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# The possible mechanism of hydroxytyrosol on reducing uric acid levels

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ABSTRACT

In this study, the inhibitory effect of hydroxytyrosol against xanthine oxidase (XOD) by *in vivo* animal model and *in vitro* inhibition assay was analysed. The results showed that hydroxytyrosol could reduce uric acid in rat serum, inhibit XOD activity in rat liver, and adjust the mRNA transcription of the renal uric acid transporters URAT1, GLUT9, OAT1, UAT and ABCG2 towards normal in the transcription level. Hydroxytyrosol was observed as an inhibitor of XOD activity, which's $IC_{50}$ value of restraining XOD activity was 8.75 mM. Circular dichroism (CD) spectroscopy indicated that the secondary structures of XOD were changed after incubation with hydroxytyrosol. The docking simulation showed that hydroxytyrosol could enter into the active site of XOD and form hydrogen bonds with amino acid residues (such as ARG880, GLU1261 and THR1010). The results suggested that hydroxytyrosol could be a potential XOD inhibitor for hyperuricaemia treatment.

## 1. Introduction

Hyperuricaemia (HUA) is the central biochemical cause of gout and has been suggested to be associated with many chronic diseases, such as obesity, hypertension, coronary heart disease, diabetes, kidney injury, etc. (Ito et al., 2011). HUA is a public health problem whichneeds to be solved because its prevalence rate is higher and higher (Adeeb et al., 2017). Exploring the pathogenesis of HUA can lead to more effective prevention and treatment of HUA. It was reported that the pathogenesis of HUA depended mainly on the related enzymes in purine metabolism and uric acid transporter in the kidney (Yang, Chen, & Peng, 2015). At the present, two targets have been used for the treatment of HUA besides reducing dietary intake of purine foods. One target is xanthine oxidase (Naoghare, Kwon, & Song, 2010; Sarawek & Butterweck, 2006; Yan, Zhang, Hu, & Ma, 2013; Zafar et al., 2017). The other is transport proteins related to uric acid metabolism inthe kidney, some of which contribute to the reabsorption of uric acid, such as URAT1 (Deeks, 2017; Hu et al., 2017), ABCG2 (Miyata et al., 2016; Qin et al., 2017), and GLUT9 (Hamajima et al., 2011; Preitner et al., 2009), some of which are secretory proteins, such as OAT1 (Sato et al., 2008) and UAT (Leal-Pinto, Cohen, & Abramson, 1999; Miao et al., 2010). URAT1 is an important transport protein for renal reabsorption of uric acid (Anzai et al., 2008; Eraly, Liu, Jamshidi, & Nigam, 2015). At the present, URAT1 inhibitors have been clinically used as drugs for the treatment of HUA. GLUT9 is responsible for transporting uric acid from the filtrate to the blood, which is widely distributed on the apical membrane and basement membrane of the epithelial cell of the renal proximal tubule in the kidney (So & Thorens, 2010). Moreover, as the most important drug efflux transporter, ABCG2 is a member of the ABC transporter family and an important component of the uric acid reabsorption system (Matsuo et al., 2014; Yamagishi et al., 2010) in the kidney; it is also considered as a target for the treatment of HUA (Woodward et al., 2009). The organic anion transporter OAT1 is located on the basal membrane of renal tubular epithelial cells and plays an important role in the secretion of uric acid (Feng et al., 2016). UAT is mainly involved in the secretion of uric acid in the proximal tubule of the kidney, and 50% of urate which enters the renal proximal tubule is mediated into the lumen by UAT (Zhu, 2012).

Hydroxytyrosol (HT), i.e., 3,4-dihydroxyphenylethanol, is a natural polyphenolic compound. Hydroxytyrosol is present in various parts of olives in the form of esterified oleuropein. Oleuropein could be hydrolysed to free hydroxytyrosol whose content is 6% of the whole phenol content in crude olive oil (Wang, Shi, & Jiang, 2010). As the active ingredient in olive oil, hydroxytyrosol has many biological and pharmacological activities, such as anti-cancer, anti-bacterial, and anti-inflammatory properties (Tuck & Hayball, 2002). Hydroxytyrosol has a higher ability to scavenge free radicals and antioxidants than other synthetic or natural compounds. It can effectively remove endogenous and exogenous free radicals as well as oxides (Aruoma et al., 1998). The LC-MS-based metabolomics of hydroxytyrosol administration in rats revealed amelioration of the metabolic syndrome (Lemonakis et al., 2017). Hydroxytyrosol can reduce not only the synthesis of unsaturated fatty acids and fatty acids, but also the metabolism of linoleic acid, vitamin A, sphingolipids and arachidonic acid. Meanwhile, it can

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increase the metabolism of glycerol esters. In our previous research work, it was found that hydroxytyrosol, as a metabolite of verbascoside, had higher *in vitro* inhibition rate on xanthine oxidase than the prototype (Wan et al., 2017). Since hydroxytyrosol is a widespread active ingredient in many plant foods especially in olive relative products, this paper aims to decipher the inhibitory mechanism of hydroxytyrosol on XOD activity *in vitro* and the mechanism of lowering blood uric acid *in vivo*, in order to evaluate the possibility and prospect of hydroxytyrosol as a potential substance for the prevention of hyperuricaemia.

## 2. Material and methods

### 2.1. Chemicals

Hydroxytyrosol (GC grade, > 98%) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Potassium oxonate (PO), xanthine and xanthine oxidase (XOD, from bovine milk) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Allopurinol and nitro blue tetrazolium chloride (NBT) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Methanol (HPLC grade) was purchased from Honeywell International Inc. (Morris Plains, NJ, USA). Uric acid assay kit, creatinine assay kit, urea nitrogen assay kit, xanthine oxidase assay kit and adenosine deaminase assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trizol and reverse transcription kit were purchased from Thermo Fisher Scientific Inc. (Hudson, NH, USA). Two× Master Mix kit were purchased from Roche Life Science Ltd. (Shanghai, China). DNA ladder, DNA loading buffer and SYBR Greenmaster mix kit were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

### 2.2. The in vitro combination ratio between hydroxytyrosol and XOD

One milliliter hydroxytyrosol solution (1 mM) was mixed with 0.2 mL XOD solution (0.05 U $mL^{-1}$) and hatched for various times (15, 30, 60, 90 and 120 min, respectively) at 37 °C. Then, the contents of free hydroxytyrosol in the mixtureswere detected by a Waters e2695 HPLC system (Waters Co., Milford, MA, USA) with a Waters Symmetry RP-$C_{18}$ column (250 mm × 4.6 mm, 5 μm) after ultrafiltration (Model 7554-95 pump, Cole-Parmer Instrument Company, IL, USA) with the membrane of the cutting molecular weight 10,000 Dalton (Millipore, MA, USA). The detection conditions were as follows: column temperature was 35 °C, flow rate was 0.7 mL $min^{-1}$, the ratio of mobile phase A (0.1% formic acid-water solution, v/v) and mobile phase B (methanol) was 60:40 (v/v). All of the eluates were monitored at 280 nm. The *in vitro* binding ratio of hydroxytyrosol to XOD was calculated by following equation.

$$\text{The } \textit{in vitro} \text{ binding ratio } \% = (1-S/S_0) \times 100\% \quad (1)$$

where $S_0$ and S are the peak areas of hydroxytyrosol when not hatched and hatched with XOD for various times, respectively.

### 2.3. The inhibition activity of hydroxytyrosol on XOD

XOD activity *in vitro* was measured with HPLC method by measuring the formation of uric acid at 290 nm wavelength with xanthine as the substrate according to the reference (Liu et al., 2014). The mixtures of different concentrations of hydroxytyrosol and XOD (0.1 U $mL^{-1}$) were first kept in a 25 °C water bath for 15 min, and then, xanthine substrate (0.189 mM final concentration) were added to initiate the reaction to make the final volume of the mixture up to 6.0 mL. After incubation for 30 min at 25 °C, 1 mL HCl (0.143 M final concentration) was added to terminate the reaction. The peak area of uric acid in the mixture was collected by a Waters e2695 HPLC system (Waters Co., Milford, MA, USA). Chromatographic separation was accomplished on a Phenomenex Luna $C_{18}$ reversed-phase column (250 mm × 4.6 mm, 5 μm) at 25 °C and monitored at 290 nm. Samples of 10 μL were injected onto the column and eluted at 1 mL $min^{-1}$ with following isometric elution program, 95% A (0.05 M $K_2HPO_4$ buffer solution, pH 7.5) and 5% B (methanol).

The inhibition rate of the enzyme without hydroxytyrosol was set as 0%, and allopurinol was the positive substance. All assays were performed in triplicates. The Inhibition rate of XOD in the system containing different concentrations of hydroxytyrosol was calculated with the following equation.

$$\text{Relative enzyme activity } \% = S/S_0 \times 100\% \quad (2)$$

where $S_0$ and S are the peak area of uric acid without and with hydroxytyrosol in the mixture, respectively.

The $IC_{50}$ of hydroxytyrosol-inhibiting XOD activity was calculated using SPSS 17.0 software.

**Table 1**
Summary of the gene-specific RT-PCR primer sequences.

| Name of the primer | Primers 5′-3′ | Product size (bp) | Annealing temperature (°C) |
|---|---|---|---|
| GAPDH | TGGAGAAACCTGCCAAGTATGAT<br>TCAAAGGTGGAAGAATGGGAGT | 142 | 60 |
| URAT1 | CCATCCAAGACATCCAGAAACA<br>CACCGCCAACCCTACCCT | 166 | 60 |
| OAT1 | CATAATACCGAAGAGCCATACGA<br>GCCTGTCTGCCGAATCACT | 133 | 60 |
| ABCG2 | CACTGACCCTTCCATCCTCTTC<br>GCCCTGTTTAGACATCCTTTTCA | 103 | 60 |
| UAT | CTGGAATCCCTCCTATGGCA<br>GTTCAGGTGGAAAGCAATGTCA | 176 | 60 |
| GLUT9 | CCAGCAGCCTTCATTATCGC<br>GTGGCAAAGACGAGGAAGCA | 116 | 60 |

**Table 2**
The peak area of free hydroxytyrosol and the binding ratio of hydroxytyrosol to XOD after incubation at different times.

| Incubation time (min) | Peak area (μV s) | The binding ratio (%) |
|---|---|---|
| 0 | 1,816,896 | 0 |
| 15 | 1,731,100 | 4.72 |
| 30 | 1,696,494 | 6.63 |
| 60 | 1,571,217 | 13.52 |
| 90 | 1,601,957 | 11.83 |
| 120 | 1,625,196 | 10.55 |

### 2.4. Circular dichroism analysis

The CD measurements were carried out in the wavelength range of 190–250 nm and scan speed of 60 nm $min^{-1}$ under constant nitrogen flush. The CD spectra of XOD incubated with hydroxytyrosol at molar ratios (r = [hydroxytyrosol]/[XO]) of 0:1, 2.5:1, 5:1, and 7.5:1 were recorded in pH 7.5 sodium phosphate buffer solution at 37 °C. All observed CD spectra were corrected for the buffer signal. The contents of different secondary structures of XOD, e.g., *α*-helix, *β*-sheet, *β*-turn and random coil, were analysed from CD spectroscopic data by the online SELCON3 program (http://dichroweb.cryst.bbk.ac.uk/htmL/home.shtmL).

### 2.5. Molecular docking study

To explore the probable binding site of hydroxytyrosol with XOD, molecular modelling studies were carried out using the docking program AutoDock (vers.4.2). The X-ray crystal structure of XOD (PDB code 3ETR) was retrieved from the RCSB Protein Data Bank (http://