SI Appendix for "Bioactive compounds from Huashi Baidu decoction possess both antiviral and anti-inflammatory effects against COVID-19"

Supplement 1: Mouse experiments

Supplement 2: Chemical identification of Huashi Baidu decoction (Q-14)

Supplement 3: *In vivo* identification of Huashi Baidu decoction (Q-14) compounds

Supplement 4: Protein expression and purification

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Supplement 8: PDE4 inhibition assay

Dataset S1: Detailed information on chemical compounds contained in Huashi Baidu decoction (Q-14) using HPLC-Q-TOF-MS/MS system

Dataset S2: The compounds in plasma after Huashi Baidu decoction (Q-14) treatment using HPLC-Q-TOF-MS/MS system

Dataset S3: Inhibition of PDE4 by 30 compounds included in Huashi Baidu decoction (Q-14)

Dataset S4: Crystallography data collection and refinement statistics

Dataset S5: Composition of Huashi Baidu decoction (Q-14)

Dataset S6: Detailed information on the reference standards for chemical identification

Dataset S7: Detailed information on the reference standards for antiviral and anti-inflammatory activities screening

1 **Supplement 1: Mouse experiments**

2 Transgenic mice were generated by microinjection of the mouse *Ace2* promoter driving
3 the human *ACE2* (*hACE2*) coding sequence into the pronuclei of fertilized ova from
4 wild-type mice, and then *hACE2* integration was identified by PCR as previously
5 described(1). Five days post infection, mice were sacrificed, and lung tissues were
6 collected for follow-up viral load determination (by quantitative real-time PCR, RT-
7 qPCR), cytokine assays, and pathological examination

8 **1) RT-qPCR**

9 Total RNA was isolated and reverse transcribed using an RNeasy Kit (Qiagen) and
10 QuantiTect Reverse Transcription Kit (Qiagen). We adopted the following qRT-PCR
11 protocol: 50°C, 2 min; 95°C, 2 min; 95°C, 15 s; and 60°C, 30 s (40 cycles); and melt
12 curve stage: 95°C, 15 s; 60°C, 1 min; and 95°C, 45 s. The SARS-CoV-2 primers 5'-
13 TCGTTTCGGAAGAGACAGGT-3' (forward) and 5'-GCGCAGTAAGGATGGCTAGT-3'
14 (reverse) were used.

15 **2) Chemokine and cytokine detection**

16 Chemokines and cytokines were measured using a LEGENDplex Mouse Th Cytokine
17 Panel (13-plex) array. Briefly, 25 μL of lung tissue homogenate was incubated with
18 microspheres. After the removal of unbound detection antibodies, Biotinylated Anti-
19 Mouse Antibodies were added. The final detection complex was formed with the
20 addition of streptavidin-phycoerythrin (SA-PE) conjugate. The fluorescence intensity of
21 PE on each microsphere was analyzed for quantitative analysis of soluble protein. Data
22 were analyzed using the LEGENDplexTM Data Analysis Software.

23 **Supplement 2: Chemical identification of Huashi Baidu decoction (Q-14)**

24 **1) Solution preparation**

25 Reference standards were diluted with methanol (MeOH) to obtain a series of control
26 solutions (1 mg/mL). To prepare Q-14 solution, 100 mg Q-14 was precisely weighed
27 and extracted with 5 mL 50% MeOH (*v/v*) under ultra-sonication (40 kHz) for 30 min,
28 followed by centrifugation at 8000 rpm for 5 min. After extraction, the filtrate was

1 collected through a 0.22-μm filter, and 1.0 μL was injected for ultra-high performance
2 liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-
3 Q-TOF-MS/MS) for analysis.

4 **2) HPLC-Q-TOF-MS/MS conditions**

5 Analysis was conducted on an ACQUITY UPLC-I-Class interfaced with a Synapt-XS
6 Q-TOF-MS (Waters, MA, USA) with an electrospray ionization source (ESI). An
7 ACQUITY UPLC HSS T3 column (2.1×100 mm, 1.8 μm) was used to perform
8 chromatographic separation with a flow rate of 0.5 mL/min at 40°C. A linear gradient
9 program with a mobile phage system including solvent A (deionized water with 0.1%
10 formic acid, *v/v*) and solvent B (acetonitrile with 0.1% formic acid, *v/v*) is described in
11 detail: 0-0.2 min, 5% B; 0.2-2.0 min, 5.0-8.0% B; 2.0-9.0 min, 8.0-13.0% B; 9.0-16.0
12 min, 13.0-20.0% B; 16.0-16.5 min, 20.0-21.0% B; 16.5-22.0 min, 21.0-30.0% B; 22.0-
13 23.0 min, 30-35% B; 23.0-28.0 min, 35-42% B; 28.0-33.0 min, 42.0-60.0% B; 33.0-
14 35.0 min, 60.0-98.0% B; 35.0-38.0 min, 98% B; 38.0-38.5 min, 98.0-5.0% B; and 38.5-
15 41.0 min, 5% B.

16 The instrumental settings of the Q-TOF-MS/MS are described as follows: capillary
17 voltage, 2.5 kV(-)/0.5 kV(+); cone voltage, 40 V; source temperature, 100°C;
18 desolvation temperature, 450°C. The solvents were removed by evaporation under
19 nitrogen with a flow rate of 900 L/h. Samples were analyzed in both positive and
20 negative ionization modes with scanning mass-to-charge (*m/z)* ranging from 50 to
21 1500 Da. Argon was used as the collision gas; the low energy was 6 eV; the high
22 energy was 50-70 eV. Leucine enkephalin was used as the reference lock mass. Mass
23 chromatograms and mass spectra were acquired using MassLynx version 4.2 (Waters,
24 MA, USA).

25 **Supplement 3: Compounds of Huashi Baidu decoction (Q-14) *in vivo***
26 **identification**

27 **1) Animal**

28 Specific-pathogen-free (SPF) male SD rats (n=8, 6- to 8-weeks-old, 250±20 g in weight)

were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Production license no.: SCCK 2021-0006, Beijing, China; n=4 per group, including a normal control group and normal control+Q-14 treatment group). Their diet was prohibited for 12 h before the experiment but water was provided *ad libitum*. In the Q-14 treatment group, the dosage selection for Q-14 was 6.165 g/kg, nearly equivalent to five times the daily dosage of COVID-19 patients in clinics. The normal control group received an equal volume of saline.

2) Collection of blood samples

Blood samples (~0.25 mL) were collected from the retinal venous plexus into heparinized 1.5-mL EDTA-K2 anticoagulant tubes before administration and 0, 0.5, 1, 2, 4, 6, 8, 12, 14, 16, 18, 24, 36, and 48 h after administration of Q-14. The plasma samples were obtained after centrifuging at 12000 rpm for 15 min and stored at -20°C prior to analysis. All samples were mixed using the AUC pooling method, and then the proteins were precipitated with 0.1% formic acid-MeOH (*v/v*). After vortexing, samples were centrifuged at 12000 rpm for 10 min at 4°C. The extract was blow-dried under nitrogen and redissolved with 100 μL 50% MeOH (*v/v*) under ultra-sonication (40 kHz) for 30 min, followed by centrifugation at 12000 rpm for 10 min. Samples (1.0 μL) were injected into the UPLC-Q-TOF-MS/MS system for analysis.

3) HPLC-Q-TOF-MS/MS conditions

The HPLC-Q-TOF-MS/MS settings were as above.

Supplement 4: Protein expression and purification

1) Protein expression and purification of SARS-CoV-2 M^{pro}

As in our previous study(2), a cDNA sequence encoding residues 3264-3569 of ORF1ab (GenBank: MN908947.3) was cloned into the *Nco*I and *Not*I sites of the pET-28b vector (Genscript) in-frame with an N-terminal His and SUMO tag. The vector was transformed into *E. coli* strain BL21 (DE3). Isopropyl-β-d-thiogalactopyranoside (IPTG; 0.2 mM) was added to the LB medium at an OD_{600} of 0.6-0.8. The cells were cultured at 16°C for 18 h and subsequently lysed by sonication and centrifuged for 30 min. Then,

the supernatants containing soluble protein were purified using 5-mL HisTrap HP columns (GE Healthcare, IL, USA). The purified proteins were incubated with 2 μL/mg SUMO protease (Beyotime, P2312M) at 30°C for 2 h after centrifugation and then purified by HisTrap HP chromatography (GE Healthcare, IL, USA) again. The native SARS-CoV-2 M^{pro} protein was obtained using a Hiload 16/600 Superdex 75 PG column (GE Healthcare, IL, USA) with a buffer of 10 mM Tris-HCl, 1 mM DTT, and 1 mM EDTA, pH 7.5.

2) PDE4D expression and purification

Briefly, the cDNA encoding the catalytic domain of human PDE4D (GenBank: NM_001197221.1; coding region: T86-S413) with a His-tag was cloned into the pET15b vector. The plasmid was then transformed into *E.coli* BL21(DE3) cells for protein expression. The expressed protein was purified by Ni NTA (GE Healthcare, Uppsala, Sweden), Q-Sepharose, and superdex200 (GE Healthcare, Uppsala, Sweden) chromatography until the purity was > 95%. The eluted protein samples were stored in a solution of 20 mM HEPES, pH 7.0, and 50 mM NaCl for the enzymatic inhibition assays and protein crystallization.

Supplement 5: Cell viability assay

First, Caco-2-N cells were seeded in 96-well plates at a density of $1×10^4$ cells/well (in DEME + 10% FBS) and incubated under 5% CO_2 at 37°C for 12 h. Then, cells were exposed to different dosages of drugs diluted in a DMEM culture medium for 48 h. After that, the contents of the wells were substituted with fresh medium containing 10% Cell Counting Kit-8 (CCK-8) solution and incubated at 37°C for 2 h. The final optical density at OD_{450} was determined using a microplate reader (Bio-Rad, CA, USA).

Supplement 6: *In vitro* polymerase activity assay

To perform the primer extension assay, template RNA and primer RNA were preheated at 65°C for 5 min to form annealed complementary RNA strands. Then, the mixture consisting of buffer, protein sample, annealed RNA, and drugs at different dosages was added to the reaction system and incubated for 15 min at room temperature, and

0.5 mM NTP in a reaction buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM beta-mercaptoethanol, and 2 mM $MgCl_2$ (freshly added prior usage) was added. The condition of the extension reaction were 30°C for 30 min. After the reaction, RNA loading buffer was added and boiled for 10 min. The samples were separated using 20% PAGE gels. Images were taken using a Vilber Fusion system and quantified with ImageJ software.

Supplement 7: High-throughput activity assay

To explore the antiviral therapy targets, a fluorescence resonance energy transfer (FRET) assay was adopted. An LC00-custom Compound Library contained Q-14 ingredients was purchased from Shanghai TOPSCIENCE (Shanghai, China). The initial specification was all 10 mM (dissolved in DMSO solution), taking 2 μL of 10 mM solution into 98 μL buffer (50 mM Tris-HCl, pH 7.3, and 1 mM EDTA) to prepare compound working solutions. SARS-CoV-2 M^{pro} (final concentration of 150 nM) and 40 μL diluted compound working solution were added to black 96-well plates, then incubated at room temperature for 10 min. Fluorescence values were determined using the CLARIOstar Plus multifunctional microplate detection system (BMG Labtech, Offenburg, Germany) after adding 100 μM MCA-AVLQSGFR-Lys(Dnp)-Lys-NH2 substrate (GenScript, Nanjing) with a volume of 20 μL. All excitation wavelengths were set at 320 nm, the emission wavelengths were 405 nm, and the fluorescence values were measured every 90 s for a total of 20 cycles. The fluorescence values in the first six cycles were collected and used to calculate the initial reaction rate $v0$ values, using the following formula to calculate the inhibition rate:

$$Inhibition\ \% = \left[1 - \frac{v0\ (compound - Mpro - substrate)}{v0\ (Mpro - substrate)}\right] * 100\%$$

To determine the concentration scope of the inhibitory effects, multiple compound concentrations, almost ranging from mM to nM, were prepared, measured as described previously in triplicate, and the IC_{50} values were calculated as the mean via nonlinear regression analysis on GraphPad Prism software 8.0.1, plotting the inhibition

1 rate curves as well.

2 **Supplement 8: PDE4 inhibition assay**

3 A protein solution with a concentration of 0.2-0.4 nM was prepared using assay buffer
4 (50 mM Tris, pH 7.5, 8.3 mM $MgCl_2$, and 1.7 mM EGTA). Assays were performed in
5 96-well plates with a total volume of 125 μL/well. Compounds were dissolved in DMSO
6 [1% (*v/v*) in the final concentration] and added to plates in a volume of 10 μL, followed
7 by the addition of 80 μL of protein solution and 10 μL of [^{3}H]-cAMP (0.5μCi/mL). The
8 reaction was stopped by the addition of 25 μL of phosphodiesterase SPA beads
9 (RPNQ0150, PerkinElmer Inc., MA, USA) after incubation at 30°C for 10 min. Twenty
10 minutes later, luminescence was measured with a MicroBeta2 (PerkinElmer Inc., MA,
11 USA). Apremilast (Shanghai yuanye Bio-Technology Co., Ltd, Shanghai, China,
12 purity≥99%) was used as a positive control. Three independent experiments were
13 conducted to determine the 50% inhibitory concentration (IC_{50}) values of each
14 compound. All experimental data were analyzed using GraphPad Prism, version 8.0
15 (GraphPad Inc., San Diego, CA, USA).

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1 **Supplemental Figures**

2

3 **Fig. S1 Therapeutic effects of Huashi Baidu decoction (Q-14) based on SARS-**

4 **CoV-2-infected *hACE2*-transgenic mice.** (**A**) Body weight changes. (**B**) Viral load of

5 lung tissues. (**C**) The expression of cytokines in lung tissues. (**D**) Histopathological

6 changes of the lung (scale bar represents 100 μm). n=6 per group. Data are expressed

7 as the mean±SEM.

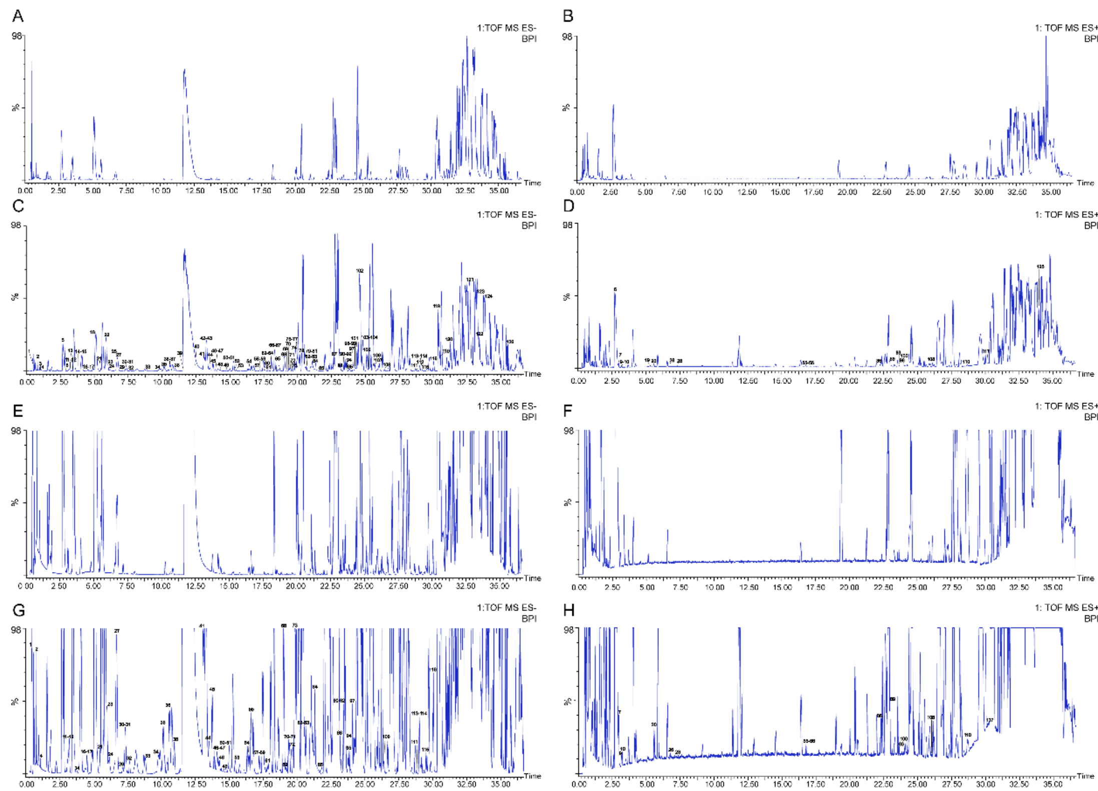
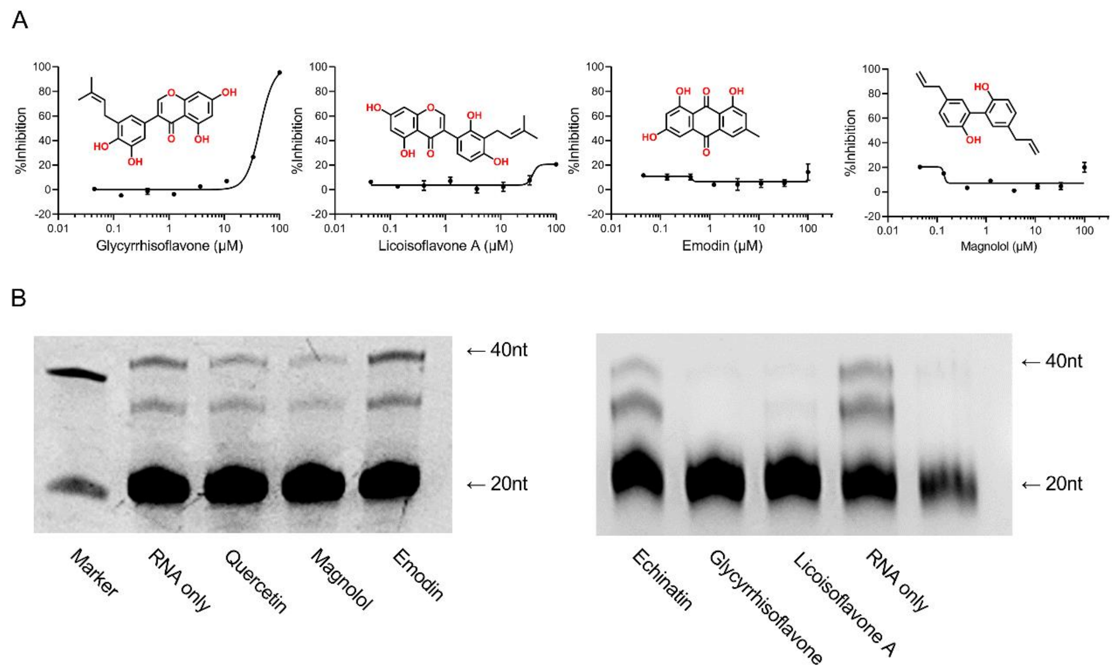


Fig. S2 The compounds in plasma after Huashi Baidu decoction (Q-14) treatment using the ultra-high performance liquid chromatography with a quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) system. (**A-B**, **E-F**) The chemical base peak ion (BPI) chromatogram in plasma after the treatment of Q-14 in the negative and positive ion modes (signals were amplified by 1 or 10, respectively). (**C-D**, **G-H**) The BPI chromatogram of blank plasma in the negative and positive ion modes (signals were amplified by 1 or 10, respectively).



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2 **Fig. S3 Screening for anti-SARS-CoV-2 bioactive compounds with prototype**
3 **structures from Q-14.** (**A**) The IC_{50} values of glycyrrhisoflavone, licoisoflavone A,
4 emodin, and magnolol on SARS-CoV-2 M^{pro}. (**B**) The inhibitory activities of quercetin,
5 magnolol, emodin, echinatin, glycyrrhisoflavone, and licoisoflavone A on RdRp. Data
6 are expressed as the mean ± SEM. Experiments were repeated in triplicate,
7 independently.

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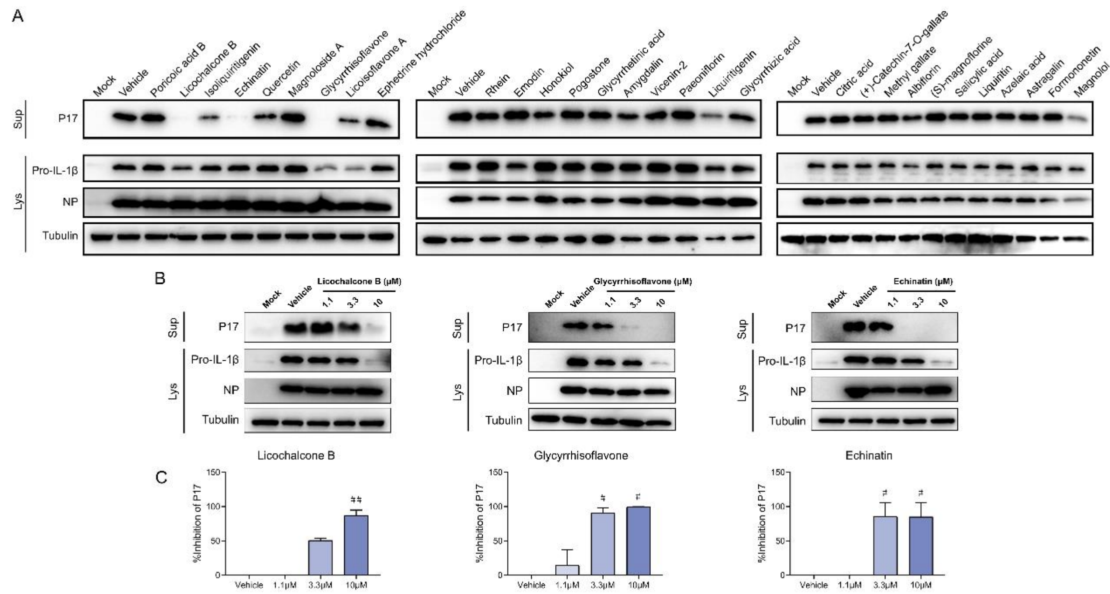

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3 **Fig. S4 Screening of anti-inflammatory compounds from Q-14. (A)** Preliminary screening of the anti-inflammatory activity of 30 compounds from Q-14. THP-1 macrophages were incubated with SFTSV (MOI = 5) for 1 h and treated with the 30 compounds in Q-14 at a concentration of 10 μM. Cells and supernatants were harvested at 48 h post infection. P17 levels in supernatants and the expression levels of Pro-IL-1β or NP in cell lysates were determined by western blotting. (**B-C**) The dose-dependent effects of licochalcone B, glycyrrhisoflavone, and echinatin on the secretion of P17 induced by SFTSV infection. THP-1 macrophages were incubated with SFTSV (MOI = 5) for 1 h and treated with licochalcone B, glycyrrhisoflavone, and echinatinat concentrations of 1.1, 3.3, or 10 μM. Cells and supernatants were collected at 48 h post infection. P17 levels in supernatants and expression levels of Pro-IL-1β or NP in cell lysates were determined by western blotting (**B**). The inhibition rates were evaluated by analysis of gray values of the P17 bands (**C**). Data are representative of two independent experiments. Error bars represent mean ± SEM. Statistical significance was analyzed by one-way ANOVA. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

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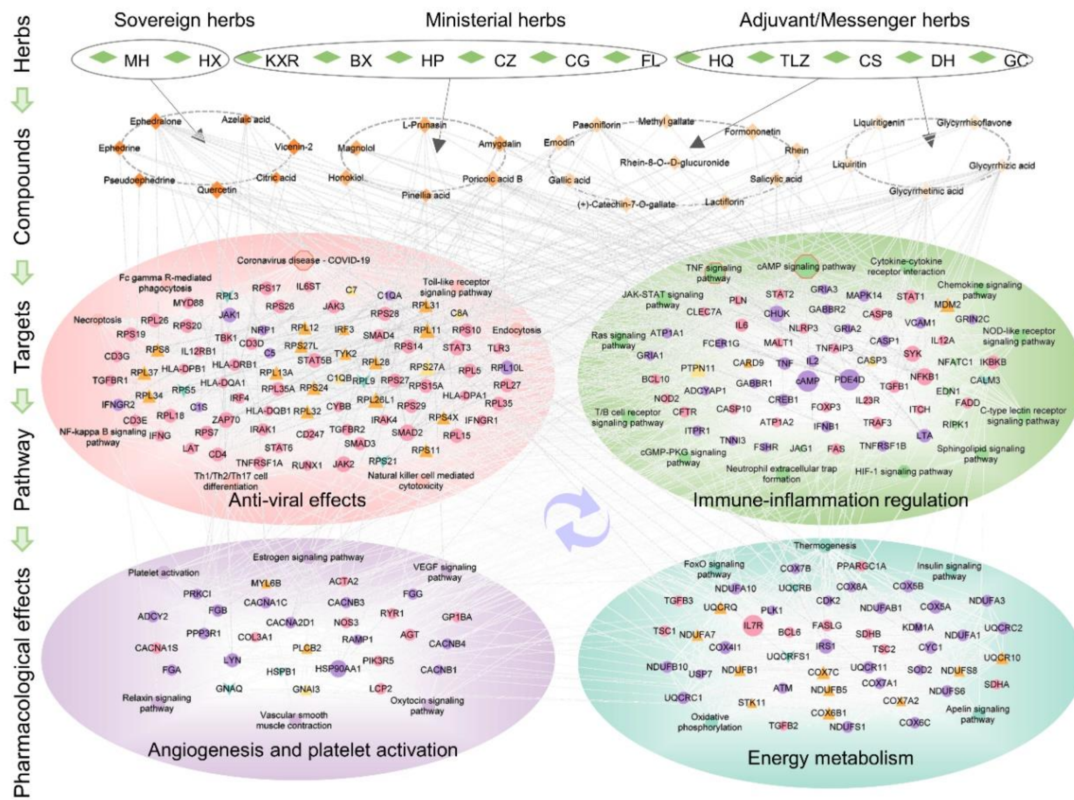

Fig. S5 Multidimensional biomolecular interaction network among "disease-clinical symptom-herbal formula" of Huashi Baidu decoction (Q-14). The green rhombuses refer to fourteen Chinese herbs. The orange rhombuses refer to compounds in plasma contained in Q-14 (the colors from dark to light are sovereign, ministerial, adjuvant, and messenger herbs, respectively). The yellow and orange triangles refer to up-regulated differentially expressed proteins and genes, respectively, while the dark and light green inverted triangles represent down-regulated differentially expressed proteins and genes, respectively. The dark pink nodes represent clinical symptom-related genes. The purple nodes represent putative targets of compounds in plasma contained in Q-14. The pink module indicates that Q-14 may exert antiviral effects; the green module indicates that Q-14 may regulate the balance of the "immune-inflammation" system; the purple module indicates that Q-14 may regulate angiogenesis and platelet activation; and the bluish-green module indicates that Q-14 may regulate energy metabolism.

1 **References:**

2 1. Yang XH, *et al.* (2007) Mice transgenic for human angiotensin-converting
3 enzyme 2 provide a model for SARS coronavirus infection. *Comparative*
4 *medicine* 57(5):450-459.
5 2. Bai Y, *et al.* (2021) Structural basis for the inhibition of the SARS-CoV-2 main
6 protease by the anti-HCV drug narlaprevir. *Signal transduction and targeted*
7 *therapy* 6(1):51.

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