Antibacterial activities of polyphenols from olive leaves against Klebsiella pneumoniae

Jiao-jiao Yuan, Han-jun Yan, Jing He, Yang-yang Liu
Department of Logistics Engineering, Dongguan Polytechnic, Dongguan City, Guangdong Province, China
The author’s e-mail: yuanjj@dgpt.edu.cn

Abstract. In this paper, the polyphenols existed in olive leaf were evaluated for its antibacterial activities against Klebsiella pneumoniae. 11 phenols all presented certain antibacterial effect against above-mentioned bacteria, and hydroxytyrosol (HT), oleuropein, 3,4-dihydroxybenzoic acid and caffeic acid, could have excellent inhibition results. The results indicated that several phenols could be a valuable source of natural agents with functional properties for the food and pharmacology industries.

1. Introduction
Olive leaf extracts (OLEs) have many phenols [1], including oleuropein (OL), hydroxytyrosol (HT), 3,4-Dihydroxybenzoic acid, tyrosol, caffeic acid, and cumaric acid. They exhibit a range of biological activities, such as an antioxidant activity (2,2-diphenyl-1-picrylhydrazyl [DPPH] radical scavenging and reducing power) [2], an anticancer activity [3], and an antimicrobial activity against Helicobacter pylori, Campylobacter jejuni, Staphylococcus aureus [4], Bacillus cereus, Escherichia coli and Salmonella enteritidis [5]. In addition, several reports have been published on the relationship between the chemical structures and the antioxidant activities of phenols [6]. Furthermore, OLEs show promise as a source of safe natural additives for functional foods [7]. Furthermore, HT, which has three free hydroxyl groups, exhibits strong bioactivity, including antioxidant [8], anti-inflammatory [9], antimicrobial [10], and antitumor [11] activities.

On our previous study [12], phenols were separated and identified from the ethyl acetate extract of olive leaves enzymatic hydrolysate. Therefore, on this basis, the antibacterial activities against Klebsiella pneumoniae of phenols in the olive leaf were evaluated.

2. Materials and Methods

2.1. Materials and Chemicals
HT and OL standards were purchased from Sigma (St. Louis, MO, USA), and others phenols were purchased from Aladdin.

Bacterial strain (K. pneumoniae CMCC 46117) was purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, Huben, China) were used. Gentamicin sulphate was provided by Guangzhou Baiyunshan Chemical Pharmaceutical Factory (Guangzhou, Guangdong, China).
2.2. Antibacterial Activity Evaluation
In order to examine the antibacterial activity of the compounds, the inhibition halos were observed by Oxford cup method and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined by broth-microdilution method [13,14].

The compounds were dissolved in 20% ethanol solution, and the solutions were then sterilized after filtration through 0.45 μm filters. Bacterial strain, with approximately $5 \times 10^5$ CFU/mL bacterial concentrations, was then spread evenly over the entire surface of the respective agar medium using a small sterile spreading rod, and the plates were dried at 25°C after inoculation. Three Oxford cups (6 mm) were added 200 μL sample in question (1 mg/mL) and put in the agar plates surface, then the plates were incubated at 37°C for 24 h. The positive control was Gentamycin sulfate (5 mg/L), and the negative control was respective solvent. The diameters of the inhibition zones were recorded in millimeters (including the disc size) after 24 h of incubation. Experiments were all done in triplicate, and the inhibition zones were presented as the mean value with standard error (SE).

Sample solutions were prepared at 11 concentrations (1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 μg/mL) using serial dilution. The media were all incubated at 37°C for 24 h. The MIC value was the lowest concentration of samples with no visible bacterial growth. Based on the MIC tests, the wells with no visible bacterial growth were all continued to culture and incubate at 37°C overnight. The highest concentration with 100% inhibition was considered to be the MBC value.

2.3. Statistical Analysis
Datas were used to statistical analysis using Origin 9.0 software (OriginLab, Northampton, MA, USA). The experiments were performed in triplicate, and data format was reported as mean ± SE.

3. Results and Discussion
The antibacterial activities of 11 phenols against *K. pneumoniae* were investigated using the Oxford cup disc diffusion method, and the inhibition halos and MIC and MBC values of the compounds were determined. As shown in Table 1, the inhibition halos show that the 11 phenols exhibit varying degrees of inhibition against the selected bacteria. MIC and MBC values were obtained by the broth-dilution method. The MIC value was defined as the lowest concentration of samples with no visible bacterial growth. The wells with no visible bacterial growth were all continued to culture and incubate at 37°C, and then MBC value was determined as the highest concentration with 100% inhibition. The obtained MIC and MBC values are presented in Table 2. The results show that the tested compounds have different MIC and MBC values against selected strain.

Against *K. pneumonia*, HT (MIC: 32 μg/mL, MBC: 64 μg/mL) exhibited the strongest inhibition effect, followed by 3,4-dihydroxybenzoic acid (64 μg/mL and 128 μg/mL), OL (64 μg/mL and 128 μg/mL), and caffeic acid (64 μg/mL and 256 μg/mL). Moreover, the MIC values for tyrosol, vanillic acid, and 4-hydroxycinnamic acid against *K. pneumoniae* were all 1,024 μg/mL, and no MBC values could be obtained for the tested concentrations. 4-Hydroxyphenylacetic acid showed no antibacterial activity against *K. pneumoniae*.

Compared to the positive control (gentamicin sulfate), HT, OL, 3,4-dihydroxybenzoic acid, and caffeic acid all have good antibacterial effects against the tested bacterial. By combined the results in Tables 1 and 2, HT and OL were both found to exhibit effective antibacterial effects against the bacterial strain, and the inhibition effect of HT was found to be better than that of OL. The results for the antibacterial activities of HT and OL against strain are consistent with those reported by other authors [15].

However, vanillic acid, and 4-hydroxycinnamic acid, 4-hydroxyphenylacetic acid, and tyrosol presented poor performance against the bacterial strains. However, it is worth that a multitude of other biological activities for these compounds have been reported. For example, recent studies have revealed that 4-hydroxycinnamic acid could inhibit mushroom tyrosinase [16]. In other studies [17], it has been demonstrated that tyrosol can scavenge reactive oxygen species and exert protective effects in different cellular types.
Table 1. Inhibition halos of different samples

| Samples               | K. pneumoniae |
|-----------------------|---------------|
| Tyrosol               | 11.2 ± 0.2    |
| HT                    | 22.4 ± 0.1    |
| Cinnamic acid         | 12.1 ± 0.1    |
| Salicylic acid        | 14.1 ± 0.1    |
| 3,4-Dihydroxybenzoic acid | 17.9 ± 0.1 |
| Caffeic acid          | 17.1 ± 0.1    |
| 4-Hydroxyphenylacetic acid | 10.2 ± 0.2 |
| 4-Hydroxycinnamic acid | 10.8 ± 0.1 |
| Vanillin              | 13.5 ± 0.1    |
| Vanillic acid         | 14.8 ± 0.2    |
| OL                    | 18.1 ± 0.1    |
| Positive control (Gentamicin sulfate) | 21.1 ± 0.1 |

Note: Tukey’s test at 5% probability

Table 2. MIC and MBC of different samples

| Samples               | K. pneumoniae |
|-----------------------|---------------|
|                        | MIC(μg/mL)    | MBC(μg/mL) |
| Tyrosol               | 1024          | -           |
| HT                    | 32            | 64          |
| Cinnamic acid         | 512           | 512         |
| Salicylic acid        | 256           | 512         |
| 3,4-Dihydroxybenzoic acid | 64         | 128         |
| Caffeic acid          | 64            | 256         |
| 4-Hydroxyphenylacetic acid | -         | -           |
| 4-Hydroxycinnamic acid | 1024        | -           |
| Vanillin              | 512           | 1024        |
| Vanillic acid         | 1024          | -           |
| OL                    | 64            | 128         |

Thus, the present results indicate that several phenols from olive leaves show potential antimicrobial activities, and the rest of the phenols in the present study show slight activity against bacteria or have no influence on the growth of the tested strains.

4. Conclusions

By determining antibacterial activities of the polyphenols from olive leaf, we have demonstrated that HT, OL, 3,4-dihydroxybenzoic acid, and caffeic acid show excellent antibacterial activities against Klebsiella pneumoniae. Therefore, they may represent excellent and economic source of functional food and pharmaceutical ingredients.

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