

# Cyclodextrin modulation of gallic acid in vitro antibacterial activity

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**Abstract** The substitution of large spectrum antibiotics for natural bioactive molecules (especially polyphenolics) for the treatment of wound infections has come into prominence in the pharmaceutical industry. However, the use of such molecules depends on their stability during environmental stress and on their ability to reach the action site without losing biological properties. The application of cyclodextrins as a vehicle for polyphenolics protection has been documented and appears to enhance the properties of bioactive molecules. Therefore, the encapsulation of gallic acid, an antibacterial agent with low stability, by  $\beta$ -cyclodextrin, (2-hydroxy) propyl- $\beta$ -cyclodextrin and methyl- $\beta$ -cyclodextrin, was investigated. Encapsulation by  $\beta$ -cyclodextrin was confirmed for pH 3 and 5, with similar stability parameters. The (2-hydroxy) propyl- $\beta$ -cyclodextrin and methyl- $\beta$ -cyclodextrin interactions with gallic acid were only confirmed at pH 3. Among the three cyclodextrins, better gallic acid encapsulation were observed for (2-hydroxy) propyl- $\beta$ -cyclodextrin, followed by  $\beta$ -cyclodextrin and methyl- $\beta$ -cyclodextrin. The effect of cyclodextrin encapsulation on the gallic acid antibacterial activity was also analysed. The antibacterial activity of the inclusion complexes was investigated here for the first time. According to the results, encapsulation of gallic acid by (2-hydroxy) propyl- $\beta$ -cyclodextrin seems to be a viable option for the treatment of skin and soft tissue infections, since this inclusion complex has good stability and antibacterial activity.

**Keywords** Gallic acid ·  $\beta$ -cyclodextrin · (2-hydroxy) propyl- $\beta$ -cyclodextrin · Methyl- $\beta$ -cyclodextrin · Benesi-Hildebrand equation · Antibacterial activity

## Introduction

In the last years, the application of cyclodextrins (CDs) as functional carriers in the pharmaceutical industry has increased. CDs are cyclic oligosaccharides having the molecular shape of a truncated cone and containing hydroxyl groups oriented to the outer molecular surface and hydrophobic groups aligned along the interior of the cavity. This creates an micro-heterogeneous environment which allow the formation of inclusion complexes (IC) with a wide range of molecules, from straight or branch aliphatic chains to polar compounds, thus changing the chemical, physical or biological properties of the included guests [1, 2].

The IC stability is extremely dependent on the 3D fit between the CDs' cavity and the guest molecule and on the specific local interactions between the CDs' surface groups and the "guest" [3]. Complex stability relies on hydrophobic forces, hydrogen bonds, van der Waals interactions, and on other factors, like the release of a ring strain and modifications in solvent surface tension. The combinations of these factors render the IC to a more stable energetic state [4, 5]. Thermodynamic factors, like enthalpy ( $\Delta H$ ), entropy ( $\Delta S$ ) and Gibbs free energy ( $\Delta G$ ), can be used as parameters to describe the complexation process, since the temperature influences the selectivity of the interaction between CD and the bioactive molecule [6]. In the case of organic compounds as "guest" molecules, additional factors such as pH and solvents seem to play a major role for IC formation [7].

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The  $\alpha$ ,  $\beta$  and  $\gamma$  CDs, differing in the cavity volume and diameter, are most used for pharmacological applications with most industrial applications involving  $\beta$ CD due to the capacity of this compound to encapsulate a wide range of molecules. This native CD has been subjected to a variety of chemical modifications in order to enhance the physicochemical properties [7]. For instance, (2-hydroxy) propyl- $\beta$ -cyclodextrin (HP $\beta$ CD) and methyl- $\beta$ -cyclodextrin (M $\beta$ CD) are more hydrophilic than  $\beta$ CD itself. Moreover, these derivatives possess higher solubility than the  $\beta$ CD (500 and 750 g L<sup>-1</sup> at room temperature compared to 18 g L<sup>-1</sup> for  $\beta$ CD), which increases the complexation of poorly water soluble molecules [8].

Wound infection has been one of the major causes of the delayed on the healing process or scar development. These infections, often associated to *Staphylococcus* and *Klebsiella* species, may range from superficial infections to life-threatening ones in compromised patients. Broad-spectrum antibiotics have been indiscriminately used for the treatment of skin and soft tissue infections, changing the normal skin flora, and leading to multi-resistant strains [9]. Therefore, the demand for new antibacterial agents has increased in recent years, and polyphenolics are a major group of compounds used for that propose [7].

Gallic acid, also known as 3,4,5-trihydroxybenzoic acid, is a simple phenolic acid frequently found in plants with several biological activities. For instance, gallic acid has been described as antioxidant, anti-inflammatory, anticarcinogenic and antimicrobial [10–13]. Nevertheless, gallic acid, as other polyphenolics, has reduced pharmacological applicability due to its low water solubility [14, 15] and sensitivity to environmental stress (pH, light, temperature), factors that cause poor bioavailability [16–18]. Thus, to maintain the gallic structural integrity and allow it to reach the physiological targets without losing any activity, an encapsulation device is necessary.

Encapsulation of phenolic acids by CDs has been reported by several authors, with native CDs being mostly used, in this context and hydroxycinnamic acids (caffeic, chlorogenic and rosmarinic acid) as guests [8, 19–22]. For these compounds IC formation with a 1:1 stoichiometry is generally reported, with the phenolic orientation within the CD depending on the structure of the guest as well as the IC stability. Moreover, the application of CD derivatives (HP $\beta$ CD and M $\beta$ CD) was reported by Çelik et al. [8]. The corresponding IC with rosmarinic acid have higher stability than the one of native CD, suggesting that the substituents facilitate encapsulation [8].

Therefore, this work aims at evaluating the effect of pH on the stability of the IC formation between gallic acid and three CDs ( $\beta$ CD, HP $\beta$ CD and M $\beta$ CD). Additionally, the

influence of gallic acids encapsulation on its antibacterial activity was assessed for the first time.

## Materials and methods

### Material

Gallic acid (3,4,5-trihydroxybenzoic acid) was purchased from Merck,  $\beta$ -cyclodextrin ( $\beta$ CD, 1135 g mol<sup>-1</sup>) and (2-hydroxy) propyl- $\beta$ -cyclodextrin (HP $\beta$ CD, 1309 g mol<sup>-1</sup>, MS  $\approx$  0.6) were acquired from AppliChem, and methyl- $\beta$ -cyclodextrin (M $\beta$ CD, 1310 g mol<sup>-1</sup>, MS  $\approx$  1.6) was obtained from Wacker. Gallic acid stock solution (2 or 10 mM) was prepared in methanol (MeOH) and kept for 30 min in an ultrasonic bath. Stock solutions for each CD ( $4 \times 10^{-2}$  M) were prepared in distilled water. The  $\beta$ CD solution was maintained at 50 °C and 200 rpm in order to improve its solubility in water.

### Buffer and pH effect

Gallic acid solutions ( $1 \times 10^{-5}$  M, 2 % MeOH) were prepared in two different buffers (H<sub>3</sub>PO<sub>4</sub>/NaOH and KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub>) at pH 3, 5, 7, and 8. The buffers were prepared as follows: the desired pH (3, 5, 7, and 8) was achieved by mixing proper amounts of H<sub>3</sub>PO<sub>4</sub> ( $1 \times 10^{-2}$  M, pH 2.05) and NaOH (1 M, pH 14) for H<sub>3</sub>PO<sub>4</sub>/NaOH buffer and K<sub>2</sub>HPO<sub>4</sub> ( $5 \times 10^{-3}$  M, pH 8.02) and KH<sub>2</sub>PO<sub>4</sub> ( $5 \times 10^{-2}$  M, pH 2.89) for the buffer KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub>. The solutions were maintained for 30 min in an ultrasonic bath to insure the total solubilisation of gallic acid. The UV–Vis absorbance spectrum of gallic acid was recorded between 200 and 360 nm for each condition.

### Inclusion complex preparation

In order to determine the stoichiometry and stability constants of the ICs between gallic acid and the three CDs, solutions with different concentrations of each CD (between 0 and  $6 \times 10^{-3}$  M) were added to a gallic acid solution ( $1 \times 10^{-5}$  M) for each pH (buffer H<sub>3</sub>PO<sub>4</sub>/NaOH). The solutions were kept for 30 min in an ultrasonic bath. Afterwards they were maintained 24 h at 25 °C and 50 rpm in the dark. Samples of each solution were taken for absorbance measurements.

The absorbance of the solutions of gallic acid or the corresponding ICs was measured at  $\lambda_{\text{max}}$  (259 nm for pH 3 or 261 nm for pH 5). The gallic acid concentration was calculated based on the previously determined calibration curve. The CDs had no influence on the gallic acid spectra taking into account the conditions used.

All absorption measurements were recorded on a Jasco V560 spectrometer using a 1 cm quartz cuvette.

#### Stoichiometry, stability constant and thermodynamic parameters calculation

The stoichiometry and stability constant ( $K$ ) of each complex at the different pH were assessed based on the modified Benesi-Hildebrand equation [23] (Eqs. 1, 2), where  $[CD]_0$  is the CD initial concentration,  $A$  is the absorbance intensities in the presence of CD,  $A_0$  is the absorbance intensities without CD, and  $A'$  is the limiting intensity of absorption. The equations were used to define the IC stoichiometry: Eq. (1) represents a 1:1 complex and the Eq. (2) 1:2 complex.  $K$  was obtained from the slope of the graph.

$$\frac{1}{A - A_0} = \frac{1}{A' - A_0} + \frac{1}{K(A' - A_0)CD_0} \quad (1)$$

$$\frac{1}{A - A_0} = \frac{1}{A' - A_0} + \frac{1}{K(A' - A_0)CD_0^2} \quad (2)$$

A double reciprocal Benesi-Hildebrand plot was drawn using both equations, i.e.  $\left(\frac{1}{A-A_0} \text{ vs } \frac{1}{[CD]}\right)$  or  $\left(\frac{1}{A-A_0} \text{ vs } \frac{1}{[CD]^2}\right)$ . The better fit (higher  $r^2$ ) of the Benesi-Hildebrand plots was used to identify the stoichiometry of the ICs. The  $\Delta G$  was calculated using  $K$  and Eq. 3, where  $R$  is the gas constant, and  $T$  the temperature.

$$\Delta G = -RT \ln(K) \quad (3)$$

#### Bacterial susceptibility to gallic acid IC

##### Bacterial suspension

The antibacterial activities of gallic acid in buffered aqueous  $H_3PO_4/NaOH$  (selected buffer) at different pH values as well as the activity of the ICs, were tested against the bacteria *Staphylococcus epidermidis* (*S. epidermidis*, ATCC 12228), *Staphylococcus aureus* (*S. aureus*, ATCC 6538) and *Klebsiella pneumoniae* (*K. pneumoniae*, ATCC 11296). The bacteria were grown in tryptic soy agar (TSA, Merck, Germany) for 24 h at 37 °C. The cells were inoculated in tryptic soy broth (TSB, Merck, Germany) and incubated for 18 h at 37 °C under agitation (120 rpm). Subsequently, bacterial concentration of each strain was adjusted to  $1 \times 10^6$  cells  $mL^{-1}$  via absorbance readings and determined with the corresponding calibration curve.

##### Susceptibility assay of gallic acid

The minimal bactericidal concentration (MBC) was obtained according to the method described by Wiegand [24], an adaptation of the standard methods published by

Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2000), using the broth micro dilution procedure. Thus, stock solutions of  $3.74 \times 10^{-3}$  M of gallic acid were prepared at pH 3, 5, 7, and 8 (aqueous  $H_3PO_4/NaOH$  buffer). Serial dilutions of these solutions were made with MHB (Mueller–Hinton broth, Merck, Germany) to a final volume of 50  $\mu L$ . Afterwards, 50  $\mu L$  of each bacterium suspension were added to give a final concentration of  $5 \times 10^5$  cell  $mL^{-1}$ . Gallic acid- and bacteria-free controls were also included. The plates were incubated for 24 h at 37 °C. The number of viable cells was assessed by determination of the number of colony forming units (CFUs), by plating 10  $\mu L$  of cell suspension from each well onto TSA, and incubated for 24 h at 37 °C.

The procedure was made in triplicate for each pH and bacterium combinations in at least three independent measurements.

##### Antibacterial activity of ICs

The ICs capacity to destroy the bacteria was also measured quantitatively. A volume of 50  $\mu L$  of each complex (IC  $\beta CD$ /gallic acid and IC HP $\beta CD$ /gallic acid) was added to 50  $\mu L$  of  $1 \times 10^{-6}$  cells  $mL^{-1}$  of each bacterium, on a 96 well plate. Bacteria and medium controls were also included. The plates were incubated for 24 h at 37 °C. The antimicrobial activity of each solution was assessed by determination of the number of CFUs as described above. The procedure was performed in triplicate for each bacterium at least in three independent assays. Solutions of buffer, gallic acid (1 mM),  $\beta CD$  (1 mM) and HP $\beta CD$  (1 mM) were also included to ensure that none of these factors alone could influence on the antibacterial activity of the ICs.

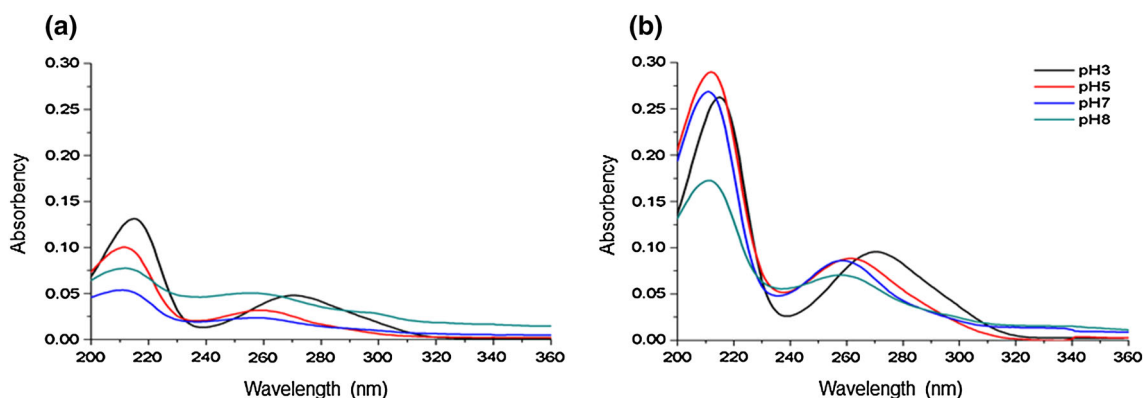
All mathematical analyses were carried out using the Origin Pro software.

## Results and discussion

The biological properties of gallic acid remain active even at low concentrations [18], however this phenolic acid is quite susceptible to degradation under environmental stress (pH, temperature, light and oxygen) [25]. Therefore, the gallic acid protection by encapsulation by CDs was studied.

##### Influence of buffer and pH on the gallic acid properties

The UV–Vis spectra of polyphenols reflect alterations of the electronic energy levels within the molecules, caused by electronic transitions between  $\pi$ -type molecular orbitals [26, 27]. The nature of the solvent, steric effects, formation



**Fig. 1** UV–Vis spectra of gallic acid ( $1 \times 10^{-5}$  M) dissolved in  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (a) and  $\text{H}_3\text{PO}_4/\text{NaOH}$  (b) buffer at pH 3 (black), pH 5 (red), pH 7 (blue), and pH 8 (green). (Color figure online)

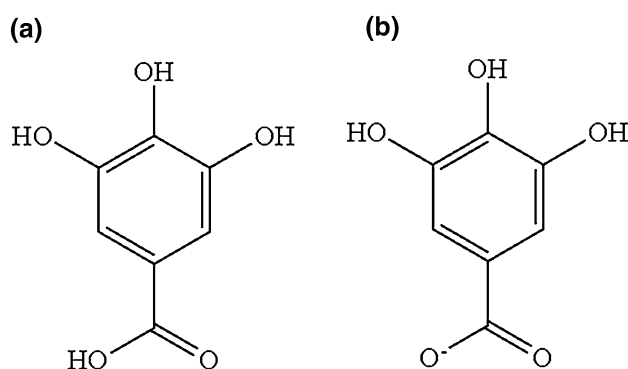
of resonance forms, intra- and inter-molecular hydrogen-bonding, electron-donating and electron-withdrawing substituents on the benzene ring are factors responsible by alterations on polyphenols spectra [25].

Therefore, the influence of two different buffers on the UV–Vis spectra of gallic acid was analysed as well as the effect of the pH for each buffer (Fig. 1).

The gallic acid UV–Vis spectra usually shows two bands between 200 and 360, a b-band (lower wavelength) near 220 nm and a c-band (higher wavelength) near 270 nm. The buffer effect on the gallic acid spectra was notorious. The intensity of the two peaks, regardless of the pH, was lower when the phenolic was dissolved in the  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  buffer (Fig. 1a). At pH 8 the peaks were not distinct, which may be the result of a higher oxidation state of the gallic acid related to its unstable nature in basic environments particularly notorious in this buffer [25, 28].

The  $\text{H}_3\text{PO}_4/\text{NaOH}$  buffer allowed higher intensity of the characteristic peaks and the changes caused by pH were also more evident. The gallic acid spectra profile was the same for the pH range tested. However, an increase of pH induced a blue shift (a shift of  $\lambda_{\text{max}}$  towards shorter wavelengths) for both bands being more notorious for the c-band (273 nm for pH 3, 261 nm for pH 5, 258 nm for pH 7, and 258 nm for pH 8) and also an increase of intensity (hyperchromic effect). The  $\text{H}_3\text{PO}_4/\text{NaOH}$  buffer was chosen for further work, since the effect of pH on the gallic acid UV–Vis spectra was more obvious when it was used.

As mentioned above, the pH is strongly linked to the stability of the polyphenols and affects their UV–Vis spectra [25]. Gallic acid has two ionisable moieties: (i) the carboxylic group and (ii) the hydroxyl groups attached to the phenolic ring (Fig. 2). The pH affects the ionization state of gallic acid. The phenolic acid is neutral (Fig. 2a) at acid pH (<4.4), contains a deprotonated carboxylate group between 4.5 and 7, and at basic pH will be deprotonated at both the carboxylic acid and hydroxyl groups [28, 29]. The



**Fig. 2** Chemical structures of neutral gallic acid (a) and the gallic acid carboxylate anion (b)

gallic acid ionisation states with increasing pH have been attributed to the three hydroxyl groups (Fig. 2). The formation of unstable quinones intermediates and other resonance forms has been linked to the reduction of intensity of the c-band as the pH increases (Fig. 1) [25]. Moreover, for basic pH (>7) gallic acid has been described as unstable, suffering fast autoxidation which results in the formation of degradation products [25, 28]. The presence of these products may explain the changes observed in the spectra at pH 8 in both buffers.

The pH-dependent antibacterial activity of gallic acid has been attributed to the ability to exchange protons with the bacteria and the environment. The antibacterial mechanism of gallic acid relies on its affinity to the lipophilic membrane layer, which enables the gallic acid transport and, consequently, cytoplasm acidification causing protein denaturation. The acidification of the cell environment causes variations in the potassium ions efflux, alters the electrical potential of the cell and improves its permeability. This cascade of events leads to irreversible alterations on the cell and, consequently, to its death [30, 31].

**Table 1** Minimal bactericidal concentration of gallic acid dissolved on H<sub>3</sub>PO<sub>4</sub>/NaOH (pH 3, 5, 7 and 8) against *K. pneumoniae*, *S. epidermidis* and *S. aureus* ( $5 \times 10^{-5}$  cell mL<sup>-1</sup>)

MBC (mM)	<i>K. pneumoniae</i>	<i>S. epidermidis</i>	<i>S. aureus</i>
pH3	0.47	0.47	0.47
pH5	0.23	0.23	0.47
pH7	0.23	0.23	0.47
pH8	0.12	0.23	0.47

In addition, the skin and soft tissues infections result from the colonization and proliferation of complex poly-microbial communities. The infections may be triggered by natural microflora such as *Staphylococcus*, *Micrococcus* and *Corynebacterium sp.* or by not typical resident microflora gram negative bacteria (*Klebsiella*, *Capnocytophaga*, *Bartonella*). The proliferation of pathogenic bacteria on the wound site has been related to the pH of the surrounding environment [32, 33]. Since the wound infection microflora depends on the environmental pH as well as the gallic acid biological properties [25], the gallic acid antibacterial activity at different pH was analysed (Table 1). Three bacteria usually isolated from infected wound, two gram positive (*S. epidermidis* and *S. aureus*) and one gram negative (*K. pneumoniae*) were used [34].

Regarding the gram positive bacteria, the pH had no influence on the MBC obtained for *S. aureus* (0.47 mM) and MBC for *S. epidermidis* was 0.47 mM, at pH 3 and 0.24 mM for higher pH. In fact, it was described that *S. epidermidis* growth is enhanced by acid environments [33]. Thus, the lower susceptibility of this bacterium at pH 3 may result from higher metabolic activity. These results suggest that all forms of gallic acid (neutral and anionic) are active against gram positive bacteria under the conditions tested. A relation between pH and *K. pneumoniae*'s susceptibility to gallic acid was observed, the MBC obtained decrease with pH basification.

The optimal growth pH for this gram negative bacterium is located near 5 and 6 [35], the pH used to access the bacterial susceptibility exceeded that pH range. Bacteria cells have mechanisms responsible for controlling the pH variation between the environment and the cytoplasm. The maintenance of optimal cytoplasmic pH comprises a combination of strategies, such as cytoplasm buffering, adaptations on the membrane structure, active ion transportation and metabolic consumption of acids and bases [36]. However, in specific situations the pH homeostasis mechanisms can fail, leading to significant changes on the pH variation thus decreasing the cell metabolism. Such situations may occur when the cell increases the uptake of weak acids to equilibrate the difference between external and internal pH. The weak acids can freely be transported

through the bacteria membrane therefore causing protons release and consequent cytoplasm acidification [37].

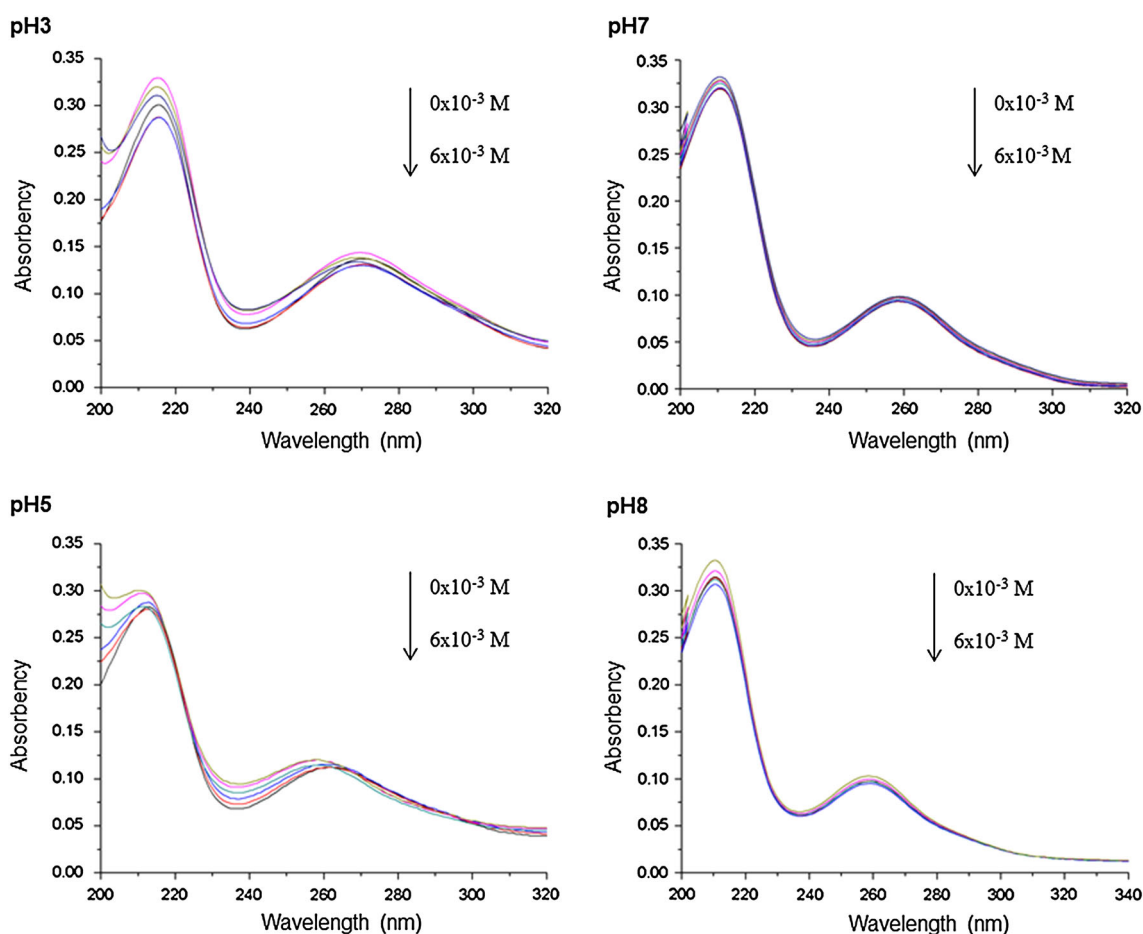
In this assay, the addition of buffers, with different pH, to the culture medium has triggered these pH homeostasis mechanisms. Thus, the pH-dependent MBC obtained for *K. pneumoniae* may be a consequence of rising uptake of gallic acid (weak acid) to keep the pH homeostasis, which resulted in the accumulation of the organic acid on the cytoplasm, hyperacidification and, ultimately, cell death [37].

#### Impact of pH on the gallic acid/ $\beta$ CD interaction

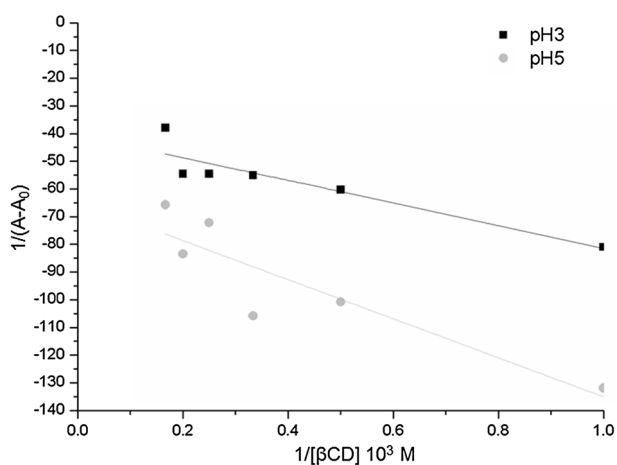
The gallic acid c-band (near 270 nm) was selected for the analysis of the interactions with CD since it is the most used for its characterization (HSDB—Hazardous Substances Data Bank). Figure 3 displays the effect of  $\beta$ CD concentration and pH on gallic acid UV–Vis absorbance spectra, collected after 24 h of complexation. Regardless of the pH, as the CD concentration increases the  $\lambda_{\max}$  intensity of gallic acid decreases. At higher pH (7 and 8) the alterations caused by  $\beta$ CD on the phenolic spectra were subtle, just a slight absorbance variation was detected (Fig. 3). The reduction of pH highlight the effects of CD on the gallic acid spectra. At pH 3 and 5, the spectra show an isosbestic point near 225 nm and an hypsochromic effect (the  $\lambda_{\max}$  shift to lower wavelength). The isosbestic point indicates the presence of gallic acid in free and encapsulated form. The hypochromic effect observed as CD concentration increase may indicate that gallic acid is totally embedded in the CD's cavity [19]. Both effects support the IC formation between  $\beta$ CD and gallic acid in this pH range and the notion that the groups involved in the complexation process are located near the chromophore [19]. Other authors observed similar effects of  $\beta$ CD on gallic acid spectra [15, 18, 29]; they also confirmed the encapsulation by SEM (scanning electron microscope), where the free gallic acid morphology was not detected after molecular inclusion [18].

As referred above, no significant effects were observed on the gallic acid UV–Vis spectra at pH 7 and 8 (Fig. 3) probably due to the absence of ICs or their lower stability. Thus, complex formation was only characterized at pH 3 and 5.

The UV–Vis absorbance spectra confirmed IC formation between gallic acid and  $\beta$ CD. However, the binding strength and the effects caused by complexation on the guest properties deserved further analysis. Therefore, complex stoichiometry and stability in terms of  $K$  and  $\Delta G$  of the ICs were evaluated [38]. The stoichiometry and  $K$  were determined UV–Vis spectroscopically by using the Benesi-Hildebrand equation (Eq. 1) and  $\Delta G$  was determined based on Eq. 3.



**Fig. 3** UV-Vis spectra of gallic acid ( $1 \times 10^{-5}$  M) at different  $\beta$ -cyclodextrin concentrations ( $0$ – $6 \times 10^{-3}$  M) at pH 3, 5, 7 and 8 (aqueous  $\text{H}_3\text{PO}_4/\text{NaOH}$  buffer)



**Fig. 4** Benesi-Hildebrand plot for the gallic acid complexation with  $\beta$ CD, at pH 3 and 5 (buffer  $\text{H}_3\text{PO}_4/\text{NaOH}$ )

A linear relation (Fig. 4) was obtained for the two pH values (3 and 5), indicating that 1:1 complexes between gallic acid and  $\beta$ CD are formed.

The pH range analysed (3 and 5) had low influence on  $K$  and  $\Delta G$ , and the values obtained were similar (Table 2). The highest values were obtained for pH 5, however with  $r^2 = 0.865$ . At pH 3, the linear relationship obtained was better ( $r^2 = 0.957$ ) but the  $K$  and  $\Delta G$  were lower. The ICs formed at pH 3 or 5 were formed spontaneously by an exergonic reaction ( $\Delta G < 0$ ) [39]. IC formation involves the replacement of polar water molecules from the hydrophobic CD cavity by gallic acid. The gallic acid neutral species ( $\text{pK} = 4.4$ ) at acid environment [28] may enhance the encapsulation process. Since  $K$  and  $\Delta G$  were similar at both pH values, pH 3 was considered as the best condition for gallic acid complexation by  $\beta$ CD due to the higher  $r^2$ .

According to the author's knowledge, there are just few publications regarding the interaction of gallic acid with  $\beta$ CD [15, 18, 29]. These papers obtained similar thermodynamic parameters for the ICs and also described analogous mechanisms of encapsulation. They all stated that the gallic acid is completely encapsulated within the  $\beta$ CD cavity with the carboxylic group oriented towards the small CD opening and the three hydroxyl groups placed near the

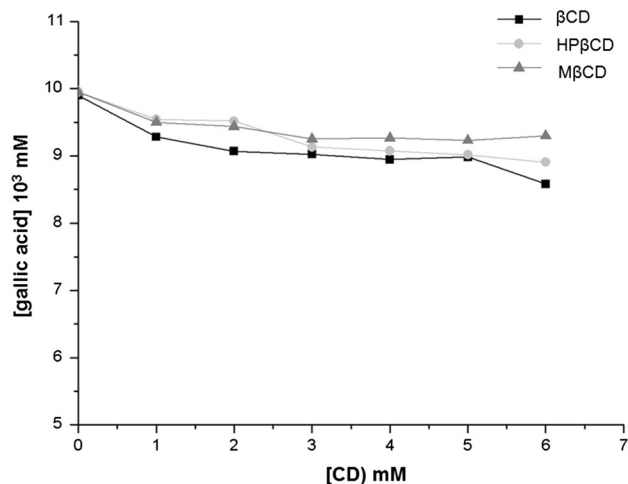
**Table 2** IC  $\beta$ CD/gallic acid characterization parameter at pH 3 and pH 5

	pH 3	pH 5
Stoichiometry	1:1	1:1
K (M <sup>-1</sup> )	41	71
$\Delta G$ (kJ)	-9.83	-10.4
r <sup>2</sup>	0.953	0.865

wider entrance (Fig. 5). Here we show that the major interaction between gallic acid and  $\beta$ CD is presumably hydrophobic, since the  $\beta$ CD is not charged at the pH range used for the measurements. Thus, the ICs stability is due to hydrophobic interactions between gallic acid and the cavity as well as by hydrogen bonds established between the gallic acid and the CD hydroxyl groups [18, 29]. Thus, the gallic acid neutral species (pH 3) may be capable of deeper insertion into the CD cavity than the ionized form (pH > 4.4) (Fig. 5). This fact explains the lower stability observed for the IC formed at pH 5 and the lack of encapsulation at higher pH. Based on our results and on those of published work, the stability of the IC between  $\beta$ CD and gallic acid may be enhanced in the acid environment (pH < 4.4).

#### Gallic acid encapsulation by HP $\beta$ CD and M $\beta$ CD

The encapsulation of polyphenolics by modified CDs has been described as leading to more stable complexes than the ones obtained with native CDs [8, 40]. Two  $\beta$ CD derivatives (HP $\beta$ CD or M $\beta$ CD) were chosen to test this assumption based on their improved solubility and also because their substituents enlarged the cavity opening reducing the intramolecular hydrogen bond network. These properties could facilitate the incorporation of the guest molecule (in this case gallic acid) to the cavity, leading to the formation of a complex with higher stability. To the best of our knowledge, the inclusion of gallic acid by

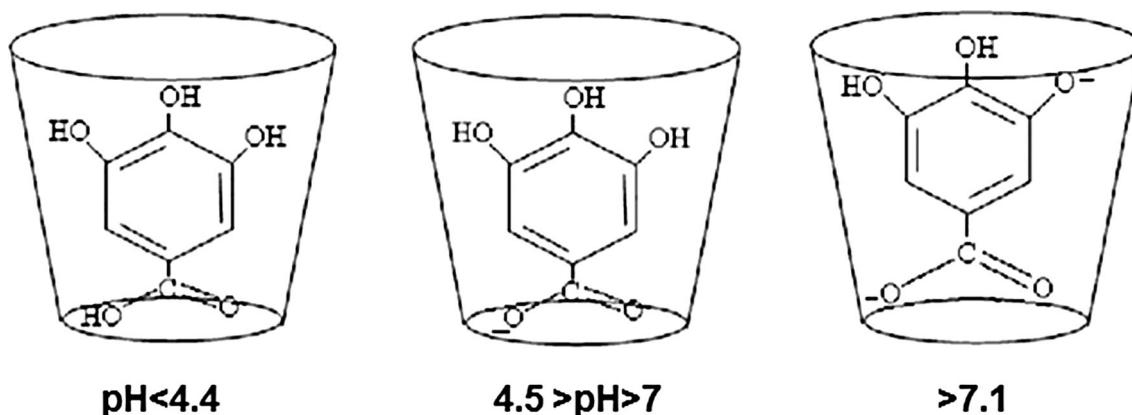


**Fig. 6** Determination of gallic acid concentration (based on the absorbance measured at the  $\lambda_{\max}$ ) after 24 h of complexation with different concentrations of cyclodextrins (0–6 mM) at pH 3 (aqueous H<sub>3</sub>PO<sub>4</sub>/NaOH buffer)

HP $\beta$ CD or M $\beta$ CD is analysed for the first time in this work.

The effect of pH (3, 5, 7, and 8) on the interaction between gallic acid and HP $\beta$ CD and M $\beta$ CD was studied. Variations on the UV–Vis spectra of the guest induced by these CDs at pH 5, 7 and 8 were not detected under the conditions used (data not shown). Since gallic acid encapsulation with native CD ( $\beta$ CD) was more efficient at pH 3 this pH value was chosen to study the effect of the  $\beta$ CD modifications on the complexation with gallic acid (Fig. 6).

The gallic acid behaviour was similar in the presence of all three CDs. A correlation between the reduction of gallic acid concentration in solution with the increase of the CDs concentration was observed. These values were obtained from the UV–Vis spectra, where an hypochromic effect



**Fig. 5** Schematic representation of location of the gallic acid molecule inside the  $\beta$ CD cavity at different pH values

**Table 3** IC HP $\beta$ CD/gallic acid and M $\beta$ CD/gallic acid characterization parameter at pH 3

	HP $\beta$ CD	M $\beta$ CD
Stoichiometry	1:1	1:1
$K$ ( $M^{-1}$ )	90	37
$\Delta G$ (kJ)	-11	-8.8
$r^2$	0.909	0.477

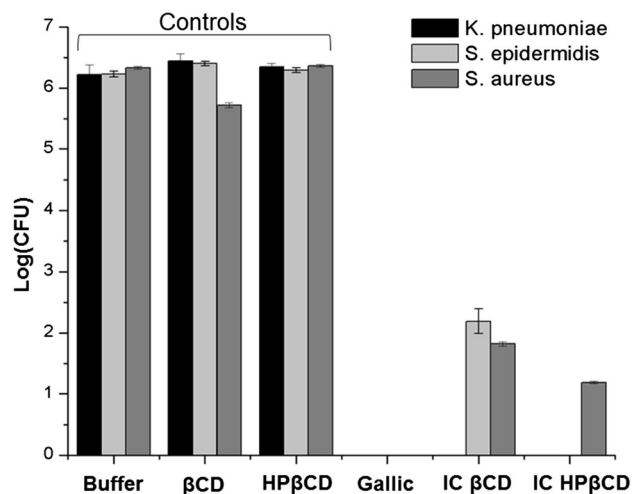
(reduction of  $\lambda_{\max}$  intensity) was observed with the increase of CDs concentration. This effect may result from the interference with molecular groups responsible for the UV-Vis absorbance induced by encapsulation [19]. The same behaviour has already been mentioned above for the native CD.

Figure 6 suggests that the IC formed by the three CDs behave similarly, but characterization (stoichiometry,  $K$  and  $\Delta G$ ) of the complexes revealed differences. The ICs obtained for the substituted CDs (HP $\beta$ CD/gallic acid and M $\beta$ CD/gallic acid) have 1:1 stoichiometry, which is consistent with the result obtained for native CD. Nevertheless, the stability and thermodynamic parameters are different for these two CDs (Table 3). The HP $\beta$ CD has a higher  $K$  than the  $\beta$ CD complex and, as a consequence, a lower  $\Delta G$ . Thus, stability of the complex between HP $\beta$ CD and gallic acid is higher in comparison to the complex of native CD. As the size of the molecular cavity of HP $\beta$ CD is similar to the one of  $\beta$ CD (7 glucopyranose units) the higher  $K$  obtained for the HP $\beta$ CD suggest that the hydroxypropyl groups play a role on the IC process. These groups seem to stabilize the gallic acid molecules trapped inside the cavity [41]. The opposite was observed for the M $\beta$ CD, since a lower stability of the IC was observed (Table 3). This CD is therefore less suited for the complexation of gallic acid when compared to native CD or HP $\beta$ CD.

Based on the results presented above, and as expected, the pH played a major role in the encapsulation of gallic acid by CDs. At pH 3, IC formation with the three CDs is best, and pH 5 creates suitable conditions for the complexation between gallic acid and native CD, with similar thermodynamic parameters obtained at pH 3.

#### Antibacterial efficacy of gallic acid/CD complexes

Encapsulation by CDs usually improves physicochemical and biological properties of included guest molecules although Zhao [21] showed that the antioxidant and antibacterial activity of chlorogenic acid is not affected by encapsulation with  $\beta$ CD. Here, the antibacterial activities of the ICs between  $\beta$ CD and gallic acid and HP $\beta$ CD and gallic acid were assessed by a quantitative method. In order



**Fig. 7** Quantitative analysis of the microbial activity of the ICs between gallic acid and  $\beta$ CD and gallic acid and HP $\beta$ CD against *K. pneumoniae*, *S. epidermidis*, and *S. aureus* ( $5 \times 10^5$  cell  $mL^{-1}$ ). The inclusion complexes were prepared at pH 3 (aqueous  $H_3PO_4/NaOH$  buffer) with equimolar amounts of gallic acid and CDs (1 mM)

to ensure that gallic acid is capable of killing all bacteria cells in an infected wound, the environmental condition less favourable to its action (pH 3) was used for this analysis. At this pH, complexation between gallic acid and  $\beta$ CD or HP $\beta$ CD is best.

The IC complexes were prepared by using solutions with the same concentrations (1 mM) of CD and gallic acid, considering the stoichiometry determined above (1:1). As expected, the controls (buffer,  $\beta$ CD and HP $\beta$ CD) had no influence on the bacteria growth.

Figure 7 displays the susceptibility of the three bacteria strains used when exposed to the ICs. Both ICs ( $\beta$ CD/gallic acid and HP $\beta$ CD/gallic acid) were capable of reducing the growths of the three bacteria, but their antibacterial activity was different for the bacteria used. Against the gram negative bacteria (*K. pneumoniae*) both ICs displayed the same effect, namely completely growth inhibition. In the case of the gram positive bacteria (*S. epidermidis* and *S. aureus*), the antibacterial activity of gallic acid is reduced upon  $\beta$ CD encapsulation. Still, the number of CFU detected was less than 4 log when compared with the control. The other IC (HP $\beta$ CD/gallic acid) retained the gallic acid activity against *S. epidermidis*, but allowed the growth of 1 log of *S. aureus*.

The gram positive and negative bacteria differ in their cell wall and, consequently, in their susceptibility to antimicrobial agents. The gram positive bacteria have a continuous cell wall of a thick layer of peptidoglycan, while gram negative bacteria have a non-continuous cell envelope formed by a thin layer of peptidoglycan covered by an



outer membrane. Hence, it is expected that gram negative bacteria are more susceptible to antimicrobial agents than gram-positive ones [42]. Therefore, the higher susceptibility of the gram negative bacterium (*K. pneumoniae*) to the ICs activity suggest that the ICs are capable of reaching the cell surface more efficiently and, consequently, with a higher uptake, when compared to other bacteria.

The reduction on the antibacterial activity of gallic acid regarding the ICs in contact with the gram positive bacteria may rely on the phenolic acid's low availability to interact with bacterial cells. As mentioned above, the antibacterial mechanism reported for gallic acid involves the interaction with the cell surface altering its electrochemical potential, reducing the membrane integrity, and causing hyperacidification of the cytoplasm via proton donation thus interfering with crucial metabolic pathways. Assuming that gallic acid is completely encapsulated in the cyclodextrin cavity forming a stable complex, proton exchange is probably lower than the one of free gallic acid. Incorporation of gallic acid within HP $\beta$ CD seems to allow a better interaction with bacteria, since the IC with this CD were capable of preserving gallic acid activity against two bacteria, and the growth of *S. aureus* obtained was minimal. In conclusion, the encapsulation of gallic acid by the  $\beta$ CD and HP $\beta$ CD may be a viable option for the application as an antibacterial agent.

## Conclusions

The role of the solvent and pH on gallic acid properties was confirmed in this study. The intensity of phenolic acid peaks was modified by the buffer. The H<sub>3</sub>PO<sub>4</sub>/NaOH buffer allowed a better detection of the pH effect on the UV–Vis spectra.

The encapsulation of gallic acid by  $\beta$ CD was studied at pH 3, 5, 7 and 8 and it was observed that the basic environment (pH 7 and 8) was less suitable for encapsulation. At pH 3 and pH 5, the ICs obtained had similar stability (similar *K* and  $\Delta G$  values) although the linear relation was better at the more acidic pH. Regarding the substituted  $\beta$ CD derivatives, encapsulation was only detected at pH 3. Therefore, the gallic acid neutral form appears to enhance its ability to form IC with HP $\beta$ CD and M $\beta$ CD. All the complexes obtained had 1:1 stoichiometry regardless of the CD, but stability of the IC was higher for HP $\beta$ CD and lower for M $\beta$ CD.

Moreover, the antimicrobial activities of the ICs between  $\beta$ CD and gallic acid and HP $\beta$ CD and gallic acid were also analysed. Phenolic activity was retained by complexation with the CDs. The IC of HP $\beta$ CD had better efficiency against the three bacteria and also exhibited higher stability. Therefore, complexation of gallic acid by

HP $\beta$ CD may be a viable option for the improvement of gallic acid applicability as antibacterial agent for the treatment of skin and soft tissue infections.

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