Investigation of Antibacterial Activity and Stability of the Polyphenol Crude Extract from Spinacia Oleracea

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Abstract. Plant polyphenols have drawn increasing scientific attention due to their multiple functions on health care. In this paper, the antibacterial properties and stability of the polyphenol crude extract from Spinacia oleracea were studied. The oxford cup method and the spread plate method were employed for the observation of antibacterial activity of spinach polyphenol against common pathogens. Results showed that the antibacterial activity of spinach polyphenol against Gram-negative bacteria was stronger than that of Gram-positive bacteria. In addition, the effects of pH value, temperature and NaCl concentration on the antibacterial stability of spinach polyphenol were also discussed. This study has a certain reference value for better research and utilization of plant polyphenols.

Introduction

Spinacia oleracea (common spinach) is an annual plant (rarely biennial) in the family Amaranthaceae, which is thought to have originated in ancient Persia. Spinach was subsequently introduced into China via Nepal in 647 AD. In 2014, the world total production of spinach was 24.3 million tonnes, with China alone accounting for 91% of this quantity [1]. Spinach is a common, vegetable in the Chinese people's daily life. It is rich in a variety of vitamins, minerals, dietary fiber, phenolic substances, etc. The intake of a certain amount of vegetables can help reduce the risk of chronic diseases [2].

In recent years, the study and utilization of bioactive substances in vegetables have been a hotspot points at home and abroad. As an important bioactive substance, plant polyphenol contains many active hydroxyl groups, which endow with a variety of physiological and pharmacological activities, such as antibacterial, antioxidant, anti-aging and so on. Therefore, the polyphenols are called as "Seventh Nutrients" [3]. The aim of this study was to investigate the antibacterial properties and stability of the spinach polyphenol. This study may be valuable for the deep processing of spinach and the application of spinach polyphenol.

Materials and Methods

Samples

The spinach used in this study was purchased from local markets. The crude extract of spinach polyphenol was obtained by ultrasonic-microwave assisted extraction process.

Reagents and Instruments

The biochemistry reagents used in this study including Beef extract, Peptone, Agar were purchased from Beijing Boxing Biotechnology Co., Ltd. Sodium chloride, Tianjin Dongtianzheng Fine Chemical Reagent Factory. Sodium hydroxide, Tianjin Kermel Chemical Reagent Co. Ltd. All other reagents used in this study were of analytical grade and bought from Xi’an Keluo Chemical Reagent
Co., Ltd. The strains of *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis* used in this study were provided by Shaanxi Microbiology Institute.

The main instruments and their manufacturers used in this study were as follows: Electronic balance (AL204, Shanghai Mettler Toledo instruments Co., Ltd), Water proof electric heating constant temperature incubator (PYX-DHS-50×65, Shanghai Yuejin Medical Instrument Factory), Stand-Drying and Air Circulation Oven (DGF-1AB, Tianjin Taisite Instruments Co., Ltd.), Automatic High Pressure Steam Sterilizer (HVE-50, Hirayama manufacturing Corporation)

**Preparation of Bacterial Suspension**

The tested strains were inoculated to the beef extract peptone medium, and each strain was cultured with five petri dishes. The strains were grown at 37°C for 24 h and then stored in the refrigerator at 4°C for use. The strains were harvested from fresh agar plates, and suspended in sterile distilled water. The bacterial suspensions (approximately 10^6 CFU mL^-1) were obtained by the appropriate sterile water dilution.

**Antibacterial Activity Assay**

Three bacteria including gram negative *E. coli* and gram positive *S. Aureus*, *B. subtilis* were used in the antibacterial assays. The antibacterial activity of spinach polyphenol was determined by the oxford cup method and the spread plate method. The cultures of three tested strains (0.1 mL, approximately 10^6 CFU mL^-1) were spread on 10 mL nutrient agar in a petri dish, respectively. Three Oxford cups (a stainless cylinder, outer diameter: 8.0 ± 0.1 mm, inner diameter: 6.0 ± 0.1 mm and height: 10.0 ± 0.1 mm) were arranged on the agar surface. The spinach polyphenol solution (200 μL) was added to the Oxford cup using a pipette and then incubated at 37°C for 24 h in a constant temperature incubator. Sterile water was used as blank control. The diameters of the inhibition zones were then measured by a caliper [4-5]. The minimal inhibiton concentration (MIC) of spinach polyphenol was tested through twice micro-dilution method. The final concentrations of spinach polyphenol in the culture medium were 60, 30, 15, 7.5, 3.75, 1.875 mg/mL individually. The bacterial suspension of three tested strains (0.1 mL) were spread on agar plate respectively and incubated at 37°C for 24 h for MIC test [6-7].

**The Stability of Antibacterial Property**

The effects of heat treatment, pH Value, and salt concentration on the antibacterial property stability of spinach polyphenol were evaluated. The sensitive *E.coli* was selected as the indicator bacteria. According to the above-mentioned method, the antibacterial activity was detected, and the diameter of the inhibition zone was measured by the cross method. In the analysis of acid-base stability, the spinach polyphenol solution was treated for 20min under different pH conditions, and the pH values were set to 3, 5, 7, 9, and 11, respectively [8]. Determination of the thermal stability was carried out by exposing the spinach polyphenol solution for 20 min at the temperatures of 4, 40, 60, 80, 100, 121°C, respectively. As for the effect of inorganic salt solution, the spinach polyphenol solution was added with sodium chloride and the salt final concentrations were 0.2%, 0.4%, 0.6%, 0.8%, 1%, respectively [9]. Each experiment was repeated three times, and the data were analyzed by Analysis of Variance and Duncan's Test by statistical software SPSS19.0 for Windows.

**Results and Discussion**

**Determination of Antibacterial Activity of Spinach Polyphenols**

The inhibition halos of the spinach polyphenols to three tested strains were determined. The results were listed in Table 1.
Table 1. Antibacterial activity of spinach polyphenol against common bacteria.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diameter of inhibition zone (mm)</th>
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</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>15.733 ± 0.642</td>
</tr>
<tr>
<td>S. aureus</td>
<td>12.933 ± 0.404</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>13.833 ± 0.473</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± standard deviation. The same superscript letter indicated no significant difference (α = 0.05). The antibacterial action of spinach polyphenol to various bacteria was different due to their different tolerance. As shown in Table 1, the inhibitory effect of spinach polyphenol on E. coli was the strongest, and B. subtilis was the second, and S. aureus was the least under the same test condition.

**Minimum Inhibitory Concentration of Spinach Polyphenol**

The minimal inhibition concentration (MIC) of spinach polyphenol on the three tested strains were showed in Table 2.

Table 2. Determination of minimum inhibitory concentration.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Spinach polyphenol concentration (mg/mL)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>E. coli</td>
<td>-</td>
</tr>
<tr>
<td>S. aureus</td>
<td>-</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>-</td>
</tr>
</tbody>
</table>

As shown in Table 2, The MICs of spinach polyphenol on E. coli, S. aureus, and B. subtilis were 15 mg/mL, 60 mg/mL, and 30 mg/mL respectively.

**The Antibacterial Property Stability of Spinach Polyphenol**

The effect of pH value on the antibacterial stability of spinach polyphenol was shown in Figure 1. Different letters (a, b, c, d) in the Figure 1 indicated significant difference (α = 0.05) and so did Figure 2 and Figure 3.

![Figure 1. The effect of pH value on the antibacterial stability of spinach polyphenol.](image)

As can be seen from Figure 1, The pH value had a great influence on the antibacterial activity of spinach polyphenol. With the increase of pH value, the diameter of inhibition zone was decreased significantly. This should be attributed to phenolic hydroxyl compounds which spinach polyphenol contained. The increase of pH led to the partial dissociation of phenolic hydroxyl groups and the destruction of the structure [10]. Therefore, under acidic conditions, the spinach polyphenol had better antibacterial activity.
The thermal stability of spinach polyphenol antibacterial stability was evaluated under different temperature conditions in conical flask with the temperature controlled using a thermostat bath. The results were shown in Figure 2. As shown in Figure 2, the strongest antibacterial activity was observed at the temperature of 4°C, and the diameter of inhibition zone decreased with the increase of temperature. Especially at 121°C, the diameter of inhibition zone was significantly reduced. This should be related to the decomposition of active substances and the destruction of the structure at high temperature.

The effect of sodium chloride concentration on the antibacterial stability of spinach polyphenol was shown in Figure 3. With the increase of sodium chloride concentration in the solution, the antibacterial activity of spinach polyphenol decreased, and sodium chloride could enhance the antibacterial activity in a certain concentration range.

Conclusions
Recently, there is an increasing interest in the study and utilization of functional compounds from natural sources [11]. Plant polyphenol is an ideal natural compound, which has many health functions.
such as preventing and curing disease, etc., and has potential wide application prospect [12]. The spinach is widely distributed in China. In this paper, the antibacterial properties and stability of spinach polyphenol were investigated. The results showed that the spinach polyphenol had a certain antibacterial activity to \textit{E. coli}, \textit{S. aureus} and \textit{B. subtilis}, and the minimum inhibitory concentrations were 15, 60, 30mg/mL individually. The value of pH had a great influence on the antibacterial activity of spinach polyphenol, and the antibacterial effect was especially significant at pH 3. With the increase of pH value, the antibacterial activity was decrease, and it was relatively weak under the alkaline condition. The effect of temperature on antibacterial activity was relatively mild, and only at 121°C there was a significant decline. Meanwhile, the result also suggested that low salt concentration promoted the antibacterial activity of spinach polyphenol. The data obtained in this study may be helpful to the further research and application of polyphenols from spinach.

\textbf{Acknowledgement}

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\textbf{References}


