Analysis of Ethyl Acetate Extract of Enzymatic Hydrolysate from High Purity Oleuropein and DPPH Radical Scavenging Capacity

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Abstract. High purify oleuropein (81.04\% OL) was hydrolyzed by hemicellulase and phenols was existed in the ethyl acetate extract of enzymatic hydrolysate (EAE). The results presented that there were hydroxytyrosol (HT), tyrosol, caffeic acid, 3,4-dihydroxybenzoic acid, 3,4-dihydroxy phenylacetic acid in EAE by HPLC, and HT content was 19.36\%. Antioxidant activities (DPPH radical scavenging capacity) were all added as the samples concentration increased, and dose-effect relationships also existed. HT possessed the highest DPPH radical scavenging capacity, followed by Vc, and eugenol, OL, caffeic acid, 3,4-dihydroxy phenylacetic acid and 3,4-dihydroxybenzoic acid.

1. Introduction

Olive leaf has many phenolic compounds [1], for example, oleuropein (OL), hydroxytyrosol (HT), caffeic acid, cumaric acid, tyrosol, ferulic acid, etc. In particular, HT possesses strong bioactivity, such as antioxidant [2, 3], anti-inflammatory [4], anti-microbial [5, 6], anti-tumor to inhibit proliferation of human promyelocytic leukemia HL60 cells [7], human colon cancer HT-29 cells [8] and MCF-7 human breast cancer cells [9]. Many reviews were reported on HT preparation from natural plant separation and chemical synthesis, but HT yield was very low, and synthesis process also needed a large amount of toxic agents, expensive catalysts and long time.

At present, many works on the biotransformation for HT had appeared. The biotransformation of tyrosol to HT was applied successfully by mushroom tyrosinase [10], Pseudomonas aeruginosa [11] and Serratia marcescens [12]. Nucci [13, 14] had used olive leaf extract (OLE) to produced high purity HT with a hyperthermophilic $\beta$-glucosidase immobilized on chitosan (pH = 7.0, 60 °C). Of course, we had reported the findings on enzymatic hydrolysis properties of high purity OL with hemicellulose [1], and it found that ethyl acetate extract of enzymatic hydrolysates (EAE) has 3.15\% polyphenol and 19.36\% HT. Therefore, the phenols composition of EAE and their biological activity had been attracted great interest. At present, gas chromatograph (GC) [15], high performance liquid chromatography (HPLC) [16], ultraviolet (UV) [17], GC-MS and HPLC-MS [18], and so on, were widely used to analyze chemical composition.

Thus, the aim of the present study was to determine the chemical composition of phenolic compounds in EAE by HPLC, at the same time, DPPH radical scavenging capacity of each compound were investigated.
2. Materials and Methods

2.1. Plant Materials and Chemicals
Olive leaf extract (OLE, 81.04% OL) was prepared with macroporous resin purification from olive leaf extract (38.6% OL) in the laboratory. DPPH (2, 2-diphenyl-1-picrylhydrazyl), BHT (butylated hydroxytoluene), Vc, 3,4-dihydroxybenzoic acid (1), 3,4-dihydroxyphenylacetic acid (2), HT (3), tyrosol (4), 4-hydroxyphenylacetic acid (5), vanillic acid (6), caffeic acid (7), vanillin (8), 4-hydroxycinnamic acid (9), sorbic acid (10), salicylic acid (11), OL (12), cinnamic acid (13), eugenol (14) were purchased from Aladdin (Aladdin Co., Ltd, and others were all analytical reagent. Hemicellulase (20 U/mg) was purchased from Aladdin (Aladdin Co., Ltd).

2.2. Enzymatic Method
High purify OL (OLE, 81.04% OL, 500 mg) was put in a 100 mL Erlenmeyer flask, then hemicellulase (55 mg) and 0.1 mol/L phosphate buffer (50 mL, pH = 5) were added. The enzymatic process were at 55 °C for 6 h [1]. Then the hydrolysates were concentrated and recorded, and the parallel test was investigated. Then, degradation rate of OL and HT content were all calculated. Degradation rate of OL = 1 – (OL content in hydrolysate/OL content in OLE) HT content = HT content in hydrolysate/weight of enzymatic hydrolysate

The enzymatic hydrolysate (EH) was extracted by petroleum ether, ethyl acetate and n-butanol successively. The pre-concentrated four extracts yields were calculated and were refrigerated for further test.

2.3. Liquid Chromatograph Analysis
The identification and quantification analysis of phenolic compounds were performed by HPLC [1]. The equipment was Shimadzu SPD-20A instrument equipped with DAD detector (280 nm). The column was a 5 μm Thermo BDS HYPERSIL C18 (250×4.6 mm), and temperature was maintained at 30°C. The mobile phase was 0.5% acetic acid in water (A) and methanol (B) (45:35, v/v) for a total running time of 20 min. The flow rate was 0.8 mL/min, and the injection volume was 10 μL. Identification analysis of HT and OL were based on their HPLC spectrum and their retention time in comparison with standards analyzed under the same conditions. Furthermore, the quantification analysis of HT and OL were based on the external calibration curves. For example, OL content had a good linear relationship with peak area in the range of 0.441-7.056 μg, and the regression equation was y = 1772846.78x+49802.31 (R²=0.9997, y stands for peak area and x stands for OL content). The HT content had a good linear relationship with peak area in the range of 0.586 - 8.9376 μg, and the regression equation was y = 1199063.90x+119947.13 (R²=0.9996, y stands for peak area and x stands for HT content).

Gradient analysis: the mobile phase was 0.5% acetic acid in water (A) and methanol (B) using a gradient programme as follows: stayed 70% B (10 min), from 70% to 55% B (25 min), from 55% to 20% B (20 min), from 20% to 70% B (5 min). The flow rate was 0.8 mL/min during a total running time of 50 min, and the injection volume was 20 μL. Monitoring was performed at 280 nm. Identification of phenols was based on their HPLC spectra and their retention time in comparison with standards analyzed under the same conditions. Furthermore, the quantifications of phenols were based on external calibration curves.

2.4. Polyphenols Content Determination
Polyphenols content was measured as gallic acid equivalents [19]. Diluted sample (1 mL) was transferred to a test tube, and distilled water (6 mL), Folin-Ciocalteu phenol reagent (1 mL) and 15% Na2CO3 (2 mL) were added, successively. Then the 10 mL total solution was well mixed and kept in the dark for 1 h. The samples were shaken and the absorbance was measured at 760 nm with an UV spectrophotometer.

2.5. Antioxidant Activities of Hydrolysate Phenols
DPPH radical scavenging capacity was used to evaluate antioxidant activity of phenols. 14 phenols
(3,4-dihydroxybenzoic acid (1), 3,4-dihydroxyphenylacetic acid (2), HT (3), tyrosol (4), 4-hydroxyphenylacetic acid (5), vanillic acid (6), caffeic acid (7), vanillin (8), 4-hydroxycinnamic acid (9), sorbic acid (10), salicylic acid (11), OL (12), cinnamic acid (13), eugenol (14)) were the tested samples. A series of samples concentration were also prepared with distilled water or 20% ethanol as solvent.

The DPPH radical scavenging effect was evaluated according to Ni et al [20]. Each diluted sample (1 mL) was added to a DPPH ethanol solution (3 mL, 40 μg/mL). Two solutions were gently mixed and left for 30 min at room temperature, and the absorbance was measured at 517 nm by a spectrophotometer. The positive controls in the assay were BHT and Vc, respectively. The antioxidant activity of each sample was expressed in terms of DPPH scavenging efficiency and calculated for the log-dose inhibition curve. Equation (1) was used to calculate the DPPH scavenging capacity as shown below:

\[ \eta = [1 - \frac{A_i - A_0}{A_0}] \times 100\% \] (1)

\( \eta \) is the DPPH scavenging efficiency of the sample;
\( A_0 \) is the absorbance with 1 mL sample and 3 mL ethanol after 30 min;
\( A_i \) is the absorbance with 1 mL ethanol and 3 mL DPPH ethanol solution after 30 min (total radical);
\( A_i \) is the absorbance with 1 mL sample and 3 mL DPPH ethanol solution after 30 min.

3. Results and Discussion

3.1. Polyphenols, OL, HT Content Analysis of Hydrolysate

At present, HT exits mainly in the olive fruit, leaf and oil waste water with the form of OL glycosides. However, due to the too low content of HT, this method does not provide HT on a large scale. Therefore, OLE with high OL was hydrolyzed by hemicellulase at 55°C for 6 h, according to our previous study [1]. Polyphenols, OL and HT content of hydrolysate were determined, and the results were presented in Table 1. Polyphenols content was decreased from 2.96% (OLE) to 0.46% (EH), but HT content was increased from 3.88% (OLE) to 8.64% (EH). OL content decreased from 81.04% (OLE) to 0.96% (EH) and it was illustrated that OL was degraded nearly fully in the enzymatic process, but OL was degraded into neutral glycosides instead of HT. EH was extracted by petroleum ether, ethyl acetate and n-butanol, successively, and the ratio were 1.41%, 21.72%, 10.39%, respectively. In the petroleum ether part, the polyphenols and HT content were very low. Water extract was the highest part, and the ratio was 64.83%. In addition, HT was concentrated in ethyl acetate part, and the content was 19.36%, which was highest in this part. There was also 7.81% HT content in n-butanol part. The results presented in this work suggested that hemicellulase had promising and attractive properties for industrial production of HT.

| Extract                  | Mass (g) | Ratio (%) | Polyphenol (%) | HT content (%) | OL content (%) |
|--------------------------|----------|-----------|----------------|----------------|----------------|
| OLE                      | 20.00    | —         | 2.96 ± 0.26    | 3.88 ± 0.24    | 81.04 ± 2.64   |
| EH                       | 18.16 ± 0.61 | —     | 0.46 ± 0.08    | 8.64 ± 0.53    | 0.96 ± 0.32    |
| Petroleum ether extract  | —        | 1.41 ± 0.24 | —              | —              | —              |
| Ethyl acetate extract    | —        | 21.72 ± 0.91 | ± 3.15 ± 0.07  | 19.36 ± 0.53   | 3.46 ± 0.33    |
| n-Butanol extract        | —        | 10.39 ± 0.37 | ± 0.96 ± 0.13  | 7.81 ± 0.42    | 1.08 ± 0.12    |
| Water extract            | —        | 64.83 ± 3.53 | ± 0.034 ± 0.04 | —              | —              |
3.2. Phenols of EAE by HPLC Analysis

14 standard phenols (3,4-dihydroxybenzoic acid (1), 3,4-dihydroxyphenylacetic acid (2), tyrosol (3), HT (4), 4-hydroxyphenylacetic acid (5), vanillic acid (6), caffeic acid (7), vanillin (8), 4-hydroxycinnamic acid (9), sorbic acid (10), salicylic acid (11), OL (12), cinnamic acid (13), eugenol (14)) were identified in EAE by HPLC gradient analysis. The phenolic contents of EAE were analyzed based on the corresponding standard concentration-response equation obtained by HPLC. From Table 2 we could see that HT content was the highest concentration in EAE and was up to 10.23%, however, the content of other phenols were quite low. 3,4-dihydroxybenzoic acid (1.82%), 3,4-dihydroxyphenylacetic acid (1.05%), tyrosol (4.37%), 4-hydroxyphenylacetic acid (2.56%), vanillic acid (0.21%), caffeic acid (3.12%), vanillin (0.17%), 4-hydroxycinnamic acid (1.14%), sorbic acid (0.58%), salicylic acid (0.23%) and eugenol (0.34%) were all detected in EAE.

Table 2. Qualitative and quantitative analysis of phenols from EAE (x ± SD, n=3)

| No. | Retention time (min) | Compounds                        | Content (%)   |
|-----|----------------------|----------------------------------|---------------|
| 1   | 3.47                 | 3,4-Dihydroxybenzoic acid        | 1.82 ± 0.16   |
| 2   | 4.01                 | 3,4-Dihydroxyphenylacetic acid   | 1.05 ± 0.11   |
| 3   | 4.38                 | HT                               | 19.36 ± 0.53  |
| 4   | 5.31                 | Tyrosol                          | 4.37 ± 0.20   |
| 5   | 6.07                 | 4-Hydroxyphenylacetic acid       | 2.56 ± 0.18   |
| 6   | 7.08                 | Vanillic acid                    | 0.21 ± 0.06   |
| 7   | 7.39                 | Caffeic acid                     | 3.12 ± 0.29   |
| 8   | 8.28                 | Vanillin                         | 0.17 ± 0.04   |
| 9   | 12.37                | 4-Hydroxycinnamic acid           | 1.14 ± 0.17   |
| 10  | 16.33                | Sorbic acid                      | 0.58 ± 0.07   |
| 11  | 19.39                | Salicylic acid                   | 0.23 ± 0.05   |
| 12  | 31.83                | Oleuropein                       | 0.047±0.002   |
| 13  | 34.60                | Cinnamic acid                    | —              |
| 14  | 44.79                | Eugenol                          | 0.34 ± 0.08   |

3.3. DPPH Radical Scavenging Capacity

DPPH radical is typically used to assess the free radical scavenging activity of antioxidants. Whether antioxidants have the ability to remove DPPH free radicals mainly depends on hydrogen capacity of the material, and the quantitative relation exists between discoloration degree and received electronic quantity. The antioxidants react with the stable free radical DPPH and convert it to 1,1-diphe-nyl-2-picryl hydrazine as evidenced through discoloration from purple to yellow. Antioxidant ability of DPPH was determined by scavenging efficiency and IC$_{50}$ value. The higher scavenging efficiency, the stronger antioxidant activity, while the higher IC$_{50}$ value, the weaker antioxidant activity. IC$_{50}$ value represented the concentration of needed antioxidant when scavenging efficiency was 50%.

Figure 1 showed the results for DPPH radical scavenging capacity of 14 tested phenols and 2 positive controls (Vc, BHT), and it was found that samples had different antioxidant ability. DPPH radical scavenging capacity were all added as the 16 samples concentration increased, and dose-effect relationship existed between samples concentration and DPPH scavenging efficiency. The reaction concentration of 3,4-dihydroxybenzoic acid (1), 3,4-dihydroxyphenylacetic acid (2), HT (3), caffeic acid (7), OL (12), eugenol (14), Vc, BHT were all microgram level, however, the reaction concentration of tyrosol (4), sorbic acid (10), 4-hydroxyphenylacetic acid (5), vanillic acid (6), vanillin (8), 4-hydroxycinnamic acid (9), salicylic acid (11), cinnamic acid (13) were all milligram level. It was therefore that DPPH radical scavenging capacity of the former was much stronger than the latter. In addition, the order of DPPH radical scavenging capacity according to IC$_{50}$ value (seen in Table 3) was: HT > Vc ≈ eugenol > OL > caffeic acid > 3, 4-dihydroxyphenylacetic acid ≈ 3, 4-dihydroxybenzoic
acid > BHT > vanillin ≈ vanillic acid > 4-hydroxycinnamic acid ≈ salicylic acid ≈ 4-hydroxyphenylacetic acid > cinnamic acid > sorbic acid ≈ tyrosol. From the order we could concluded that HT possessed the highest DPPH radical scavenging capacity, followed by Vc, and eugenol. OL, caffeic acid, 3,4-dihydroxy phenylacetic acid and 3,4-dihydroxybenzoic acid were all higher than BHT. From ANOVA data, 3, 4-dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid, eugenol and Vc had no significant difference \((p > 0.05)\). 4-hydroxycinnamic acid, 4-hydroxyphenylacetic acid and salicylic acid, vanillin and vanillic acid, sorbic acid and tyrosol all had no significant difference, respectively \((p > 0.05)\).

Figure 1. DPPH radical scavenging capacity of phenols
Table 3. Phenols products of DPPH IC_{50} value (x ± SD, n=3)

| Phenols               | IC_{50}(μg/mL) | Phenols               | IC_{50}(mg/mL) |
|-----------------------|----------------|-----------------------|----------------|
| Eugenol               | 3.67 ± 0.15 e  | 4-Hydroxyphenylacetic acid | 11.38 ± 0.75 b |
| 3,4-Dihydroxyphenylacetic acid | 9.54 ± 0.14 b | 4-Hydroxycinnamic acid   | 9.06 ± 1.06 b  |
| 3,4-Dihydroxybenzoic acid  | 10.94 ± 0.51 b | Sorbic acid           | 16.06 ± 1.49 a |
| Caffeic acid          | 7.82 ± 0.17 c  | Salicylic acid        | 10.78 ± 1.23 b |
| OL                    | 4.97 ± 0.14 d  | Cinnamic acid         | 15.49 ± 0.86 a |
| HT                    | 1.07 ± 0.16 f  | Vanillin             | 4.03 ± 0.52 c  |
| BHT                   | 30.27 ± 1.09 a | Vanillic acid        | 4.92 ± 0.27 c  |
| Vc                    | 3.31 ± 0.17 e  | Tyrosol              | 16.87 ± 0.78 a |

Note: The same letter showing a no difference (Tukey test, P>0.05), and the different letter showing a significant difference (Tukey test, P<0.05).

4. Conclusions
To sum up, the polyphenols, HT and OL content of OLE, EH, and EAE were analyzed, and HT content improved to 19.36% after enzymatic hydrolysis. There were 14 phenols were verified in EAE by HPLC. In addition, DPPH radical scavenging capacity of each compound was tested. Antioxidant activities were all added as 14 samples concentration increased, and dose-effect relationships existed between samples concentration and antioxidant activities. Moreover, HT possessed the highest DPPH radical scavenging capacity in 14 compounds.

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