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Screening and determination of potential xanthine oxidase inhibitors from *Radix Salviae Miltiorrhizae* using ultrafiltration liquid chromatography–mass spectrometry

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ABSTRACT

Xanthine oxidase (XOD) inhibitors play an important role in the treatment of gout and many other diseases related to the superoxide anion metabolism. In this study, an ultrafiltration-liquid chromatography–mass spectrometry (UF-LC–MS) method was developed for the screening and identification of potential XOD inhibitors from *Radix Salviae Miltiorrhizae* extract. Eleven lipophilic diterpenoid quinines were identified as XOD inhibitors from the extract. The relationship between the structure and activity of the detected compounds was estimated on the basis of the UF-LC–MS data. The results demonstrate that the 1,2-naphthoquinone group is necessary for the XOD inhibitory activity of the compounds, and that furan and hydroxyl on the alicyclic ring could enhance the activity of the compounds at different levels. These results may explain and support the medical use of the extract of *Radix S. Miltiorrhizae* for the prevention and treatment of hyperuricemia and gout.

1. Introduction

Gout, a fairly common metabolic disease, has become a health problem worldwide. It affects more than 1% of adult men in Taiwan [1,2] and the USA [3,4]. The prevalence of gout in the UK and Germany during 2000–2005 was 1.4% [5]. Global studies have found an increase in mean serum urate in both sexes during the past four decades. Two general strategies based on the pathogenesis of gout have been adopted to develop therapies for chronic gout. The first strategy is to introduce uricosuric agents such as probenecid to increase uric acid excretion in the urine. The second approach is to use xanthine oxidase (XOD) inhibitors to decrease the circulating levels of uric acid by blocking the production of uric acid [6,7].

The role of XOD is situated at the end of the purine catabolic pathway in humans and other uricotelic species. Xanthine oxidase plays a key role in uric acid biosynthesis by converting hypoxanthine to xanthine and further converting xanthine to uric acid [8,9]. Decreasing the production of uric acid by XOD inhibitors has been proven to be one of the most effective treatment strategies for hyperuricemia and chronic gout in previous studies [10,11]. Some synthetic XOD inhibitors such as allopurinol [12,13], Y-700 [14–16], and febuxostat [17–19] have shown good efficacies against hyperuricemia and chronic gout. However, they may also cause side effects such as skin rash, systemic vasculitis, and renal failure [20]. For this reason, XOD inhibitors from natural products have been explored as viable, harmless, and nontoxic alternatives for the treatment of hyperuricemia and gout [21–23].

Radix Salviae Miltiorrhizae, a well-known traditional Chinese medicine, has been used to treat coronary heart disease, heart stroke, and cerebrovascular diseases. It also has good clinical efficacy against hepatitis, hepatocirrhosis, and chronic renal failure [24–26]. The main bioactive chemical constituents in *Radix S. Miltiorrhizae* are diterpenoid quinones and water-soluble phenolic acids [27]. Our preliminary in vitro screening study revealed that the extract from the *Radix S. Miltiorrhizae* has potent XOD inhibitory activity. However, it is still unclear which compounds are the active ingredients in the extracts, and their degrees of XOD inhibitory activity are unknown. The present study seeks to investigate the potent XOD inhibitors from *Radix S. Miltiorrhizae* extracts and to rank their XOD inhibitory activity according to their structure. The results of this study would explain and support application of the extract of *Radix S. Miltiorrhizae* for the prevention and treatment of hyperuricemia and gout. The results could also provide a guide for the design of anti-gout drugs.

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In vitro methods have been commonly used to screen XOD inhibitors from fractionated extracts of medicinal herbs. However, assays based on fractionation require multiple-step isolations of active compounds and conventional analyses for elucidation, which are time-consuming and labor-intensive [28]. To overcome the limitations of in vitro screening assays and enhance the throughput of the drug discovery, a method based on ultrafiltration liquid chromatography–tandem mass spectrometry (UF-LC–MS) was proposed. UF-LC–MS has been proven to be a powerful tool for screening biologically active compounds from botanical extracts because the ultrafiltration step facilitates the separation of ligand–receptor complexes from unbound compounds, and the subsequent LC–MS step could identify the ligands. Low sample consumption, the obviated need for immobilization, and the reuse of enzymes are the most important advantages of UF-LC–MS for high-throughput screening and identification of active compounds [29–32].

In this study, we used UF-LC–MS to screen potential XOD binding agents from *Radix S. Miltiorrhizae* extract. Eleven lipophilic diterpenoidal quinines and a hydrophilic depside were identified from the extract of *Radix S. Miltiorrhizae*. The relationship between the chemical structures and inhibitory activities associated with the functional groups of the inhibitors were also determined through this method. The results demonstrate that a 1,2-naphthoquinone group is necessary for the XOD inhibitory activity of the compound, and that furan and hydroxyl substituents on the alicyclic ring could enhance the activity of the compounds at different levels. These results may explain and support the use of *Radix S. Miltiorrhizae* extract for the prevention and treatment of hyperuricemia, gout, and other cardiovascular diseases.

2. Materials and methods

2.1. Materials

Radix S. Miltiorrhizae was purchased from a drugstore (Tongrentang, Changchun, PR China). Xanthine oxidase (E.C. 1.1.3.22) from bovine milk was obtained from Sigma (St. Louis, MO). HPLC-grade methanol and acetic acid were purchased from Fisher Scientific (Loughborough, UK). Standards of salvianolic acid B, tanshinone II A, tanshinone I, and cryptotanshinone were acquired from the Chinese Authenticating Institute of Material Medical and Biological Products (Beijing, China). All other standard compounds were provided by Prof. Houwei Luo of the Natural Products Laboratory, China Pharmaceutical University. Water was purified through a Milli-Q water purification system (Milford, MA). Solvents and all other chemicals were of analytical grade and were purchased from Beijing Chemical Engineering Company (Beijing, China).

2.2. Preparation of extract of Radix S. Miltiorrhizae

A powdered sample (2 g) of *Radix S. Miltiorrhizae* was extracted two times by ultrasonication in 50 volumes of 60% ethanol for 1 h. After filtration, the combined extracts were concentrated under reduced pressure. The resulting *Radix S. Miltiorrhizae* extract powder was dissolved in 1 mL of dimethylsulfoxide (DMSO). The DMSO solution was ultrasonicated for 10 min, and then 39 mL of water was added to it. The solution had a final concentration of 0.05 g crude herb extract per milliliter. The solution was filtered through a 0.45 μm membrane filter and was used in LC–MS, XOD inhibition assay, and UF-LC–MS.

2.3. Determination of XOD inhibitory activity

The XOD inhibition assay was performed according to the method modified by our group [33]. A 0.2 mL portion of the reaction mixture containing 50 mM Tris–HCl buffer (pH 8.7), 0.5 mM EDTA, 20 U XOD, and 25 μM WST-1 was used. The reaction was initiated by adding an appropriate concentration of xanthine. The reaction was allowed to proceed at 35 °C for 5 min, and then halted by adding 0.8 mL of methanol. Sodium tauroursodeoxycholic acid (1 μM) was added as the internal standard. Afterward, the production of uric acid and superoxide was indirectly determined by measuring the chromatographic peak area of xanthine and WST-1 obtained by ultra-high performance liquid chromatography–triple quadrupole-mass spectrometry (UPLC–TQ-MS). Allopurinol was used as the positive control. The XOD inhibition was calculated using the following equation: $[(C_1 - C_2) - (C_1 - C_3)]/(C_1 - C_2) \times 100\%$, where C_1 is the xanthine concentration of the control, C_2 is the xanthine concentration of the sample without inhibitor, and C_3 is the xanthine concentration of the sample with inhibitor.

2.4. Screening procedure of UF-LC–MS

The principle of the UF-LC–MS screening based on MS is described as follows. The mixture of compounds was injected into the ultrafiltration cell containing a solution of macromolecular receptor (XOD). Those components with an affinity for the receptor bound to XOD. The solution was subjected to ultrafiltration, which facilitated the removal of the unbound compounds of low molecular weight. Subsequently, destabilizing conditions were used to facilitate the release of the bound ligands from the receptor. Specifically, receptor–ligand binding was disrupted through pH change or the addition of an organic solvent. The released ligands were further analyzed via LC–MS.

The *Radix S. Miltiorrhizae* extract sample (50 μL) was incubated in a solution consisting of 50 μL of 20 μM xanthine oxidase (EC 1.1.3.22) in 50 mM Tris–HCl buffer (pH 8.7) for 0.5 h at 37 °C. After incubation, the mixture was filtered through an ultramembrane filter (Microcon YM-100, Millipore, MA) according to the method modified by Sun et al. [31], and then centrifuged at 7000 rpm for 5 min at room temperature. The filter was washed three times by centrifugation with 100 μL aliquots of Tris–HCl buffer (pH 8.7) to remove the unbound compounds. The bound ligands were released by adding 100 μL of a methanol–water mixture (50:50, v/v, pH 3.30) followed by centrifugation at 8000 rpm for 7 min, which was repeated three times. Solvent in the ultrafiltrate was removed under vacuum, and the released ligands were used for further LC–MS analysis. The control experiments were carried out in a similar manner using denatured enzyme. All the binding assays were performed in duplicate and performed three times.

2.5. Ultraperformance liquid chromatography–diode array detection–electrospray ionization–tandem mass spectrometry (UPLC–DAD–ESI–MS^n)

The released ligands were redissolved in 50 μL of methanol–water mixture (50:50, v/v). Aliquots (10 μL) of this reconstituted ligand solution were analyzed by LC–MS. The instrument used consisted of a Waters Acquity H-Class UPLC system (Milford, MA) coupled to an LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific Inc., CA). The UPLC separation was carried out using an ACQUITY UPLC™ BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm; Waters Corp., MA). The column temperature was controlled at 25 °C. Methanol (A) and 0.5% acetic acid aqueous solution (B) comprised the mobile phase. The flow rate was set to 0.3 mL/min and the eluting gradient was as follows: t = 0–4 min, 40–70% A; t = 4–7 min, 70% A; t = 7–11 min, 70–80% A; t = 11–13 min, 80–100% A; t = 13–15 min, 100–40% A. The mass spectrometer was operated both in the negative ion and positive ion modes. The spray voltage was 4.5 kV for the negative ion mode and 5.0 kV for the positive ion mode. The capillary voltage was set