PREPARATION AND PROCESS OPTIMIZATION OF POMEGRANATE ELLAGIC ACID-HYDROXYPROPYL-β-CYCLODEXTRIN INCLUSION COMPLEX AND ITS ANTIBACTERIAL ACTIVITY IN VITRO

GAOFU FAN1, YUHUA CAI1*, ENTAO FU1, XING YUAN1, JIE TANG1, HUI SHENG1, JUMEI GONG1
1School of Bioscience Engineering, Hefei technology college - 1*Medical school, Hefei technology college

ABSTRACT

Finding new antibacterial drugs or reversing bacterial resistance has become a hot spot in international research. A lot of studies have shown that Chinese medicines have antibacterial activity or reverse bacterial resistance. The aim of the present paper is to select preparation process of pomegranate ellagic acid-hydroxypropyl-β-cyclodextrin inclusion complex and its antibacterial effect in vitro. The inclusion complex was prepared by stirring-ultrasonic method. The structure of the inclusion complex was identified by X-ray diffraction and scanning electron microscopy. The antibacterial activity of the inclusion complex was determined by the Oxford cup method. The inclusion ratio of pomegranate and hydroxypropyl-β-cyclodextrin was 1:2, the concentration of ethanol was 60%, the inclusion temperature was 20 °C, and the inclusion time was 36 h. The inclusion rate was 88.9%; pomegranate ellagic acid-hydroxypropyl-β-cyclodextrin inclusion complex inhibited Staphylococcus aureus (Gram-positive bacteria) and Escherichia coli (Gram-negative bacteria). The preparation process of pomegranate ellagic acid-hydroxypropyl-β-cyclodextrin inclusion complex is simple and stable, and the inclusion rate is high. The obtained inclusion compound has antibacterial activity and provides prospect for the application and development of pomegranate ellagic acid.

Keywords: Pomegranate ellagic acid (PEA), Hydroxypropyl-β-cyclodextrin (HP-β-CD), Inclusion complex, Orthogonal design, Scanning electron microscopy, Stirring-ultrasonic method, Antibacterial effect.

DOI: 10.19193/0393-6384_2019_1_63

Received July 17, 2018; Accepted September 20, 2018

Introduction

With the wide application of antibiotic drugs, microbial resistance has become a hot issue of global concern1. In recent years, the search for pure plant-derived monomeric drugs from natural plants, especially medicinal plants, has become a research hotspot2. Pomegranate ellagic acid (PEA) is one of the important active substances in the pomegranate plant. It is a naturally occurring polyphenolic compound in pomegranate, which has various pharmacological effects such as anti-oxidation, anti-tumor, anti-inflammatory, antibacterial and skin whitening3,4. However, pomegranate acid is almost insoluble in water, unstable to light, poor in permeability, and low in vivo absorption, which limits the clinical application of pomegranate acid5,6. The inclusion technique is a technique for embedding a poorly soluble drug of a guest molecule into a molecular capsule inclusion compound by using a large cavity of the main molecule of the inclusion material, thereby improving the solubility and stability of the poorly soluble drug. Hydroxypropyl-β-cyclodextrin (HP-β-CD) is one of the commonly used inclusion materials. It is non-toxic, has good water solubility, thermal stability, and poorly soluble drugs. After the inclusion of the inclusion compound, the solubility, bioavailability and stability of the drug can be improved.

However, there are no reports on the inclusion of hydroxypropyl-β-cyclodextrin inclusion pomegranate in domestic and foreign countries. Therefore, in this experiment, HP-β-CD was used to study the inclusion of pomegranate, and the inclusion compound was prepared by solution agitation-ultrasonic method, ultrasonic method and grinding method. The feed ratio and ethanol concentration were used. The inclusion time and inclusion temperature were ana-
alyzed, and the orthogonal design experiment was established to optimize the pomegranate hydroxypropyl-β-cyclodextrin (PEA-HP-β-CD) package. The best preparation process of the compound, in order to improve the bioavailability, solubility and stability of pomegranate acid; using the Oxford cup method to determine the PEA-HP-β-CD inclusion complex against Gram-positive bacteria (such as golden yellow grapes) Antibacterial activity of cocci) and Gram-negative bacteria (such as Escherichia coli); in order to provide a theoretical basis for the development of preparations for pomegranate ellagic acid.

**Material**

*Materials and reagents*

Pomegranate ellagic acid homemade (batch number: 20151230, which pomegranate was purchased from Anhui Province, the city of medicinal materials company, identified by Professor Peng Huasheng of the College of Pharmacy of Anhui University of Traditional Chinese Medicine as pomegranate pomegranate pomegranate, pomegranate scorpion acid dry powder by Anhui University of Traditional Chinese Medicine Anhui Provincial Key Laboratory Testing, purity ≥90%); Staphylococcus aureus (ATCC 6538) and E. coli (ATCC 6539) were purchased from China National Institute for the Control of Pharmaceutical and Biological Products, agar medium, Sabour's medium (Shanghai Xinran) Biotechnology Co., Ltd.); Pomegranate scorpion acid reference substance: purity ≥95% (batch number: 2075083, US sigma); HP-β-CD (Shanghai Boao Biotechnology Co., Ltd.); methanol, acetonitrile for chromatographic purity; other reagents All are analytically pure.

*Instruments and equipment*

Thermo-U3000 UHPLC System: equipped with TCC-3000RS UV detector and DAD-3000 diode array detector, American Thermo Fisher Company; Clear-D 24UV pure water / ultra pure water integrated system (Merck Chemical Technology (Shanghai) Ltd.); X'Pert PRO MPD ray diffractometer (Panaco, the Netherlands); SU8020 field emission scanning electron microscope (Japan Hitachi); KQ2200E ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.); V-1700 UV-visible Spectrophotometer (Shanghai Spectrometer Co., Ltd.); RE-52AA Rotary Evaporator (Shanghai Chubai Laboratory Equipment Co., Ltd.); HWS24 Electric Thermostatic Water Bath (Shanghai Yiheng Technology Co., Ltd.); SHB-III circulating water Multi-purpose vacuum pump (Zhengzhou Great Wall Science and Education Instrument Co., Ltd.); FD-1 type freeze dryer (Beijing Detianyou Technology Development Co., Ltd.); ZYW-100H constant temperature culture oscillator (Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.); FA1004 type electronic Balance (Tianjin Tianma); SPECTRAMAX 190 microplate reader ( Molecular Devices, USA); YJ-875A medical purification workbench (Suzhou gold) Yan Purification Equipment Factory), DNP-9162 Electrothermal Constant Temperature Incubator (Shanghai Jinghong Experimental Equipment Co., Ltd.), 3110 Series CO2 Incubator ( Thermo Scientific Water Jacketed Incubator, USA), LS-30CA High Pressure Steam Sterilizer (Shanghai Green Yu Biotech Co., Ltd.); Oxford Cup (outer diameter 8mm, inner diameter 6mm, height 10mm) Beijing Pioneer Weifeng Technology Development Company.

**Methods**

*Chromatographic conditions*\(^7\)

Column: Hypersil C18 (250 mm × 4.6 mm, 5 μm); mobile phase: acetonitrile: 0.3% trifluoroacetic acid (TFA) (20:80); column temperature 30 °C; The flow rate was 1.0 mL/min; the injection volume was 10 μL; the detection wavelength was 254 nm. Under this chromatographic condition, the characteristic peak shape of PEA is good, and its chromatogram is shown in Figure 1.

![Figure 1: HPLC chromatograms.](image)

Notice: PEA reference substance (A), negative control (B), PEA (C), and PEA-HP-β-CD inclusion complex (D)
Preparation of PEA reference
Solution accurately weigh 10.48 mg of PEA reference substance, place it in a 10 mL volumetric flask, dissolve it with methanol and dilute to volume, and prepare a reference stock solution with a concentration of 0.1048 mg/mL.

Drawing of the standard curves
Accurately draw the appropriate amount of PEA reference solution, and dilute to obtain a series of standard solutions with concentrations of 0.1048, 0.524, 1.048, 2.096, 5.240, 10.48, 20.96 μg/mL, and perform the chromatography under “3.1”. Conditional injection high performance liquid chromatography, recording chromatogram, with peak area as abscissa (X), series standard solution concentration as ordinate (Y), draw the standard curve of PEA, PEA regression equation is: Y=0.6587X+0.0491, r = 0.9999 (n = 7), the linear range is 0.1048 ~ 20.96 μg/mL.

Precision test
Take a PEA reference solution with a concentration of 1.048 μg/mL and inject it continuously for 6 times. Each injection volume is 10 μL, and the RSD of the peak area of PEA was 0.31%, indicating that the precision of the instrument is good.

Stability test
The test solution was injected at 0, 1, 2, 4, 6, 8, 12, 24 h according to the above chromatographic conditions, and the injection volume was 10 μL, and the RSD on the peak area of PEA-HP-β-CD was 0.48%, indicating that the sample is stable within 24 h.

Reproducibility test
Six samples of the test solution were prepared in parallel and injected separately. The injection volume was 10 μL, and the RSD of the PEA peak area was 1.03%. The repeatability was good.

Sample recovery rate test
Take a known concentration (1.048 μg/mL) of PEA-HP-β-CD test solution 10 mL, a total of 9 parts, according to the low, medium and high concentrations of the appropriate amount of PEA The reference solution was prepared according to the method of 1.3.3. And the injection analysis, the average recovery of PEA-HP-β-CD was calculated to be 99.46%, and the RSD was 1.25%.

Selection of the inclusion method
Stirring-Ultrasonic method
Dissolve pomegranate acid with a small amount of ethanol, slowly drip into HP-β-CD aqueous solution with stirring, ultrasonic (300 W, frequency 40 kHz) for 20 min, stir at room temperature (frequency 149 r/min) 18 h, microporous membrane (0.45 μm) was filtered, concentrated to about 10 mL with a rotary evaporator, and the concentrate was vacuum dried to obtain an inclusion compound.

Grinding method
Take appropriate amount of HP-β-CD dissolved in aqueous solution, while ultrasonically, accurately weigh ellagic acid, dissolve it with a small amount of ethanol and slowly add HP-β-CD solution dropwise, Under (25±1) °C, Continue to ultrasonic (300 W, frequency 40 kHz) to 30 min, filter the reaction mother solution through a microporous membrane (0.45 μm), and continue to concentrate the solvent to about 10 with a rotary evaporator. In mL, the concentrate is freeze-dried to obtain a clathrate.

Analysis of inclusion complexes
3 parts of PEA and HP-β-CD were accurately weighed according to the ratio of substances: PEA-HP-β-CD prepared by stirring-ultrasonic method, ultrasonic method and grinding method respectively. Place an appropriate amount in a 10 mL volumetric flask, add an appropriate amount of methanol, sonicate for 20 min, dilute to the mark with methanol, and shake well. The analysis was carried out according to the above chromatographic conditions, the content of PEA was calculated according to the external standard method, and the inclusion ratio was calculated according to the following formula. The inclusion ratio = (the content of PEA in the inclusion compound / the input amount of PEA) × 100%, the inclusion ratio of pomegranate in the inclusion compound prepared by the above three preparation methods was (83.64±0.21)% , (40.08±1.22)% and (55.14±0.885)% (n=3). The results showed that the inclusion rate of PEA-HP-β-CD prepared by stirring-
ultrasonic method was higher, so this method was chosen as the best method for preparing PEA-HP-β-CD inclusion complex.

Orthogonal test optimization of PEA-HP-β-CD preparation process

According to the literature and pre-test, the molar ratio of PEA to HP-β-CD (A), ethanol concentration (B), inclusion time (C), inclusion temperature (D) The influence was greater. Therefore, the inclusion rate of the inclusion compound was used as an indicator to carry out the L9(34) orthogonal test design optimization preparation preparation process for the above factors. The test design and results are shown in Table 1, and the variance analysis is shown in Table 2.

<table>
<thead>
<tr>
<th>Test number</th>
<th>A/(mol:mol)</th>
<th>B/%</th>
<th>C/h</th>
<th>D/℃</th>
<th>Inclusion rate/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1 (1)</td>
<td>40 (1)</td>
<td>12(1)</td>
<td>20(1)</td>
<td>78.16</td>
</tr>
<tr>
<td>2</td>
<td>1:1 (1)</td>
<td>50(2)</td>
<td>24(2)</td>
<td>30(2)</td>
<td>80.08</td>
</tr>
<tr>
<td>3</td>
<td>1:1 (1)</td>
<td>60(3)</td>
<td>36(3)</td>
<td>40(3)</td>
<td>79.44</td>
</tr>
<tr>
<td>4</td>
<td>1:2 (2)</td>
<td>40(1)</td>
<td>24(2)</td>
<td>40(3)</td>
<td>82.29</td>
</tr>
<tr>
<td>5</td>
<td>1:2 (2)</td>
<td>50(2)</td>
<td>36(3)</td>
<td>20(1)</td>
<td>87.32</td>
</tr>
<tr>
<td>6</td>
<td>1:2 (2)</td>
<td>60(3)</td>
<td>12(1)</td>
<td>30(2)</td>
<td>80.21</td>
</tr>
<tr>
<td>7</td>
<td>1:3 (3)</td>
<td>40(1)</td>
<td>36(3)</td>
<td>30(2)</td>
<td>77.08</td>
</tr>
<tr>
<td>8</td>
<td>1:3 (3)</td>
<td>50(2)</td>
<td>12(1)</td>
<td>40(3)</td>
<td>70.41</td>
</tr>
<tr>
<td>9</td>
<td>1:3 (3)</td>
<td>60(3)</td>
<td>24(2)</td>
<td>20(1)</td>
<td>80.48</td>
</tr>
<tr>
<td>K1</td>
<td>237.68</td>
<td>237.53</td>
<td>228.78</td>
<td>245.96</td>
<td></td>
</tr>
<tr>
<td>K2</td>
<td>249.82</td>
<td>237.81</td>
<td>242.85</td>
<td>237.37</td>
<td></td>
</tr>
<tr>
<td>K3</td>
<td>227.97</td>
<td>240.13</td>
<td>243.84</td>
<td>232.14</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>21.85</td>
<td>2.6</td>
<td>15.06</td>
<td>13.82</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Design and results of L9(34) orthogonal test (n=3).

Notice:F₀.₀₅(2, 2) = 19.00 F₀.₀₀(2, 2) = 99.0

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>sum of squares</th>
<th>n</th>
<th>MS</th>
<th>F</th>
<th>Significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>79.9</td>
<td>2</td>
<td>39.95</td>
<td>58.75</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>B</td>
<td>1.36</td>
<td>2</td>
<td>0.68</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>47.31</td>
<td>2</td>
<td>23.66</td>
<td>34.79</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>D</td>
<td>32.46</td>
<td>2</td>
<td>16.23</td>
<td>23.87</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>E (error)</td>
<td>1.36</td>
<td>2</td>
<td>0.68</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Analysis of variance (n=3).

Notice:F₀.₀₅(2, 2) = 19.00 F₀.₀₀(2, 2) = 99.0

Antibacterial effect research

Preparation of the bacterial suspension

After the bacteria were taken out from the -80 °C refrigerator, they were streaked onto the LB agar medium to activate them, and then the bacteria were wiped off with a sterile cotton swab and sterilized into sterile physiological saline. The absorbance of the bacterial suspension was adjusted to OD530 equal to 0.2, diluted 100 times (about 106 CFU/mL containing bacteria), and used.

Determination of antibacterial activity

Antibacterial activity was determined by the Oxford Cup method. Take 100 μL of the above bacterial suspension, apply it on LB agar plate, take the autoclaved Oxford cup with sterile forceps, place it on the surface of the culture dish with the coated bacterial solution, gently press, and place each dish. Three Oxford cups were injected with 200 μL of PEA-HP-β-CD clathrate solution with mass concentrations of 20, 10, 5, 2.5, and 1 mg/mL, respectively. Three parallel experiments were performed at each concentration and placed at 37 °C. After 24 hours of incubation in the chamber, the diameter of the zone of inhibition was measured.

Determination of growth curve

The PEA-HP-β-CD inclusion compound was dissolved in sterile LB broth and formulated into a 20 mg/mL solution, which was diluted by a half-dilution method to obtain 10, 5, 2.5 mg/mL of solution. The bacteria were inoculated into LB broth and cultured until the OD530 was equal to 0.2. In the experimental group, 100 μL of different concentrations of PEA-HP-β-CD clathrate solution and 100 μL of bacterial suspension were added to the 96-well plate, and the control group was added with 100 μL. Different concentrations of PEA-HP-β-CD clathrate solution and 100 μL of sterile LB broth were made in three parallels, and the absorbance at 530 nm was measured by a microplate reader every 1 h. The experimental results were subtracted from the absorbance of the experimental group. Group absorbance is indicated.

Statistical analysis

This study was analyzed by SPSS19.0 statistical software package. The data were analyzed by single factor analysis. The difference was statistically significant by P <0.05; and Data processing and analysis were performed using orthogonal design assistant II3.1 software.
Results

Orthogonal test optimization of the best process

It could be seen from Table 3 that the influence degree of each factor is A>C>D>B, and the best inclusion process is A2B3C3D1, and the feed ratio has the greatest influence on the inclusion effect. The optimum conditions for the agitation-ultrasonic method were obtained by orthogonal test: the molar ratio of PEA to HP-β-CD was 1:2, the ethanol concentration was 60%, the inclusion time was 36 h, and the inclusion temperature was 20 °C. The optimum process conditions were verified and the inclusion ratio was 88.92%. The result was higher than any of the orthogonal experimental design conditions.

Vera acid inclusion complex verification

X-ray diffraction method Cu target / graphite monochromator, tube pressure / tube flow: 40 kV / 40 mA, scan rate / sampling width: 5 °min⁻¹ / 0.01 °, scanning range 5 °~ 85 °X-ray diffraction powder analysis of PEA, HP-β-CD, physical mixture, and PEA-HP-β-CD inclusion complex, respectively, and the results were shown in Figure 2.

Comparing the X-ray diffraction pattern, it could be seen that the X-ray diffraction pattern of PEA had multiple specific crystal diffraction peaks; the X-ray diffraction patterns of HP-β-CD and PEA-HP-β-CD inclusion complex have similarity. There is no obvious crystal form peak; while the peak shape of the physical mixture of PEA and HP-β-CD was a superposition of molecules due to the interaction between host and guest molecules, resulting in the disappearance of the crystalline form of PEA, indicating the formation of a new phase.

Scanning electron microscopy method

The sample was prepared and analyzed using a support film dispersion powder method with an accelerating voltage of 5.0 kV. The appearance of PEA, HP-β-CD, physical mixture, and PEA-HP-β-CD inclusion complex was shown in Figure 3.

The results showed that the morphology of PEA was irregular flocculent spherical crystal particles, and the HP-β-CD morphology showed a spherical shape with pores. The physical mixture of PEA and HP-β-CD was filled with solid spherical shape of PEA and simply filled in HP-β-CD cavity. There are also a small number of PEA adhered to the crystalline particles on the surface of HP-β-CD; while the morphological appearance of PEA-HP-β-CD inclusion complex had no typical structure of PEA and HP-β-CD, indicating that the inclusion complex had formed.

Antibacterial effect

Diameter of inhibition zone

According to Figure 4, PEA-HP-β-CD inclusion complex (component A) can produce obvious inhibition zone against Staphylococcus aureus, indicating PEA-HP-β-CD package. The compound has a good antibacterial effect. It could be seen from Table 4 that the diameter of the inhibition zone was positively correlated with the concentration of the inclusion complex, and the diameter of the inhibition zone at the concentration of 10 mg/mL is 20.5 mm.
Growth curve

The effect of purified material on bacterial growth was shown in Figure 5. It could be seen from Figure 5 that the control group of S. aureus reached the logarithmic growth phase 4 h after the transfer, and reached the stable phase after 5-12 h, and the golden yellow grape was added after the addition of PEA-HP-β-CD inclusion complex. The growth of cocci was inhibited, and although the number of cells increased, it did not reach the normal growth peak and then stabilized. The PEA-HP-β-CD inclusion complex inhibited the growth of S. aureus in a dose-dependent manner, with significant inhibition at 20 mg/mL and almost no effect at 0.1 mg/mL.

Current problems and future directions

In this experiment, PEA-HP-β-CD inclusion complex was prepared by the preferred stirring-ultrasonic method. The frequency, time and temperature of the ultrasonic wave had a great influence on the formation of the inclusion compound, because the cavitation effect was generated by the ultrasonic process\textsuperscript{(12)}. It can reduce the interfacial tension between the phases, which is conducive to the inclusion formation. However, if the ultrasonic frequency is too large and the time is too long, the particles will continue to act together, and the heat generation will be enhanced, resulting in the formation of the inclusion complex due to the stability of the interaction between the host and the guest. Destruction reduces the inclusion efficiency of the clathrate. In addition, the method of oscillating agitation can enhance the particle contact area of the dispersed phase in the dispersion medium and reduce the phenomenon of local mixing unevenness, which is beneficial to the formation of the clathrate.

It has been reported that ellagic acid is made into ellagic acid-phospholipid complex nanoemulsion by phospholipid complex technology, which effectively improves its solubility and permeability, but the solubility in water increases only about 2 times\textsuperscript{(13)}, which is significantly lower than Hydroxypropyl-β-cyclodextrin formed 10 times the inclusion complex. Hydroxypropyl-β-cyclodextrin is a commonly used water-soluble inclusion material. As a main molecule in the inclusion process, it is used to encapsulate the pomegranate acid drug molecule molecular flocculent spherical particles into a clathrate. The intermolecular cooperation between the host and the guest may be the hydrogen bonding interaction between the pomegranate phenolic polyphenolic group and the hydrophilic group of the hydroxypropyl-β-cyclodextrin molecule, thereby improving the water solubility of the pomegranate ellagic acid. Stability. As for the molecular structure and inclusion principle of pomegranate acid inclusion complex, further research is needed.

In this experiment, the PEA-HP-β-CD inclusion complex was prepared by an optimized process, and the antibacterial results by Gram-positive antibacterial (S. aureus) and Gram-negative bacteria (E.coli) showed a certain antibacterial effect. The antibacterial effect is positively correlated with the concentration of pomegranate acid. As for the antibacterial mechanism of PEA-HP-β-CD inclusion complex, it can be related to the multi-target effect of Chinese herbal extracts\textsuperscript{(14)}, which needs further study.
References


Acknowledgement
This study was supported by the Key Projects of Excellent Young Talents Support Program of Colleges and Universities in Anhui Province (gxydZD2017128); Natural Science Foundation of Anhui Province Key Project (KJ2018A0825); Provincial Quality Engineering in Anhui Province (2015ckj157; 2016msgz062). Gaofu Fan was responsible for the design and writing of the thesis; Yuhua CAI and Xing YUAN put forward constructive suggestions on this paper and made some modifications; Entao Fu, Jie Tang and Jumei Gong worked for part of the experimental; Hui SHENG was responsible for the literature review of the article. No potential conflicts of interest are disclosed.

Corresponding author
Email: fgf@htc.edu.cn
Email: chzyjsxy2006@126.com (China)