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Affinity ultrafiltration and UPLC-HR-Orbitrap-MS based screening of thrombin-targeted small molecules with anticoagulation activity from *Poecilobdella manillensis*

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Abstract

This study aims to screen potential anticoagulant components from leeches, a representative animal-sourced traditional Chinese medicine using thrombin (THR)-targeted ultrafiltration combined with ultrahigh performance liquid chromatography and high-resolution Orbitrap mass spectrometry (UPLC-HR-Orbitrap-MS). As a result, five small molecules in leech extract were discovered to interact with THR for the first time. Among them, two new compounds were isolated and their structures were identified by IR, HR-MS and NMR data. Furthermore, their THR inhibitory activity was confirmed with IC_{50} values of 4.74 and 8.31 μM, respectively. In addition, molecular docking analysis showed that the active (catalytic) site of THR might be the possible binding site of the two hits. Finally, reverse screening analysis indicated that LTA4-H, ACE and ALOX5AP were potential anticoagulant targets of the two new compounds. This study will broaden our understanding of the medicinal substance basis in leeches and further contribute to the discovery and development of clinical anticoagulant drugs from leeches.

Keywords: UPLC-HR-Orbitrap-MS; Ultrafiltration; Leech; Thrombin inhibitory activity; Molecular docking

Introduction

Cardiovascular disease (CVD) is the leading cause of death in China [1, 2]. Blood coagulation was implicated to play significant roles in the occurrence and development of CVD, and the corresponding pathways are the primary targets for CVD treatment [3]. For CVD treatment, on the one hand, clopidogrel and aspirin are widely used in the clinic to inhibit platelet aggregation, and thrombus formation and the subsequent risk of thromboembolism events [4], However, they have side effects such as bleeding and resistance, thereby limiting their clinical application [5, 6]. On the other hand, vitamin K antagonists such as heparins and warfarin are commonly used worldwide as anticoagulants [7]. However, they have several undesirable effects, such as possible bleeding due to overanticoagulation or thrombosis [8, 9], which has prompted to the search for alternative sources of anticoagulants [10].

Thrombin (THR), the key serine proteinase involved in the coagulation cascade [11, 12], has become an effective drug target and hot spot for the research of new anticoagulants [13], due to its vital role in many diseases related to CVD [14]. Consequently, inhibiting the activity of THR is currently an ideal and effective strategy for the treatment of thrombotic diseases [15, 16]. Direct THR inhibitors are divided into bivalent inhibitors and univalent inhibitors. Bivalent inhibitors bind to the THR-active (catalytic) site and positively charged exosite I, while univalent inhibitors only act on the active (catalytic) site of THR. Hirudin is a bivalent inhibitor and the binding of hirudin to THR is essentially irreversible. However, this characteristic may lead to the occurrence of side effects of hirudin bleeding [17]. Argatroban is a univalent inhibitor of small molecules. Compared with macromolecular THR inhibitors, it can not only combine with free THR, but also penetrate deep into the thrombus and interact with agglomerated THR or THR bound to fibrin, possessing the advantages of a smaller required dose, easier crossing across the blood-brain barrier, safer and better efficacy [18, 19]. Consequently, small molecules

are ideal candidates as THR inhibitors.

Leech, a representative animal-sourced TCM, has the effect of promoting blood circulation and removing blood stasis [20-23]. To date, 650 species of leeches have been found worldwide [24]. Among them, in addition to three legally listed Chinese species, leech *Poecilobdella manillensis* is the most commonly available from the Chinese commercial leech market. Currently, many studies have focused on the analysis of macromolecules including bufrudin, poecistasin and some other active peptides in leech *Poecilobdella manillensis* [25-28]. However, there is little research on the active components of small molecules.

Bioaffinity-based methods, also called ligand fishing, have been developed for fast screening and identification of specific target components in complex natural products. The principle is based on the reversible interaction between biomolecules. After the affinity interactions between ligands and proteins, THR and ligands are dissociated and eluted. It is possible to screen components that have reversible binding effects with the target protein. In ligand fishing experiments, compounds that have affinity for the target will be acquired and further analysed, while compounds not bound to the target will be discarded [29-31]. Ultrafiltration affinity combined with other advanced analytical methods is a powerful tool for fishing biologically active compounds from complex natural products, which is simple, time-saving, and requires less sample pretreatment [32, 33]. Analytical methods have evolvedfrom common liquid chromatography (LC) to liquid chromatography mass spectrometry (LC-MS). Among them, ultrahigh-performance liquid chromatography (UPLC) coupled with high-resolution mass spectrometry (HR-MS) has been extensively used in the detection and identification of complex mixtures due to the efficient physical separation capability of UPLC and the excellent identification ability of HR-MS [34]. In particular, the HR-Orbitrap-MS can provide accurate mass data of multistage MS (from MS^2 to MS^n)

data, which can be important when attempting to discriminate compounds with the same molecular formula and similar chemical structures [35].

In our previous work, we used a high-throughput screening method based on the principle of affinity to investigate potential bioactive components in animal based TCMs [30, 31]. This study aimed to screen the potential THR-targeting small molecules from *P. manillensis* based on ultrafiltration and UPLC-HR-Orbitrap-MS. As a result, a series of new small molecules were discovered with high THR inhibitory activity from leeches for the first time. Furthermore, their THR inhibition activity was analysed *in vitro* and molecular docking was employed to investigate their ligand-THR interactions and potential pharmacodynamic mechanisms (Fig. 1). The THR-targeting based screening method coupled with UPLC-HR-Orbitrap-MS can be used as a useful tool to rapidly screen and identify the bioactive constituents in natural products, which are very useful for drug discovery from TCM.

2. Materials and methods

2.1 Chemicals and materials

The dried materials of *P. manillensis* were collected from Fuyang, Anhui Province, China, and authenticated as *P. manillensis* (voucher specimen number: No.20190506) by Dr. Rong Luo from the School of Traditional Chinese Medicine, Capital Medical University (Beijing, China). THR from bovine plasma was obtained from Sigma Aldrich Chemical Co. LLC. (St. Louis, MO, US). Argatroban (AG) and ticagrelor (TC) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Hirudin was obtained from Melone Pharmaceutical Co., Ltd. (Dalian, China).

Acetonitrile and methanol were of MS grade and purchased from Merck (Darmstadt, Germany). MS grade formic acid was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corporation, Billerica, MA,

USA). All other chemicals and reagents were of high analytical grade.

2.2 Preparation of leech extract and stock solution

Dried leech materials were crushed into a coarse powder and accurately weighed. First, the powders were added to 10 times water with reflux extraction for 1.5 h, and the extraction was repeated 3 times. The filtrate was collected and concentrated using a rotary evaporator at 60 °C. Furthermore, the extract was precipitated by 80% ethanol, and the supernatant was dried by vacuum freeze-drying. The dried powder was stored at -20 °C before analysis.

Standard stock solutions were prepared by dissolving the respective working standard substance in methanol solution, and diluted with PBS (100 mM, pH 7.4) to generate a concentration of 40 μg mL^{-1} AG (positive control) and TC (negative control) in the mixed standard solution. The working solutions of leech extracts were prepared by dissolving the freeze-dried powder in pure water.

2.3 Ligand screening of leech extract by THR-based affinity ultrafiltration

The screening method is mainly divided into three steps: incubation, washing and elution. First, 20 μL of standard solution and leech extract working solution were separately incubated with 250 μL of THR solution at 37 °C for 90 min under shaking. Next, ultrafiltration was performed through an Amicon®Ultra-0.5 centrifugal filter device with a 10,000 MW cut-off at 14000×g for 10 min at 4 °C, followed by washing with phosphate buffer (100 mM, pH 7.4) five times via centrifugation at 14000×g for 10 min to remove the unbound components. The bound small molecules were extracted by methanol under sonication for 30 min to destroy the binding of THR to the ligands. After centrifugation at 17,000×g for 15 min, the supernatant was dried by speed-vacuum and reconstituted in 200 μL of methanol for UPLC-HR-Orbitrap-MS analysis. THR denatured in boiling water was used as a nonspecific binding control group. All screening trials were independently performed three times and analysed in duplicate.

The peak area discrepancy of compounds in the leech extract between the experiment and control (ΔP) was calculated with the following formula:

$$\Delta P = \frac{(PA - PB)}{PA} \times 100\%$$

where PA and PB are the peak areas of the compounds in the leech extract reacted with active THR and denatured THR, respectively. Compounds with ΔP higher than 20% indicated specific binding and were considered potential bioactive compounds binding to the target [36].

2.4 UPLC-HR-Orbitrap-MS conditions

The ultimate 3000 hyperbaric LC system coupled with high resolution Orbitrap Fusion Lumos TribridTM via an electrospray ionization (ESI) interface from Thermo Fisher Scientific (Bremen, Germany) was used for compound identification of leech elution. The chromatography system was equipped with an autosampler, a diode-array detector, a column compartment, and two pumps. The chromatographic conditions were optimized and a BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters) maintained at 35 °C was finally chosen for separation of the *P. manillensis* extract. The mobile phase was composed of water (0.1% formic acid, A) mixed in gradient mode with acetonitrile (0.1% formic acid, B), at a flow rate of 200 μL/min. The elution gradient procedure was optimized as follows: 0-3 min, 3% B; 3-8 min, 3% to 14% B; 8-22 min, 14% to 25% B; 22-26 min, 25% to 55% B; 26-28 min, 55% to 100% B; 28-35 min, 100% B. The injection volume was 3.0 μL and the sampler was set at 4 °C.

For identification of the components in leech elution, positive full scan modes within the range of *m/z* (mass/charge ratio) 150-1500 at a resolution of 120,000 were used for acquisition of accurate molecular ions. The other parameters were set as follows: spray voltage, +3.0 kV; sheath gas flow rate, 35 arb; aux gas flow rate, 10 arb; sweep gas, 2 arb; ion transfer tube temperature, 320 °C; vaporizer temperature, 275 °C; cycle time, 3 s; RF lens, 50%. The fragment ions in MS/MS data obtained by higher

energy collision dissociation (HCD) at proper collision energy were further utilized to confirm the structures of the components. Xcalibur 3.0 software (Thermo Fisher) was used for UPLC-HR-Orbitrap-MS control and data handling.

2.5 Isolation of compounds from P. manillensis

Among the discovered potential active compounds, two compounds were isolated and purified using preparative HPLC byin our group. Ten grams of *P. manillensis* was crushed and extracted with 10 times water for 1.5 h, which was repeated 3 times. The supernatants were combined and concentrated to 20 mL under vacuum. Then, 80 mL ethanol was added to precipitate the proteins and peptides in the leech. After overnight incubation, the precipitates were discarded and the supernatants were collected and freeze-dried under vacuum. After dissolving with methanol, the target compounds were purified by preparative HPLC. The HR-MS data were recorded on an Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). IR spectra were obtained on a Thermo Nicolet iS50 FTIR spectrometer (Thermo Nicolet, Madison, USA). NMR spectra were acquired with Bruker AV 800 spectrometers (Bruker BioSpin Group, Faellanden, Switzerland).

2.6 THR inhibitory activity assay

To evaluate the inhibitory effects against THR, the two isolated hits were measured as a fluorescence assay utilizing a SensoLyte 520 trombin activity assay kit (AnaSpec, Inc., San Jose, CA). It was performed following the manufacturer's instructions. AG, a known direct THR inhibitor, was used as a positive control for the THR activity assay.

2.7 Identification of potential anticoagulation targets by inverse docking

Prior to docking, key protein targets from the literature related to thrombotic disease were summarized [38-41, 50, 51, 55]. In total, 30 protein targets were selected as the key targets of leech

compounds. All protein (receptor) structure files were downloaded from the pdb database (http://www.rcsb.org/) and preprocessed in pyMOL, including deleting crystal water and irrelevant small molecules, and adding hydrogen atoms. Furthermore, the protein files were saved in pdb format and loaded into the autodocktools 1.5.6 program to add atomic charges and assign atom types, respectively. Then, the files were saved in pdbqt format as a recipient. The structures of the two new compounds were drawn by Chemdraw12 and loaded into chem3D in cdx format. The MM2 force field was used for energy optimization and the two compounds were further loaded into the autodocktools 1.5.6 program in pdb format to add atomic charges, assign atom types and set all rotatable bonds to flexible, which were further saved in pdbqt format as docking ligands. On this basis, autodock AutoDock Vina1.1.2 was used for molecular docking. The ligand was set to be flexible and the receptor was rigid. The search accuracy exhaustiveness was set to 64, and other parameters were default. The docking box was set to exactly contain the receptor protein binding pocket. Furthermore, the conformation with the highest score (lowest affinity value) was selected as the docking conformation. Finally, ligplot1.4 was used for visual analysis and drawing 2D interaction views.

2.8 Statistical analysis

All data are presented for at least three different experiments. The statistical analysis was performed with GraphPad Prism software (version 5, GraphPad Software Incorporation, USA).

3. Results and discussion

3.1 Evaluation of the THR-based screening method

To obtain reliable screening results, the suitability of the target THR-based screening method was evaluated using standard solutions containing the positive control AG and the negative control TC. AG is a direct THR inhibitor, that reversibly binds specifically to the catalytic domain of THR. This

reversible binding effect with THR can also be one of the reasons for reducing the risk of bleeding after withdrawal [41, 42]. The affinity selection strategy is based on the reversible interaction between biomolecules. During the elution process, targets and compounds were dissociated when the solution environment was changed [43]. Therefore, AG was selected as a positive control in the ligand fishing experiments. It is expected to obtain components possessing reversible binding properties with THR to avoid side effects such as bleeding. TC was used as the negative control because it can significantly inhibit platelet aggregation induced by adenosine diphosphate, instead of binding to THR [44]. As shown in Fig. 2, the positive control AG showed relevant area enhancement compared with the denatured THR control group (ΔP = 21.52%). However, the peak area of the negative control TC was almost the same with ΔP close to zero. Compounds with ΔP >20% would specifically bind to THR, which was consistent with other reports [40, 45], demonstrating the reliability of the established THR-based screening method to screen ligands targeting THR [34]. And a diagram (Electronic Supplementary Material (ESM) Fig. S1) is also shown to exhibit statistically and analyze the binding activities of AG and TC samples upon active and denatured THR methods, respectively. Consequently, active compounds from leech extracts targeting THR were further investigated using the proposed method.

3.2 Screening of THR-based ligands from P. manillensis

Leech has a long history for the treatment of thrombotic disease. However, bioactive components, especially small molecules in leeches have rarely been studied previously [24]. In this study, a target THR-based screening method was used to screen active constituents from leech extracts. TIC of the leech extract is shown in Fig. 3. The concentrations of leech extract (12.5, 25 and 50 mg mL^{-1}) were optimized prior to experiments. As shown in ESM Fig. S2, the elution peak was the largest when the sample concentration was set at 25 mg mL^{-1}. Furthermore, the effect of ultrasonication on the extraction of

binding ligands was investigated. The area of the elution peak was larger after sonication (ESM Fig. S3). Consequently, the incubation concentration of the leech extract was optimized to 25 mg mL^{-1} and methanol extraction under sonication was chosen for the extraction of binding constituents. Finally, the THR-targeted constituents were screened by UPLC-HR-Orbitrap-MS and their binding percentages were determined accurately on the basis of the peak area.

Compared with the denatured THR (black line), a total of five constituents in the leech extract (compounds **1**-**5**) showed specific binding activity with THR (red line) based on UPLC-HR-Orbitrap-MS analysis (Fig. 4). The RSDs (n = 3) of all hit compounds were <16% and the detailed numerical results are shown in Table 1 and Table 2. To comprehensively detect the hit compounds, both negative and positive ion modes were used in UPLC-HR-Orbitrap-MS analysis, and the positive ionization mode was more suitable for the detection of the hit components (ESM Fig. S4). The UPLC-HR-Orbitrap-MS data and the structural characteristic ions of the corresponding constituents are summarized in Table 1 and Table 2. Finally, according to the database search of SciFinder, it was deduced that the screened small molecules in the leech were all new and further compound isolation was performed for their structure identification.

3.3 Isolation and identification of the screened compounds from P. manillensis

Among the five compounds binding with THR, two main components, compounds **2** and **3,** showed high binding activity towards THR, which were consequently isolated for subsequent experiments by preparative HPLC and were identified by HR-MS, IR and NMR spectra. Compound **2** was isolated as a brown, amorphous powder with the molecular formula $C_{18}H_{22}N_8O_5S_2$ with an $[M+H]^+$ ion at *m/z* 495.1236 (calcd for 495.1227) and an $[M-H]^-$ ion at *m/z* 493.1075 (calcd for 493.1071) in its HR-MS spectrum. The IR absorption bands at 1718 and 1670 cm^{-1} suggested the presence of carbonyl groups. IR

absorption bands at 1636 and 1507 cm^{-1} indicated the presence of carbon nitrogen double bonds (ESM Fig. S5A). Detailed NMR identification results are described in the Supplementary Document. In fact, the structure of compound **2** is similar to that reported for whitmanine A [45], except for the difference in the carbon chain at C-14. As a result, compound **2** was elucidated as a pteridine alkaloid and named bdelline A.

Compound **3** was isolated as a brown, amorphous powder. The molecular formula of compound **3** was determined to be $C_{20}H_{26}N_8O_5S_2$ based on the HR-MS data with an $[M+H]^+$ ion at *m/z* 523.1547 (calcd for 523.1540) and an $[M-H]^-$ ion at *m/z* 521.1386 (calcd for 521.1383). The NMR data of **3** in Table **3** were similar to compound **2**, except for the two methyl groups at N-22. Therefore, compound **3** was elucidated as a pteridine alkaloid and named bdelline B (Fig. 5).

On the basis of the isolation and structure identification of compounds **2** and **3**, ESI-HR-MS/MS dissociation pathways in positive ionization mode were proposed first to confirm the structure identification by NMR data and further assist the structure identification of the other compounds. As shown in ESM Figs. S12 and S13, neutral losses of NH_3 and other carbon chains in part B were the main fragmentation patterns of compounds **2** and **3**. In addition, identical characteristic ions at *m/z* 478.0991 ($C_{18}H_{20}O_5N_7S_2$), 436.0770 ($C_{17}H_{18}O_5N_5S_2$), 435.0928 ($C_{17}H_{19}O_4N_6S_2$), 392.0869 ($C_{16}H_{18}O_3N_5S_2$), 321.0129 ($C_{12}H_9O_3N_4S_2$), 320.0291 ($C_{12}H_{10}O_2N_5S_2$) and 295.0336 ($C_{11}H_{11}O_2N_4S_2$) were found in their MS/MS spectra, which were attributed to the loss of side chains in part B with part A residue, demonstrating their structural similarity. As the minor constituents screened from the leech extract, compound **1** was detected with an $[M+H]^+$ ion at *m/z* 495.1196 ($C_{18}H_{23}O_5N_8S_2$, calcd for 495.1227), which was an isomer of compound **2**. In addition, compounds **4** and **5** were detected with $[M+H]^+$ ions at *m/z* 451.1333 ($C_{17}H_{23}O_3N_8S_2$, calcd for 451.1329) and 479.1646 ($C_{19}H_{27}O_3N_8S_2$, calcd for 434.2652),

which were 44 Da (CO_2) less than those of compounds **2** and **3**, respectively. In their MS/MS spectra (ESM Fig. S14), the fragments at *m/z* 392.0846, 321.0110, 320.0271 and 295.0318 were identical to those of the fragments of compounds **2** and **3**, indicating that compounds **4** and **5** shared the same skeleton. In addition, the fragments at *m/z* 434.1063 and 409.1109 were observed in their MS/MS spectra. By comparison with the proposed fragmentation pathways of compounds **2** and **3**, the structures of compounds **4** and **5** were deduced (ESM Fig. S15). Additionally, compounds **1-5** all had sulfur structures similar to those of other antiplatelet aggregation agents, such as ticlopidine, clopidogrel, and prasugrel [46], exhibiting great potential for the treatment of CVD-related diseases.

3.4 THR inhibitory activity assay

An *in vitro* enzyme activity assay was performed to confirm the inhibitory activity of compounds **2** and **3** towards THR. Prior to experiments, the reliability of the assay was evaluated by AG, a THR inhibitor. The results showed that AG had obvious inhibitory activity towards THR (ESM Fig. S16). As shown in Fig. 6, the two new compounds had strong THR inhibition effects with IC_{50} values of 4.74 and 8.31 μM, respectively, exhibiting orders of magnitude difference from those of cryptotanshinone (IC_{50}=81.11 μM) and tanshinone IIA (IC_{50}=66.60 μM), which were screened from Salvia miltiorrhiza [48] and isochlorogenic acid C (IC_{50}=206.48 μM) and senkyunolide I (IC_{50}=197.23 μM) that was discovered from *Ligusticum chuanxiong* [32]. The masses of compounds **2** and **3** were similar to that of the direct THR inhibitor argatroban (*m/z* 508.63). Consequently, it can be inferred that compounds **2** and **3** were active small molecules in *P. manillensis* and had great potential for anticoagulation drug development and application in the future.

3.5 Identification of the THR binding site and other potential anticoagulation targets by inverse docking

To further investigate the interactions between the two compounds and THR, molecular docking analysis was performed. Fig. 7 shows that the binding energies of compounds **2** and **3** with THR were both -7.4 kJ/mol, interacting with THR to form hydrophobic interactions with His57 and strong hydrogen bonding with Ser195. Because residues His57 and Ser195 are the active (catalytic) site, the molecular docking results showed that compounds **2** and **3** blocked the active (catalytic) site, one of the three main active domains of THR [49]. Consequently, we speculate that compounds **2** and **3** were attributed to univalent inhibitors of THR.

As new small molecules in *P. manillensis* exhibiting anticoagulant activity, further identification of their potential targets is necessary. To comprehensively demonstrate the potential anticoagulant targets of compounds **2** and **3**, 30 anticoagulant-related proteins reported in the literature were included using reverse screening technology [38-41, 50, 51, 55]. As shown in Table S1, the two hit compounds showed high affinity for many anticoagulant targets. They both exhibited high binding affinity with LTA4-H, ALOX5AP and ACE. For compound **2**, the binding energies were -10.4, -10.2 and -9.1 kJ/mol, respectively. For compound **3**, the binding energies were -10.1, -8.8 and -9.0 kJ/mol, respectively, which were all higher than that of THR. LTA4-H is the key enzyme in the process of leukotriene generation. LTA4-H not only participates in the regulation of inflammation, but is also an important lipid inflammatory mediator, that affects coronary vessel tone and the pathogenesis and progression of atherosclerosis [50]. ACE can convert angiotensin I to angiotensin II and then play a vital role in regulating blood pressure. In addition, ACE-inhibitors and angiotensin receptor blockers are widely used clinically in the treatment of cardiovascular diseases [51]. The proteins that interact with it are angiotensinogen (AGT) [52], angiotensin II receptor type 1 (AGTR1) [53], angiotensin II receptor type 2 (AGTR2) [54], etc. These proteins were involved in the regulation of biological processes, such as

blood vessel diameter, the complement and coagulation cascades and calcium signalling pathways. ALOX5AP has been proposed to play an important role in the pathogenesis of atherosclerosis and ischaemic stroke [49]. Pathway analysis of ALOX5AP indicated that its related target protein could act on the arachidonic acid metabolism pathway and affect the body's coagulation regulation. Further analysis showed that hydrogen bonds and hydrophobic contact played an important role in their binding with these proteins (ESM Fig. S18). In short, the results of molecular docking made further predictions on the potential mechanism and targets of the two small molecule compounds discovered by this topic.

4. Conclusions

In this study, THR-targeting small molecules from *P. manillensis* were screened based on ultrafiltration and UPLC-HR-Orbitrap-MS analysis. A series of small molecules were discovered to be potential THR inhibitors in leeches for the first time. Among the small molecules, two new compounds were isolated and their structures were identified by HR-MS and NMR data. Furthermore, their THR inhibitory activity was confirmed with IC_{50} values of 4.74 and 8.31 μM, respectively. In addition, molecular docking analysis showed that the possible binding site of the two hit compounds was the catalytic site, which is a univalent THR inhibitor. Reverse screening analysis for a possible mechanism showed that LTA4-H, ACE and ALOX5AP were potential anticoagulant targets of the two new small molecules. The present research sheds new light on the active material basis of leeches and their further efficient utilization, which is of great significance for the research and development of anticoagulants in medicine.

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- **Figure captions**

Table 1 Compounds identified by UPLC-HR-Orbitrap-MS/MS

Table 2 MS/MS fragments of identified compounds

Table 3 NMR data of compounds **2** and **3** in DMSO-d_6

Fig. 1 Scheme of the ligand fishing procedure for THR-based inhibitor screening from leeches

Fig. 2 Extracted ion chromatograms of the positive control AG and the negative control TC using the THR-based screening method obtained with active (red line) and denatured THR (black line)

Fig. 3 TIC of the leech extract (A) Positive ion mode; (B)Positive ion mode

Fig. 4 Analysis of THR-targeted constituents (compounds **1**-**5**) in leech extract by ultrafiltration and UPLC-HR-Orbitrap-MS obtained with denatured (black line) and active THR (red line)

Fig. 5 Key ^{1}H-^{1}H COSY and HMBC correlations of compounds **2** and **3**

Fig. 6 THR inhibitory activity of compounds **2** (A) and **3** (B)

Fig. 7 Interaction of hit compounds with THR: (A) Compound **2** binding with THR; (B) Compound **3** binding with THR

Table 1 Compounds identified by UPLC-HR-Orbitrap-MS/MS

Compound NO.	tR (min)	ΔP% (n = 3)	RSD%	Ion form	Measured mass	Calculated mass	Proposed fomula	Error (ppm)
1	12.84	83.74±10.35	12.35	$[M+H]^+$	495.1196	495.1227	$C_{18}H_{23}O_5N_8S_2$	0.578
				$[M+H]^+$	495.1236	495.1227	$C_{18}H_{23}O_5N_8S_2$	1.299
2	16.38	78.43±10.24	13.06	$[M+Na]^+$	517.1054	517.1047	$C_{18}H_{22}O_5N_8S_2Na$	1.427
				$[M-H]^-$	493.1075	493.1071	$C_{18}H_{21}O_5N_8S_2$	0.804
				$[M+H]^+$	523.1547	523.1540	$C_{20}H_{27}O_5N_8S_2$	1.197
3	17.91	77.9±12.42	15.95	$[M+Na]^+$	545.1366	545.1360	$C_{20}H_{26}O_5N_8S_2Na$	1.140
				$[M-H]^-$	521.1386	521.1384	$C_{20}H_{25}O_5N_8S_2$	0.434
				$[M+H]^+$	451.1333	451.1329	$C_{17}H_{23}O_3N_8S_2$	-0.359
4	18.53	84.08±9.09	10.81	$[M+Na]^+$	473.1153	485.1148	$C_{18}H_{22}O_3N_8S_2Na$	-0.289
				$[M-H]^-$	449.1178	449.1173	$C_{17}H_{21}O_3N_8S_2$	-0.027
				$[M+H]^+$	479.1646	479.1642	$C_{19}H_{27}O_3N_8S_2$	-0.327
5	20.11	80.58±11.63	14.43	$[M-H]^-$	477.1491	478.1564	$C_{19}H_{26}O_3N_8S_2$	-0.139

Table 2 MS/MS fragments of identified compounds

NO.	MS/MS fragments
1	478.0929、436.0718、435.0873、392.0824、306.9932、281.0142、84.0803
2	478.0991、436.0770、435.0928、392.0846、**321.0110**、320.0291、295.0318
3	478.0960、436.0744、435.0906、**321.0110**、320.0270、295.0318、70.0653
4	434.1063、409.1109, 392.0846、**321.0110**、320.0271、295.0318、115.0868
5	462.1378、434.1063、409.1109、392.0846、**321.0110**、320.0271、295.0318

Bold: the most abundant product ion

Table 3 NMR data of compounds **2** and **3** in DMSO-d_6

Position	Compound 2		Compound 3	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
2		150.2		150.3
4		159.2		159.4
4a		126.9		127.1
5a		144.6		144.6
6		126.5		126.5
7		141.9		141.7
8a		156.4		156.3
9a		145.9		146.1

10	3.58, 3H, s	29.5	3.59, 3H, s	29.6
11	3.37, 3H, s	28.6	3.36, 3H, s	28.6
13	2.69, 3H, s	18.0	2.74, 3H, s	18.0
14		158.8		158.6
15	9.32, 1H, d (5.6)		9.39, 1H, d (5.6)	
16	4.19, 1H, q (5.6, 11.2)	54.9	4.11, 1H, q (6.4, 11.6)	54.8
17	1.87, 2H, m	29.5	1.89, 2H, m	29.4
18		25.0	1.66, 2H, m	24.8
18a	1.64, 1H, br s			
18b	1.50, 1H, br s			
19	3.11, 2H, m	40.4	3.24, 2H, m	41.8
21		157.2		155.8
20	9.27, 1H, br s		8.28, 1H, t (5.2)	
23		174.0		172.1
22	7.48, 2H, s			
24	7.73, 2H, s		7.64, 2H, s	
25			2.96, 6H, s	38.2
26			2.96, 6H, s	38.2

^{1}H NMR recorded at 800 MHz; ^{13}C NMR recorded at 200 MHz.

Highlights

1) Discovery of a series of new ingredients and two direct thrombin inhibitors in the animal-sourced traditional Chinese medicine.

2) Bio-specific thrombin-targeted affinity ultrafiltration and UPLC-HR-Orbitrap-MS screening.

3) The ingredients found in this study were helpful in studying leeches.

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Declaration of interests

☑ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: