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ORIGINAL ARTICLE

Effect of hydroxypropyl-β-cyclodextrin complexation on the aqueous solubility, structure, thermal stability, antioxidant activity, and tyrosinase inhibition of paeonol

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Abstract The objective of this paper is to study the effect of hydroxypropyl-β-cyclodextrin (HP-β-CD) complexation on the aqueous solubility, structure, thermal stability, antioxidant activity, and tyrosinase inhibition of paeonol (PAE). The inclusion complex (PAE-HP-β-CD complex) of HP-β-CD and PAE was prepared by a freeze-drying method. Phase solubility tests showed that the stability constant of the inclusion complex was about 33.8 M^{-1} at 25 °C. The experimental results of proton nuclear magnetic resonance (H-NMR) spectroscopy, differential scanning calorimetry (DSC) and X-ray diffraction (XRD) suggested that PAE was included by HP-β-CD to form the PAE-HP-β-CD complex. Furthermore, the thermogravimetric analysis (TGA) results showed that the thermal stability of PAE was improved when it was complexed with HP-β-CD. Comparing the antioxidant activity of PAE with that of the PAE-HP-β-CD complex at the same concentration revealed that the complex of PAE with HP-β-CD was better able to eliminate radical. Furthermore, the experimental results revealed that the formation of a complex with HP-β-CD increased the water solubility of PAE, improving its apparent inhibitive activity of tyrosinase.

Keywords Paeonol · Hydroxypropyl-β-cyclodextrin · Inclusion complex · Chinese traditional medicine

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Introduction

Naturally occurring cyclodextrins (CDs) are cyclic oligosaccharides that are made of (α-1,4)-linked-glucopyranose units. Each has a hydrophilic outer surface and a hydrophobic cavity at its center [1–3]. The most common CDs are α-, β- and γ-CDs, which consist of six, seven and eight glucopyranose units, respectively. The special feature of these CDs is their ability to act as host molecules or containers in the formation of inclusion compounds with many quest molecules in aqueous solution. Inclusion complexes of native and modified CDs with many species have been widely described in the fields of supramolecular chemistry [4], pharmacology [5], food science [6] and cosmetics [7].

Paeonol (2′-hydroxy-4′-methoxyacetophenone, PAE), as shown in Fig. 1a, is the main active compound of the Paeonia suffruticosa Andrews, a traditional Chinese herb that is used in Asia and Europe [8]. PAE can improve blood flow; down-regulate transcription factors NF-κB and AP-1

[9, 10]; suppress the expression of cyclooxygenase-2, nitric oxide synthase, cell surface adhesion molecules, TNF-α and IL-1β [10, 11], and inhibit the activity of extracellular signal-regulated kinase and p38 [10, 12]. This compound has antioxidant and anti-inflammatory activity and suppresses tumor formation [10, 11, 13]. PAE can also inhibit melanin synthesis and down-regulate melanin transfer [14], which effects can be exploited in cosmetic applications.

Although PAE has many characteristics that make it potentially suitable for medical use, it is lipophilic and has a low solubility in water, which may limit its range of applications. Therefore, the formation of an inclusion complex with CD to increase its solubility has been reported. To the best of our knowledge, these investigations are focus on the use of β-CD to include PAE, such as the identification of the complex by using UV–visible absorption spectrometry [15], isothermal titration calorimetry [16, 17], nuclear magnetic resonance (H-NMR) spectroscopy [16–18], thermogravimetric analysis (TGA) [19], two-dimensional rotating frame spectroscopy [19], differential scanning calorimetry (DSC) [18] and X-ray diffraction (XRD) [18].

2-Hydroxypropyl-β-cyclodextrin (HP-β-CD), as presented in Fig. 1b, is a hydroxyalkylated CD derivative and is widely used to increase the solubility, stability and bioavailability of drugs, because of its relatively higher water solubility and lower toxicity than β-CD [20]. Therefore, the use of HP-β-CD to form an inclusion complex (PAE-HP-β-CD complex) with PAE is of interesting. The aims of this investigation are to study the effect of HP-β-CD complexation on the aqueous solubility, structure, thermal stability, antioxidant activity, and tyrosinase inhibition of PAE.

In this study, the PAE-HP-β-CD complex is prepared by a freeze-drying method and is characterized by H-NMR spectroscopy, DSC, TGA and XRD. The antioxidant activity of PAE and PAE-HP-β-CD complex and their ability to inhibit tyrosinase are determined by using 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) free radical and mushroom tyrosinase, respectively.

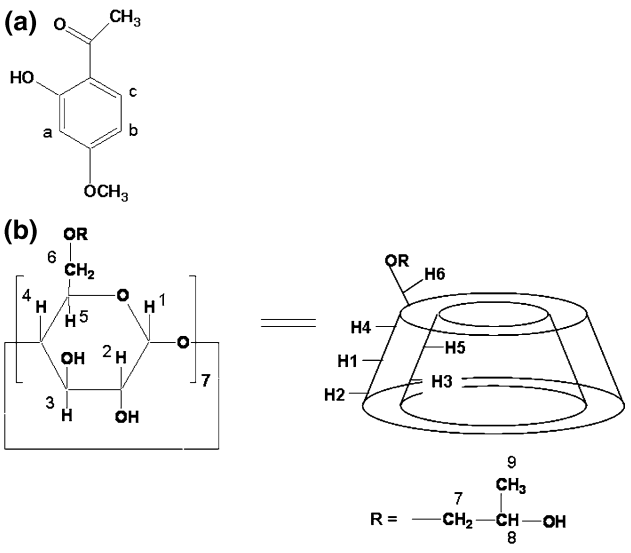

Fig. 1 Chemical structures of **a** PAE and **b** HP-β-CD

Materials and methods

Materials

PAE, HP-β-CD (an average $M_w \sim 1{,}460$ with 0.8 molar substitution, hydroxypropyl moiety), mushroom tyrosinase, L-tyrosine, sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), DPPH free radical, dimethyl sulfoxide (DMSO) and hydroquinone (HQ) were obtained from Aldrich (St. Louis, MO, USA). All of these chemicals were of analytical grade. Ethanol (95%, v/v) was purchased from Merck Co. (Santa Ana, CA, USA). Sorensen's buffer solution (PBS) was prepared by adding 3.4 g of KH_2PO_4 and 3.55 g of Na_2HPO_4 to 1000 mL of water. The water was doubly distilled and deionized.

Phase solubility

Phase solubility studies were performed using the method of Higuchi and Connors [21]. An excess of PAE was mixed in aqueous solutions that contained increasing amounts of HP-β-CD using a laboratory shaker at 25 °C. After each solution had been equilibrated for 2 days, it was filtered through 0.45 μm PTFE membrane filters to remove undissolved PAE. 0.1 mL of filtrate was diluted to 25 mL by adding deionized water, and the amount of dissolved PAE was spectrophotometrically determined by measuring the absorbance at 275 nm using a PerkinElmer (Lambda 25) UV/Vis spectrometer. The experiments were carried out in triplicate. The stability constant (Kc) of the PAE-HP-β-CD complexed was determined from the slope and intercept of the straight line (So) of the phase solubility diagram, using the equation

$$Kc = \text{slope}/So(1 - \text{slope})$$

Preparation of PAE-HP-β-CD complexes and physical mixtures

PAE-HP-β-CD complex was prepared by a freeze-drying method. 4.6 g of HP-β-CD (FW = 1540) was dissolved in 10 g of distilled water. 0.5 g of PAE (FW = 166) in 10 g of ethanol was slowly added to the HP-β-CD solution with continuous agitation. The molar ratio of PAE to HP-β-CD was 1:1. The mixture was stirred continuously for 24 h at room temperature and then filtered through a 0.45 μm membrane filter. Filtrates were frozen at −40 °C and

lyophilised in a freeze dryer to produce the PAE-HP-β-CD complexes. A physical mixture of HP-β-CD and PAE in the same molar ratio as that of the PAE-HP-β-CD complex was prepared using a mortar and pestle for 2 min to obtain a homogeneous physical mixture.

H-NMR

H-NMR spectra were obtained using a Bruker Avance 600NMR spectrometer with D_2O as a solvent. The chemical shifts (δ) were presented as ppm and referenced to the residual water signal (4.75 ppm).

DSC

Thermal analyses were performed using a DSC TA Q2000. 10 mg of sample was sealed into aluminum pans. Samples were heated over a temperature range of −20–350 °C at a heating rate of 10 °C min^{-1} under flowing nitrogen gas.

XRD

X-ray powder diffraction patterns were obtained using a Rigaku-D/MAX-IIIV diffractometer with Ni-filtered, Cu Kα radiation, a voltage of 40 kV and a current of 300 mA. The scanning rate was 0.020 s^{-1} over a 2θ range of 10–60°.

TGA

TGA was performed using a thermigravimetric analyzer (TA Q5000). After the sample (about 10 mg) had been loaded on the platinum pan of the analyzer, the analysis was carried out at a heating rate of 20 °C/min in an atmosphere of nitrogen.

Antioxidant activity of PAE-HP-β-CD complex and PAE

The DPPH free radical scavenging test was conducted as described elsewhere [22]. The PAE dose of the PAE-HP-β-CD complex was defined as the amount of PAE that was incorporated in the complex [22, 23]. The formula weights of PAE and HP-β-CD are 166 and 1540, respectively. 1706 g/mol (166 + 1540 = 1706) was taken as the formula weight of the PAE-HP-β-CD inclusion complex in the experimental work. Various concentrations of PAE-HP-β-CD inclusion complex (or PAE) solutions were prepared by dissolving the desired amount of PAE-β-CD inclusion complex (or PAE) in 100 mL of a mixed solvent (water: ethanol = 50: 50, v/v). 1 ml of each of these inclusion complex (or PAE) solutions was added to 2 mL of the DPPH solution (water: ethanol = 50: 50, v/v) to yield the final concentration of inclusion complex (or PAE). The initial concentration of the DPPH solution was 0.4 mM. After incubation for 30 min at room temperature in the dark, the free radical scavenging activity of the drug was measured by monitoring the decay of absorbance of the DPPH solution at 517 nm in the presence of the drug solution. All data were presented as means of at least three replicates. The scavenging activity was calculated as follows.

$$\%\text{Scavenging activity} = 100 \times (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}$$

where A_{control} is the absorbance of the DPPH solution without the drug, and A_{sample} is the absorbance of the DPPH solution with the drug.

Inhibition of tyrosinase

The oxidation of L-tyrosine by tyrosinase was spectrophotometrically monitored, as described elsewhere [24]. Mushroom tyrosinase was used without further purification. The assay was conducted in a 3.5 mL quartz cell, and a series of concentrations of the drug (inclusion complex or PAE) in a mixed solvent (PBS: DMSO = 90: 10, v/v) was pre-incubated with 0.3 mL of tyrosinase (230 units/mL) for 10 min at 37 °C. 0.3 mL L-tyrosine was added to initiate the reaction and the assay mixture was incubated for 30 min. The amount of dopachrome that was generated in the reaction mixture was measured using a UV–visible spectrometer at a fixed wavelength of 475 nm. All data were reported as means of at least three replicates. The inhibition of tyrosinase was calculated as follows.

$$\%\text{Inhibition} = 100 \times (A_0 - A_S)/A_0$$

where A_0 is the absorbance of the assay solution without the drug after incubation, and A_S is the absorbance of the assay solution with the drug after incubation.

Results and discussion

Phase solubility study

Phase solubility tests were performed at 25 °C. The absorbance of PAE at 275 nm was measured in the presence of increasing concentrations of HP-β-CD. Figure 2 presents the phase solubility diagram of PAE with HP-β-CD, in which the solubility of PAE increased linearly with HP-β-CD concentration, which relationship is a feature of AL-type complexes [21]. The stability constant, Kc, was about 33.8 M^{-1}.

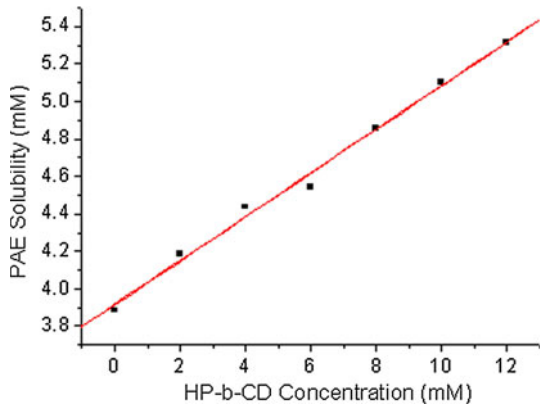

Fig. 2 Phase solubility diagram of PAE with HP-β-CD at 25 °C

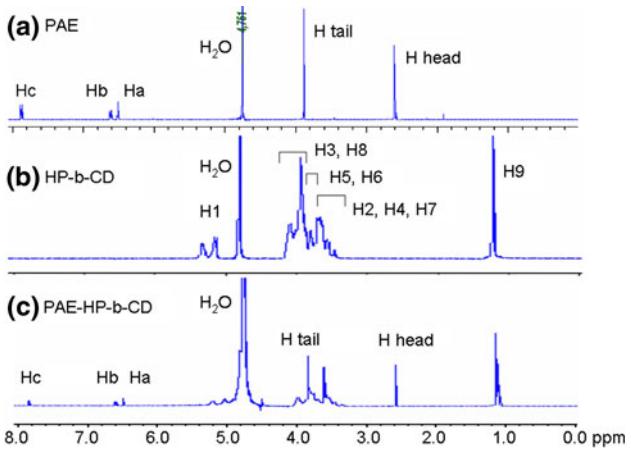

Fig. 3 H-NMR spectra of **a** PAE, **b** HP-β-CD and **c** PAE-HP-β-CD complex

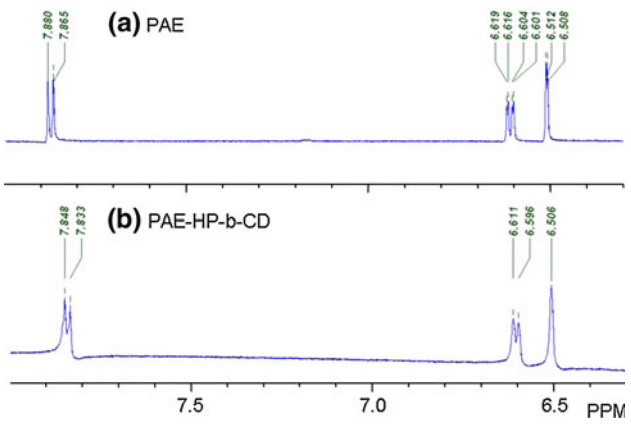

Fig. 4 Comparison of H-NMR spectra of **a** PAE and **b** PAE-HP-β-CD complex

H-NMR

Figure 3a, b and c present the H-NMR spectra of PAE, HP-β-CD and PAE-HP-β-CD complex, respectively. The proton resonances of HP-β-CD were assigned based on the relevant literature [25–28]: chemical shifts in the range 3.85–3.95 ppm were those of H3 and H8, those in the range 3.40–3.75 ppm were those of H2, H4 and H7, and those in the range 3.70–3.85 ppm were those of H5 and H6. The appearance of chemical shifts at 5.51, 6.65 and 7.84 ppm in Fig. 3c corresponded to Ha, Hb and Hc of PAE, indicating the presence of PAE in the PAE-HP-β-CD complex. As well as providing information on the composition of the inclusion complex, H-NMR spectroscopy provides direct evidence of the inclusion of a guest molecule inside the CD cavity, which can be identified by comparing the chemical shifts of the free guest molecule and CD with those of its complex [27]. Such evidence is based on the fact that, if an inclusion is present, then the physical or chemical environment is affected by the hydrogens at the internal surface of the cavity (H3 and H5 of the CD) and the included guest molecule. Since the H-NMR spectral peaks of H3 and H5 of HP-β-CD overlapped those of the other hydrogen, the H-NMR spectral peaks of PAE were used to analyze the inclusion of PAE with HP-β-CD. Figure 4 present the partial H-NMR spectra of PAE and PAE-HP-β-CD complex, in which changes in the chemical shifts of Ha (~ -0.006 ppm), Hb (~ -0.008 ppm) and Hc (~ -0.032 ppm) of PAE were observed, suggesting that PAE was included by HP-β-CD to form the PAE-HP-β-CD complex.

DSC

DSC analysis is often performed to characterize inclusion compounds with CDs by comparing the thermal behaviors of the individual components, their physical mixtures and inclusion compounds. Figure 5 shows the DSC curves of PAE, HP-β-CD, PAE-HP-β-CD complex and the physical mixture of PAE and HP-β-CD. HP-β-CD dehydrated at 75–120 °C. PAE was a crystalline compound, in which an endothermic peak appeared at its melting temperature around 50 °C. The physical mixture of PAE and HP-β-CD had the same endothermic peak as PAE. When a crystallizable sample shows amorphous due to the freezing process, this sample will have exothermic annealing (or crystallizing) peak, which is followed by endothermic melting peak, during the DSC heating process. In this study, neither exothermic annealing peak nor endothermic melting peak of PAE was observed, indicating no free drug (PAE) was present (i.e. HP-β-CD in the PAE-HP-β-CD complex destroyed the interaction of PAE molecules to form a crystalline structure). These DSC results confirmed that PAE was no longer present as a crystalline material

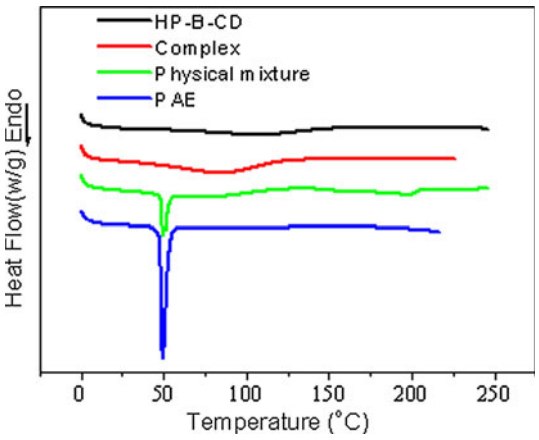

Fig. 5 DSC curves of PAE, HP-β-CD, PAE-HP-β-CD complex and physical mixture of PAE and HP-β-CD

and its HP-β-CD solid complexes were present in the amorphous state because an HP-β-CD molecule encapsulated each PAE molecule [28].

XRD

The powder X-ray diffraction spectra verified the formation of the inclusion complex. Figure 6 shows the XRD patterns of PAE, HP-β-CD, PAE-β-CD complex and the physical mixture of PAE and HP-β-CD. In the X-ray diffractogram of PAE powder, sharp peaks appeared at diffraction angles of 2θ at 12, 24 and 26°, suggesting that PAE is a crystalline material. Crystallinity peaks of PAE were still obtained from the physical mixture of PAE and HP-β-CD. In contrast, these characteristic peaks were not obtained from the PAE-HP-β-CD complex, revealing that PAE was present in the cavity of HP-β-CD and PAE was completely amorphized in the PAE-HP-β-CD complex. These XRD results were consistent with the DSC results.

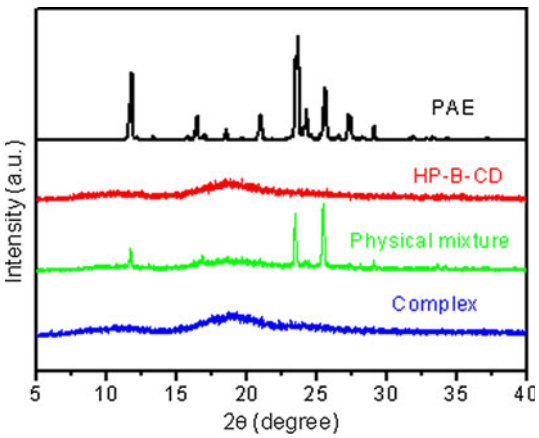

Fig. 6 XRD patterns of PAE, HP-β-CD, physical mixture of PAE and HP-β-CD and PAE-β-CD complex

TGA

Thermogravimetric analysis was performed at a heating rate of 20 °C/min in an atmosphere of nitrogen. Figure 7 presents the TGA profiles of HP-β-CD, PAE, PAE-HP-β-CD complex and the physical mixture of PAE and HP-β-CD. HP-β-CD lost weight in two steps at 30–100 °C and 330–380 °C. The former was associated with the evaporation of water that had been adsorbed by HP-β-CD; the latter was caused by the decomposition of HP-β-CD. PAE had a single weight loss step at 120–180 °C, associated with the evaporation and/or decomposition of PAE. The physical mixture exhibited a similar three-step weight loss (30–100, 120–180 and 330–380 °C). These steps, at increasing temperatures, yielded similar profiles to those of PAE and HP-β-CD, indicating that water, PAE and HP-β-CD were the major components of this physical mixture.

Although PAE-HP-β-CD complex had the same number of weight loss steps as the physical mixture, the weight loss temperatures of PAE-HP-β-CD complex (30–100, 120–240 and 330–380 °C) differed from those of the physical mixture. In particular, the weight loss of PAE in the complex occurred at 120–240 °C; the ranges for both the physical mixture and PAE were about 120–180 °C. This observation indicated that the formation of PAE-HP-β-CD complex retarded the weight loss of PAE during heating. These findings were consistent with the literature, in which the thermal stability of quest molecules was improved when it was complexed with HP-β-CD [29, 30].

Antioxidant activity

The DPPH method is used worldwide to evaluate free radical scavenging activity. The method is based on the loss of color when the unpaired electron of the nitrogen atom in the DPPH radical is reduced by receiving a hydrogen atom from an antioxidant compound. Therefore,

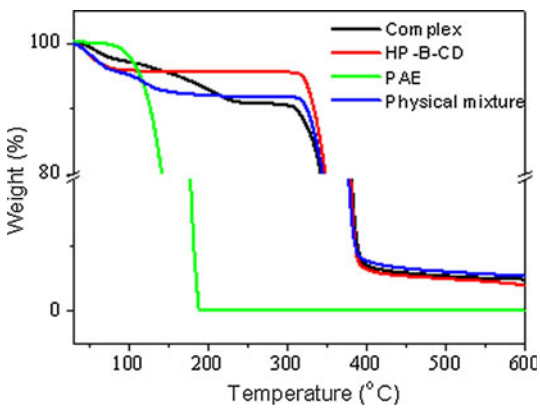

Fig. 7 TGA profiles of HP-β-CD, PAE, PAE-HP-β-CD complex and physical mixture of PAE and HP-β-CD

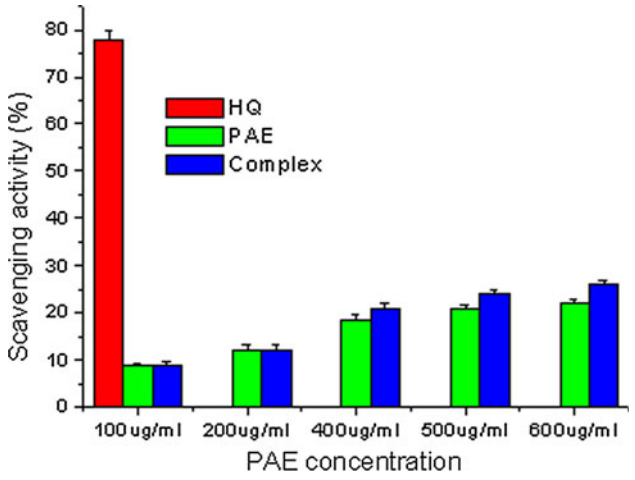

Fig. 8 Antioxidant activity of PAE and PAE-HP-β-CD complex at various concentrations

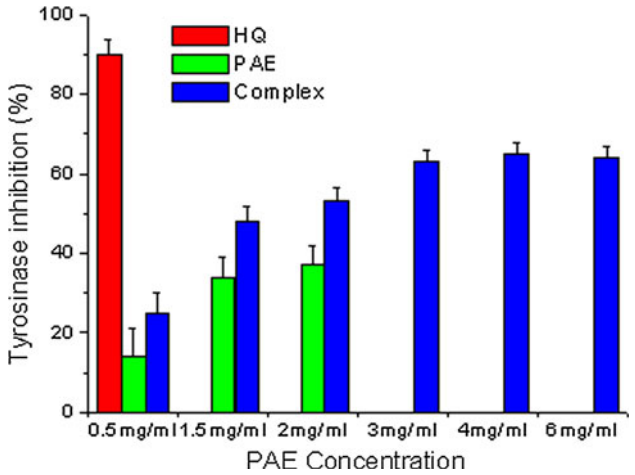

Fig. 9 Tyrosinase inhibition by PAE and PAE-HP-β-CD complex at various concentrations

the antioxidant activity of phenolic compounds can be determined by monitoring the drop in absorbance of the DPPH radical at 517 nm.

Figure 8 presents the antioxidant activity of PAE and PAE-HP-β-CD complex at various concentrations, with HQ as a positive control. The antioxidant activities of both PAE and PAE-HP-β-CD complex increased with concentration up to 22 and 28%, respectively, indicating that the practical use of both was limited. The relatively low radical scavenging activity of PAE was consistent with the literature [31], in which its 50% scavenging concentration is reported to exceed 400 mM [66.4 mg/mL]. However, comparing the antioxidant activity of PAE and PAE-HP-β-CD complex at a particular concentration revealed that the PAE-HP-β-CD complex was better able than PAE to eliminate the DPPH radical. This effect of inclusion on activity has been observed in several phenolic compounds, such as morin [23], quercetin [32], resveratrol [33] and coumaric acids [22]. It has been proposed to involve either the effective stabilization of radical species in the CD cavity [23] or the formation of a hydrogen bond between secondary hydroxyl groups of CD and –OH on the aromatic ring of the quest molecule [22].

Inhibition of tyrosinase

The inhibition of tyrosinase by PAE and PAE-HP-β-CD complex was identified by measuring the amount of dopachrome that was generated in the reaction mixture in a buffer solution, using a UV–visible spectrometer with a fixed wavelength of 475 nm. Figure 9 presents the percentage inhibition of tyrosinase. The inhibition of tyrosinase by both PAE and PAE-HP-β-CD complex increased with concentration. Furthermore, the inhibition of tyronsinase by PAE-HP-β-CD complex exceeded that by PAE at the same concentration. When the concentration of PAE was higher than 2 mg/ml, the testing medium became turbid because of the limited water solubility of PAE; the inhibition of tyrosinase could therefore not be quantified. In contrast, the PAE-HP-β-CD complex was soluble, even at a concentration of 6 mg/mL, at which its tyrosinase inhibition rate was more than 60%. Although the effects of CD on inhibition (or promotion) depend strongly on the substrates, enzymes and inhibitors [34–37], the experimental results in this study revealed that the formation of a complex with HP-β-CD increased the water solubility of PAE, improving inhibition activity.

Conclusions

The inclusion complex of PAE and HP-β-CD was synthesized by a freeze-drying method. The PAE-HP-β-CD complex was characterized by H-NMR, DSC and XRD. The thermal stability and antioxidant activity of PAE and its ability to inhibit tyrosinase were improved when it was complexed with HP-β-CD. These results suggested that the use of HP-β-CD to improve the water solubility of PAE without sacrificing its activity was feasible.

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