^{-1}, confirmed by the band at 1069 cm^{-1} (in the case of plane deformation) and at 900-690 cm^{-1} (in the case of out-of-plane deformation), respectively, bands in the range 854-821 cm^{-1}.

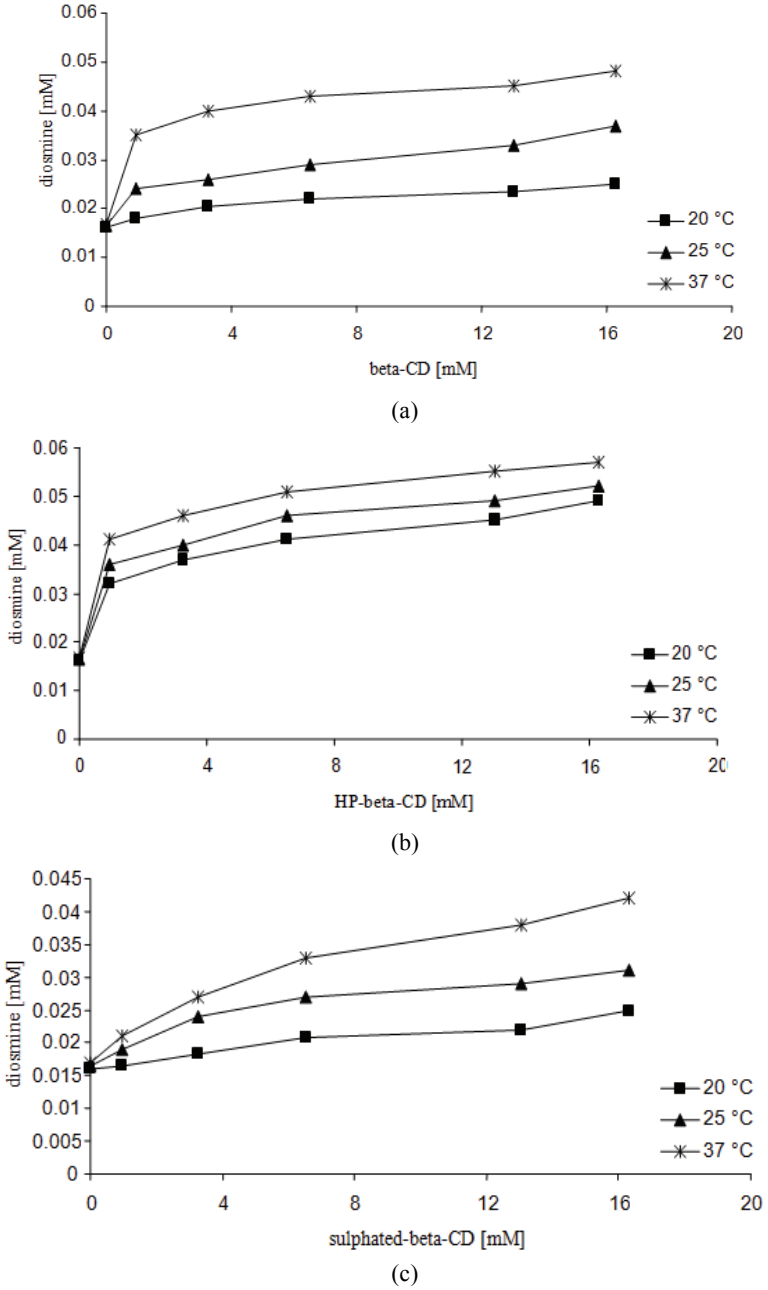

Fig. 1. Phase diagram of diosmin with β-CD (a), HP-β-CD (b) and sulphated-β-CD (c) in water, at different temperatures

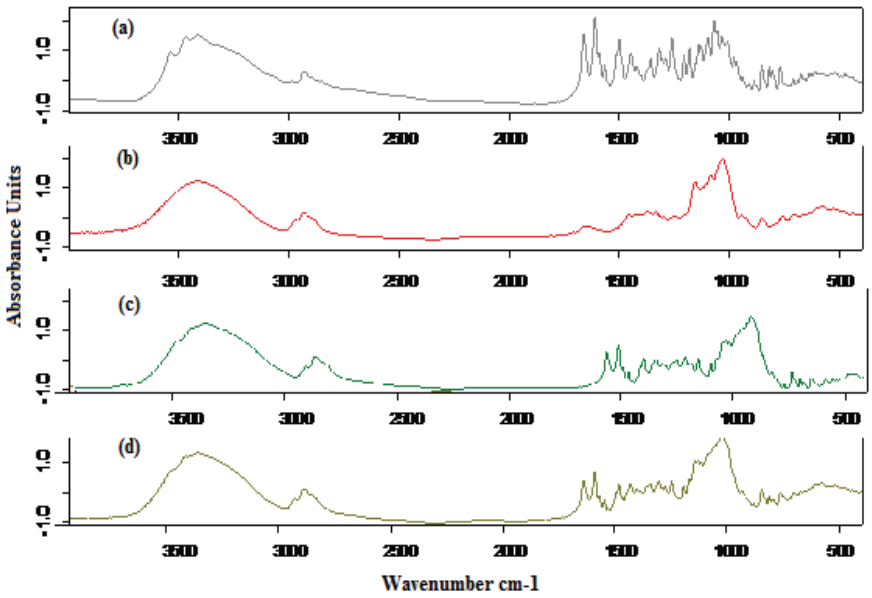

Fig. 2. FTIR spectra of diosmin (a), HP-β-CD (b), inclusion compound CP (c), inclusion compound CV (d)

The spectral line with average intensity is typical of carbonyl group and it is associated with the stretching vibration of the group. Hydroxypropyl-cyclodextrin (Fig. 2b.) shows absorption maxima with higher intensity of bands 865 and 2934 cm^{-1} corresponding to vibrations of asymmetrical stretching for methylene groups and to deformation vibrations of the same C-H bonds.

FTIR spectrum of CP sample (Fig. 2c.) is distinguished by the asymmetry of signal 3410 cm^{-1} that masks OH groups on the surface of the substrate, the shift to 3410 cm^{-1} being also associated with the hydrogen bridges that were created inside the complex. The presence of diosmin is further confirmed by the characteristic signals, 1661, 1610 1501, 1448 cm^{-1}. The substrate is still highlighted by the intense band at 1070 cm^{-1}. CV sample (Fig. 2d.) is similar to the CP sample, but the absorption maxima from 1660 to 1448 cm^{-1} show a lower intensity, confirming the inclusion.

3.2.2. Mass spectroscopic analysis

Prior to the analysis of inclusion complex, was performed the analysis of diosmin in the mass range 100-700 m/z. We noticed the presence of signal 607.8 corresponding to total mass (Fig. 3a). Polarization was negative. MS / MS evaluation (Fig. 3b) at a collision energy of 75EV produced further fragmentation, so fragments are observed at 284.76, 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyranone, and 175.75 (theoretical mass 176), 5-hydroxy-1-benzopyranone. With a larger error D-glucopyranosyl was identified at mass 149.67 (d = -2.67) (Colombo et al., 2008). Fig. 4a shows the spectrum of the inclusion complex formed by co-precipitation method. Ion 2119.94 is distinguished in the mass spectrum. It can be the adduct (Diosmine + HP-β-CD) which has theoretical mass 2071 at equimolar ratio. For further confirmation, a residual signal is present at 606.9 that belongs to residual diosmin. High intensity is determined by the higher ionization ability of the substance than of the complex.

Similarly, in case of complex formed by co-evaporation method (Fig. 4b), the signal with mass 2137 is distinguished, whose probability is associated with the mass of theoretical complex 2071. The difference may be associated with Na adduct, since sodium acetate (20 mM) and methanol (50:50) is the dispersion medium (Biernacka et al., 2014; García et al., 2014, Lee et al., 2009).

3.3. Biological characterization

3.3.1. Antioxidant activity

In order to determine the antioxidant activity (Malterud and Rydland, 2000), solutions of the tested samples (CV, CP and D) in DMSO were prepared, with concentrations ranging between 312.5-10000 µg/ml diosmin.

Fig. 5 shows the ability of tested samples to inhibit the activity of lipoxygenase, depending on the concentration of samples. As can be seen from Fig. 5, lipoxygenase activity is influenced by the increase in diosmin concentration. At the same diosmin concentration, inclusion compounds exhibit more intense activity than free diosmin, which demonstrates that the antioxidant activity was influenced by the presence of HP-β-CD.

Calculating IC_{50} values (the ability to inhibit 50 % of lipoxygenase activity) of the analyzed compounds, it was observed that the capacity to inhibit lipoxygenase increased from 76.50 ± 3.89 for free diosmin to 15.73 ± 0.57 (CP) and 16.106 ± 0.586 (CV), showing a 4-fold increase in activity in case of diosmin inclusion in HP-β-CD. Between the inclusion compounds obtained, the differences are insignificant.

3.3.2. Antimicrobial activity

We compared the antibacterial activity against Gram positive bacteria *S. aureus*, *S. lutea*, *B. cereus* and the antifungal activity against *C. glabrata* and *C. parapsilosis* (Fig. 6.) of inclusion compounds in relation to free diosmin. (Atilano et al., 2011; LaJean Chaffin, 2008)

When comparing the antimicrobial activity of inclusion compounds relative to free diosmin, against all tested microorganisms the inclusion compounds showed higher activity due to improved solubility, thus increasing the amount of compound that crosses the microbial membrane. After inclusion, antifungal activity is stronger than antibacterial activity, probably due to the different composition of the cell wall.

Antifungal activity was influenced by the method of preparation of inclusion compounds, those obtained by co-evaporation showing increased activity, as demonstrated by the size of the inhibition zone.

3.4. In vitro dissolution studies

In vitro dissolution tests were performed according to European Pharmacopoeia, 8th edition, at physiological pH simulating the pH of the stomach (Fig. 7a. - acidic pH 1.2) and intestinal pH (Fig. 7b. - alkaline pH 6.8). It can be observed that the dissolution of inclusion compounds was improved compared to the unincluded substance, with similar values for both types of preparation methods

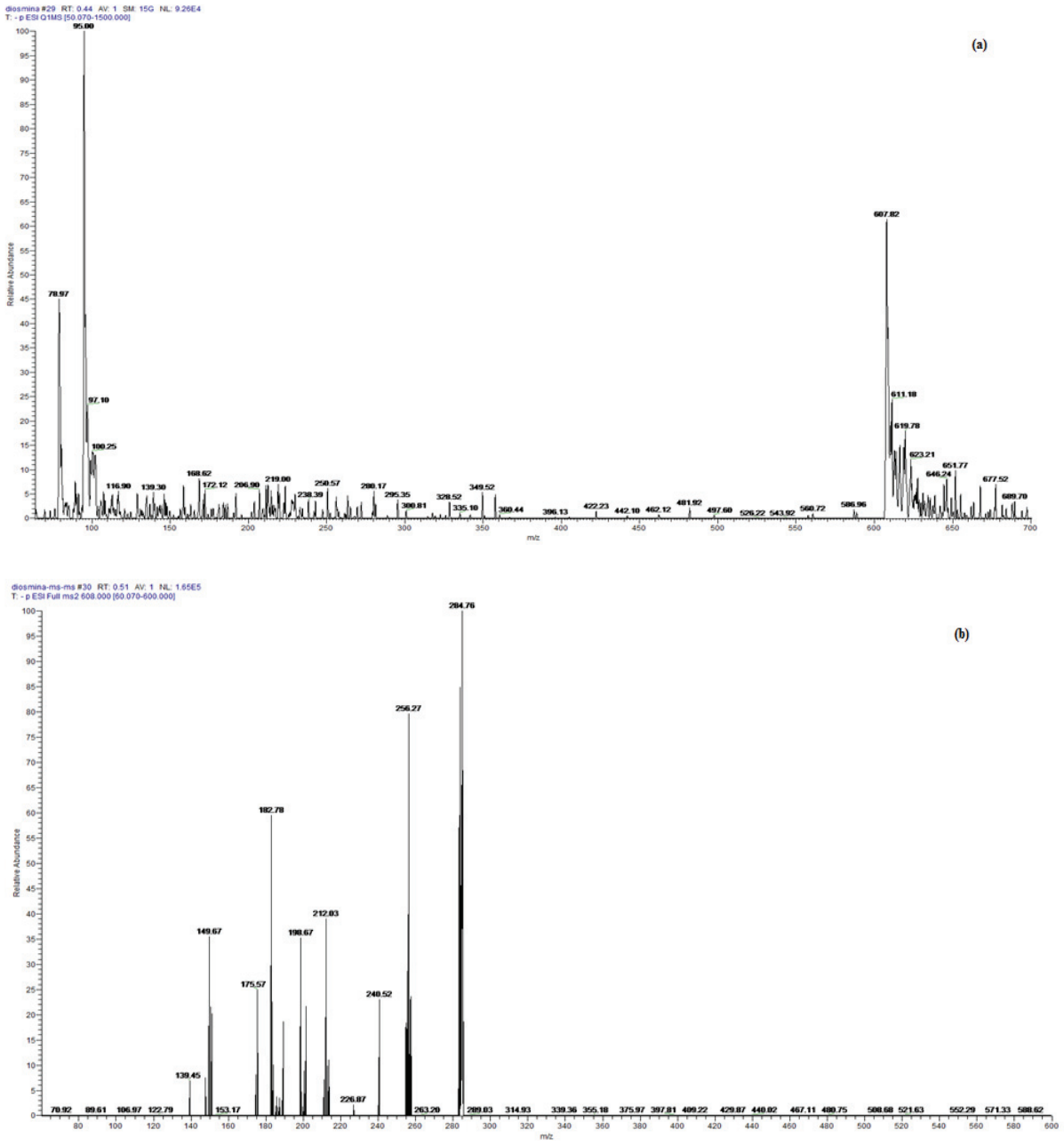

Fig. 3. Full scan MS (a) and MS/MS spectrum (b) of diosmin

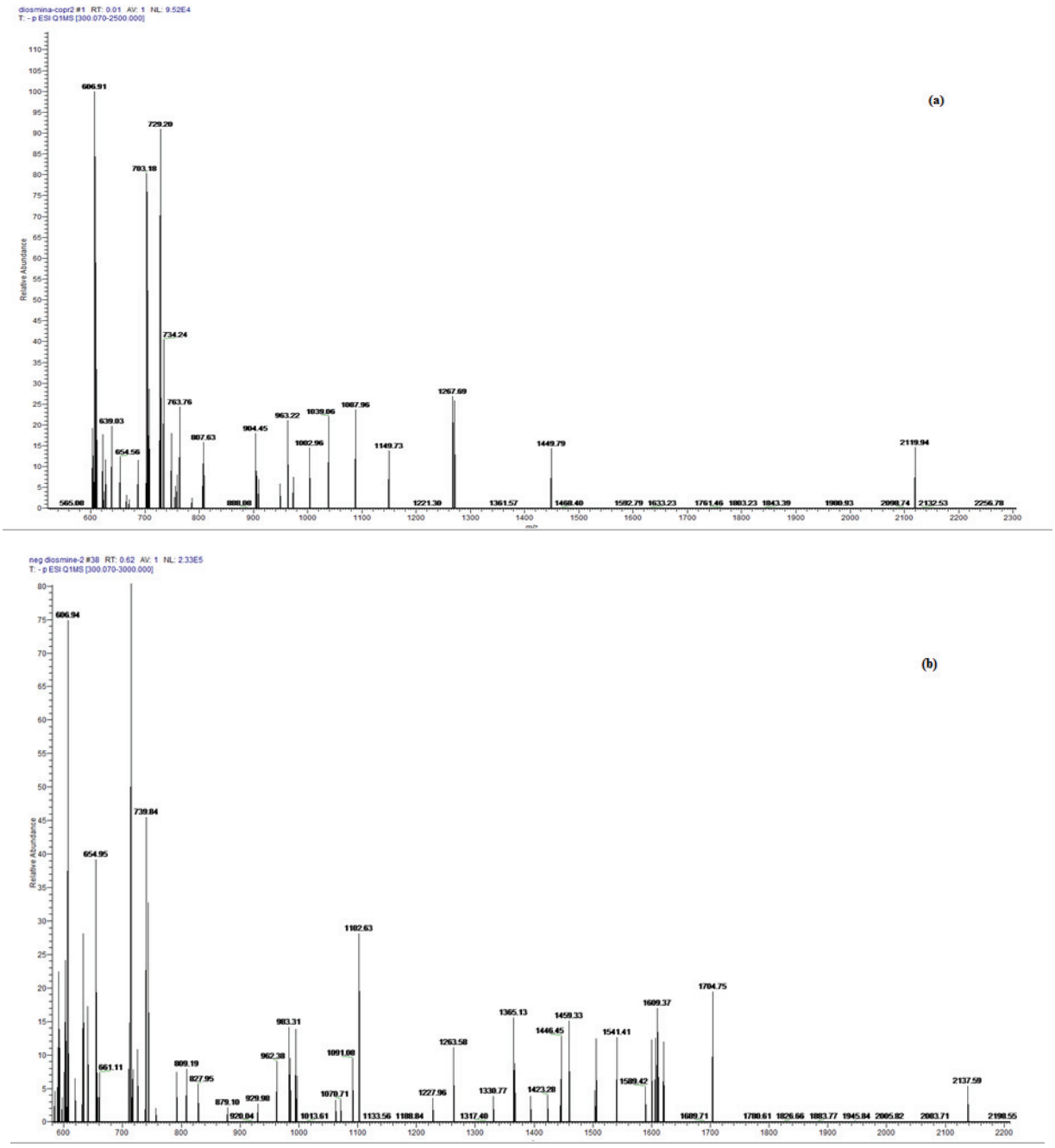

Fig. 4. Full scan MS of complex CP (a), CV (b)

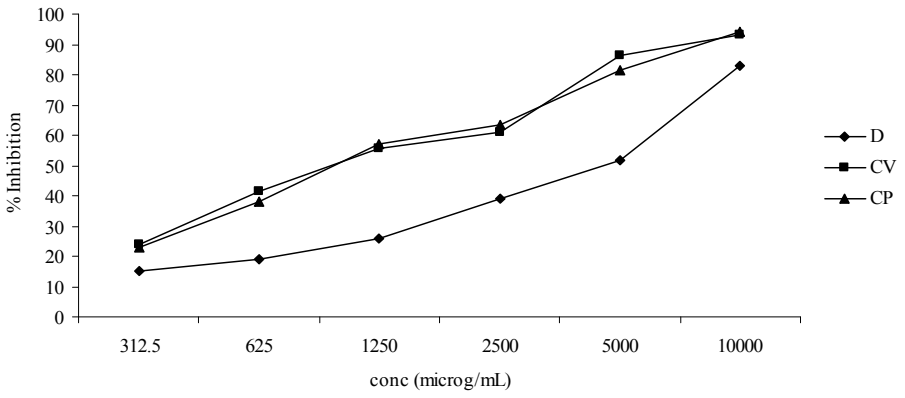

Fig. 5. Lipoxygenase inhibition by diosmin and by inclusion compounds

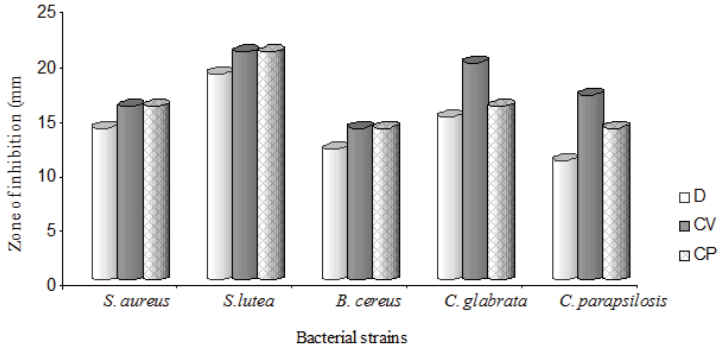

Fig. 6. Antibacterial and antifungal activity of inclusion compounds, relative to free diosmin

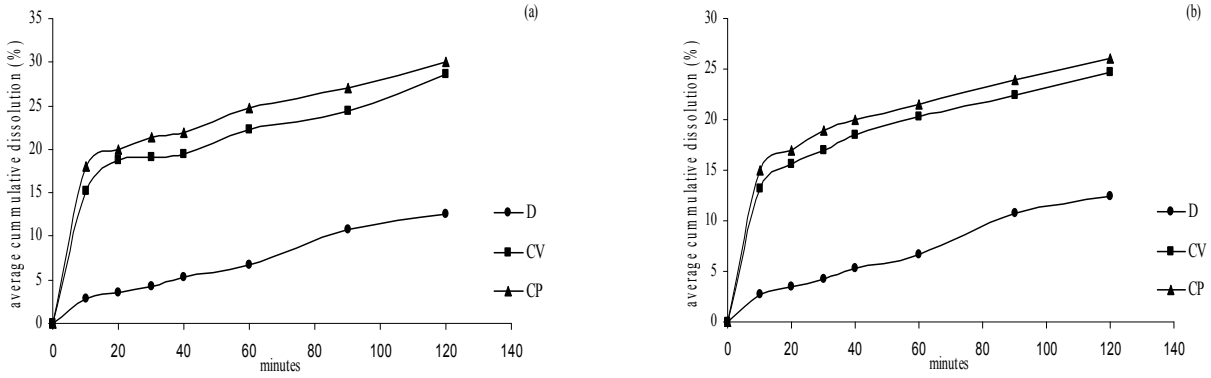

Fig. 7. The in vitro dissolution profile of diosmin and inclusion compounds, at pH 1.2 (a) and pH 6.8 (b)

4. Conclusions

In this work we determined the influence of HP-β-CD on a pharmacologically important flavonoid, diosmin. The phase solubility diagrams in aqueous solution showed a linear increase in water solubility with the increase of cyclodextrin amount and temperature.

The best solubility and stability were obtained in the presence of HP-β-CD at 37 ^{0}C. The processe takes place spontaneously and it is controlled by enthalpy. The inclusion compounds were characterized physicochemically by FTIR and MS analysis.

Furthermore, antioxidant and antimicrobial activity enhanced after inclusion in HP-β-CD, thereby expanding the possibility of using the inclusion compounds in order to improve pharmacological effects.

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