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Article

# Inclusion complexes of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin with 2-hydroxypropyl-β-cyclodextrin: solubility and antimicrobial activity

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The aim of the present study is to improve the solubility and antimicrobial activity of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin by formulating its inclusion complexes with 2-hydroxypropyl-β-cyclodextrin in solution and in solid state. The phase solubility study was used to investigate the interactions between 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin and 2-hydroxypropyl-β-cyclodextrin and to estimate the molar ratio between them. The structural characterization of binary systems (prepared by physical mixing, kneading and solvent evaporation methods) was analysed using the FTIR-ATM spectroscopy. The antimicrobial activity of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin and inclusion complexes prepared by solvent evaporation method was tested by the diffusion and dilution methods on various strains of microorganisms. The results of phase solubility studies showed that 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin formed the inclusion complexes with 2-hydroxypropyl-β-cyclodextrin of AP type. The solubility of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin was increased 64.05-fold with 50% w/w of 2-hydroxypropyl-β-cyclodextrin at 37 °C. The inclusion complexes in solid state, prepared by the solvent evaporation method, showed higher solubility in purified water and in phosphate buffer solutions in comparison with 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin alone. The inclusion complexes prepared by solvent evaporation method showed higher activity on *Bacillus subtilis* and *Staphylococcus aureus* compared to uncomplexed 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin due to improved aqueous solubility, thus increasing the amount of available 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin that crosses the bacterial membrane.

**Keywords**: 3-(3-(2-Chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin. 2-Hydroxypropyl-β-cyclodextrin. Inclusion complexes. Phase solubility. Antimicrobial activity.

## INTRODUCTION

Cyclodextrins represent one of the most commonly complexing agents used in the pharmaceutical industry because of being inexpensive and non-toxic to humans and having the capability of improving the physical, chemical and biological properties of bioactive molecules. 2-Hydroxypropyl-β-cyclodextrin (2-HP-β-CD) (Figure 1) is produced from β-cyclodextrin (β-CD) by addition of propylene oxide to some of the hydroxyl groups of β-CD. This modification results in greater solubility of 2-HP-β-CD and its complexes compared to β-CD (Muankaew, Loftsson, 2018).

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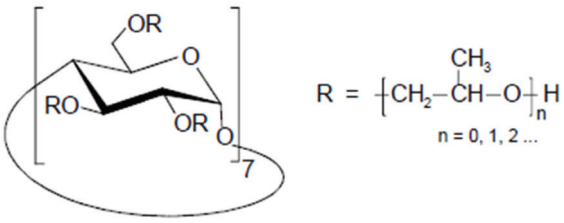



**FIGURE 1 -** The chemical structure of 2-hydroxypropyl-β-cyclodextrin.

Hydroxypropyl-β-cyclodextrin has been considered to be the most accepted representative of hydroxyalkylated derivatives as hydrophilic drug carrier, because of its amorphousness, higher water solubility (about 60%), solubilizing power and lower toxicity compared to the parent β-CD and other hydrophilic derivatives of β-CD (Muankaew, Loftsson, 2018). 2-HP-β-CD has a hydrophobic/non-polar inner cavity and hydroxyl groups placed on the hydrophilic outer surface. The inclusion of hydrophobic compounds takes place mainly by hydrophobic interactions between guest molecules (drugs) and the walls of cyclodextrin cavity. Permeation of the molecules of biologically active substances ("guest") into a macrocyclic cavity ("host") and their arrangement within it are provided by nonvalent interactions (van der Waals, hydrophobic, electrostatic, hydrogen bonding and steric effects). Besides, if the "guest" molecule has hydrophobic and hydrophilic fragments of a similar size, cyclodextrins prefer bonding with the former. Despite the number of factors and different forces involved in the complexation with cyclodextrins, the production of complexes is a rather simple process. There are several methods to obtain cyclodextrin-guest complexes depending on the properties of the guest and the nature of the chosen cyclodextrin (Cheirsilp, Rakmai, 2016). Complexation with 2-HP-β-CD, as modified β-CD, can enhance the solubility, dissolution rate and bioavailability of poorly water-soluble drugs, enhance stability, reduce unpleasant odours and tastes of drugs (Saokham *et al.*, 2018).

In the last decade, cyclodextrins have been widely used to form inclusion complexes with various compounds for different purposes (Abdel-Mottaleb *et al.*, 2018). The investigation of such interaction of new potential drugs or existing ones is of great importance and this includes the subject of this work, such as 3-substituted derivative of 4-hydroxycoumarin. Significant antimicrobial activities and interesting physicochemical properties of 3-substituted derivatives of 4-hydroxycoumarin have been reported (Špirtović-Halilović *et al.*, 2014; Završnik, Špirtović-Halilović, Softić, 2011). 3-(3-(2-Chlorophenyl) prop-2-enoyl)-4-hydroxycoumarin (CPHC) (Figure 2), as a 3-cynnamoyl-4-hydroxycoumarin derivative, is biologically active toward some bacteria strains. It was prepared by the reaction of nucleophilic addition from 3-acetyl-4-hydroxycoumarin reacting with appropriate aromatic aldehyde, with pyridine and piperidine as catalysts (Završnik, Špirtović-Halilović, Softić, 2011).

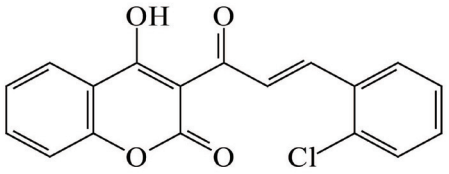



**FIGURE 2 -** The chemical structure of 3-(3-(2-chlorophenyl) prop-2-enoyl)-4-hydroxycoumarin (Špirtović-Halilović *et al.*, 2014).

Density functional theory (DFT) global chemical reactivity descriptors: chemical hardness, total energy, electronic chemical potential and electrophilicity were calculated for synthesized compound, 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin, and used to predict its relative stability and reactivity. The diffusion and dilution methods showed that 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin inhibited the growth of the Gram-positive aerobic bacteria *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 11778 (Špirtović-Halilović *et al.*, 2014). The objective of this study was to prepare and characterize the physical mixtures and inclusion complexes of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin with 2-hydroxypropyl-b-cyclodextrin using kneading and solvent evaporation methods, and to reassess their antimicrobial activity. By inclusion complexation of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin with 2-hydroxypropyl-b-cyclodextrin, we tried to improve its antibacterial activity.

## MATERIAL AND METHODS

### Material

Chemicals and other ingredients used were all of analytical grade or higher purity. Acetic acid (glacial, pro analysi), Alkaloid (Makedonia), 4-hydroxycoumarin, Sigma-Aldrich GmbH (Germany) and phosphoryl chloride, Sigma-Aldrich GmbH (Germany), were used for the synthesis of 3-acetyl-4-hydroxycoumarin. Pyridine (99+%, pro analysi), ACROS Organics™ (India) and piperidine (anhydrous, 99.8%), Sigma-Aldrich GmbH (Germany) as catalysts, were used to obtain a 3-cinnamoyl-4-hydroxycoumarin derivative, 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin. Dimethyl sulfoxide (99.5%) and ethanol (96% v/v) were purchased from Carlo Erba (France). Hydrochloric acid (37%, pro analysi), potassium dihydrogen phosphate and sodium dihydrogen phosphate dihydrate were purchased from Merck KgaA (Germany). 2-Hydroxypropyl-β-cyclodextrin (Cavasol® W7 HP, DS 6.8, Mr ~ 1410 g $mol^{-1}$) was purchased from ISP A.G. (Germany). Acetonitrile was purchased from Fluka, Chemika (Switzerland).

### UV spectrophotometry

*Stock solutions of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin*: About 5 mg of CPHC (Analytical balance type XS 205DU/A, Mettler Toledo GmbH, Germany) weighted average was dissolved in 50 mL of acetonitrile and another 5 mg in 50 mL of ethanol, 96.0% v/v, in the ultrasonic bath (Sonorex Digitec DT 512 H, Bandelin, GmbH, Germany) for 5 minutes (solution A). Volumes of 1 mL of stock solutions were further diluted in volumetric flasks with 20 mL of the mentioned solvents. Solutions of CPHC were freshly prepared, protected from direct sunlight, after which UV spectra were recorded to determine the absorption maximum/absorption maxima of the test substance (UV 1601, UV/Visible spectrophotometer, Shimadzu, Japan). The wavelength at which the test substance (CPHC) exhibits an absorption maximum (Figure 3) will later serve to determine its content in the test samples.

### Calibration curve of CPHC

From the CPHC ethanol stock solution, concentration of 0.1033 ± 0.0001 mg $mL^{-1}$, five working CPHC dilutions were prepared and their absorbances were measured at wavelengths of 280.5 nm and 360.0 nm in quartz cells, 1 cm thick (Figure 4). The molar absorption coefficient of CPHC in ethanol solutions was calculated from the concentration range (from $3.1218 \times 10^{-6}$ to $3.1279 \times 10^{-5}$ mol $L^{-1}$ at $\lambda = 280.5$ nm, and from $6.3353 \times 10^{-6}$ to $4.7469 \times 10^{-5}$ mol $L^{-1}$ at $\lambda = 360.0$ nm) and the measured absorbances of ethanol solutions of CPHC at given wavelengths (Figure 5).

### Preparation of physical mixtures of CPHC with 2-HP-β-CD

Solid physical mixtures (PM) of the test substance and 2-HP-β-CD (PM CPHC : 2-HP-β-CD 1:1 and PM CPHC : 2-HP-β-CD 1:2) were prepared simply by mechanical trituration. CPHC (Mr = 326.74 g $mol^{-1}$) and 2-HP-β-CD (Mr = 1410.0 g $mol^{-1}$) in molar ratio 1:1 and 1:2, were weighed accurately, pulverized and then mixed thoroughly (30 min) by light trituration in a ceramic mortar until a homogenous mixture was obtained. The resultant mixtures were sieved through the sieve No. 20 (WS TylerR, Ohio, USA) and stored in dark glass bottles until analysis (Iacovino *et al.*, 2013).

### Preparation of inclusion complexes of CPHC with 2-HP-β-CD by kneading method

Kneading method is suitable for poorly water-soluble drugs, because the guest (test substance) is dissolved slowly during the formation of complex. It affords a very good yield of inclusion formation but it is unsuitable for large scale preparation (Cheirsilp, Rakmai, 2016). In laboratory, scale kneading can be achieved by using a mortar and a pestle. In large scale, kneading can be done by utilizing the extruders and other machines. This is the most common, simple and also low-cost method used to prepare the inclusion complexes. The physical mixtures of CPHC and 2-HP-β-CD in 1:1 and 1:2 molar ratios were prepared as earlier described, in a mortar and pestle (by

mechanical trituration), which were then kneaded with a 1/3 mixture of water and ethanol (1:1, in mass ratio) relative to the weight of the physical mixtures of CPHC and 2-HP-β-CD, for 60 minutes (Agarwal, Gupta, 2012). Kneading was done until the product (the paste) started to dry on the walls of the mortar. The products were further dried in a vacuum oven VD 23 (Binder, USA) at 80 °C ± 0.5 °C (boiling point(ethanol) = 78.5 °C), at pressure of 6 ± 2 mbar, until a constant weight was obtained. Ethanol, which was used for preparation of inclusion complexes of CPHC and 2-HP-β-CD, is classified as a class 3 solvent. As solvent in class 3 it may be regarded as less toxic and of lower risk to human health and is accepted in pharmaceuticals (ICH, 2016). The resultant products (KN CPHC : 2-HP-β-CD 1:1 and KN CPHC : 2-HP-β-CD 1:2) were sieved through the sieve No. 20 (WS TylerR, Ohio, USA) and stored in dark glass bottles until analysis (Agarwal, Gupta, 2012).

### Preparation of inclusion complexes of CPHC with 2-HP-β-CD by solvent evaporation

The solvent evaporation method is one of the most commonly used methods in the pharmaceutical industry for improving solubility of poorly water-soluble drugs. This method is suitable for heat unstable components. The basic principle of this method is that drug and carrier are dissolved in a volatile solvent for homogeneous mixing (Tran *et al*., 2019). For preparation of the binary systems of CPHC and 2-HP-β-CD in the molar ratios of 1:1 and 1:2 by solvent evaporation method, ethanol was used as a solvent for CPHC and 2-HP-β-CD (Agarwal, Gupta, 2012). CPHC and 2-HP-β-CD were separately dissolved in ethanol on the ultrasonic bath (Sonorex Digitec DT 512 H, Bandelin, GmbH, Germany) for 15 minutes at room temperature (at 25 °C). A minimum volume of ethanol, for achieving the unsaturated solution, was used in order to dissolve the substances. The ethanolic solution of 2-HP-β-CD was added to the clear ethanolic solution of CPHC, and the solution was homogeneously mixed on the magnetic stirrer**,** C-MAG HP 7 (IKA, Werke GmbH & Co.KG, Germany), for 30 minutes. The solvent was evaporated from ethanolic solution of CPHC and 2-HP-β-CD mixture on Rotavapor R-205 (Buchi, Switzerland). The water temperature of the rotavapor bath was set to 40 °C ± 0.5 °C. The given temperature was lower than the boiling point of ethanol. Evaporation of ethanol was carried out under reduced pressure of 337 mbar. The solvent was evaporated at 120 rpm $min^{-1}$. Time of evaporation of the solvent depended on its volume (i.e. the mass of the sample). During the evaporation of ethanol in the sample with CPHC and 2-HP-β-CD, a yellow layer of solid inclusion complex was formed. The samples were subjected to drying for a period of 5 hours in the vacuum oven VD 23 (Binder, USA) at 80 °C ± 0.5 °C, at pressure of 6 ± 2 mbar. After drying in oven, yellow crystals of the newly formed compound were obtained. Completely dry samples (SE CPHC : 2-HP-β-CD 1:1 and SE CPHC : 2-HP-β-CD 1:2) were sieved through the sieve No. 20 (WS TylerR, Ohio, USA) and stored in dark glass bottles until analysis.

### Determination of the percentage of CPHC content in physical mixtures and inclusion complexes

Each inclusion complex (prepared by different methods) and physical mixtures containing 5 mg/10 mg of CPHC (calculated based on the amount of substances added in preparation) were accurately weighed and placed in 10 mL glass flasks. The samples were dissolved with 10 mL of ethanol using an ultrasonic bath for 30 min. The solution was filtered through a 0.2 μm filter (Cellulose acetate filter, Sartorius, Germany) and the filtrate was diluted with 96% v/v ethanol to obtain a suitable concentration (within the calibration range). The content of CPHC was quantified by using UV/VIS spectroscopy (UV 1601, Shimadzu, Japan) at a wavelength of 360.0 nm. At the wavelength of 360.0 nm which is the $\lambda^A$ (maximum absorption wavelength) of physical mixtures and inclusion complexes of CPHC, there was no interference absorption from 2-HP-β-CD (Khalafi, Rafiee, 2013). Each sample was carried out in triplicate.

### Phase solubility studies

Solubility measurements and the determination of saturation concentrations were carried out by adding excess amounts of CPHC to purified water/aqueous buffer

solutions/aqueous solutions of 2-HP-β-CD. Preliminary determination of solubility of CPHC in purified water was conducted by sampling of aqueous solutions of CPHC in selected time intervals during 5 days (after 84, 90, 96, 108 and 120 hours). All samples were taken by a pipette, then filtered by filter paper with pore diameter of 0.2 μm (Cellulose acetate filter, Sartorius, Germany). The concentration of dissolved CPHC in purified water was determined by absorption spectroscopy using the Shimadzu UV 1601, UV/VIS spectrophotometer (Shimadzu, Japan).

As a medium for solubilization of CPHC, the chloride buffer solution, as an acidic medium that simulates gastric fluid without enzymes, and phosphate buffer solutions, simulating intestinal fluid without enzymes, were selected, with pH values ranging from 1.2 to 7.4 (pH meter SevenMultiTM S47-K**,** Mettler Toledo GmbH, Germany) (Guidance for Industry, 2017). Solutions with 2-HP-β-CD were prepared at concentrations from 1% to 50% (w/w). Concentrations of 2-HP-β-CD were selected based on their solubility in water (solubility more than 50%). The CPHC powder was added into dark, glass flasks containing already mentioned percentages of 2-HP-β-CD.

Solubility measurements and the determination of saturation concentrations of physical mixtures and inclusion complexes were carried out by addition of excess amounts of physical mixtures and inclusion complexes to purified water and aqueous buffer solutions (Gupta, 2013).

All samples (powder of CPHC/physical mixtures/inclusion complexes) were added to dark, glass flasks in 50 mL of the test mediums. The samples were shaken at 300 rpm $min^{-1}$, during 108 hours on the thermostated shaking bath (BDL, Type: GFL 1083, Czech Republic), to reach equilibrium, at 25 °C ± 0.1 °C and 37 °C ± 0.1 °C. Preliminary studies were carried out using different equilibration periods (after 84, 90, 96, 108 and 120 hours) confirming that equilibrium was reached within 4 days. Longer equilibration did not result in increased CPHC solubility. Undissolved substance was visualized before and after the achievement of equilibrium. After mixing, the solutions were left for 24 hours at room temperature in order to achieve equilibrium between the dissolved and undissolved CPHC in solutions (saturated aqueous solutions) (Higuchi, Connors, 1965). After reaching equilibrium, all suspensions (samples) were filtered through a 0.2 μm pore size membrane filter (Cellulose acetate filter, Sartorius, Germany). The concentrations of the dissolved test sample (CPHC) in the tested mediums (purified water, aqueous buffer solutions, aqueous solutions of 2-HP-β-CD) and tested samples (physical mixtures and inclusion complexes) in the tested mediums (purified water, aqueous buffer solutions) were determined by absorption spectroscopy using the Shimadzu UV 1601, UV/VIS spectrophotometer at 280.5 nm, in ethanol, 96.0% v/v (which was previously used to develop the calibration curves). To nullify the absorbance due to the presence of aqueous buffer solutions/2-HP-β-CD, the apparatus was calibrated with the corresponding blank in every assay. All experiments were carried out in triplicate and the results are presented as the mean values (Figures 6-8).

The changes in the solubility of CPHC resulting from the addition of various concentrations of 2-HP-β-CD were used to plot phase-solubility diagrams and to evaluate the stoichiometry and apparent stability constants/encapsulation constant (*Ks*) of the resultant complexes. The most common type of cyclodextrin complexes is the apparent 1:1 drug/cyclodextrin complex (*D/CD*) where one drug molecule (*D*) forms a complex with one cyclodextrin molecule (*CD*). According to the phase solubility diagram, the encapsulation constant *Ks* of the complex having a stoichiometric ratio of 1:1 can be determined using the Equation 1 (Conceicao *et al*., 2018; Muankaew, Loftsson, 2018; Higuchi, Connors, 1965):

$$K_s = \frac{slope}{S_o\ (1 - slope)} \qquad \text{Equation 1}$$

where *Ks* is the apparent stability constant (L $mol^{-1}$ i.e. $M^{-1}$), *slope* denotes the slope of the straight line, and *So* is the solubility of CPHC in aqueous solution without the existence of 2-HP-β-CD (the saturation concentration of CPHC in pure water). The value of the stability constant is used to compare the affinity of CPHC for 2-HP-β-CD. A more reliable method for evaluation of cyclodextrins and their solubilizing potentials is to determine the

complexation efficiency (*CE*), which is equal to the complex to free cyclodextrin concentration ratio and can be obtained from the *slope* of their phase solubility profile. For 1:1 drug/cyclodextrin complexes the *CE* can be calculated from the *slope* of the phase solubility diagram (Equation 2) (Conceicao *et al*., 2018; Muankaew, Loftsson, 2018; Loftsson, Hreinsdóttir, Másson, 2007; Higuchi, Connors, 1965):

$$CE = \frac{[D/CD]}{[CD]} = S_o \cdot K_s = \frac{slope}{(1 - slope)} \qquad \text{Equation 2}$$

where [*D/CD*] is the concentration of dissolved drug-cyclodextrin complex, [*CD*] is the concentration of dissolved free cyclodextrin and *slope* is the slope of the phase solubility profile (Higuchi, Connors, 1965). The *CE* was calculated from *slope* of the phase solubility profiles where the molar cyclodextrin (2-HP-*β*-CD) concentration is on the x-axis and the molar drug (CPHC) solubility on the y-axis. The *CE* was used to calculate the drug : cyclodextrin ratio (*D* : *CD*), which can be correlated to the expected increase in formulation bulk (Equation 3) (Conceicao *et al*., 2018; Higuchi, Connors, 1965):

$$D:CD = 1:\left(\frac{CE+1}{CE}\right) \qquad \text{Equation 3}$$

The determination of the value of apparent stability constants (*Ks*) is one of the critical steps to judge whether cyclodextrin can be applied to pharmaceutical preparations. Therefore, it usually acts as an initial point for drug-cyclodextrin application research. In general, if the *Ks* value is too large, the balance will move into the formation of complex (Jambhekar, Breen, 2016).

### FTIR-ATR absorption measurements

Fourier transformation-infrared (FTIR) - attenuated total reflectance (ATR) spectroscopy (FTIR-ATR) was used as part of the qualitative analysis of physical mixtures and inclusion complexes, in solid state (Figure 9). Shift of the absorption bands of tested powders toward other frequency (different spectral area) compared to CPHC, can indicate complexation with 2-HP-β-CD (Pralhad, Rajendrakumar, 2004). One of the main advantages of this configuration, besides the usual non-destructiveness of the technique in itself, is that it requires only few micrograms of the sample avoiding the possible saturation of the absorbance signal. Infrared spectra of all samples were obtained by using the Perkin Elmer, Frontier FT-IR/NIR spectrometer (Waltham, MA, USA). The samples (about 200 mg) were placed directly on the sample pan and analysed from 450 to 4000 $cm^{-1}$ spectral range with 16 scans and spectral resolution of 4 $cm^{-1}$. Recorded FTIR-ATR spectrum of CPHC, with its specific absorption band intensity was used for identification of the inclusion in 1 mol and 2 mols of 2-HP-β-CD after recording FTIR-ATR spectra of physical mixtures and inclusion complexes obtained by kneading method and solvent evaporation method.

### The antimicrobial activity of the CPHC and inclusion complexes of CPHC with 2-HP-β-CD prepared by solvent evaporation method

Antimicrobial activity of inclusion compounds (SE 1:1 and SE 1:2) compared to the uncomplexed/free substance (CPHC) was evaluated by the agar diffusion method (Špirtović-Halilović *et al*., 2014; Ph.Eur. 2014) and dilution method (Završnik, Špirtović-Halilović, Softić, 2011). The test microorganisms used were: laboratory American Type Culture Collection (ATCC) reference strains, Gram - positive aerobic bacteria or facultative anaerobic *Bacillus subtilis* ATCC 6633, facultative anaerobic *Staphylococcus aureus* ATCC 25923, Gram - negative bacteria, facultative anaerobic *Escherichia coli* ATCC 25922, and fungal strains *Saccharomyces cerevisiae* ATCC 9763 and *Aspergillus brasiliensis* ATCC 16404. The nutrient agar (Liofilchem, Italy) and Sabouraud maltose agar (Liofilchem, Italy), as the mediums for bacterial and fungi growth, were sterilized in an autoclave at 121 °C under 110 kPa for 15 min. The inoculum of the culture (100 µL) was added to 10 mL of the medium and poured into the Petri dishes. The test samples (CPHC, SE 1:1 and SE 1:2) were dissolved in dimethyl sulfoxide (99.5 % DMSO) to obtain 1 mg $mL^{-1}$ stock solutions (ZX3 Advanced Voretex Mixer, Velp Scientifica®, DQS GmbH, Germany).

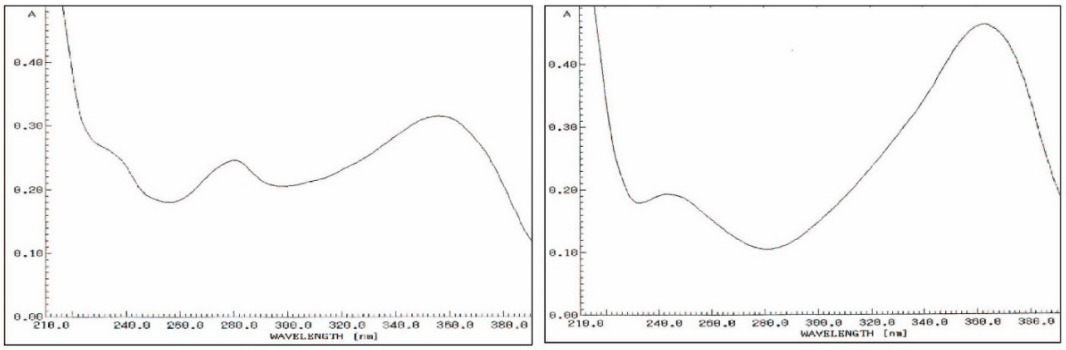
On the surface of the Petri plates, with nutrient agar for bacterial growth and Sabouraud maltose agar for fungi growth, inoculated with suspension of the test microorganisms, the sterilized stainless-steel cylinders (Schleicher and Schuell, Dassel, Germany) were placed, with an inner diameter of 6 mm in which 100 µL of solutions of CPHC and its inclusion complexes were deposited. For the purpose of comparison, standard antibiotic drug tablets of penicillin and gentamicin were used, as positive controls, in concentrations of 6 µg and 30 µg, respectively. DMSO and 2-HP-β-CD were used as negative controls because no response was expected in their cases. The period of diffusion prior to incubation was 2 hours. The incubation was carried out at 37 °C/18 hours for bacterial growth and at 25 °C/2 days for the fungi growth. After incubation, the diameters of inhibition zones of microbial growth were registered. The inhibition zones diameters (*I*) for bacterial and fungal strains were measured and expressed in millimetres (mm) (Ph.Eur., 2014). The results represent the mean diameters registered on three plates (Figure 10). Antibacterial activity of all test samples was also analysed by the dilution method. The aim of this method was to determine concentrations of the tested samples which will have an inhibitory effect on the growth of the test bacteria. For analysis by the dilution method, solutions of the test samples (CPHC, SE 1:1 and SE 1:2) were prepared, which were then followed by preparation of a series of twelve dilutions with a liquid nutritious base, concentrations range from 500 µg $mL^{-1}$ to 0.24 µg $mL^{-1}$. Casein soybean digest broth (Tryptic soybean bouillon/Tryptic soy broth, BD™ Tryptic Soy Broth, Becton Dickinson GmbH, Germany) was used in the dilution method. 2.0 mL of casein soybean digest broth was added to 2.0 mL of the starting solution of the test sample, thus forming the first dilution (Završnik, Špirtović-Halilović, Softić, 2011). Negative controls were prepared containing inoculum and free 2-HP-β-CD at test concentrations (4.32 mg $mL^{-1}$ and 8.63 mg $mL^{-1}$) to guarantee 2-HP-β-CD had no effect on antimicrobial effect. After a 24-hour incubation, using the dilution method, minimum inhibitory concentration (MIC) expressed in µg $mL^{-1}$, and the minimum bactericidal concentration (MBC), expressed in µg $mL^{-1}$ were determined. The MIC was determined as the lowest concentration of an antimicrobial agent that inhibited the visible growth of a microorganism after incubation. The lowest concentration of an antimicrobial agent needed to kill 99.9% of the final inoculum after incubation for 24 h was considered the MBC, also known as the minimum lethal concentration (MLC) (Balouiri, Sadiki, Ibnsouda, 2016).

## RESULTS AND DISCUSSION

3-(3-(2-Chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin, derivative of 3-cynnamoyl-4-hydroxycoumarin, was prepared by electrophilic reagents. Due to the presence of the hydroxyl group at the C4 position, which is the electron donor, the electrophilicity of the α-pyrone is increased so that the substitution reaction is directed to the C3 atom of 4-hydroxycoumarin. Aromatization and complete stabilization of the coumarin system occurs only when it is possible to extend the conjugated π-linkage system to the side chain, as is the case with 3-substituted derivatives such as 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin (Kulkarni, Patil, 1981).

### UV spectra of CPHC

Figure 3 shows UV spectra of CPHC in 96.0% v/v ethanol (Figure 3-A) and in acetonitrile (Figure 3-B). On the UV spectrum of ethanol solution of CPHC, concentration of 4.90 µg $mL^{-1}$, two absorption maxima were observed at 280.5 nm (A = 0.2466) and 360.0 nm (A = 0.3167) (Figure 3-A). Two absorption maxima, at 243.0 nm (A = 0.1814) and 364.0 nm (A = 0.4354) were observed on UV spectrum of acetonitrile solution of CPHC at the concentration of 5.04 µg $mL^{-1}$ (Figure 3-B).




A) solvent - 96.0% ethanol B) solvent - acetonitrile

**FIGURE 3 -** UV/VIS absorption spectra of CPHC in 96.0% and acetonitrile solutions.

The specificity of the coumarin ring is coumarin (4-hydroxy-benzopyran-2-one) - chromonic tautomerism, that can significantly affect the absorption and spectral properties of coumarin derivatives. Since the test substance consists of coumarin and cinnamoyl parts, its spectral properties can be affected by both parts of the molecule. The coumarin derivative 3-(3- (2-chlorophenyl) prop-2-enoyl)-4-hydroxycoumarin is substituted at the C3 position of the 4-hydroxycoumarin by cinnamoyl substituent. Usually the absorption maxima of the compound originate from the coumarin ring, at the lower wavelength, and from the phenyl substituent at the C3 position, at the higher wavelength. The electrophilic chlorine (Cl) substituent on the phenyl ring of the cinnamoyl portion of the test substance molecule does not affect the position of the absorption maximum of the phenyl ring (Kulkarni, Patil, 1981).

The position of the absorption maximum depends on the energy required to excite the ground state in the excited state of the compound. The amount of required energy depends on the transition. The width of the absorption band is greater if the duration of the excited state is shorter. The coumarin ring is dominated by $\pi \rightarrow \pi^*$ transition that is weakly dependent on the polarity of the solvent (Ammar, Forgues-Fery, El Gharbi, 2003).

When selecting a solvent, care was taken that the test substance is soluble in it. Ethanol is the most commonly used solvent for UV/VIS spectroscopic testing, dielectric constant of 24.3. Acetonitrile is a polar aprotic solvent, with dielectric constant of 35.85 at T = 298.15 K and p = 101.33 kPa (Côté *et al.*, 1996). Acetonitrile was not selected as the solvent for the spectrophotometric study of samples of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin with 2-HP-β-CD, since it forms the white precipitate of 2-HP-β-CD. 2-HP-β-CD also sticks on the walls of the weighing vessel in the form of a white layer. Aqueous solutions of 2-HP-β-CD and 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin diluted with acetonitrile, irrespective of the dilution factor, give milky solutions that clear up after shaking but to an opalescent appearance with a visible glassy transparent, thin border between aqueous solution and acetonitrile (Das *et al.*, 2013).

### Calibration curve of CPHC

Figure 4 shows the CPHC calibration curves used to quantify the results.

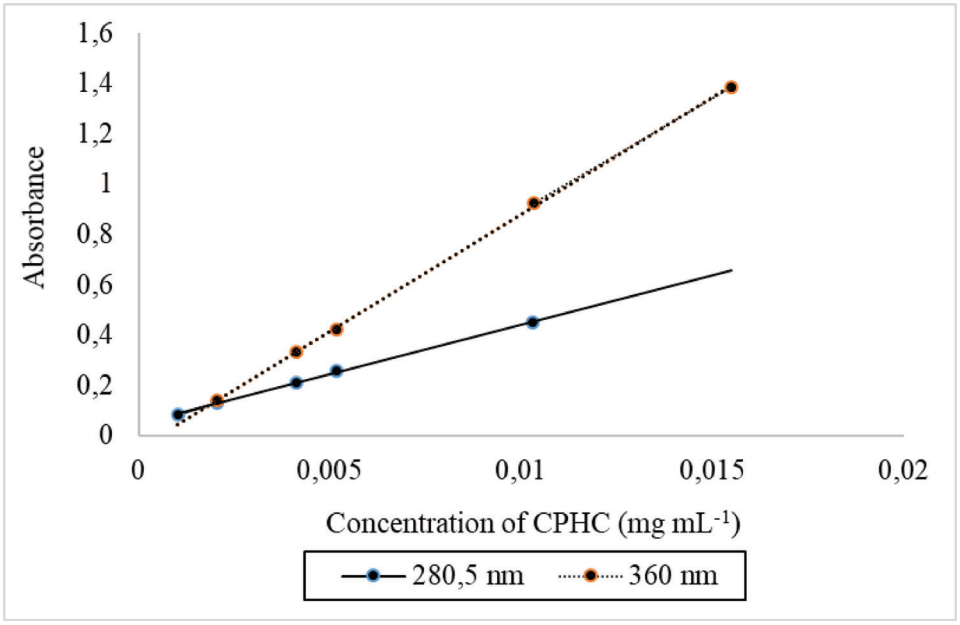



**FIGURE 4 -** Calibration curves of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin.

In the concentration range from 1.03 to 10.32 μg $mL^{-1}$ of ethanol solution of CPHC, the linear calibration curve ($R^2$ = 0.9994) was obtained at 280.5 nm, and for the concentration range from 2.07 to 15.51 mg $mL^{-1}$ at 360.0 nm ($R^2$ = 0.9997). The results showed that there is a highly linear relation of concentrations and absorptions in the test concentration range. UV/VIS spectroscopy can be used to determine the concentration of CPHC.

Figure 5 shows the absorbances of ethanol solutions of CPHC versus the obtained concentrations (mmol $L^{-1}$) of ethanol solutions of CPHC.

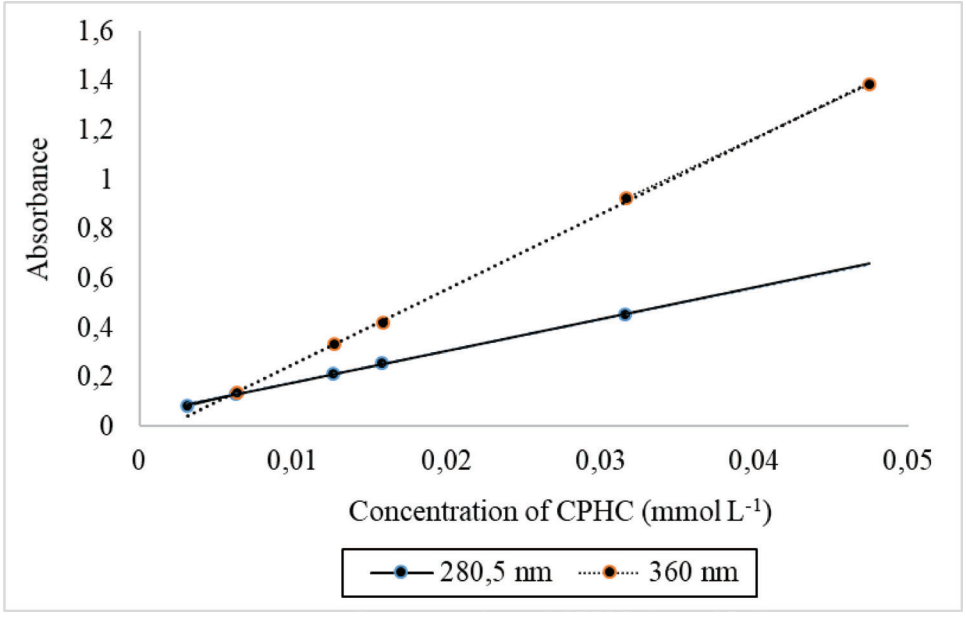



**FIGURE 5 -** Absorbances versus concentration.

The wavelength-dependent molar absorptivity coefficient was obtained using the Beer-Lambert's law: $A = \varepsilon \times l \times c$, where $A$ is absorbance (dimensionless), $\varepsilon$ - the molar absorptivity coefficient, which expresses the absorption intensities of the test compound, or its molar absorption capacity ($dm^3\ mol^{-1}.\ cm^{-1}/L\ mol^{-1.}\ cm^{-1}$), $l$ - the path length of the cell filled with the sample (cm), and $c$ - the concentration of the compound in the solution (in $mol\ dm^{-3}/mol\ L^{-1}$) (Abdul, 2018; Feigenbrugel *et al.*, 2005). The sensitivity of the UV/VIS spectrophotometric method for the test substance was determined by calculating the molar absorption coefficients.

The molar absorption coefficient ($\varepsilon$) of the ethanol solution of CPHC is $12.97 \times 10^3$ L $mol^{-1.}\ cm^{-1}$ at 280.5 nm, and the logarithmic value log$\varepsilon$ is 4.11, while at 360.0 nm $\varepsilon$ is $31.22 \times 10^3$ L $mol^{-1.}\ cm^{-1}$ and log$\varepsilon$ is 4.49. $\varepsilon$ values are in range from 0 to $10^6$ L $mol^{-1.}\ cm^{-1}$, where values above $10^4$ L $mol^{-1.}\ cm^{-1}$ indicate high molar absorptivity of the molecule, while values below $1 \times 10^3$ L $mol^{-1.}\ cm^{-1}$ indicate low absorption intensity, so the method is of low sensitivity (Abdul, 2018). The obtained molar absorption coefficient values indicate relatively intense molar absorption of the CPHC molecule dissolved in 96.0% v/v ethanol. In particular, highly conjugated molecules such as aromatic hydrocarbons and heterocycles typically exhibit absorption bands characteristic for $\pi \rightarrow \pi^*$ transitions and have significantly higher $\varepsilon$ values. Most of the coumarin derivatives, due to the $\pi \rightarrow \pi^*$ transition that dominates the coumarin ring, have high values of the molar absorption coefficient, with values ranging from $13.3 \times 10^3$ to $50 \times 10^3$ L $mol^{-1.}\ cm^{-1}$, and from $5.7 \times 10^2$ to $54 \times 10^3$ L $mol^{-1.}\ cm^{-1}$ when ethanol/methanol is used as the solvent (Taniguchi, Lindsey, 2018; Ammar, Forgues-Fery, El Gharbi, 2003).

## Content of CPHC in the physical mixtures and inclusion complexes

All the inclusion complexes prepared using kneading method (KN) and solvent evaporation method (SE) were found to be slightly yellow, free-flowing powders and easier to manipulate with than the test substance alone (3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin). The UV/VIS spectroscopy method for the assay of CPHC content in the physical mixtures (PM) and inclusion complexes gave percentage contents of 97.33 ± 1.124, 97.01 ± 1.590, 96.18 ± 0.882, 96.07 ± 0.924, 99.67 ± 0.380, 99.58 ± 0.771 for PM CPHC : 2-HP-β-CD 1:1, PM CPHC : 2-HP-β-CD 1:2, KN CPHC : 2-HP-β-CD 1:1, KN CPHC : 2-HP-β-CD 1:2, SE CPHC : 2-HP-β-CD 1:1, SE CPHC : 2-HP-β-CD 1:2, respectively. All the binary systems showed the presence of high CPHC content and good uniformity of the method employed for preparation. The CPHC content of physical mixtures and inclusion complexes prepared by kneading shows slightly less CPHC content as compared to that prepared by using the solvent evaporation method. The inclusion complexes prepared by the solvent evaporation method showed nearly 100% uniform CPHC content (with a low standard deviation). The method used in this study appears to be reproducible for preparation of CPHC binary systems. In order to compare antimicrobial activity, it was necessary to determine the CPHC content of the formed complexes.

## Solubility measurements

### *Solubility of CPHC in purified water*

The solubility values of CPHC (concentrations of dissolved CPHC) in purified water, at 25 °C ± 0.1 °C and 37 °C ± 0.1 °C, as functions of time are presented in Figure 6.

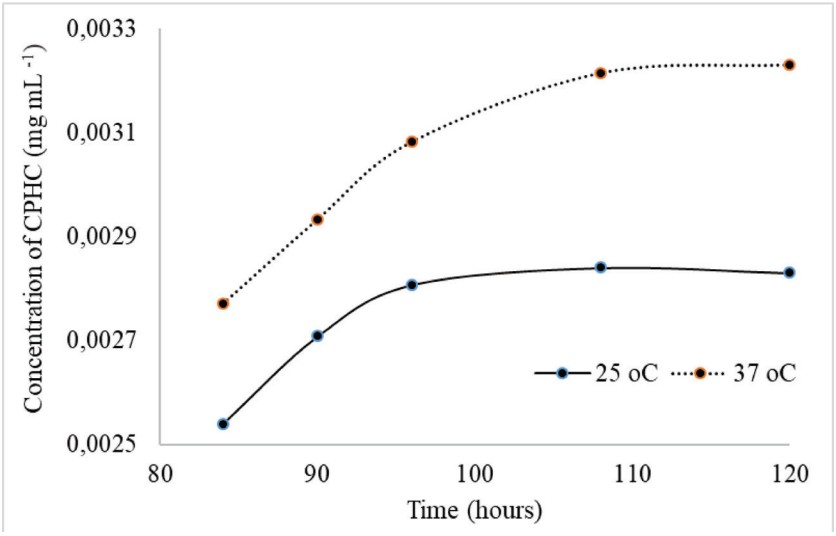



**FIGURE 6 -** Concentration of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin depending of in time.

CPHC is a voluminous powder that is not hydrophobic, so when in contact with purified water it is easily wetted. CPHC is practically insoluble in water. To dissolve 1 g of CPHC in water, 352.11 L of water (at 25 °C) and 311.53 L of water (at 37 °C) are required (Ph. Eur., 2014; Gupta 2013). The aqueous solubility of CPHC is 0.00321 mg $mL^{-1}$ (9.82 × $10^{-6}$ mol $L^{-1}$) at 37 °C ± 0.1 °C. The solubility of CPHC in purified water is 1.13 times higher at 37 °C than at 25 °C.

*CPHC solubility in aqueous buffer solutions*

Table I shows the change of CPHC solubility in aqueous buffer solutions with pH values from 1.2 to 7.4, compared to its solubility in purified water.

**TABLE I -** Influence of pH values on solubility of CPHC at 25 ± 0.1 °C and at 37 ± 0.1 °C

| pH value of aqueous buffer solutions | 1.2 | 4.5 | 6.8 | 7.4 |
|---|---|---|---|---|
| Solubility change of CPHC (fold), at 25 ± 0.1 °C | 0.36 | 1.65 | 2.06 | 11.42 |
| Solubility change of CPHC (fold), at 37 ± 0.1 °C | 0.76 | 2.31 | 2.53 | 12.86 |

Based on the results, it was found that CPHC solubility was increased with an increasing pH of the media, being more soluble in slightly alkaline medium, compared to the acidic medium. The results of the solubility testing of CPHC indicate that CPHC was practically insoluble in testing aqueous buffer solutions. At the pH 1.2, the solubility of CPHC (at 37 °C) decreased by 24%, while at the pH value of 7.4 (intestinal pH/colon, duodenum), the solubility of CPHC increased by 28.6% at 37 °C.

CPHC is weakly dissociated acid with pKa value of 4.22, determined using the CspKa computer program. During the dissolution, acids (as well as weak bases) partially dissociate, and their solubility in bodily fluids is strongly dependent on the acidity of the media. Only the non-ionized substance will be available for the absorption. Concentration of the non-ionized form will depend on the pH of the environment (Jayashree *et al*., 2014).

CPHC in chloride buffer solution pH 1.2 was 99.90% in the non-ionized form or 0.1% (0.099%), in dissociated form (Florence, Attwood, 2006). Since the absorption rate of the active substance dissolved in biological fluids depends on the concentration of non-ionized lipophilic part, and the concentration of non-ionized form of the active substance at the site of absorption depends on the degree of ionization and the pH at the site of absorption, based on the results we could expect good absorption of CPHC at pH 1.2 (as in case of gastric pH). In acid pH, solubility of CPHC (at 37 $^{o}$C) was $2.44 \times 10^{-3}$ mg mL$^{-1}$ which could be considered as the solubility of its non-ionized form (*Cs*). In this way, one could only approximate prediction of absorption, given that it was almost impossible to predict absorption for each drug molecule.

CPHC had low saturation solubility, *Cs*, $2.44 \times 10^{-3}$ mg mL$^{-1}$ (37 $^{o}$C) which hampers drug dissolution. If aqueous solubility of a substance/drug is below 0.1 - 0.05 mg mL$^{-1}$, the dissolution of the drug particles will be slow, thus limiting the dissolution rate. Rapid absorption requires rapid dissolution, which depends on greater aqueous solubility (Issa, Ferraz, 2011). According to the biopharmaceutics classification system (BCS), a drug substance is considered highly soluble in 250 mL or less of aqueous medium over the pH range (Guidance for Industry, 2017). Because dissolution depends on the drug solubility, and absorption depends on the intestinal permeability, these are the main factors that determine the bioavailability of an orally administered solid formulation. The BCS defines tree dimensionless numbers, dose number, dissolution number and absorption number, to characterize drug substances. These numbers are combinations of physicochemical and physiological parameters and are the most fundamental to the oral absorption process. The dose number is defined as the ratio of dose concentration to drug solubility (Equation 4) (Issa, Ferraz, 2011):

$$D_o = \frac{M / V_o}{C_s} \qquad \text{Equation 4}$$

where *Do* is the dose number, *M* is the dose of administered drug (mg), *Vo* is the volume of water taken with the dose which is generally set to be 250 mL, *Cs* is the saturation solubility of the drug/test substance in aqueous fluid at 37 $^{o}$C (maximum solubility of CPHC in an aqueous medium of pH 1.2 at 37 °C, mg mL$^{-1}$).

Probability of bioavailability problems requires consideration of both drug dose and its solubility, as well as permeability. Very poorly soluble substances which have very low therapeutic doses, should be completely dissolved under physiological conditions. The substance is considered to be poorly soluble if its highest dose is soluble in a larger volume of 250 mL of water or a suitable aqueous medium whose pH varies in the range of pH values of the gastrointestinal tract at 37 °C (within the range of pH 1 - 6.8, preferably at pH 1.2, 4.5 and 6.8) (Guidance for Industry, 2017; CHMP, 2010). *Do* is the ratio of drug concentration in the administered volume (250 mL) to the saturation solubility of the drug in water, that may also be viewed as the number of glasses of water (about 8 oz) required to dissolve the drug dose. A dose number equal or lower than 1 indicated high solubility, and $Do > 1$ signified a low solubility test substance (Issa, Ferraz, 2011).

Because CPHC is a newly synthesized compound (unknown therapeutic dose), the dose of administered drug, *M* (mg), is determined based on the desirable value of the dosage number, *Do* (to be the dose number equal or lower than 1), the volume of the medium required to completely dissolve the dose at minimum physiologic solubility, *Vo* (250 mL), and the determinated saturation solubility, *Cs* (mg mL$^{-1}$) of CPHC in aqueous buffer solutions whose pH varies in the range of pH values of the gastrointestinal tract at 37 °C. The lowest value of the physiological solubility of CPHC was taken as a reference value of the saturation solubility of the test substance in an aqueous medium, at 37 °C ($Cs = 2.44 \times 10^{-3}$ mg mL$^{-1}$ at pH 1.2). Given that the value of *Cs* of CPHC was less than $5 \times 10^{-2}$ mg mL$^{-1}$, the dissolution rate will be a limiting factor for absorption after oral administration of the drug substance. It was observed that CPHC administered in the therapeutic dose of 0.6 mg (or less) would be completely absorbed along whole gastrointestinal tract, since its dose number *(Do*) is not greater than 1 (or is less than 1). Water-soluble cyclodextrin complexes of drugs (such as a complex with 2-HP-β-CD) can increase their apparent *Cs* value, thus facilitating dissolution, leading later to

the enhanced oral bioavailability (Loftsson, Brewster, Masson 2004).

*Solubility of CPHC in aqueous solutions of 2-HP-β-CD*

Cyclodextrins in aqueous solution are capable of forming inclusion complexes with many drugs by accepting a drug molecule into their central cavity or, more frequently, only the lipophilic portion of the therapeutic moiety. The interactions of CPHC : 2-HP-*β*-CD in aqueous solution were investigated by the phase solubility analysis (Higuchi, Connors, 1965). The solubility of CPHC in the binary system containing 2-HP-β-CD of various concentrations (0% - 50% w/w) is illustrated in the phase solubility diagram, in Figure 7.

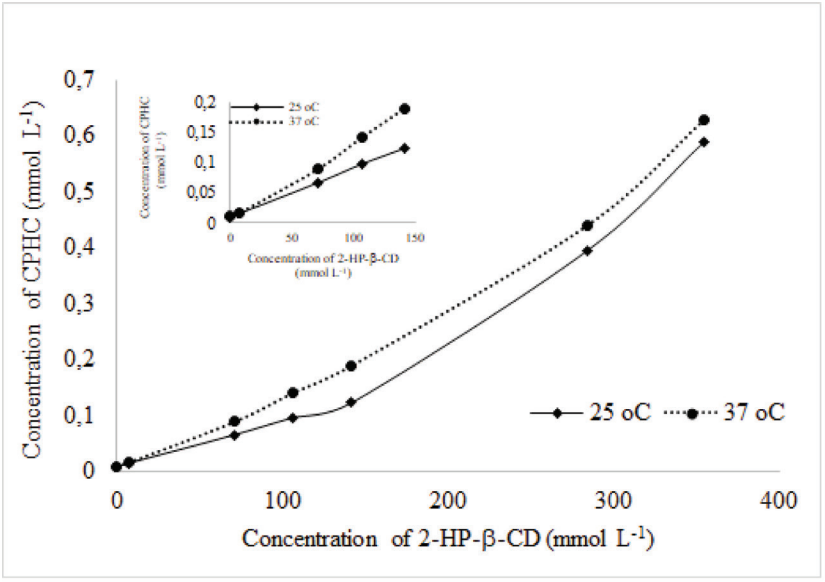



**FIGURE 7 -** Phase solubility diagram of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin and 2- hydroxypropyl-β-cyclodextrin in purified water at 25 °C and 37 °C ± 0.1 °C.

In the case of CPHC and 2-HP-β-CD in concentration range 0% - 50% w/w, the phase solubility curve was positively deviated from linearity: $R^2 = 0.9543$ at 25 °C, $R^2 = 0.9827$ at 37 °C. It was assigned as an AP - type. This indicates the formation of higher order inclusion complexes. The complex formed may be of second order or more with respect to 2-HP-β-CD concentrations (i.e., one CPHC binds to more than one 2-HP-β-CD). However, at a lower concentration of up to 20% w/w (141.84 mmol $L^{-1}$), as illustrated in the insert of Figure 7, a linear phase solubility curve was obtained: $R^2 = 0.9997$ at 25 °C, $R^2 = 0.9968$ at 37 °C. This suggests the formation of a first order complex between CPHC and 2-HP-β-CD. Linearity was characteristic of the AL - type system and suggested that water-soluble complexes were formed in the solution. The first part of the plot, used to calculate the apparent stability constants *Ks/K1:1*, can be used in the future experimental work for comparing with results of some other cyclodextrins that will be investigated in the concentration range of 0% - 20% w/w. The results of the change in CPHC solubility in purified water, depending on 2-HP-β-CD concentration, are listed in Table II.

**TABLE II -** Solubility of CPHC in aqueous solutions depending on the concentration of 2-HP-β-CD at 25 °C and 37 °C ± 0.1 °C

| S[a] | $S_{CPHC}$[b] (at 25 °C) | $S_{2\text{-HP-β-CD}}/S_o$[c] (at 25 °C) | $S_{CPHC}$[b] (at 37 °C) | $S_{2\text{-HP-β-CD}}/S_o$[c] (at 37 °C) |
|---|---|---|---|---|
| 7.092 | 0.0151 | 1.73 | 0.0160 | 1.63 |
| 70.92 | 0.0660 | 7.59 | 0.0889 | 9.05 |
| 106.38 | 0.0967 | 11.11 | 0.1410 | 14.34 |
| 141.84 | 0.1233 | 14.18 | 0.1897 | 19.30 |
| 283.69 | 0.3948 | 45.37 | 0.4396 | 44.72 |
| 354.61 | 0.5902 | 67.84 | 0.6296 | 64.05 |

[a]S - Concentration of 2-HP-β-CD (mmol $L^{-1}$); [b]SCPHC - Concentration of CPHC (mmol $L^{-1}$); [c]S2-HP-β-CD/*So* - Solubility enhancement factor calculated as the ratio of CPHC solubility in 2-HP-β-CD solution (S2-HP-β-CD) versus CPHC solubility value (*So*) measured in the absence of 2-HP-β-CD (values of *So* are listed in Table III)

The increase in solubility of CPHC displayed the concentration dependency on 2-HP-β-CD. The results of the phase solubility showed a relative positive effect of 2-HP-β-CD on CPHC in aqueous solutions (Table II). Hydroxypropyl-β-cyclodextrin, in concentration 1% - 50% w/w, increased the solubility of CPHC 1.63- to 64.05-fold at 37 °C. This may be caused by the release of water molecules bound in the cyclodextrin cavity (Ol'khovich *et al.*, 2014). The solubility of CPHC (from 0.00321 mg $mL^{-1}$) was increased 19.30-fold (up to 0.06306 mg $mL^{-1}$) with 20% w/w of 2-HP-*β*-CD at 37 °C.

The *So* (the solubility of CPHC in aqueous solution without the existence of 2-HP-β-CD expressed in mmol $L^{-1}$ i.e. mM), *slope* (the slope of the straight line), *Ks*/*K1:1* (the apparent stability constant/stability constant of the 1:1 complex, L $mol^{-1}$, calculated according to Equation 1), *CE* (the complexation efficiency calculated from the *slope* of the phase solubility diagram according to Equation 2) and *D* : *CD* (the drug/test substance : cyclodextrin/CPHC : 2-HP-β-CD molar ratio is based on the calculated complexation efficiency according to Equation 3) values of CPHC : 2-HP-β-CD complexes are summarized in Table III.

**TABLE III -** The saturation concentrations of CPHC in pure water, CPHC : 2-HP-β-CD s*lopes,* apparent stability constants, the complexation efficiency of CPHC : 2-HP-β-CD complexes and the CPHC: 2-HP-β-CD ratio, at 25 °C and 37 °C ± 0.1 °C

| Temperature (°C) | $S_o$ (× $10^{-3}$ mM) | *slope* | $K_{1:1}$ ($M^{-1}$) | *CE* (× $10^{-3}$) | *D* : *CD* |
|---|---|---|---|---|---|
| 25 | 8.6919 | 0.0008 | 92.0598 | 0.8002 | 1 : 1250.69 |
| 37 | 9.8243 | 0.0013 | 132.5043 | 1.3018 | 1 : 769.17 |

The *slope* of the resulting phase solubility profile can give valuable information on the type of complex formed in terms of stoichiometry. The steeper the *slope*, the better is the complexation, and at values of 1.0, 1.0 mole of cyclodextrin ("host") is complexing 1.0 mole of drug ("guest"). Although the *slope* value was lower than 1, the inclusion complexes in molar ratio of 1:1 were formed between the "guest" (CPHC) and "host" molecule (2-HP-β-CD) (Jambhekar, Breen, 2016). If the *slope* of a linear diagram is greater than 1, but less than 2, the complex formed is likely to be of the second, or higher, order with respect to the drug, but of the first order with respect to cyclodextrin (Saokham *et al.*, 2018).

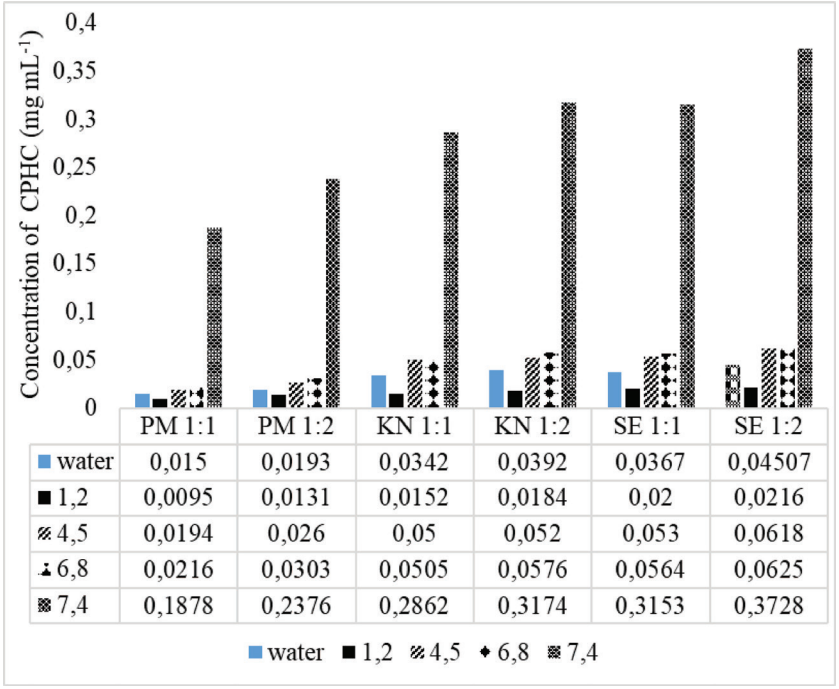
The apparent stability constant (*Ks*) is a fundamental property that describes the strength of an interaction between a drug and a cyclodextrin. For

many drug/cyclodextrin complexes, apparent stability constant values are in the range of 50 to 2000/5000 $M^{-1}$. Small *Ks* values (CPHC : 2-HP-β-CD, at 25 °C) indicate too weak interaction, whereas a larger value indicates the possibility of limited drug release from the complex. Weak interaction of the very labile complexes (*Ks* < 100) results in premature release of the "guest" and an insignificant improvement in solubility. In cases of very high *Ks* (*Ks* > 5000), the complexes are very stable and the release of the "guest" from the cyclodextrin cavity is incomplete or obstructed. Optimal values of the apparent stability constant are from 100 to 1000 $M^{-1}$ (Jambhekar, Breen, 2016; Loftsson, Brewster, 2011). The apparent stability constants (*Ks*/*K1:1*) of the binary complexes, CPHC : 2-HP-β-CD, were calculated using the linear regression analysis method from the diagrams according to the Equation mentioned above (Equation 1). The value of *Ks*/*K1:1* with CPHC : 2-HP-β-CD binary complex at 37 °C was 132.50 $M^{-1}$ and it is slightly higher in comparison to the value of *Ks*/*K1:1* of CPHC : 2-HP-β-CD complex at 25 °C which is typical of the endothermic process of complexation. The calculated apparent stability constant was low for CPHC: 2-HP-*β*-CD, indicating that a relatively high amount of 2-HP-*β*-CD is required to achieve complexation of CPHC (Ol'khovich *et al.*, 2014).

Determination of *CE* is a simple method for quick evaluating the solubilizing effects of different cyclodextrins. The *CE* and *D : CD* of binary systems as a result of effect of the 2-HP-*β*-CD on the saturation concentration (*So*), at 25 °C and 37 °C, are presented in Table III. From our phase solubility profile of CPHC with 2-HP-*β*-CD (37 °C ± 0.1°C), the *CE* of $1.3 \times 10^{-3}$ was calculated, indicating that approximately one of every 769 cyclodextrin molecules forms a complex with CPHC (Loftsson, Hreinsdóttir, Másson, 2007).

### *Solubility of physical mixtures and inclusion complexes CPHC with 2-HP-β-CD in purified water and aqueous buffer solutions*

The concentration of dissolved binary physical mixtures (PM 1:1 and PM 1:2) and inclusion complexes (KN 1:1, KN 1:2, SE 1:1 and SE 1:2) of CPHC with 2-HP-β-CD in purified water and aqueous buffer solutions at 37 °C ± 0.1 °C are shown in Figure 8.




| | PM 1:1 | PM 1:2 | KN 1:1 | KN 1:2 | SE 1:1 | SE 1:2 |
|---|---|---|---|---|---|---|
| water | 0,015 | 0,0193 | 0,0342 | 0,0392 | 0,0367 | 0,04507 |
| 1,2 | 0,0095 | 0,0131 | 0,0152 | 0,0184 | 0,02 | 0,0216 |
| 4,5 | 0,0194 | 0,026 | 0,05 | 0,052 | 0,053 | 0,0618 |
| 6,8 | 0,0216 | 0,0303 | 0,0505 | 0,0576 | 0,0564 | 0,0625 |
| 7,4 | 0,1878 | 0,2376 | 0,2862 | 0,3174 | 0,3153 | 0,3728 |

**FIGURE 8 -** Solubility of physical mixtures and inclusion complexes of 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin with 2- hydroxypropyl-β-cyclodextrin in purified water and aqueous buffer solutions at 37 °C ± 0.1 °C.

The solubility change factor of physical mixtures and inclusion complexes of CPHC with 2-HP-β-CD in purified water and aqueous buffer solutions at 37 °C ± 0.1 °C are summarized in Table IV. The solubility change factor was calculated as the ratio of CPHC solubility in purified water/aqueous buffer solutions at 37 °C ± 0.1 °C versus solubility of physical mixtures and inclusion complexes of CPHC with 2-HP-β-CD in purified water/aqueous buffer solutions at 37 °C ± 0.1 °C.

**TABLE IV -** Solubility change factor of binary physical mixtures and inclusion complexes of CPHC with 2-HP-β-CD in purified water and aqueous buffer solutions at 37 °C ± 0.1 °C

| Sample | Solubility change factor in medium (purified water/aqueous buffer solutions) | | | | |
|---|---|---|---|---|---|
| | Water | pH 1.2 | pH 4.5 | pH 6.8 | pH 7.4 |
| PM 1:1 | 4.67 | 3.89 | 2.62 | 2.65 | 4.50 |
| PM 1:2 | 6.02 | 5.35 | 3.51 | 3.73 | 5.76 |
| KN 1:1 | 10.66 | 6.23 | 6.75 | 6.21 | 6.63 |
| KN 1:2 | 12.20 | 7.55 | 7.02 | 7.09 | 7.69 |
| SE 1:1 | 11.44 | 8.21 | 7.15 | 6.64 | 7.64 |
| SE 1:2 | 14.04 | 8.89 | 8.34 | 7.69 | 9.03 |

The best CPHC solubility in purified water was achieved with SE 1:1 and SE 1:2 of all the tested samples. Table IV shows that the solubility of SE 1:1 and SE 1:2 in purified water is increased 11.44/14.04-fold (0.037 mg of SE 1:1/0.045 mg of SE 1:2/mL purified water) compared to SE 1:1/SE 1:2 solubility in purified water. The SE 1:1 and SE 1:2 solubility in phosphate buffer solution pH 7.4 is increased 98.22/116.14-fold (0.315 mg of SE 1:1/0.373 mg of SE 1:2/mL phosphate buffer solution pH 7.4) compared to CPHC solubility in purified water ($3.21 \times 10^{-3}$). If the solubility of inclusion complexes of CPHC with 2-HP-β-CD, SE 1:1 and SE 1:2, was expressed in descriptive terms, then these inclusion complexes were very slightly soluble at pH 7.4 (1000 to 10000 parts of solvent required for 1 part of solute), practically insoluble in purified water (≥ 10000 parts of solvent/water required for 1 part of solute/CPHC). To dissolve 1 g of SE 1:1/SE 1:2 in phosphate buffer solution pH 7.4, 3.17 L/2.68 L of phosphate buffer solution pH 7.4 at 37 °C is required (Ph.Eur. 2014).

It was determinated that the *Cs* of SE 1:1 and SE 1:2 in chloride buffer solution pH 1.2 at 37 °C is 20.03 $\times 10^{-3}$ mg mL$^{-1}$ (*Cs*(SE 1:1)) and 21.68 $\times 10^{-3}$ mg mL$^{-1}$ (*Cs*(SE 1:2)) (Figure 8). Given that the value of *Cs* of SE 1:1 and SE 1:2 was less than $5 \times 10^{-2}$ mg mL$^{-1}$, the dissolution rate will be a limiting factor for absorption after oral administration of the complexed CPHC. If the SE 1:1 and SE 1:2 inclusion complexes would be applied in therapeutic doses of 5.01 mg and less, and 5.42 mg and less of CPHC, these complexes could be well absorbed from gastrointestinal tract ($Do \leq 1$) (Loftsson, Brewster, Masson, 2004). 2-HP-β-CD, as a complexing agent, was shown to increase the solubility of CPHC and increase the therapeutic dose of CPHC (from 0.6 mg to 5 mg) to get the dose number to be value up to 1 and volume of fluid required for complete dissolution of CPHC administered in a single dose.

**FTIR-ATR absorption measurements of solid binary systems of CPHC and 2-HP-β-CD**

A significant shift in intensity of characteristic absorption bands for CPHC in FTIR range, whether toward lower or higher frequencies, confirms

its complexation into a molecule of 2-HP-β-CD. Disappearance and widening or CPHC's absorption band indicate the inclusion of CPHC into 2-HP-β-CD molecule (Pralhad, Rajendrakumar, 2004). In the present study, the FTIR-ATM technique is used to assess the possible formation of inclusion complexes that might arise due to the interaction between the 2-HP-β-CD and the CPHC. The FTIR-ATM spectra of CPHC, its physical mixtures with 2-HP-β-CD (PM CPHC : 2-HP-β-CD 1:1 and PM CPHC : 2-HP-β-CD 1:2), the inclusion complexes prepared by different methods (KN CPHC : 2-HP-β-CD 1:1, KN CPHC : 2-HP-β-CD 1:2, SE CPHC : 2-HP-β-CD 1:1, SE CPHC : 2-HP-β-CD 1:2) are given in Figure 9.

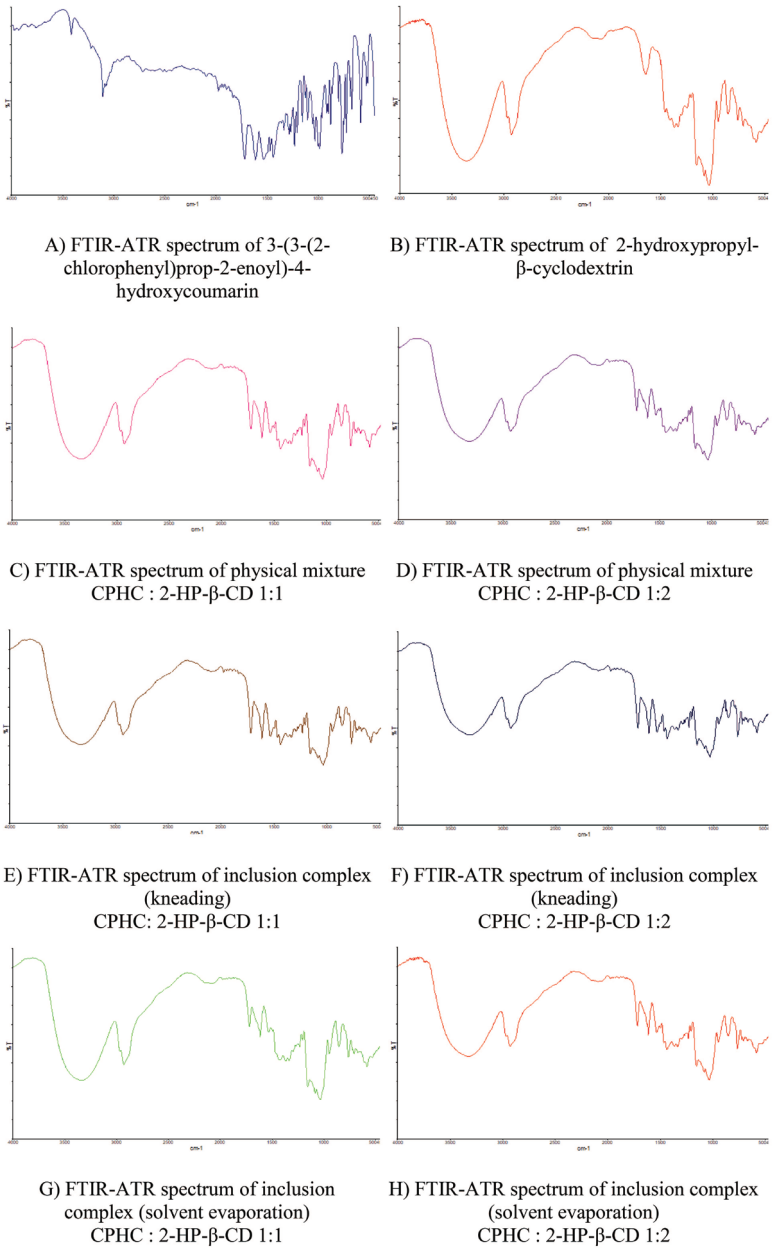



**FIGURE 9 -** FTIR-ATR spectra in the 4000 - 450 $cm^{-1}$ range of A) CPHC, B) 2-HP-β-CD, C) PM CPHC : 2-HP-β-CD 1:1, D) PM CPHC : 2-HP-β-CD 1:2, E) KN CPHC : 2-HP-β-CD 1:1, F) KN CPHC : 2-HP-β-CD 1:2, G) SE CPHC : 2-HP-β-CD 1:1, H) SE CPHC : 2-HP-β-CD 1:2.

Absorption bands at wave numbers of 3102 $cm^{-1}$, 1579.27 $cm^{-1}$, 1480.68 $cm^{-1}$, 1456.33 $cm^{-1}$, 1439.93 $cm^{-1}$, characteristic of aromatic C - H bonds, were observed on the FTIR-ATM spectrum of CPHC (Figure 9-A). At 1718 $cm^{-1}$, an absorption band corresponding to the lactone C = O group and the carbonyl group at position 2 in the pyrone ring of 4-hydroxycoumarin was observed. An absorption band originating from (C (= O) CH = CH) groups was observed at the wavenumber of 1614.54 $cm^{-1}$. An absorption band originating from the hydroxyl (OH) group at position 4 of coumarin moiety was observed at wave number of 1230.34 $cm^{-1}$. At 766.13 $cm^{-1}$, an absorption band occurs, characteristic for the elongation vibration of chlorine atom at the *meta* position of the phenyl ring (*m*-Cl) (Stuart, 2004).

FTIR-ATM spectrum of 2-HP-β-CD (Figure 9-B) shows characteristic, wide and intensive radius of vibrations frequencies for hydroxy groups in the range between 3791 $cm^{-1}$ - 3017.67 $cm^{-1}$. Characteristic absorption bands for 2-HP-β-CD at 2929.82 $cm^{-1}$ come from stretching vibrations of –CH and –CH2 groups. At 1154.77 $cm^{-1}$ there is an absorption band as a result of ether-like bonds between cyclically linked glucose molecules, as well as a band at 1071.59 $cm^{-1}$ and 1034.78 $cm^{-1}$ coming from stretching vibrations of hydroxy groups. Band at 757.38 $cm^{-1}$ is a result of the deformation vibrations outside of the plane (δ), flexion of –C – H bonds of aromatic core.

At FTIR spectra of CPHC inclusion complexes: 2-HP-β-CD prepared by kneading (Figure 9-E and 9-F), a wide, intense absorption band was identified in the spectral range from 3791 $cm^{-1}$ to 3015.95 $cm^{-1}$/3016.87 $cm^{-1}$, similar to physical mixtures of CPHC: 2-HP-β-CD (Figure 9-C and 9-D), which originates from the vibration of the stretching of primary hydroxy groups on C6 atoms of glucose molecules located on the narrower side of the 2-HP-β-CD ring which covers the absorption band at 3102 $cm^{-1}$ originating from the aromatic C – H bond of the CPHC. An absorption band was identified at wavelength of 2929.81 $cm^{-1}$/2930.19 $cm^{-1}$ originating from -CH and -CH2 stretching vibrations of the 2-HP-β-CD molecule and at 1154.38 $cm^{-1}$/1154.18 $cm^{-1}$ originating from ether-like bond between cyclically linked glucose molecules of 2-HP-β-CD (for KN 1:1/KN 1:2, similar to PM 1:1/PM 1:2). At KN 1:1/KN 1:2 the absorption bands of 2-HP-β-CD hydroxy groups were observed at 1071.89 $cm^{-1}$/1071.68 $cm^{-1}$ and 1031.66 $cm^{-1}$/1032.01 $cm^{-1}$. In both KN 1:1/KN 1:2 and PM 1:1/PM 1:2, 2-HP-β-CD increased CPHC hydrophilicity. The absorption band of the CPHC lactone C = O group at 1718 $cm^{-1}$ was slightly shifted to 1717.28 $cm^{-1}$ at KN 1:1/1717.56 $cm^{-1}$ at KN 1:2 and it is of lower intensity (CPHC - 9.11 %T; KN 1:1/PM 1:1 - 40.09 %T/34.59 %T; KN 1:2/PM 1:2 - 46.62 %T/not identified). The CPHC absorption band at the 1414.54 $cm^{-1}$ of (C (=O) CH = CH) group and at 766.13 $cm^{-1}$ of *m*-Cl was slightly shifted and of lower intensity, as was the absorption band at 1230.34 $cm^{-1}$ of the OH group of CPHC.

Tables V and VI show the difference in frequencies between 2-HP-β-CD and the inclusion complexes prepared by the solvent evaporation method (SE), and between CPHC and inclusion complexes prepared by the same method.

**TABLE V -** Comparison between the intensity of FTIR signals of 2-HP-β-CD and the inclusion complexes prepared by solvent evaporation method

| Functional group | Wavenumber ($cm^{-1}$) | | Changes |
|---|---|---|---|
| | 2-HP-β-CD | Inclusion complex SE 1:1/SE 1:2 | Δδ (SE 1:1/SE 1:2) |
| ν[OH] symmetric and antisymmetric | 3364.06 | 3342.12/3326.95 | - 21.94/- 37.11 |
| ν[$CH_2$] | 2929.82 | 2929.74/2930.46 | - 0.08/0.64 |
| ν[C–C] | 1154.77 | 1154.43/1154.27 | - 0.34/- 0.50 |
| ν[O–H] bending vibration | 1034.78 | 1032.49/1032.02 | - 2.29/- 2.72 |

**TABLE VI -** Comparison between the intensity of FTIR signals of CPHC and the inclusion complexes prepared by solvent evaporation method

| Functional group | Wavenumber ($cm^{-1}$) | | Changes | Transmittance (%) |
|---|---|---|---|---|
| | CPHC | Inclusion complex SE 1:1/SE 1:2 | $\Delta\delta^d$ (SE 1:1/SE 1:2) | CPHC/SE 1:1/ SE 1:2 |
| **ν[C = O lactone]** | 1718 | 1717.28/1717.26 | - 0.72/- 0.74 | 9.11/53.99/53.93 |
| ν[(C (= O) CH = CH)] | 1614.54 | 1614.42/1614.46 | - 0.12/- 0.08 | 8.53/48.65/49.05 |
| ν[OH] | 1230.34 | 1231.66/1231.03 | 1.32/0.69 | 15.82/43.19/47.41 |
| ν[m-Cl] | 766.13 | 763.88/765.54 | - 2.25/- 0.59 | 11.71/37.73/41.84 |

$^{d}D\delta = \delta_{(\text{inclusion complex SE 1:1/SE 1:2})} - \delta_{(\text{uncomplexed /free CPHC})}$

Tables V and VI show some decrease and increase in intensity changes, Δδ. The decrease in the frequency between the inclusion complexes and their constituent molecule is due to the changes in the microenvironment which lead to the formation of hydrogen bonding and the presence of van der Waals forces during their interaction to form the inclusion complex. The increment is due to the insertion of the benzene part ring into the electron rich cavity of 2-HP-β-CD and will increase the electron cloud density, which leads to an increase in frequency (Sambasevam *et al.*, 2013).

At the FTIR spectra of CPHC inclusion complexes with 2-HP-β-CD prepared by solvent evaporation (Figures 9-G and 9-H), the absence of an absorption band at the wave number 3102 cm-1 is noticed, originating from the C - H bond of the CPHC aromatics. Based on the identified absorption bands originating from the stretching vibrations of the OH groups, the conservation of the hydrophilic outer part of the 2-HP-β-CD molecule was confirmed. The CPHC absorption bands compared to the SE 1:1/SE 1:2 absorption bands are slightly shifted and of lower intensity (Table VI). The SE 1:1/SE 1:2 absorption bands in the spectral range from 707.19 $cm^{-1}$/718.14 $cm^{-1}$ to 461.4 $cm^{-1}$/462.17 $cm^{-1}$ were identified, characteristic of the 2-HP-β-CD molecule. These samples resulted in partial incorporation of the CPHC molecule into the 2-HP-β-CD molecule.

The FTIR-ATM spectra of physical mixtures/ inclusion complexes of CPHC and the 2-HP-β-CD revealed no significant changes in absorption bands. However, the FTIR spectra of inclusion complexes prepared by solvent evaporation method revealed that the major peaks corresponding to the CPHC were affected. From the FTIR studies it can be concluded that the aromatic functional groups of the CPHC could have interacted with OH groups of the 2-HP-β-CD through hydrogen bonding thereby forming partially the inclusion complexes (variations in relative intensities, shifts). Some of the peaks of CPHC were slightly shifted and found to be attenuated. The binary systems of CPHC and 2-HP-β-CD did not show any new peaks, indicating noncovalent interaction in the inclusion complex.

FTIR-ATR technique has been found a suitable method for the characterization of inclusion phenomena, especially if used in conjunction with other experimental techniques and/or numerical approaches (Khalafi, Rafiee, 2013).

**Biological characterization of CPHC and inclusion complexes of CPHC with 2-HP-β-CD prepared by solvent evaporation method**

The diffusion method in antimibacterial activity assessment is based on monitoring the growth inhibition of a specific microorganism caused by a certain concentration of the tested sample. Figure 10 summarizes the results of antimicrobial/antibacterial activity for uncomplexed CPHC and its inclusion complexes (SE 1:1 and SE 1:2) against the various strains of microorganisms.

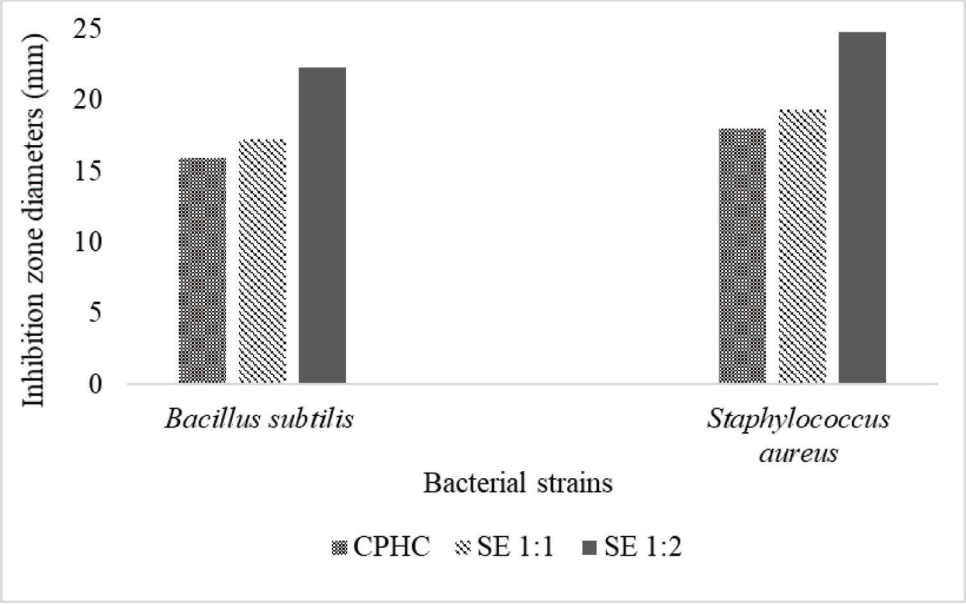



**FIGURE 10 -** Antibacterial activity of inclusion complexes prepared by solvent evaporation method compared with uncomplexed 3-(3-(2-chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin.

The biological activity of CPHC and its complexes (SE 1:1 and SE 1:2) was determined to estimate the possible differences in their antimicrobial activity. The tested samples (CPHC, SE 1:1, SE 1:2) were ineffective against fungi - *Saccharomyces cerevisiae* and *Aspergillus brasiliensis* and against Gram-negative type of bacteria - *Escherichia coli* (data not shown). Gram-negative bacteria are much more resistant to antimicrobial agents than Gram-positive bacteria because those have more lipophilic membrane than Gram-positive bacteria. Penicillin and gentamicin were used as a positive control because their activity against the tested strains is already known and could be compared with the activity of the investigated samples (Završnik, Špirtović-Halilović, Softić, 2011). DMSO and 2-HP-β-CD**,** as negative controls, did not inhibit the growth of the test organisms. The diffusion method showed that the test samples (CPHC, SE 1:1 and SE 1:2) have growth inhibition zones when it comes to Gram-positive aerobic bacteria *Bacillus subtilis* (*I* = 15.9 - 22.3 mm - week to high antibacterial activity) and *Staphylococcus aureus* (*I* = 18 - 24.8 mm - moderate to strong antibacterial activity) (Savić *et al*., 2019). CPHC inhibited the growth of *Bacillus subtilis* and *Staphylococcus aureus*, but the inhibition zones were approximately 16 mm for *Bacillus subtilis* (week antibacterial activity), apropos less than 19 mm for *Staphylococcus aureus* (moderate antibacterial activity). Unlike CPHC, the inclusion complexes SE 1:1 and SE 1:2 expressed the activity against *Bacillus subtilis*/ *Staphylococcus aureus* with the inhibition zones higher than 17 mm/19 mm (SE 1:1 - moderate antibacterial activity) i.e. slightly higher than 22 mm (SE 1:2 high antibacterial activity)/higher than 24 mm (SE 1:2 - strong antibacterial activity). The strain *Staphylococcus aureus* showed higher sensitivity to test samples. The greater antibacterial activity of inclusion complexes (SE 1:1 and SE 1:2) compared with uncomplexed CPHC was probably the result of the ability of 2-HP-β-CD to release the CPHC readily from the inclusion complexes. The improvement of antibacterial activity of inclusion complexes could be caused due to the increase of aqueous solubility of CPHC (Leclercq, 2016).

MICs and MBCs of CPHC and its 2-HP-β-CD inclusion complexes against *Bacillus subtilis* and *Staphylococcus aureus* are shown in Tables VII and VIII, respectively. The values shown refer to the lowest concentration of uncomplexed or 2-HP-β-CD-complexed CPHC for which a change was observed after 24 h incubation at 37 °C in tryptic soy broth. MIC and MBC values are given based on CPHC concentration.

**TABLE VII -** Minimum inhibitory and bactericidal concentration (MIC, MBC) against *Bacillus subtilis* for uncomplexed CPHC and inclusion complexes (SE 1:1 and SE 1:2)

| Antibacterial test samples | MIC (μg $mL^{-1}$) | MBC (μg $mL^{-1}$) |
|---|---|---|
| CPHC | 3.90 | 7.81 |
| SE 1:1 | 1.95 | 7.81 |
| SE 1:2 | 0.97 | 3.90 |

**TABLE VIII -** Minimum inhibitory and bactericidal concentration (MIC, MBC) against *Staphylococcus aureus* for uncomplexed CPHC and inclusion complexes (SE 1:1 and SE 1:2)

| Antibacterial test samples | MIC (μg $mL^{-1}$) | MBC (μg $mL^{-1}$) |
|---|---|---|
| CPHC | 3.90 | 15.63 |
| SE 1:1 | 0.97 | 3.90 |
| SE 1:2 | 0.49 | 1.95 |

MICs values for uncomplexed CPHC were equivalent, 3.90 μg $mL^{-1}$, for both *Bacillus subtilis* and *Staphylococcus aureus*. MICs values for SE 1:1 showed improvement in antibacterial inhibition (MIC) ranging from 50.00 to 75.13% better activity than uncomplexed CPHC for *Bacillus subtilis* while MICs values for SE 1:2 showed improvement in inhibition ranging from 75.13 to 88.97% better activity than uncomplexed CPHC for *Staphylococcus aureus*. MBCs values indicating CPHC's bactericidal (complexed: SE 1:1 and SE 1:2) activity ranged in concentrations from 7.81 to 3.90 μg $mL^{-1}$ for *Bacillus subtilis* and from 3.90 to 1.95 μg $mL^{-1}$ for *Staphylococcus aureus*. The results of the negative controls indicated complete absence of inhibition toward both bacteria species (data not shown).

By complexation of CPHC into the hydrophobic cavity of 2-HP-β-CD, its water solubility is enhanced, thanks to the hydrophilic surface of the used 2-HP-β-CD. That way, CPHC is more available by diffusion for cell's membrane of Gram-positive aerobic bacteria *Bacillus subtilis* and *Staphylococcus aureus*. The SE 1:1 and SE 1:2 showed better antibacterial activity than uncomplexed CPHC for both bacteria, indicating that 2-HP-β-CD increased water solubility and consequently increased contact between CPHC and bacteria in the medium. The MIC and MBC values of SE 1:1 and SE 1:2 were lower for both bacteria species probably due to the increase in the CPHC-carrier contact surface as a consequence of the interaction of 2-HP-β-CD with the lipid membrane components. Formation of water-soluble drug/cyclodextrin inclusion complexes can increase the aqueous solubility of the drug, increase its chemical and physical stability, and enhance drug permeation through biological membranes (Saokham *et al.*, 2018; Leclercq, 2016). In relation to the biological activities of compounds complexed with cyclodextrins, it is essential to evaluate the effect of the drug in the complexed (SE 1:1 and SE 1:2) and uncomplexed (CPHC) states, using an equivalent dose of the drug (CPHC). *In vitro* studies, such as diffusion and dilution methods, of inclusion complexes with cyclodextrins may provide evidence of their therapeutic effect (Carneiro *et al.*, 2019; Leclercq, 2016). Since it is possible to use a lower concentration of CPHC (in complexed form), these results provide guidance for the future preparation of pharmaceutical dosage forms which contain this antibacterial compound.

## CONCLUSION

3-(3-(2-Chlorophenyl)prop-2-enoyl)-4-hydroxycoumarin (CPHC) is an almost water insoluble

substance and weakly dissociated acid. The aqueous solubility of CPHC is $9.82 \times 10^{-6}$ mol $L^{-1}$ at 37 °C ± 0.1 °C. The solubility of non-ionized form of CPHC was $2.44 \times 10^{-3}$ mg $mL^{-1}$ at 37 °C. The results of phase solubility studies showed that CPHC formed the inclusion complexes of AP type with 2-HP-β-CD in a concentration of up to 354.61 mmol $L^{-1}$. The phase solubility diagrams of the studied CPHC showed that its solubility rises linearly as the 2-HP-*β*-CD concentration increases up to 141.84 mmol $L^{-1}$. The linear solubility growth can be explained by the formation of inclusion complexes between CPHC and 2-HP-*β*-CD in molar ratio of 1:1. The value of the apparent stability constant of CPHC and 2-HP-β-CD at 37 °C, calculated from the phase solubility diagram using the linear regression analysis method, was 132.50 $M^{-1}$ which is typical of the endothermic process of complexation of CPHC into the hydrophobic cavity of 2-HP-β-CD. The solubility of CPHC was increased 64.05-fold in the presence of 354.61 mmol $L^{-1}$ of 2-HP-*β*-CD dissolved in purified water, at 37 °C. All the inclusion complexes prepared using the kneading method and the solvent evaporation method were found to be slightly yellow, free-flowing powders and easier to manipulate with than the CPHC alone. The inclusion complexes, in solid state, prepared by the solvent evaporation method (SE) showed better results, i.e. nearly 100% uniform CPHC content, higher solubility of CPHC in purified water and in aqueous buffer solutions. The FTIR-ATM spectra of solid CPHC : 2-HP-β-CD inclusion complexes prepared by the solvent evaporation method present a high resemblance with FTIR-ATM spectrum of 2-HP-β-CD because of significant decreases in intensity of CPHC peaks, and showed the formation of new hydrogen intermolecular bonds between the two molecules (CPHC and 2-HP-β-CD). CPHC, SE 1:1, SE 1:2 showed antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* while all other strains remained resistant. When comparing the antibacterial activity of the inclusion complexes prepared by the solvent evaporation method with uncomplexed CPHC, the inclusion complexes showed higher activity on *Bacillus subtilis* and *Staphylococcus aureus*. The improvement of antibacterial activity could be caused due to the increase of aqueous solubility of CPHC with 2-HP-β-CD and enhanced CPHC availability and permeation through the bacterial membrane of. The *Staphylococcus aureus* showed higher sensitivity to test samples (CPHC, SE 1:1, SE 1:2). The antibacterial activity of CPHC was enhanced after inclusion in 2-HP-β-CD, thereby expanding the possibility of using the CPHC inclusion complexes in order to improve the pharmacological effects of CPHC. Further experiments are needed in order to clarify the possible mechanism of action of the inclusion complexes of CPHC and 2-HP-β-CD on bacterial species. The results of the study showed that it is possible to use 2-HP-β-CD to increase the solubility of CPHC in aqueous solutions (water/biological fluids), which would reduce the required dose and thus the toxicity of the tested compound.

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