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PREPARATION OF INCLUSION COMPLEX BETWEEN ELLAGIC ACID AND HYDROXYPROPYL-β-CYCLODEXTRIN

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SUMMARY

In recent years, the research and discovery of antioxidants of natural origin, such as those found plants, have increased dramatically. Ellagic acid is a bioactive compound found in many fruits and vegetables, which carries many biological activities, such as antioxidant, anti-inflammatory, anti-cancer and antibacterial activities. However, the low solubility of ellagic acid in water decreases its practical application. In this study, a complex of ellagic acid with hydroxypropyl-cyclodextrin was synthesized in the water-ethanol solvent. The results showed that the solvent with a volume content of EtOH of 20% was the most suitable for complex formation, with complexation yield of 46%. The complex was characterized by FTIR, DSC methods. The infrared spectrum of the complex is similar to that of HP-β-CD, however, the intensity and position of some oscillations in the complex have changed significantly, compared to spectra of EA and HP-β-CD. The sharp adsorbance band at 3475 cm^{-1} of the O-H bond of EA was not observed in the spectrum of the complex, indicating that the O-H group participated in the bonding and covered by the hollow cavity of the HP-β-CD molecules. The DSC curve of the complex shows that the melting points of EA and HPβCD in the complex are declined in terms of temperature and intensity. This is evidence that there is a complex interaction between EA and HPβCD. The complexation improved the solubility and antioxidant activity of EA. Especifically, the solubility of EA was increased by 3.2 times compared to raw EA; EC_{50} value of EA was reduced from $5.3x10^{-5}$ to $4.9x10^{-5}$mol. L^{-1} after complexation.

Keywords: antioxidant activity, ellagic acid, hydroxypropyl-β-cyclodextrin, inclusion complexation

INTRODUCTION

Reactive oxygen species (ROS) are produced inside cells when exposed to a number of endogenous and exogenous agents. If ROS are not efficiently scavenged by cellular components, cell disorder can occur (Wulf D., 2002). All aerobic organisms have antioxidant system including antioxidant enzymes to remove or repair damaged molecules. According to Lobo's definition, antioxidants are molecules that are stable enough to accept or donate electrons to free radicals and

neutralize them; thereby, reducing or inhibiting ROS ability to damage cells (Lobo *et al*., 2010). In recent years, the research and discovery of natural antioxidants, especially of plant origin, have increased dramatically.

Ellagic acid (EA) is a polyphenol containing two gallic acid radicals (Figure 1), found in many fruits and vegetables: raspberries, strawberries, pomegranates, etc. (Kilic I. *et al*., 2014).

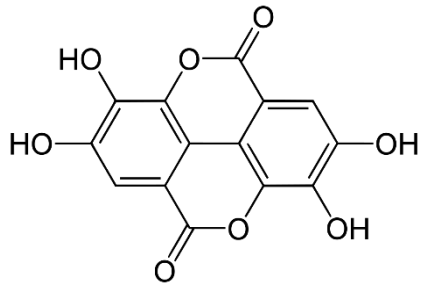

Figure 1. Molecular structure of ellagic acid.

EA has several valuable biological activities such as: antioxidant, anti-inflammatory, anti-cancer and antibacterial activities (Sharifi-Rad J. *et al.,* 2022; Nyamba I. *et al*., 2021; Shakeri A. *et al.,* 2018; Alfei S. *et al.,* 2019). However, the low solubility of EA in water limits its practical application, due to reduced bioavailability and pharmacological activity (Nyamba I. *et al*., 2021; Tambosi G. *et al*., 2018). There have been many studies aiming to improve the solubility of EA, among them, forming complex with cyclodextrins (CD) is a method that does not require complicated techniques, it is also safe and highly effective (Challa R. *et al*., 2005).

CD can form complexes with a wide variety of biologically active compounds, and alters their disadvantageous properties like: unpleasant odor and taste, low water solubility and chemical instability (Li J., Loh X. J., 2008). Some previous studies demonstrated that, after forming complexes with cyclodextrins, the properties of EA including bioavailability, anti-arthritic, anti-inflammatory and antioxidant were significantly improved (Savic I. M. *et al*., 2019; Bulani V. D *et al*., 2015)

Among the methods of CD complex synthesis, the co-precipitated method is commonly used, however, the complexation efficiency is low. Therefore, in this study, the inclusion complex of EA with hydroxypropyl-β-cyclodextrin (HPβCD) was prepared by the co-precipitation in the mixed water-ethanol solvents for improving the complexation efficiency. The obtained complex was characterized by several techniques, determined the antioxidant activity and compared to raw EA.

MATERIALS AND METHODS

Materials

Ellagic acid and HPβCD from Sigma-Aldrich, ethanol (EtOH), dimethylsulfoxide (DMSO) from Xilong Scientific Co. (China) were used as received without further purification. All experiments were carried out in distilled water.

Synthesis of the complex [EA-HPβCD]

The inclusion complex of ellagic acid with HPβCD was synthesized using the co-precipitation method, with a 1:1 molar ratio of ellagic acid to HPβCD. The synthesis was carried out in mixed water-ethanol solvents with varying volume ratios of ethanol and water (10/90, 20/80, 30/70, 40/60, 50/50). The concentration of ethanol in the binary solvent could not be increased due to the poor solubility of HPβCD in ethanol. A solution of HPβCD with a concentration of $5x10^{-3}$ $mol.L^{-1}$ was added to a solution of ellagic acid with the same concentration, and the resulting mixture was stirred by a magnetic stirrer for 24 hours at 25°C. The reaction solution was settled for 48 h at 4°C to obtain a cream-color precipitate. The precipitate was washed several times with dimethyl sulfoxide (DMSO), then evaporated and dried at 60°C for 24 hours.

Determination of complexation efficiency

The complexation efficiency of [EA-HPβCD] complex with different composition of

ethanol in the mixed solvents was determined by the following equation:

$$CE = \frac{m_{complex}}{m_{EA} + m_{HP\beta CD}} x\ 100\% \quad (1)$$

Where $m_{complex}$, m_{EA}, $m_{HP\beta CD}$ are the mass of the complex, EA and HPβCD.

Fourier transforms infrared (FTIR) spectroscopy

Fourrier transform infrared spectroscopy (FTIR) is used to determine the characteristic oscillation of chemical bonds between atoms. In this study, the complex sample was weighed and mixed with KBr then pelleted and performed on a Nicolet iS10 (Thermo Scientific, USA. The EA and HPβCD samples were also analyzed simultaneously as control.

Differential scanning calorimetry (DSC)

EA, HPβCD and the [EA-HPβCD] complex were analyzed for DSC with a differential calorimeter (DSC, DSC204F1 (NETZSCH-Germany). Solid sample was approximately weighed of 3-4 mg in an aluminum cup (Al_2O_3), and scanned over a temperature range: 25°C (room temperature) to 250°C with a constant heating rate of 10°C/min in an inert gas atmosphere (nitrogen).

UV-Vis spectroscopy analysis

The solubility of EA before and after complexation was determined by instrument CINTRA 40, GBC (USA). The experimental procedure was as follows: an excess of EA or the complex is placed in a flask containing 25 mL of distilled water. The flask was then shaken for 24 hours at 25±1°C. The resulting solution was collected and filtered through a 0.45 μm filter. The solution was then diluted to the required concentration, and the absorbance was measured at 277 nm using UV-Vis spectroscopy.

Determination of antioxidant activity of EA before and after complexation

The experiment to investigate the antioxidant capacity of EA or [EA-HPβCD] complex was conducted as follows: 2 mL of the bioactive solution (EA or [EA-HPβCD] complex) with concentration in the range from $2x10^{-6}$ to $1.5x10^{-5}$ M was dripped into a reaction flask containing 5 mL of DPPH solution $1.8x10^{-4}$ M (stored in a dark room, mixed immediately before experiment). The obtained solution was shaken well and left for 30 minutes in the dark. After 30 minutes, UV-Vis measurement was carried out at λ_{max} = 517 nm.

The antioxidant capacity (C) of the samples was calculated according to the following formula:

$$C = \frac{A_0 - A}{A_0} x\ 100\% \quad (2)$$

Where A_o is optical density of initial DPPH solution and A is optical density of samples.

From the C values, a linear correlation equation was established showing the dependence between the solution concentration of the samples and the percentage of antioxidant; thereby, determining the EC_{50} value (which is the concentration of the samples that capture or react with 50% of the DPPH free radicals). This is the basis for comparing the oxidation resistance between samples. The lower the EC_{50} value of the sample, the higher the antioxidant activity.

RESULTS AND DISCUSSION

Determination of complexation efficiency

From Figure 2, it can be seen that the complexation efficiency increased when adding ethanol to the mixed solvent. The maximum value of complexation efficiency (CE) reached 46% when the ethanol content in the mixed solvent was 20%.

FTIR analysis

The FTIR analysis is an useful tool to demonstrate the occurrence of “envelope complexation” reaction. After the reaction, the characteristic oscillations of EA molecules usually disappear, attenuate the intensity or to be displaced.

The infrared spectrum of the complex was similar to those of HP-β-CD, however, the magnitude and position of some EA oscillations in the complex have changed significantly. The sharp valence band at 3475 cm^{-1} of the O-H bond of EA was not observed in the spectrum of the complex, indicating that the O-H group participated in the bonding and covered by the hollow cavity of the HP-β-CD molecules. The oscillations of the O-H group in the spectrum of complex (3323 cm^{-1}) were shifted to the region of lower wave number than HP-β-CD (3417 cm^{-1}). The bands at wave numbers 1613 and 1580 cm^{-1} of EA were displaced (1620 and 1577 cm^{-1}) and markedly declined, indicating that the C=C bond of the EA aromatic ring participated in the complexation. The bands at 1255 and 1107 cm^{-1} of EA were very strongly declined in the spectrum of the complex.

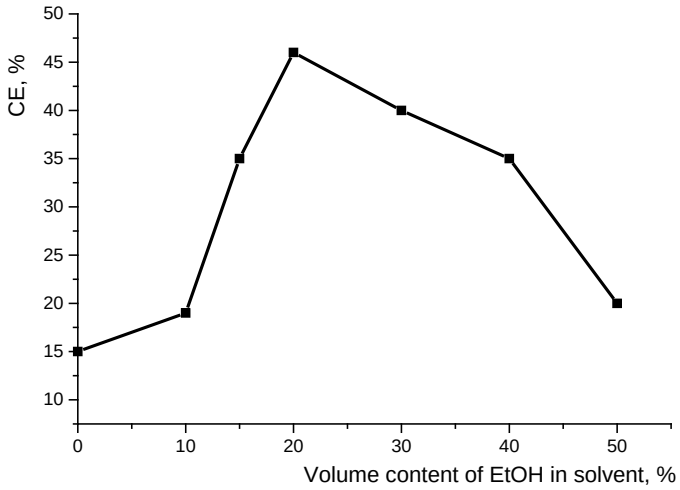

Figure 2. The complexation efficiency of [EA-HPβCD] in the solvents with different ethanol content.

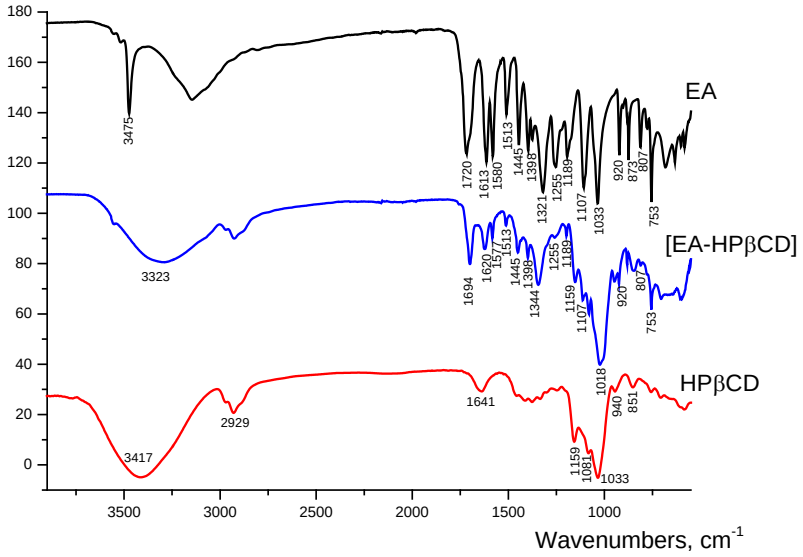

Figure 3. FT-IR spectra of raw EA, HPβCD and [EA-HPβCD) complex.

DSC analysis

Differential scanning calorimetry (DSC) is commonly used in supramolecular complex chemistry to demonstrate differences in the composition of the "guest" molecules, such as EA, before and after complexation. In particular, when the melting points of the reaction products are disappeared or reduced in intensity, that is the evidence of complexation. The graph of Figure 4 showed the heat curves of EA, HPβCD and the analyzed complex in the temperature range from 25 to 250°C in an inert gas (nitrogen) atmosphere. The result of DSC analysis of EA was a large thermal region with a peak at 134°C which could be considered as the melting process of EA. In the case of HPβCD, the DSC spectrum was a wider region corresponding to the release of water molecules from the inner cavity of HP-βCD. The melting point of hydroxypropyl-β-cyclodextrin was 95°C.

The DSC curve of the [EA-HPβCD] complex showed that, the melting peaks of EA and HPβCD in the complex curve were 118°C and 75°C, respectively, which were declined in both temperature and intensity, compared with the corresponding points when EA and HPβCD in the pure form. This is the evidence that there is an interaction between EA and HPβCD.

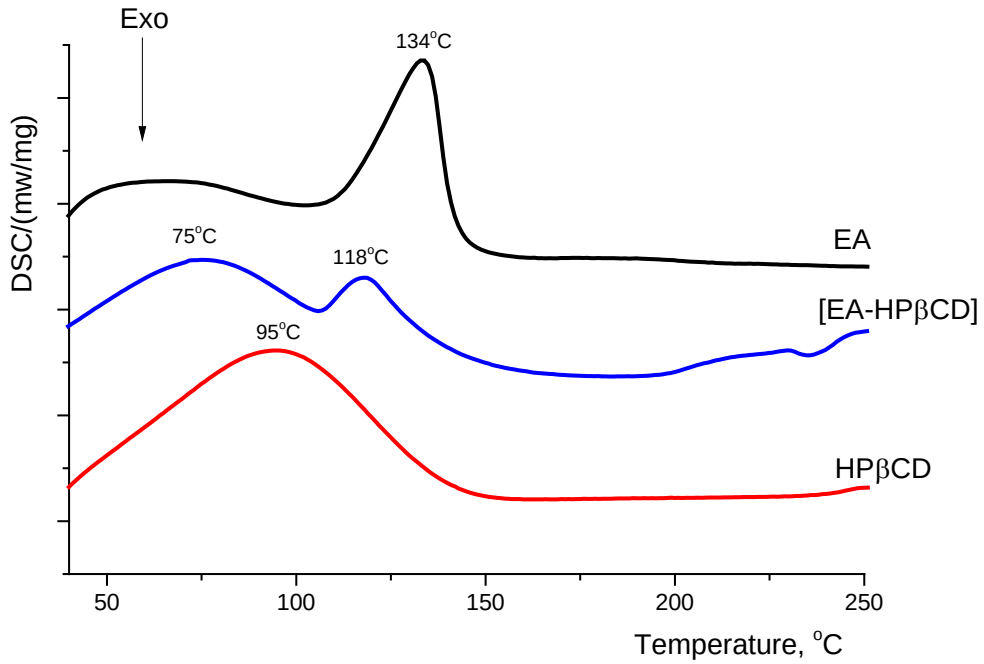

Figure 4. The DSC curves of EA, HPβCD and [EA-HPβCD] complex.

Determination of EA solubility before and after complexation

Establishing a calibration equation for EA in water

The optical density (Abs) of the EA solution was determined by the multi-concentration dilution method (Table 1).

From the data in Table 1, the calibration equation of EA in the water was established based on Origin software: y = 11122 + 0.0379*x, with regression coefficient R^2 = 0.9996, where "x" was the concentration of EA in solution (mol.L^{-1}), "y" was the optical density. This equation was used to determine the exact concentration of EA in solution in subsequent studies.

Table 1. The optical density (A) corresponds to the concentrations (C) of EA in solution at wavelength λ = 277 nm.

Sample	$C_{EA.}$ mol.L^{-1}	Abs
1	9.50E-06	0.120
2	1.24E-05	0.201
3	1.71E-05	0.217
4	1.90E-05	0.254
5	2.28E-05	0.290
6	2.85E-05	0.353
7	4.75E-05	0.570
8	5.70E-05	0.675
9	7.60E-05	0.884
10	9.50E-05	1.095
11	1.33E-04	1.527
12	1.66E-04	1.874

Determination of solubility of EA before and after complexation

Using the standard equation of EA in water, the solubility of EA and the complex [EA-HPβCD] in water was 12.2 mg.L^{-1} and 39.0 mg.L^{-1}, respectively. Thus, the solubility of the complex increased 3.2 times (Figure 5).

Figure 5. Sample of EA (2) or [EA-HPβCD] (2) in water.

Determination of antioxidant activity of EA and the complex

The results of determining the correlation between the percentages of DPPH free radical scavenging of EA or [EA-HPβCD] with the concentration of EA were shown in Figures 6-9.

From the regression equations, the corresponding EC_{50} values of EA and the complex were determined as concentrations at which the active ingredient captures 50% of the free radicals of DPPH. These data were listed in Table 2.

The data from Table 2 showed that after forming complex with HPβCD, the antioxidant activity of EA improved. Specifically, the EA50 value reduced from 5.3 10^{-5} to 4.9 10^{-5} mol.L^{-1}. The antioxidant capacity of polyphenols, including EA, is mainly contributed by the molecule contains hydroxyl groups that directly linked to the aromatic ring, which are able to give hydrogen, enable these active substances to participate in the redox reactions and capture free radicals. Several studies have demonstrated that the free radical scavenging ability of some antioxidants is closely related to the ability to donate/remove hydrogen atoms. Min Liu suggested that, the formation of intermolecular hydrogen bonds weakens the intramolecular hydrogen bonds, thereby, the ability to donate hydrogen of polyphenols has been improved (Liu *et al.*, 2013). Thus, the obtained results of this study are in consistent with several previous publications (Savic *et al.*, 2019). In this study, the solubility of EA after complexation increased by 3.2 times, while Savic *et al.* (2019) improved the solubility by 2.2 (when complexed with β-CD) and 2.6 times (when complexed with HP-β-CD) (Savic *et al.*, 2019). Especially, if the complex is synthesized by the freeze-drying method, the solubility of EA increases by 3.77 times, and even 5.1 times if the complex is converted into nanosponges.

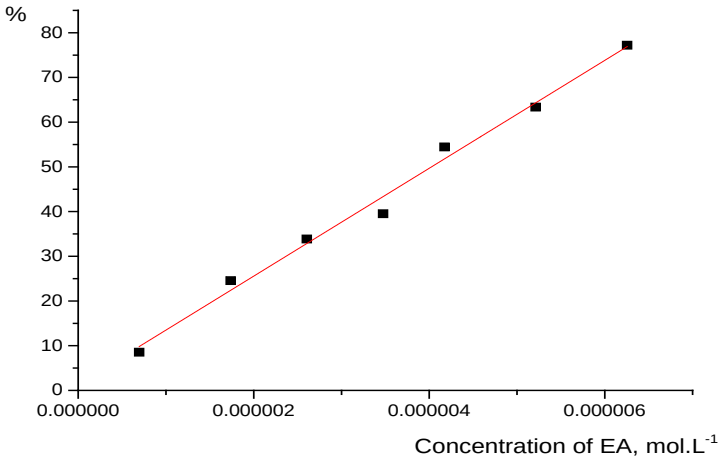

Figure 6. The graph of the correlation between the percentages of free radical scavenging of DPPH with the concentration of EA.

Figure 7. Reaction flasks of free radical scavenging of DPPH by EA. From left to right corresponds to samples from 0 to 7 of Figure 6, with increasing EA concentration. 0 is blank sample without EA.

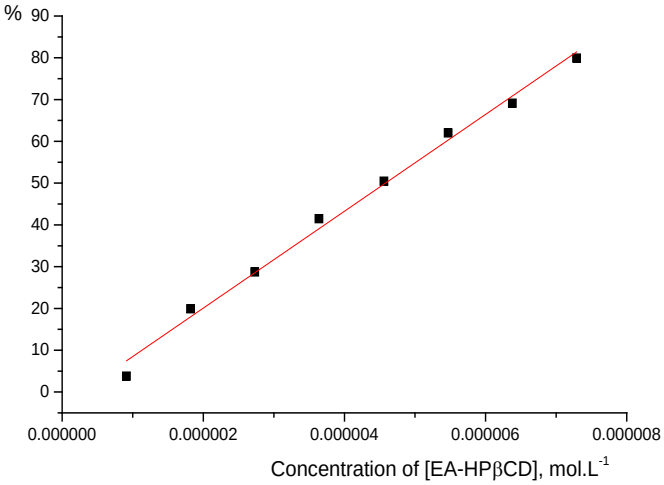

Figure 8. Graph of the correlation between the percentage of free radical scavenging of DPPH and the concentration of the [EA-HPβCD] complex.with increasing EA concentration. 0 is blank sample without EA.

Figure 9. Reaction flasks of free radical scavenging of DPPH by EA. From left to right corresponds to samples from 0 to 7 of Figure 8, with increasing [EA-HPβCD] concentration. 0 is blank sample without [EA-HPβCD].

Table 2. Correlation equation between percent DPPH free radical capture and concentration of EA and complex and corresponding EC_{50} value.

	Correlation equation	**Regression oefficient**	**EC_{50}, mol.L^{-1}**
EA	$y=1.15933\cdot10^{7}x-3.10765$	0.993	$5.3\cdot10^{-5}$
[EA-HPβCD]	$y=1.20674\cdot10^{7}x+1.42517$	0.989	$4.9\cdot10^{-5}$

CONCLUSION

The simple procedure for preparation of inclusion complex between ellagic acid with hydroxypropyl-β-cyclodextrin was successfully developed. The results of FTIR and DSC analysis confirmed that EA formed the complex with HPβCD. Obtained complexation efficiency was improved using mixtures of water and ethanol. Its maximal values were observed in solvent with volume ethanol content of 20%. The solubility and antioxidant activity of the complex were increased after complexation.

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