Journal of Chromatography B, 923–924 (2013) 48–53

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

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ARTICLE INFO

Article history:
Received 19 November 2012
Accepted 6 February 2013
Available online 16 February 2013

Keywords:
Radix Salviae Miltiorrhizae
Gout
Xanthine oxidase inhibitors
Screening
Ultrafiltration LC–MSS

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.

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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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http://dx.doi.org/10.1016/j.jchromb.2013.02.009

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

to–20 V in the negative ion mode and 20 V in the positive ion mode. The tube lens voltage was set to–110 V in the negative ion mode and 100 V in the positive ion mode. The capillary temperature was set to 250 °C. The sheath gas (N_2) flow rate was 30 units and the auxiliary gas flow was set at 5 units. The scan range was *m*/*z* 100–1000 Da. The chosen tandem mass spectrometry precursor ions were first isolated and then fragmented at a normalized collision energy of 20%. The UPLC was connected to the mass spectrometer via the UV cell outlet.

2.6. Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS)

Mass spectrometry experiments at high mass resolution were performed on an Ion-Spec Ultima 7.0 T FT-ICR-MS instrument (Ion-Spec, USA) with a Waters Z-spray source. The capillary voltage was set at −3.0 kV. The source heater and probe heater were set at 100 and 80 °C, respectively. The operating software IonSpec99 version 7.5.10.64 was used in the analysis. All acquisitions were performed on a 1024 K data set and one scan.

3. Results and discussion

3.1. Evaluation of XOD inhibitory activity

The XOD inhibitory activity of the extract was 50.83% at 1 mg/mL, as evaluated by in vitro assays. Compared with the activity of the well-known, synthetic XOD inhibitor, allopurinol (60.04% inhibition at 20 μM), the obtained data demonstrate that the extract had considerable XOD inhibitory activity. Therefore, it would be worthwhile to screen and identify the active compounds in the extract of *Radix S. Miltiorrhizae*.

3.2. Screening of XOD ligands in the extract of Radix S. Miltiorrhizae by UF-LC–MSS

Fig. 1 shows the UPLC chromatogram of *Radix S. Miltiorrhizae*. The 14 constituents were separated and detected within 10 min. After the extract was incubated with XOD and purified by affinity ultrafiltration, the trapped ligands in *Radix S. Miltiorrhizae* were analyzed by UPLC. Compounds specifically binding to XOD that were incubated with XOD show chromatogram peaks with intensities higher than those of the control sample incubated with denatured enzyme. Larger peaks of the 12 trapped ligands (compounds 1–11and 13) were observed in the chromatogram (Fig. 2). Compounds **12** and **14** were not considered as XOD ligands because they could not be distinguished from the control sample in the ultrafiltration screening assay. Compound **1** may have bound non-specifically because of its high concentration in the original solution and the multiple hydrogen bonds formed between the hydroxyl group of phenolic acid and proteins. Therefore, detection of its

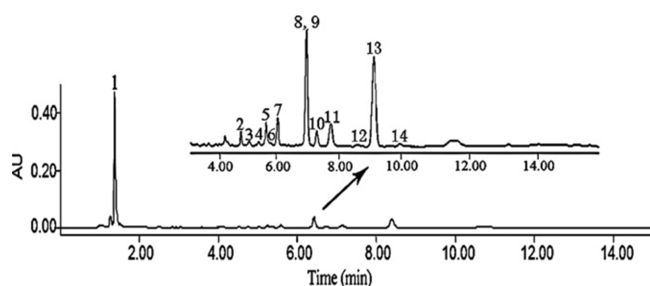

Fig. 1. Ultra-high performance liquid chromatography–ultraviolet chromatograms (at 260 nm) of the *Radix Salviae Miltiorrhizae* extract.

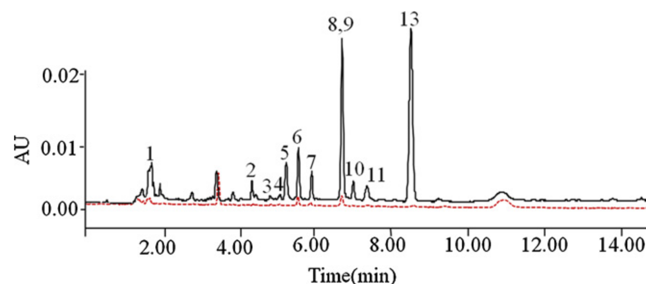

Fig. 2. The UV chromatogram obtained by ultrafiltration ultra-high performance liquid chromatography–tandem mass spectrometry of *Radix Salviae Miltiorrhizae* extract (10 μM); analysis was a screening procedure for binding to XOD. The solid line represents the experiment with XOD, and the dashed line represents the experiment without XOD.

binding to the enzyme does not necessarily mean that it is a ligand for the enzyme.

3.3. Identification of XOD inhibitors by UPLC–DAD–ESI–MS^n and FT-ICR-MS

The multistage mass spectra (MS^n, $n \geq 2$) provided by ion trap mass spectrometry (IT-MS) could confirm the relationship between precursor and daughter ions. This type of analysis is very helpful for the structural determination of unknown compounds. FT-ICR could give high-resolution mass spectra of all ions and the elemental composition of the ions [34]. In this study, UPLC–DAD–ESI–MS^n and FT-ICR-MS were carried out to identify the ligands screened from *Radix S. Miltiorrhiza*. Prior to ESI–MS, parameters such as electrospray voltage, capillary voltage, and capillary temperature were optimized for the ligands. The mass spectral data of the *Radix S. Miltiorrhiza* extract were obtained in positive ion mode, which provided more structural information on most compounds in the extract (except for salvianolic acid B) than those obtained in the negative ion mode. Data on the retention time (t_R) in FT-ICR-MS and ESI–MS^n are summarized in Table 1. Twelve ligands were identified by comparing the t_R and FT-ICR-MS and ESI–MS^n fragmentation patterns of those compounds against those of the corresponding reference compounds and literature data (chemical structures of the compounds are shown in Fig. 3). Eleven compounds were lipophilic diterpenoidal quinines and one was a hydrophilic depside (Fig. 3). The structural analysis of compounds **5**, **6**, and **11** were taken as examples to illustrate our paradigm.

Under the ESI–MS conditions, compounds **5**, **6**, and **11** were strongly predominating species in the positive ion mode; all ESI mass spectra of these compounds are dominated by peaks of the same $[M+Na]^+$ ions at *m*/*z* 301, indicating that they are isomers. When tandem mass spectrometry was used to distinguish these three isomers, they gave the same fragment ions, but the ability of the parent ion to dissociate at a normalized collision energy of 20% in tandem mass spectrometry was different. The order of relative abundance of the three parent ions is compound **6** (100%) > compound **11** (50%) > compound **5** (<15%) (Fig. 4). This shows that the structure of compound 6 is the most stable among the three isomers. In a further multistage tandem MS, the three isomers generated the same fragment ions, i.e., the ion at *m*/*z* 261 further fragmented by the loss of CO to form the ion at *m*/*z* 233; the ion at *m*/*z* 233 further fragmented by the continuous loss of CO and CH_3, as well as CO and CH_3 (Fig. 5). On the basis of the ESI–MS^n fragmentation patterns of the corresponding reference compounds and the literature data, the three isomers were identified as 1,2-dihydrotanshinone I, 15,16-dihydrotanshinone I, and methylenetanshinquinone.

Table 1
The LC–MS^n data of XOD ligands identified from *Radix Salvia Miltiorrhizae* extract.

Peak No.	Compound	Retention time (min)	$[M+H]^+$ or $[M-H]^-$	MS^2	MS^n	FT-ICR-MS	Formula element
1	Salvianolic acid B	1.49	717	519	519; 339; 321	717.14487	$C_{36}H_{29}O_{16}{}^{-1}$
2	Tanshinone II B	4.15	311	293	293;275; 278; 265; 251	311.12743	$C_{19}H_{19}O_4{}^{+1}$
3	Tanshindiol B	4.89	313	295; 265; 247	295; 277; 267; 249; 253	313.10759	$C_{18}H_{17}O_5{}^{+1}$
4	Tanshindiol A	5.01	313	298; 267; 277	298; 267; 277	313.10768	$C_{18}H_{17}O_5{}^{+1}$
5	15,16-Dihydrotanshinone I	5.62	279	279 (100%); 261	233; 218; 205; 190	279.10149	$C_{18}H_{15}O_3{}^{+1}$
6	1,2-Dihydrotanshinone I	5.23	279	279 (<15); 261	233; 218; 205; 190	279.10127	$C_{18}H_{15}O_3{}^{+1}$
7	Danshenxinkun B	6.01	281	266; 253; 239; 235; 211		281.11751	$C_{18}H_{17}O_3{}^{+1}$
8	Cryptotanshinone	5.23	297	279; 251; 264; 261; 237	264; 233; 218; 205; 190	297.14898	$C_{19}H_{21}O_3{}^{+1}$
9	Tanshinone I	6.98	277	249; 231; 259		277.08547	$C_{18}H_{13}O_3{}^{+1}$
10	3-Hydroxy methylene tanshinquinone	7.21	295	277; 249; 267; 253		295.09849	$C_{18}H_{15}O_4{}^{+1}$
11	Methylene tanshinquinone	7.90	279	279 (50%); 261	233; 218; 205; 190	279.10124	$C_{18}H_{15}O_3{}^{+1}$
13	Tanshinone II A	9.28	295	277	277; 262; 259; 249; 235	295.13078	$C_{19}H_{19}O_3{}^{+1}$

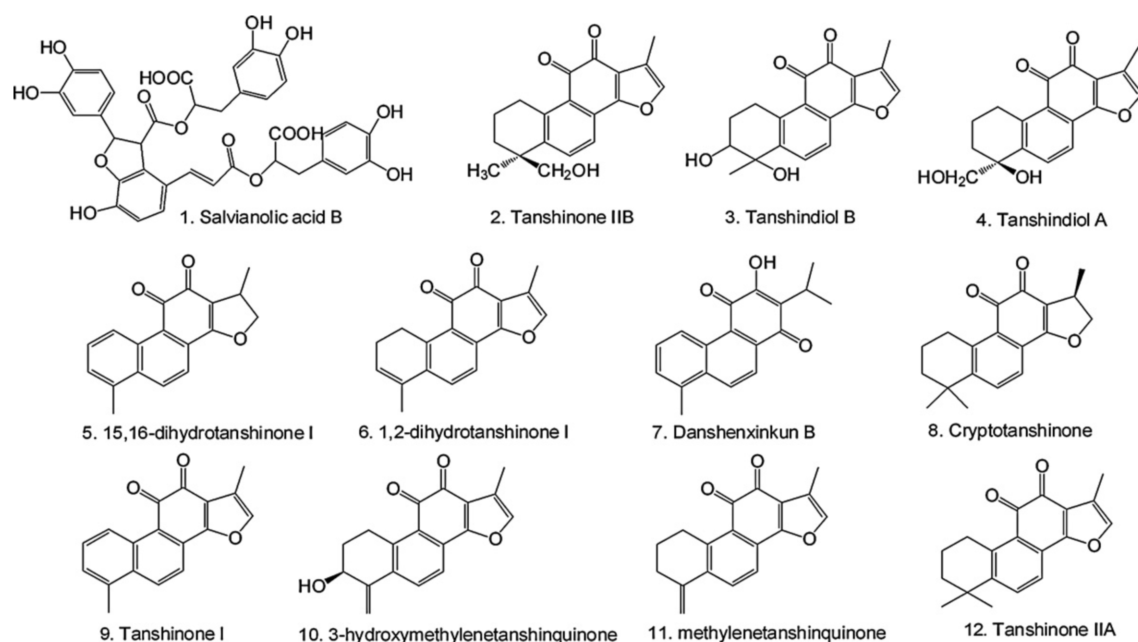

Fig. 3. Chemical structures of compounds identified from *Radix Salviae Miltiorrhizae* (Danshen).

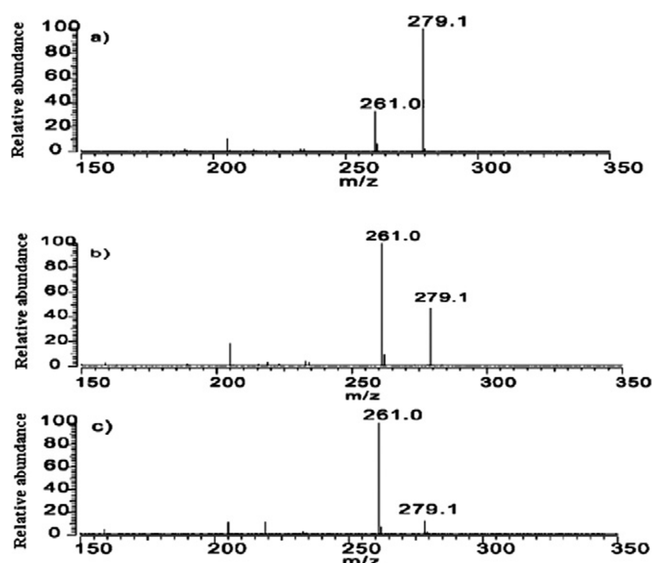

Fig. 4. MS^2 spectra of compounds **5**, **6** and **11** obtained by IT-MS in positive ionization mode: (a) compound **6**; (b) compound **11**; and (c) compound **5**.

3.4. Evaluation of relative binding affinity of the ligands

The relative binding affinities of the ligands from *Radix S. Miltiorrhizae* toward XOD were compared using the values of the "enrichment factors" as defined by Nikolic et al. [29]. Based on the liquid chromatograms, enrichment factors that could represent the specific binding affinity of each ligand to XOD could be obtained by dividing the amount of specific binding by the original amount of each compound in the incubation solution. The enrichment factors were calculated based on the following equation: $(A1-A2)/A3\times 100\%$, where $A1$ is the peak area obtained from the experiment with XOD, $A2$ is the area of the control without XOD, and $A3$ is the area of the compounds in the original solution. The results obtained from the above calculations are shown in Table 2. Based on Table 2, salvianolic acid B could not bind to XOD. This means that the 1,2-naphthoquinone group is necessary for the XOD inhibitory activity of these compounds. The structure of the 1,2-naphthoquinone group is similar to those of xanthine and hypoxanthine, which are the substrates of XOD. Thus, these 11 lipophilic diterpenoid quinines are likely to occupy the active site of the enzyme and bind to the active site of XOD. The affinity ranking of the other ligands for XOD was

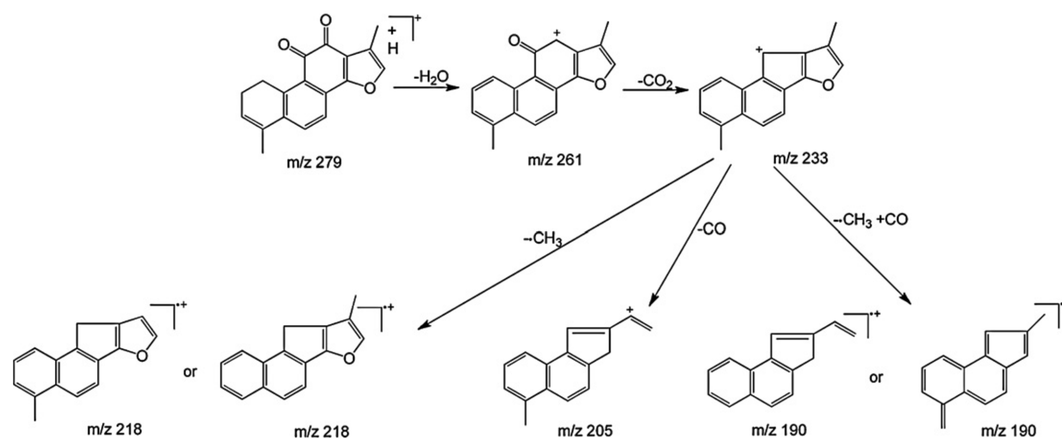

Fig. 5. Proposed MS fragmentation pathway for the $[M-H]^-$ ions of compounds **5**, **6**, and **11**.

Table 2
Ultrafiltration LC–MS signal enrichment factors for Ligands to XOD[a,b] (%).

Peak No.	Compound	Enrichment factors (XOD 10 μM)
1	Salvianolic acid B	<1
2	Tanshinone II B	25.60 ± 1.5
3	Tanshindiol B	33.69 ± 0.9
4	Tanshindiol A	25.25 ± 1.7
5	15,16-Dihydrotanshinone I	53.24 ± 0.5
6	1,2-Dihydrotanshinone I	20.13 ± 1.6
7	Danshenxinkun B	17.45 ± 2.1
8	Cryptotanshinone	70.50 ± 0.6
9	Tanshinone I	
10	3-Hydroxy methylene tanshinquinone	81.35 ± 0.7
11	Methylene tanshinquinone	63.53 ± 0.9
13	Tanshinone II A	61.35 ± 1.1

[a] Enrichment factors = amount of compound specifically bound/total amount of compound in incubation.
[b] Mean ± SD (*N* = 3).

3-hydroxymethylenetanshinquinone ≫ methylenetanshinquinone ≈ tanshinone II A > 15,16-dihydrotanshinone I ≫ tanshindiol B > tanshinone II B ≈ tanshindiol A > 1,2-dihydrotanshinone I > danshenxinkun B. The different binding affinities of these ligands demonstrate that the furan ring substituent and hydroxyl substituent on the alicyclic ring group could enhance the activity of the compounds at different levels. To some degree, small molecules that have stronger binding affinity are more likely to have higher XOD inhibitory activity. Thus, this assay not only can be used for screening of XOD inhibitors from mixtures, but also for providing useful information for determining their strength of inhibitory activity.

4. Conclusions

The present work demonstrated that UF-LC–MS was a powerful tool for the rapid screening and characterization of XOD inhibitors from *Radix S. Miltiorrhizae* extracts. Eleven compounds were screened for XOD inhibitory activity and their structures were further identified by LC–MS^n and FT-ICR-MS. Furthermore, the relative binding affinities of the 11 ligands were estimated and the structural features necessary for their XOD binding affinity were elucidated. The obtained results are expected to be valuable for discovering XOD inhibitor candidates from *Radix S. Miltiorrhizae* and efficiently designing drugs for the prevention and treatment of hyperuricemia and gout.

Acknowledgements

The authors would like to thank the National Natural Science Foundation of China (No. 21175128, 21073178, 81073040), the Innovation Method Fund of China (2009IM030400), the Science and Technology Foundation of Jilin Province (No. 200905104, 20110928), and the Natural Science Foundation of Jilin Province (No. 201015101) for financial support.

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