INTRODUCTION

Investigations of molecular recognition have attracted much attention in supramolecular chemistry involving natural and artificial host-guest systems.

Apigenin (AP), (4',5,7-trihydroxyflavone, [Figure 1]), is one of the active ingredients found naturally in a variety of fruits and vegetables, including parsley, onions, orange, tea, wheat sprouts, and so on [1,2]. As an important food functional factor and potential therapeutic drug for some diseases, it has been widely investigated, including anti-inflammatory effects [3], free radical scavenging effects [4], growth inhibitory properties in several cancer lines including breast [5], colon [6], skin [7], myeloid leukemia cells [8], and pancreas [9]. Influences on membranes [10-15] have been studied. Moreover, recent clinical studies demonstrated that AP intake was associated with a suggestive decrease in woman’s risk of ovarian cancer [16,17], which indicated AP could be a very promising drug candidate for future drug development.

However, both of the basic physicochemical and biopharmaceutical properties of AP were still limited in clinical application.

Cyclodextrins (CDs) are polysaccharides made up of 6-8 D-glucose monomers connected at the 1 and 4 carbon atoms. They have the property of forming inclusion complex with various guest molecules with suitable polarity and dimension due to their special molecular structure-hydrophobic internal cavity and hydrophilic external surface [18-21]. This ability has been widely used in pharmaceutical industries and has also been used for analytical purposes [22]. Furthermore, the CDs have been used as models for proteins and enzymes because they interact with many substances in a manner similar to proteins and enzymes [23]. In addition, especially in pharmaceutical industries,
since the inclusion process of pharmaceutical molecules with CDs led to important modifications of pharmaceutical properties of guest molecules, the pharmaceutical interest in CDs extends to enhance solubility, chemical stability, and bioavailability of poorly soluble drugs, to reduce toxicity and to control the rate of release so on and so forth [24]. Therefore, it is essential to comprehensively understand the effects of inclusion about pharmaceutical molecules.

Since many guest compounds present fluorescent properties, it is interesting to analyze the changes produced in such properties when these compounds form inclusion complexes. The nonradioactive decay process of analyst is often significantly attenuates as the fluorescence emission increases [25,26]. Due to its sensitivity, selectivity, and instrumental simplicity, fluorimetric method can be used as a resource to improve the performance of analytical methods and to determine the association constants of complexes [27,28].

1, 1-Diphenyl-2-picryl-hydrazyl, (DPPH) [Figure 1] is a stable free radical which has an unpaired valence electron at one atom of the nitrogen bridge [29]. Scavenging of DPPH radical is the basis of the popular DPPH antioxidant assay [30-32].

The inclusion of AP with biologic-molecular, for example, bovine serum albumin [33] has been researched by many groups, but the inclusion of AP with CDs has not been investigated. Such, this paper has definite guidance purport in clinic pharmaceutics.

In this paper, the interaction of AP with β-cyclodextrin (β-CD) and 2-hydroxylpropyl (HP)-β-CD was studied in detail based on fluorescence method and determined the effect of the complex process on their antioxidant capacity.

EXPERIMENTAL

Instrumentation and Materials

UV-7504 PC spectrophotometer (Shanghai Xinmao Instrument Co., Ltd.). Fluorescence measurements were performed by F-2500 fluorescence spectrophotometer (HITACHI) using 1 cm quartz cell and both the slits were set at 5 nm with an excitation wavelength at 270 nm and the emission at about 330 nm.

A stock solution of 1.0 × 10^{-4} mol/L AP (purity ≥98%, purchased from Nanjing Zelang Medical Technology Co., Ltd.) was prepared by dissolving and diluting its crystals in ethanol. CDs and DPPH• were purchased from Shanghai Yuanye Biotechnology Co., Ltd. All other reagents were of analytical-reagent grade and were used without purification. Ultrapure water was used throughout. All experiments were carried out at room temperature.

Procedure

A 1 mL aliquot of the stock solution (1.0 × 10^{-4} mol/L) of AP was transferred into a 10 mL volumetric burette, and an appropriate amount of 1.0 × 10^{-2} mol/L CDs (β-CD and HP-β-CD) was added. The mixed solution was diluted to the final volume with ultrapure water, and ultrasonic handled for 6 h, equilibrated for the whole night at room temperature. Then the solution was analyzed by fluorescence method.

Phase-solubility Study

Solubility measurements were based on the phase solubility technique [34]. Namely, the excess amount of solid AP (8 mg) was added to a series of 10 mL stopper burette that contained increasing amount of CDs (1.0 × 10^{-2} mol/L, 0-9 mL, including β-CD and HP-β-CD). These obtained suspensions were shaken by ultrasonic method for 6 h at room temperature, and then were filtered after being placed for 7 days. The filtrate was diluted and analyzed through ultraviolet method. Phase-solubility profile was obtained by plotting the solubility of AP versus the concentration of CDs.

The apparent stability constant (Ks) of the complexes were calculated according to the following equation:

\[ K_s = \frac{\text{Slope}}{S_0 (1 - \text{Slope})} \]

Where \( S_0 \) is the solubility of AP at room temperature in the absence of CDs and slope means the corresponding slope of the phase-solubility diagrams.

The Scavenging Activity of the Stable Radical DPPH•

The antioxidant activity was measured, wherein the bleaching rate of a stable free radical, DPPH• is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH• absorbs at 517-520 nm, but upon reduction by an antioxidant or a radical species its absorption decreases.

Stock solutions of DPPH were prepared in methanol and ethanol (1.0 × 10^{-4} M), respectively. The sample bottles were wrapped with aluminum foil and used as the samples stored in the dark [35]. Spectrophotometric measurements were
done at 517 nm. The reaction was started by adding a series of concentration of AP \((1.0 \times 10^{-4} \text{ M})\), 3 mL of CDs (β-CD, or HP-β-CD, \(1.0 \times 10^{-2} \text{ M}\)) and a certain amount of DPPH. The mixed solution was diluted to the final volume with methanol or ethanol. After shaking up, the mixed solution was equilibrated for 30 min at room temperature.

The results were expressed as percentage DPPH elimination calculated according to the following equation [36]:

\[ I(\%) = \left(1 - \frac{[A_{\text{sample}} - A_{\text{blank}}]}{A_{\text{control}}} \right) \times 100 \quad \text{(eq.A1)} \]

Where, \(I\) is radical-scavenging activity, \(A_{\text{sample}}\) is absorbance of the sample, \(A_{\text{blank}}\) is absorbance of the blank sample without DPPH, and \(A_{\text{control}}\) is absorbance of DPPH only.

**RESULTS**

**Fluorescence Study**

Figure 2 shows fluorescence spectra of AP in the absence and presence of CDs (including β-CD, and HP-β-CD). The excitation wavelength was 270 nm; the maximum emission wavelengths for the HP-β-CD were 326 nm and 335 nm, respectively. And for the β-CD, the maximum emission wavelengths were 299 nm and 324 nm, respectively. With increasing concentration of CDs, the emission peaks intensity increasing. These data suggest that stable complexes were formed between AP and CDs. The CDs cavities provide an apolar environment for the AP molecular and thus, increase the quantum yield of the fluorescence of AP.

The formation constant (Ks) and the ratio of the complex were calculated from these data by use of the modified Benesi–Hildebrand equation [37]:

\[ \frac{1}{(F-F_0)} = \frac{1}{(|CDs| K\alpha)} + \frac{1}{\alpha} \quad \text{(eq.B1)} \]

Where, \(F\) and \(F_0\) represent the fluorescence intensity of AP in the presence and absence of CDs, respectively; \(K\) is a forming constant; \(\alpha\) is a constant.

Table 1: Apparent stability constant (Ks) of AP inclusion

<table>
<thead>
<tr>
<th>CDs complex</th>
<th>Linear equation</th>
<th>Ks (M⁻¹)</th>
<th>(r^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-CD</td>
<td>y=0.0005x+0.0235</td>
<td>2000</td>
<td>0.9905</td>
</tr>
<tr>
<td>HP-β-CD</td>
<td>y=2.2E-05x-0.0008</td>
<td>4.55×10⁴</td>
<td>0.9941</td>
</tr>
</tbody>
</table>

AP: Apigenin, HP: 2-hydroxylpropyl, β-CD: β-cyclodextrins

**Phase-solubility Measurements**

Figure 4 shows that CDs enhanced the poor aqueous solubility of AP, thus proving a certain degree of its inclusion complexes in aqueous solutions, the results observed showed a linear behavior for β-CD (\(r^2 = 0.9960\)) and HP-β-CD (\(r^2 = 0.9975\)), and consistent with 1:1 molecular complex formation for CDs and AP. The binding constant (Ks) of the complexes is shown in Table 2. As shown in Table 2, the binding constant and solubility of AP determined with CDs followed the rank order HP-β-CD>β-CD. The results were as the same as fluorescence results.

**Scavenging of DPPH Free Radical**

The absorbance profiles of DPPH in methanol and ethanol are shown in Figure 5. The order of absorbance was higher in methanol solution than in ethanol. Higher absorbance in methanol solution implies better sensitivity vis-à-vis ethanol.
solution of DPPH, but the linearity is better in ethanol solution ($r^2 = 0.9990$) than in methanol solution ($r^2 = 0.9984$). Hence, we choose ethanol as solvent. The range of accuracy for spectrophotometric measurements falls within an absorbance of 0.221-0.735 which corresponds to the DPPH concentration of nearly 20-60 μM [Figure 5].

Further, we determine the DPPH radical scavenging activity of AP, AP-β-CD inclusion, and AP-HP-β-CD inclusion as shown in Figure 6. In the range of the experimental concentration, the median inhibitory concentration for AP, AP-β-CD and AP-HP-β-CD are 5.96 μM, 7.54 μM, and 14.41 μM, respectively. DPPH radical scavenging activity is influenced by the polarity of the reaction medium, chemical structure of the radical scavenger and the pH of the reaction mixture [35,38,39]. Furthermore, the antioxidant activity of phenolic compounds depends on the position and degree of hydroxylation, as well as the nature of radicals of the ring structure. Anti-oxidative activity is intensified by the presence of a second hydroxy group, through the formation of an intra-molecular hydrogen bond [40]. It might be that the -OH positions of AP molecules is close enough to secondary -OH groups of CDs to form hydrogen bonds and contribute to antioxidant activity. Therefore, the formation of an “intra-molecular” hydrogen bond of the inclusion complex is possible and consequently an increase of antioxidant capacity is expected.

Besides, our data on the comparative reaction of AP, AP-β-CD, and AP-β-CD [Figure 7] indicate that the time course of inhibition also have to be determined. The radical scavenging reaction of AP, AP-β-CD, and AP-β-CD with DPPH are, essentially, instantaneous. On the other hand, the order of the radical scavenging reaction with DPPH are AP-HP-β-CD inclusion > AP-β-CD inclusion > AP, and the absorbance remain unchanged till a period of 90 min of observation. It is important to do a time course of radical scavenging activity while using DPPH radical for the assay of antioxidant activity.

CONCLUSION

The present study demonstrates the inclusion complex interaction between AP with β-CD and HP-β-CD in the solution. Among CDs, the inclusion ability of HP-β-CD is stronger than that of β-CD. The antioxidant assay based on scavenging of DPPH radical at a DPPH concentration of 40 μM in ethanol, depending upon the solubility of the compound under investigation, is recommended. And the activity of eliminating free radical DPPH• was HP-β-CD inclusion complex > β-CD inclusion complex > free AP. In addition, the fluorescence spectroscopy and Phase-solubility measurements data show the formation of 1:1 stoichiometric complex of AP with β-CD and HP-β-CD over the concentration range.
evaluated. Moreover, the study demonstrated that CDs served as drugs carrier system in a dosage-controlled manner and can increase the solubility, stability, and antioxidant activity of guest molecular.

REFERENCES

23. Li, et al.: Inclusion complex of apigenin with β-cyclodextrins evaluated. Moreover, the study demonstrated that CDs served as drugs carrier system in a dosage-controlled manner and can increase the solubility, stability, and antioxidant activity of guest molecular.

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