Supporting Information

Targeted screening of natural thrombin inhibitor from herbal extracts using enzyme-immobilized microfluidic reactor

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Supporting experimental section

Reagents and Materials. Glass slides (75×25 mm) coated with an indium-tin-oxide (ITO) layer were obtained from Bruker Daltonics (USA). Tetraethoxysilane (TEOS, ≥99.0%), ammonia aqueous solution (25 wt%), thrombin (EC 3.4.21.5, 1 kU), bovine serum albumin (BSA), argatroban (>99% purity), hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$, ≥99.9% purity), 10× phosphate-buffered saline (PBS, PH=7.4), tris(hydroxymethy)aminomethane-HCl (Tris-HCl), 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), and 9‑aminoacridine (9-AA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-Trimethoxysilylpropyl-N,N,N-trimethylammonium chloride (TMAC) was purchased from Alfa Aesar (USA). HPLC-grade ethanol, acetonitrile, dimethyl sulfoxide (DMSO), and methanol were obtained from Merck (Darmstadt, Germany). Cetyltrimethylammonium bromide (CTAB, ≥99.0% purity) was purchased from Acros (Belgium). Sodium borohydride ($NaBH_4$), hydrochloric acid (HCl, 36.0-38.0 wt%), sodium hydroxide (NaOH), isopropanol, acetone, and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Thrombin fluorescent-labeled substrate peptide FITC-Gly-D-Phe-Pip-Arg-Ser-Gly-Gly-Gly-Gly-Lys-Cys-NH_2 (FITC-GC-11 NH_2, >95% purity), FITC-Gly-D-Phe-Pip-Arg (>97% purity), DNA aptamer 5'GGTTGGTGTGGTTGG-C_6-SH3' (TBA_{15}) was synthesized by Sangon Biotech (Shanghai, China). Thrombin chromogenic substrate S2238 (HD-Phe-Pip-Arg-pNA) was purchased from Shanghai Boat-man Biotech Co., Ltd (Shanghai, China). All medicinal herbs were purchased from Tonghetang Traditional Chinese Medicine Clinic Co., Ltd (Jiangsu, China). Hydroxysafflor yellow A, quercetin, berberine, hyperoside, luteolin, baicalin, tenuifolin, and sibiricaxanthone A were purchased from Yuanye Bio-Technology (Shanghai, China). SU-8 2075 photoresist and its developers were purchased from MicroChem (MA, USA). Sylgard 184 polydimethylsiloxane (PDMS) pre-polymer and curing agent were purchased from Momentive Performance Materials (Dow Corning, USA). Deionized water (18.2 MΩ·cm at 25 °C) was prepared by a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals and solvents were of analytical grade and used without further purification.

PDMS microchannel plate. A 4-inch silica wafer master (University Wafer, USA) was cleaned thoroughly with acetone, isopropanol, and deionized water in sequence, and dehydrated at 105 °C for 5 min. The master structure was first fabricated using the negative photoresist SU-8 spinning at 3500 rpm to achieve a height of 65 μm. Standard protocols, including pre- and post-exposure heat treatment, UV exposure, and development, were then performed to obtain a positive relief pattern of the photoresist over a silica wafer of desired dimensions. The PDMS microchannel plate was prepared by casting an optically transparent soft elastomer, PDMS, onto the patterned master template. The PDMS and crosslinking agent were thoroughly mixed at a mass ratio of 10:1, stirred vigorously for 5 min, and degassed to remove bubbles. Subsequently, the prepolymer solution of PDMS was transferred to the master template and cured at 80 °C for 2 h. Following curing, the sample was placed in the vacuum chamber for about 30 min to expel trapped air bubbles. The PDMS layer was then peeled off from the master template carefully, followed by cutting and hole punching (1 mm inner diameter biopsy puncher; Miltex, USA). The reservoirs were fabricated by punching holes at the ends of microchannels.

Characterization of AuNPs@FPSF/ITO Glass. High-resolution transmission electron microscopy (HRTEM) images and energy-dispersive X-ray spectroscopy (EDS) images were collected on a Tecnai G2 F20 (FEI Co., USA) system operated at 200 kV.

Fluorescence Assay. Fluorescence spectra were obtained using the FLS-1000 PL spectrometer (Edinburgh Instruments, UK). AuNPs@FPSF/ITO glass-PDMS hybrid microfluidic chip was imaged by a Nikon Eclipse Ti2 inverted fluorescence microscopy (Nikon Corporation, Tokyo, Japan). The fluorescence signal in the channels was acquired using a fluorescence filter (EX 450~490 nm, 50 ms exposure). Fluorescence microscopy was used to monitor the enzymatic reaction. The obtained fluorescence images were evaluated using ImageJ software (v1.41, NIH, Bethesda, MD, USA) to quantify the fluorescence intensity. The fluorescence experiment was carried out in triplicate, and the data were shown as the mean.

MALDI MS. MALDI MS detection was performed on an UltrafleXtreme MALDI

TOF/TOF MS (Bruker Daltonics, USA) with a frequency tripled Nd:YAG solid-state laser (λ = 355 nm). Mass spectrometer calibration was performed using DHB matrix ions and a Peptide Calibration Standard Kit II (Bruker Daltonics, USA). Samples were analyzed in positive reflectron ion mode with 200 laser shots fired at 1000 Hz. The laser was set to the “Ultra” footprint setting at ~100 μm diameter. *In situ,* tandem MS (MS/MS) was performed using MALDI LIFT-TOF/TOF MS to confirm the structure of the enzymatic hydrolysate and potential thrombin inhibitors. MS data were analyzed using flexAnalysis 3.4 (Bruker Daltonics, USA). CHCA matrix (10 mg/mL) was prepared in water/acetonitrile (2:8, v/v). DHB matrix (30 mg/mL) and 9-AA matrix (10 mg/mL) were prepared in water/methanol (3:7, v/v). All experiments were carried out in triplicate, and the data were shown as the mean.

Animals and Establishment of the tMCAO Model. Male Sprague Dawley (SD) rats, weighing 250–280 g, were provided by Xipuer-BiKai (Shanghai, China). Animals were maintained in an environmentally controlled breeding room (12 h dark/light cycle at 24 °C) for at least one week before experiments. Animal experiments were conducted following the Guidelines for Animal Experimentation of China Pharmaceutical University (Nanjing, China) and were approved by the Animal Ethics Committee of this institution. In this study, an experimental model of cerebral I/R injury was induced in rats by transient middle cerebral artery occlusion (tMCAO). The surgical procedure was performed according to a previous work[1]. In brief, after anesthetization with 10% urethane (i.p., 10 mL/kg), the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were fully exposed. A nylon filament (A5-2636, Beijing Xinong Biotech Co. Ltd., China) was inserted into the ICA and advanced to the origin of the middle cerebral artery (MCA). The filament was then left in place for about 1.5 h and then removed to achieve reperfusion. Throughout the procedure, the rat’s body temperature was kept constant at ~37 °C. Following surgery until recovery from anesthesia, animals were kept in a closed chamber heated with a lamp. Rats were killed 24 h. Before sacrifice, motor deficits were measured for each animal and scored using a 5-point neurologic severity score described as follows: 0, no neurologic deficit;1, mild (failure to extend left

forepaw fully); 2, moderate (circling to the left); 3, severe (falling to the left); 4, very severe (depressed level of consciousness and failure to walk spontaneously). This procedure resulted in moderate neurological deficits (median = 2). The brain of each rat was immediately removed and placed in a steel brain matrix (1 mm, Coronal; Stoelting; IL, USA). First, the coronal slices of the brain tissues were continuously cut starting at its anterior side (starting at slice # 1, 2 mm anterior to the bregma), into six slices (2 mm thick). Then, the brain was cut sagittally in its midline so the left contralateral and the right ischemic hemispheres were separated. Thrombin activity levels 24 h after tMCAO in rats were measured (slice # 4), and the other slices were stained with 2% TTC solution (#T8877, Sigma- Aldrich) in a constant temperature shaker at 37 °C for 15 min to examine the infarct area assessment in the ischemic versus contralateral hemisphere by the Image J software. Animals with complications such as excessive bleeding, filament displacement into the pterygopalatine artery, and those that died during or less than 24 h after surgery, were excluded from the experiment. In total, four rats successfully underwent surgery.

Sample Preparation. Luteolin and baicalin were dissolved in ethanol at a concentration of 10 mM. Hyperoside, tenuifolin, and sibiricaxanthone A were dissolved in DMSO at a concentration of 10 mM. Hydroxysafflor yellow A, berberine, and quercetin were dissolved in deionized water at a concentration of 10 mM. All solutions were stored in the refrigerator at 4 °C before use. Herbal medicines, including Carthami Flos, Coptidis Rhizoma, Polygalae Radix, Ligustri Lucidi Fructus, and Hyperici Perforati Herba, were ground into fine powder. 1 g of each was accurately weighed, ultrasonically extracted with 10 mL methanol/water mixture (8:2, v/v) for 30 min (×2), centrifuged at 13000 r/min for 10 min, collected the supernatant, and freeze-dried. The freeze-dried herbal extracts were stored at -20 °C away from light before use.

Data analysis. The experimental data were subjected to statistical analysis using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The percentage inhibition of each compound at different concentrations was calculated by the formula:

$$\text{Percentage inhibition (\%)} = (1\text{-}r_1/r_2) \times 100\%$$

Where r_1 and r_2 are the MS S/N ratio of enzymatic products (FITC-Gly-D-Phe-Pip-Arg) with and without inhibitors, respectively. The mean MS S/N ratio was obtained from ten mass spectra.

References

[1] E.Z. Longa, P.R. Weinstein, S. Carlson, R. Cummins, Reversible middle cerebral artery occlusion without craniectomy in rats, Stroke, 20 (1989) 84-91.

Supporting Figures

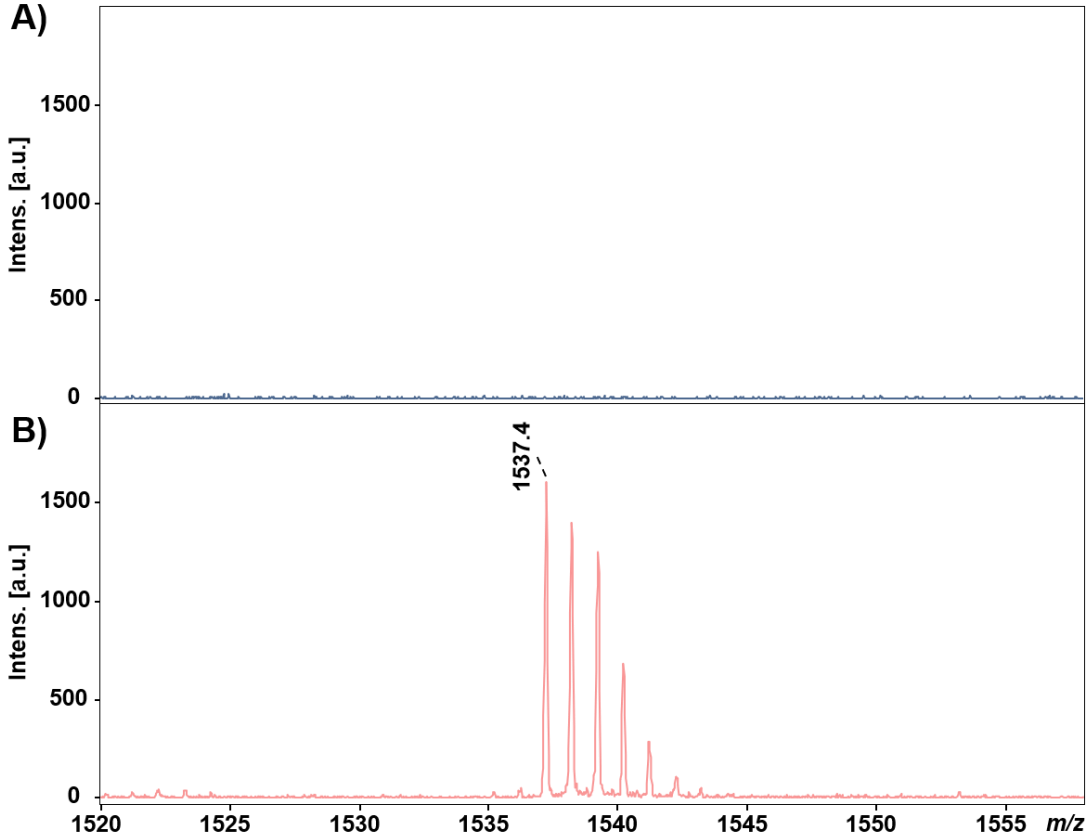

Fig. S1. MALDI Mass spectra of (A) blank DHB and (B) FITC-GC-11 NH_2 (30 μM).

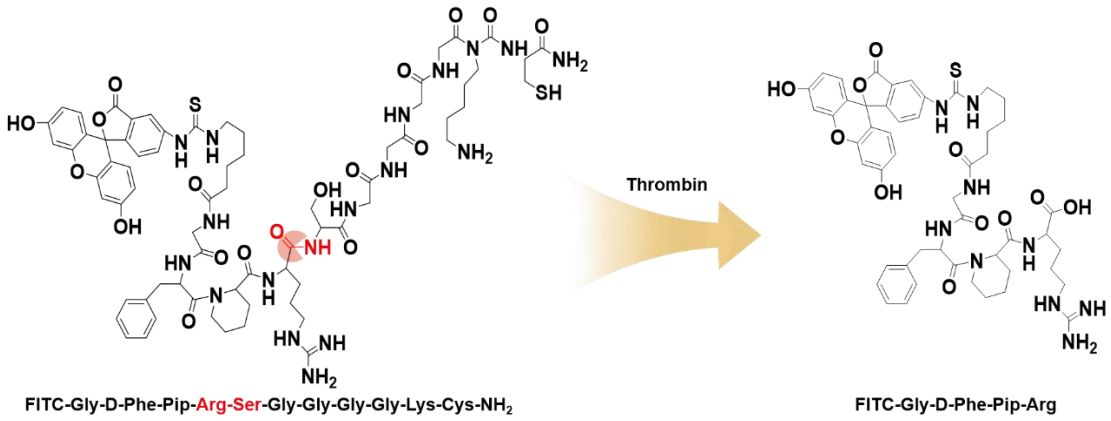

Fig. S2. Schematic illustration of the hydrolysis of FITC-GC-11 NH_2 by thrombin.

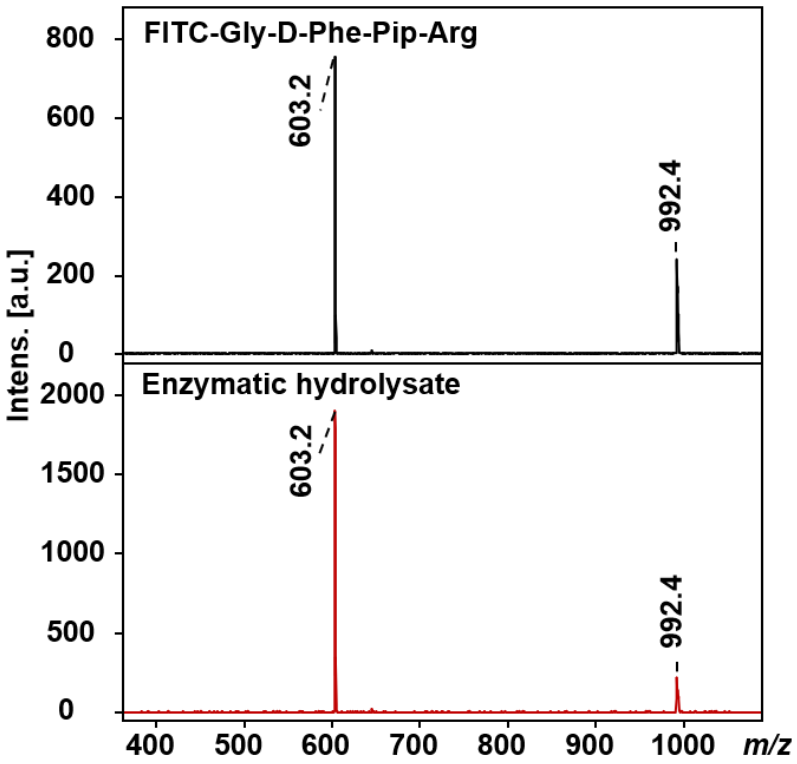

Fig. S3. *In situ* MALDI LIFT-TOF/TOF MS/MS spectra of FITC-Gly-D-Phe-Pip-Arg (1 mM) (upper one) and enzymatic hydrolysate of FITC-GC-11 NH_2 in positive ion mode (lower one).

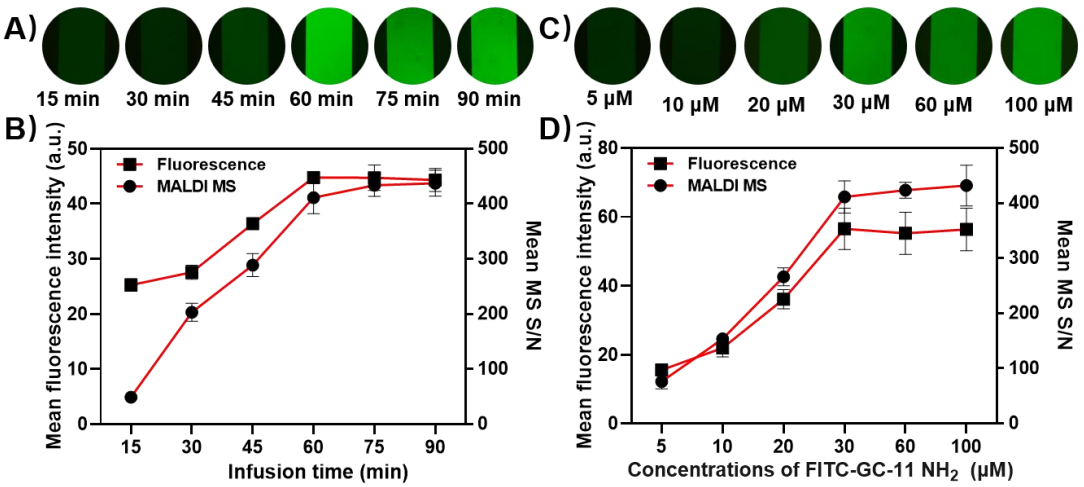

Fig. S4. (A) Fluorescence images of enzymatic hydrolysis after thrombin immobilized by TBA_{15} for different time in microchannels. (B) Fluorescence intensities and MS S/N ratios of FITC-Gly-D-Phe-Pip-Arg obtained after enzymatic hydrolysis of thrombin immobilized by TBA_{15} for different time in microchannels. (C) Fluorescence images obtained after enzymatic hydrolysis with different concentrations of FITC-GC-11 NH_2 in microchannels. (D) Fluorescence intensities and MS S/N ratios of FITC-Gly-D-Phe-Pip-Arg obtained after enzymatic hydrolysis with different concentrations of FITC-GC-11 NH_2 in microchannels. The width of the channels is 1 mm. Error bars correspond to a standard deviation of fluorescence intensities of three fluorescence images obtained from one microchannel and MS S/N ratios of FITC-Gly-D-Phe-Pip-Arg obtained from ten mass spectra in one microchannel, respectively.

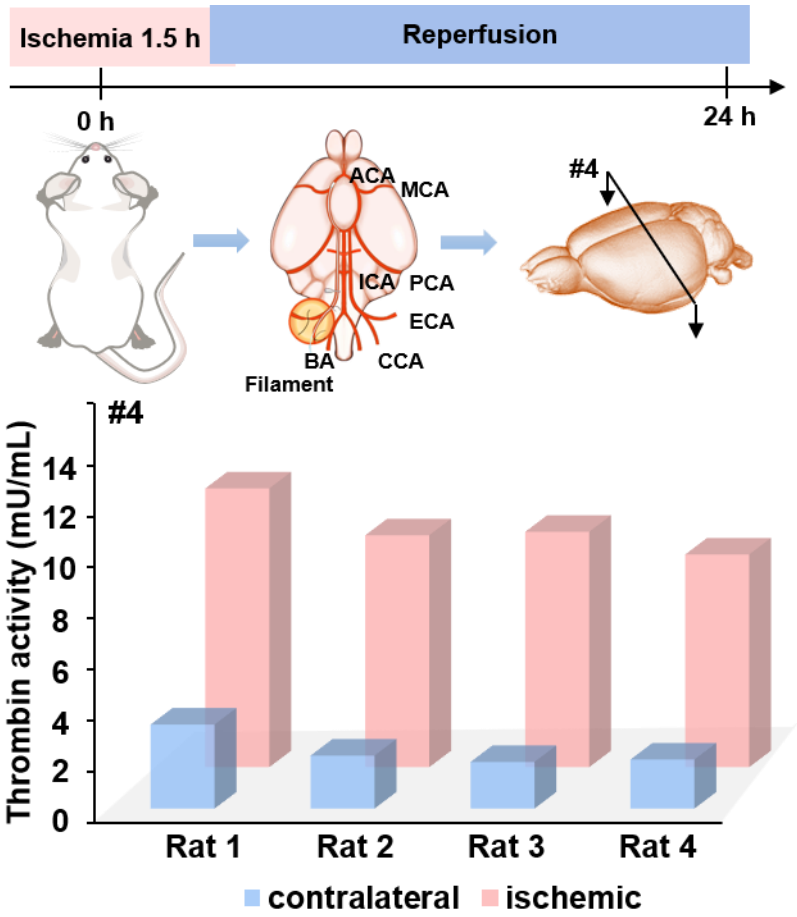

Fig. S5. Measurement of thrombin activity of brain sections of four tMCAO rats.

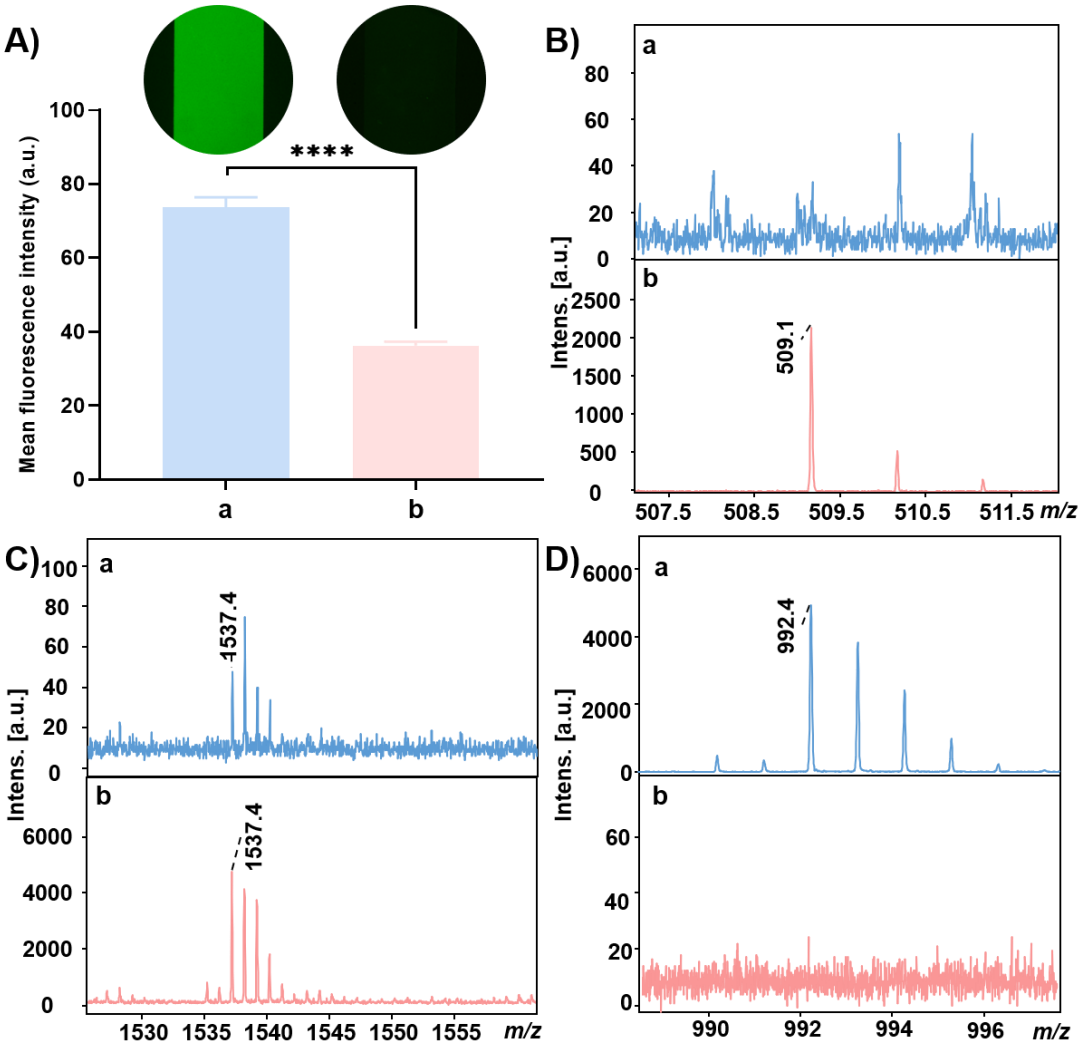

Fig. S6. (A) Fluorescence images and corresponding mean fluorescence intensity, and MALDI mass spectra of (B) argatroban, (C) FITC-GC-11 NH_2, and (D) FITC-Gly-D-Phe-Pip-Arg obtained from different channels infused with FITC-GC-11 NH_2: (a) channel without the infusion of inhibitor, and (b) channel with the infusion of argatroban, respectively. Error bars corresponding to a standard deviation of fluorescence intensity obtained from three positions of one channel. ****, $p<0.0001$.

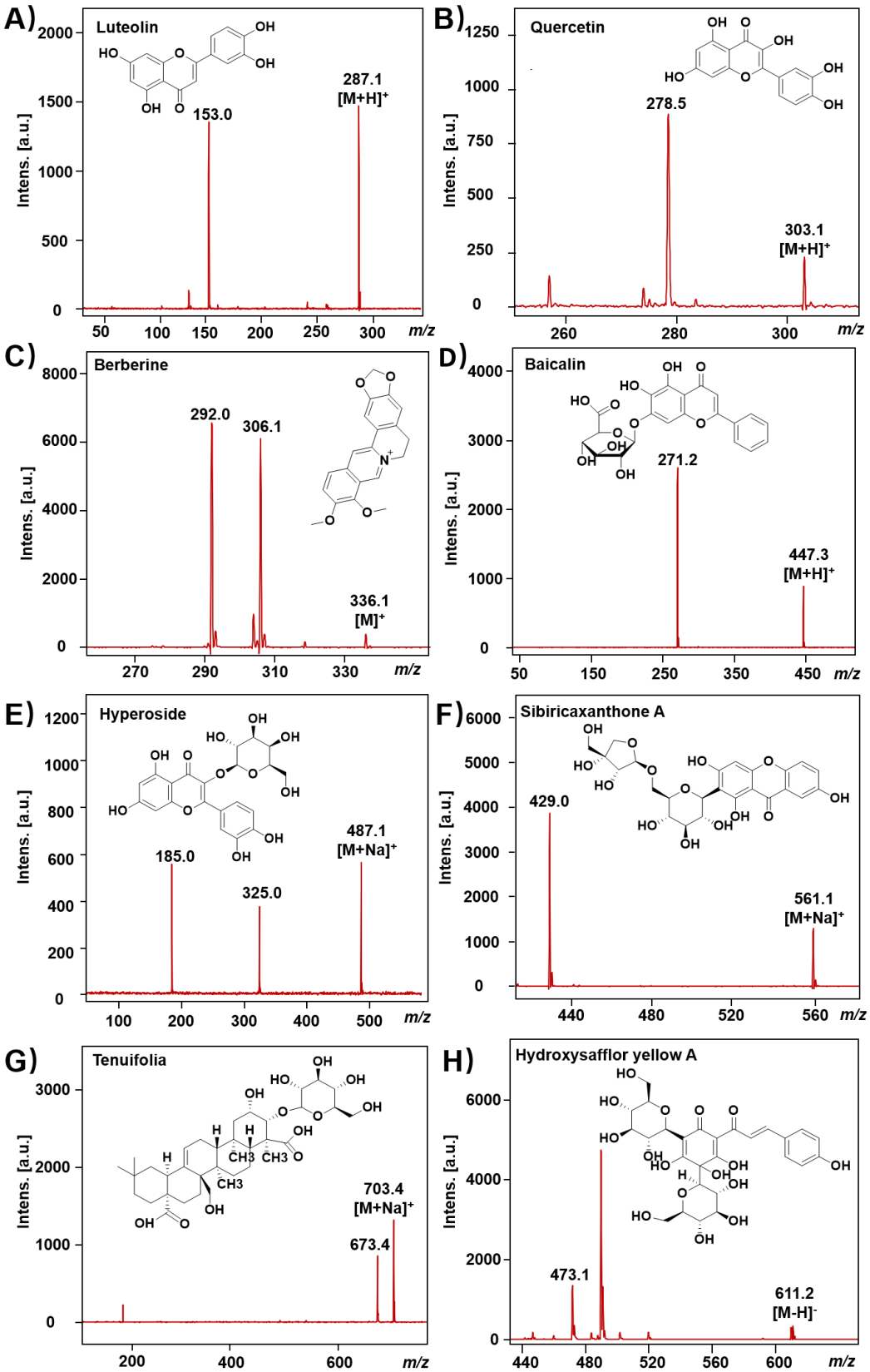

Fig. S7. *In situ* MALDI LIFT-TOF/TOF MS/MS spectra of (A) luteolin, (B) quercetin, (C) berberine, (D) baicalin, (E) hyperoside, (F) sibiricaxanthone A, (G) tenuifolin, and (H) hydroxysafflor yellow A obtained from the channels infused with five herbal extracts.

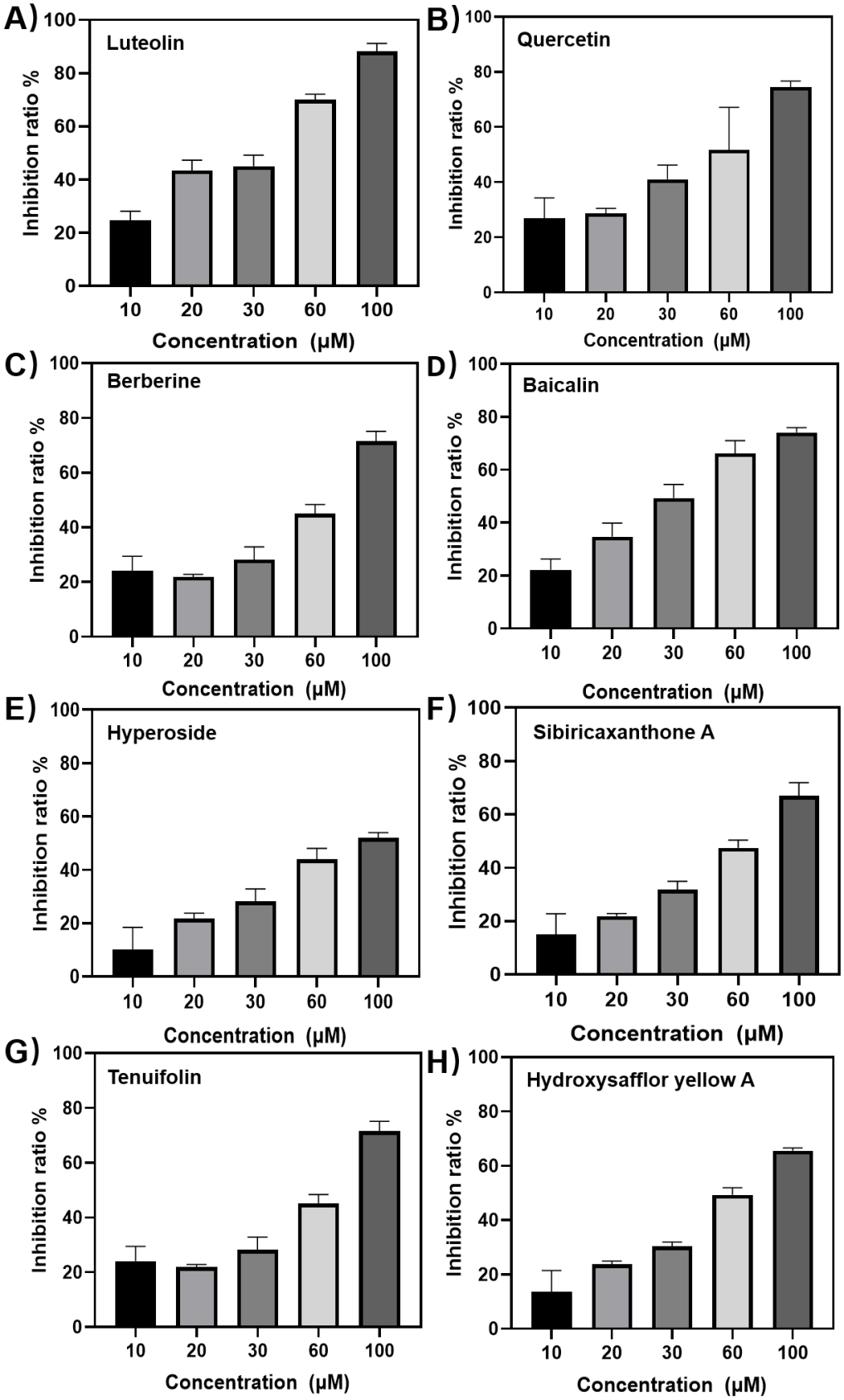

Fig. S8. In-vitro validation of thrombin inhibitors activity of eight active compounds.

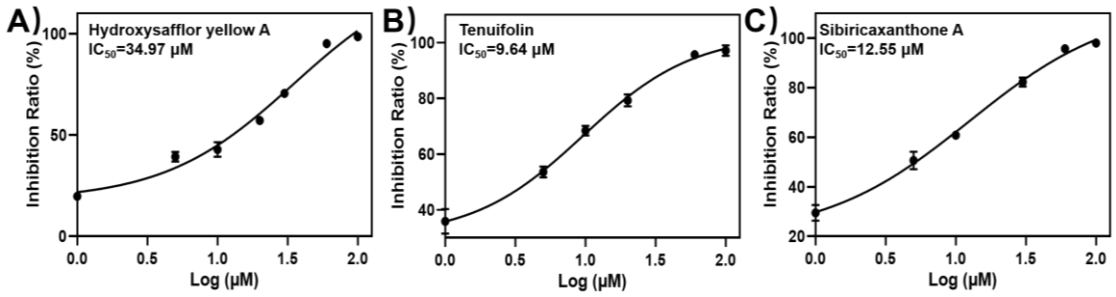

Fig. S9. Determination of IC_{50} of (A) hydroxysafflor yellow A, (B) tenuifolin, and (C) sibiricaxanthone A by MALDI MS. All experiments were done in triplicate. Error bars corresponding to a standard deviation of three individual experiments.

Supporting Tables

Table S1. Evaluation of the channel-to-channel reproducibility.

Channels	Mean Fluorescence Intensity	RSD % (n=3)	MS S/N ratio	RSD % (n=10)
1	52.1	2.6	538.3	8.9
2	54.3	0.8	543.5	6.2
3	55.9	1.0	548.9	8.8

Table S2. Evaluation of the reactor-to-reactor reproducibility.

Reactors	Mean fluorescence intensity	RSD % (n=3)	MS S/N ratio	RSD % (n=10)
1	54.3	0.8	531.3	6.1
2	54.5	1.5	535.8	7.3
3	53.1	0.7	533.8	9.9

Table S3. Measurement of thrombin activities in the ischemic hemisphere (right) and contralateral hemisphere (left) of brain sections of four tMCAO rats.

Rats	Left contralateral hemisphere (mU/mL)	Right ischemic hemisphere(mU/mL)
1	3.8	12.7
2	2.4	10.6
3	2.1	9.6
4	2.2	11.0