

Potential Anti-Proliferative, Anti-Inflammatory and Anti-Viral Components from *Sinopodophyllum Hexandrum* Explored Using Bio-Affinity Ultrafiltration with Multiple Drug Targets

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Research

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Abstract

Background: *Sinopodophyllum hexandrum* (*S. hexandrum*) is a typical Chinese herbal medicine with numerous components and remarkable pharmacological activities. However, the specific phytochemicals responsible for its anti-proliferative, anti-inflammatory and anti-viral effects remain unexplored.

Methods: The integrated analytical strategy combining bio-affinity ultrafiltration with multiple drug targets was developed to rapidly screen and identify bioactive ligands from *S. hexandrum*. The *in vitro* anti-proliferative and COX-2 inhibitory assays of bioactive ligands screened were further verified by sulforhodamine B (SRB) cell proliferation and cytotoxicity detection and COX-2 inhibitor screening kits, respectively. Molecular docking analysis was also implemented by the AutoDockTools 1.5.6 software.

Results: 10, 7, 9 and 9 phytochemicals were screened out and identified as the potential Topo I, Topo II, COX-2 and ACE2 ligands, respectively. Hereinto, podophyllotoxin and quercetin with higher EF values displayed strong inhibitory effects on A549 and HT-29 cells comparable with etoposide and 5-FU. Furthermore, compared with indomethacin at 0.73 ± 0.07 mM, podophyllotoxin and kaempferol with higher EF values exerted stronger inhibitory effects with IC_{50} values at 0.36 ± 0.02 mM and 10.49 ± 0.61 mM, respectively. Additionally, the optimal binding sites and mode of action between bioactive ligands and multiple drug targets were determined by molecular docking. Wherein, isorhamnetin showed a stronger affinity to ACE2 with the binding energy of -5.72 kcal/mol and the IC_{50} value at 63.95 mM, lower than MLN-4760 (-4.27 kcal/mol and 738.62 mM).

Conclusions: The integrative strategy combining multiple drug targets and bio-affinity ultrafiltration LC-MS in the present study showed very promising potential for the quick screening and identifying bioactive ligands in *S. hexandrum* for Topo I, Topo II, COX-2 and ACE2, and some bioactive compounds screened out from this work were verified with other *in vitro* assays, and even better than those positive drugs of interest. Based on these findings, we then first constructed an interacting network among multi-components and multi-targets. In this way, we showcased a quick and reliable experimental strategy for uncovering the underlying mechanism of the empirical traditional applications of *S. hexandrum* which could also provide valuable information for better understanding the therapeutic targets and therapeutic ligands of other herbal medicines.

Background

Sinopodophyllum hexandrum (Royle) Ying (*S. hexandrum*), belonging to *Sinopodophyllum* genus, Berberidaceae Family, is a rare perennial herbaceous medicinal herb with extensive applications as a traditional Chinese medicine. It is mainly distributed in Yunnan, Tibet, Sichuan, Hubei, Shaanxi, Qinghai, Gansu, Ningxia and other provinces of China [1]. *S. hexandrum* embodies abundant pharmacological effects such as anti-proliferative, anti-viral, anti-inflammatory, anti-bacterial, insecticide and cytotoxic activities [2]. In recent decades, the main researches on *S. hexandrum* have been chiefly focused on the establishment of chromatographic methods to quantitatively analyze and measure the content of podophyllotoxin, the chemical compositions and pharmacological effects, and how to prompt the sustainable development and utilization of this endangered plant [3–5]. So far, numerous chemical components have been isolated and

identified from *S. hexandrum*, namely lignans, flavonoids, saponins, polysaccharides and tannins, among which lignans are primarily composed of podophyllotoxin [3, 6]. However, owing to its complicated and diversified chemical components, the specific bioactive phytochemicals of *S. hexandrum* responsible for its anti-tumor, anti-inflammatory and anti-viral effects in the empirical applications, their potential target-ligand interactions, and their possible mechanism of action remain unexplored at present. Therefore, an integrative strategy to rapidly screen and identify latent bioactive ligands in *S. hexandrum* against specific drug targets is in great need, which can be very helpful to further decipher the material basis for their efficacy in the development and utilization of this medicinal plant.

The bioactive components in traditional Chinese medicine are considered to be the material basis for the prevention and treatment of diseases. Compared with modern drugs, traditional Chinese medicine is characterized to take its pharmacological effects through the interactions among multiple components and multiple targets [7]. For multi-causal diseases regulated by complicated pharmacological networks, single-target remedies rapidly develop resistance, bring about poor clinical therapeutic effects, and eventually lead to treatment failure on the basis of the strategy of “one disease-one target-one therapeutic drug” [8]. In response to the limitations of single-target drugs, the development of multi-target natural agents, especially with synergistic effects, will provide greater benefits in enhancing efficacy and lowering drug resistance [9]. Additionally, nearly half of small molecule drugs were developed as enzyme inhibitors according to current statistics. These small molecule drugs exerted their pharmacological effects by inhibiting the biological activities of key enzymes or other significant biological macromolecules in a certain process of biochemical reactions in the body [10]. Consequently, the affinity activity between target biological macromolecules and potential small-molecule ligands has become one of the dominating decisive factors in new drug development [11]. Taking the above research ideas into account, four well-known drug targets closely correlated to the empirical applications of *S. hexandrum* as a traditional Chinese medicine, including DNA topoisomerase I (Topo I), DNA topoisomerase II (Topo II), cyclooxygenase-2 (COX-2) and angiotensin-converting enzyme II (ACE2), were chosen to actively explore the correlation between potential bioactive constituents screened from *S. hexandrum*, and its multiple pharmacological effects of anti-proliferative, anti-inflammatory and anti-viral activities. DNA topoisomerase (Topo) is a type of ribozyme that can control and change the topoisomeric state of DNA. Topo is divided into Topo I and Topo II in accordance with the instantaneous break of single-strand and double-strand during DNA allosteric action [12]. Moreover, Topo, a momentous anti-tumor molecular target, exhibits high-level expression in tumor cells, and numerous links to the action of anticancer drugs, such as DNA replication, DNA damage and repair, gene recombination and transcription, and cell mitosis and differentiation [13, 14]. As regards COX-2, it has been considered as a vital target for new anti-inflammatory and auxiliary anti-cancer remedies. Selective COX-2 inhibitors were characterized by retaining all the benefits of classic non-steroidal anti-inflammatory agents, involving plenty of pathophysiological processes such as inflammation, cancer, and neurodegenerative diseases [15, 16]. As a homolog to the carboxypeptidase ACE, ACE2 can act as a basic functional receptor for the SARS-CoV-2, regulate the renin-angiotensin system (RAS) as a puissant negative regulator, and participate in the absorption of amino acids by the kidneys and intestines [17–19].

In vitro screening test is commonly applied to screen enzyme inhibitors from medicinal plants, and its orthodox procedures comprise biometric-guided separation, purification and structural identification of

purified compounds followed by the activity test of individual compounds. However, the whole process of operation is quite laborious, cost-prohibitive and time-consuming, and cannot truly reflect the interactions between the receptor and the natural conformation of active ingredients [20, 21]. In order to overcome these limitations, a potent and efficient screening strategy based on bio-affinity ultrafiltration combined with liquid chromatography mass spectrometry technology (AUF-LC/MS) has been actively developed for rapidly screening and identification of potential bioactive ligands from complex systems such as botanical extracts [11, 22]. AUF-LC/MS is a membrane separation technology similar to the dialysis method combining ultrafiltration device with mass spectrometry. This approach is suitable for high-throughput screening and rapid identification of potentially active small molecules from complicated matrixes such as natural product extracts [23, 24]. During the AUF-LC/MS procedures, potential small-molecule ligands in a mixture selectively bind to macromolecule target enzymes based on the principle of affinity, the unbound ligands are eluted from the enzyme-ligand complexes, and then the LC-MS/MS is applied to detect and identify the retained ligands from the denatured receptor [23, 25]. To the best of our knowledge, the AUF-LC/MS strategy with four drug targets (Topo I, Topo II, COX-2 and ACE2) was first introduced and developed in the present study for the quick screening and characterization of their respective ligands from *S. hexandrum* in an effort to explore the specific bioactive components responsible for anti-proliferative, anti-inflammatory and anti-viral activities in the traditional applications. In this way, we could further construct a network based on interactions among multi-components in *S. hexandrum* and multi-targets by evidence-based experimental studies, and a quick and reliable experimental strategy should also be very helpful to uncover the underlying mechanism of the empirical traditional applications of *S. hexandrum*. More strikingly, this could be a showcased strategy to offer direct experimental evidences on the application of traditional herbal medicine, and facilitate to provide valuable information for better understanding the main therapeutic targets and therapeutic roles of other herbal medicines.

Methods

Reagents and apparatus

DNA topoisomerase I, topoisomerase II, cyclooxygenase-2 and angiotensin-converting enzyme II were obtained from New England Biolabs (Ipswich, MA, USA), Sigma-Aldrich (St Louis, MO, USA) and Novoprotein (Shanghai, China), respectively. The standards of rutin, quercetin, isorhamnetin, kaempferol, kaempferol 3-*O*-glucoside, quercetin 3-*O*-glucoside were provided by Shanghai Tauto Biotech (Shanghai, China). 5-Fluorouracil, indomethacin, podophyllotoxin and etoposide were bought from Shanghai Aladdin Bio-Chem Technology (Shanghai, China) and Shanghai Yuanye Biotechnology (Shanghai, China). Ultra-pure water was provided by EPED Co., Ltd. (Nanjing, China). Chromatographically pure reagents for HPLC and HPLC-ESI/MS (acetonitrile, methanol, formic acid) were offered by TEDIA Company Inc. (Fairfield, OH, USA). Sulforhodamine B cell proliferation and cytotoxicity detection kits were purchased from Shanghai Beyotime Biotechnology (Shanghai, China). COX-2 (human) inhibitor screening assay kits were obtained from Shanghai Beyotime Biotechnology (Shanghai, China). The 30 kDa (YM-30) ultrafiltration membranes were obtained from Millipore (Bedford, MA, USA). The optical density values were measured on a Tecan Infinite M200 PRO multi-functional microplate reader (Männedorf, Switzerland). The HPLC-UV/ESI-MS/MS analysis

was implemented by a Thermo Access 600 HPLC system connected with a TSQ Quantum Access MAX mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Analytical HPLC was performed applying an Agilent 1220 liquid chromatography with a Waters Symmetry RP-C18 column (250 mm × 4.6 mm, 5 mm).

Plant material

The roots of *S. hexandrum* were purchased from Panzihua (Sichuan, China), and kindly authenticated by Prof. Guan wan Hu, a professional plant taxonomist from the Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture (Wuhan Botanical Garden), Chinese Academy of Sciences.

Preparation of samples

The air-dried root powders (200 g) were smashed, soaked in 90% ethanol overnight, and ultrasonically extracted 3 times (30 minutes for each time) at room temperature. Then, the total filtrates were collected and concentrated with a reduced pressure evaporator, and finally to obtain the crude extracts of *S. hexandrum*.

Affinity ultrafiltration with Topo I, Topo II, COX-2 and ACE2

The extracts obtained above were screened by affinity ultrafiltration method with four drug targets selected (Topo I, Top II, COX-2 and ACE2) according to our previous study with slight modifications [26]. Briefly, the principle of affinity ultrafiltration method primarily involves three steps, including incubation, interception and release. Firstly, 100 µL tested sample solution (8 mg/mL) was mixed with 10 µL Topo I (5 U), Topo II (2 U), COX-2 (4 U) and ACE2 (0.5 µg) in 2.0 mL EP tubes, and incubated at 37 °C for 40 minutes in the dark. Meanwhile, the incubation procedures of inactivated enzyme solution (obtained by boiling water for 10 minutes) were consistent with the activated enzyme solution. Afterwards, the incubated solutions were turned over to the ultrafiltration tubes with 30 kDa ultrafiltration membranes, followed by centrifugation at 10,000 rpm for 10 minutes at 25°C. The unbound components were eluted with 200 mL phosphate buffer saline (PBS, pH 7.04) or tri(hydroxymethyl) aminomethane hydrochloride (Tris-HCl, pH 7.80) 3 to 4 times by centrifugation at 10,000 rpm for 10 minutes at 25°C. Critically, 200 mL 90% MeOH-H₂O (v/v) was added and incubated at room temperature for 10 minutes, and the mixed filtrates were centrifuged 3 to 4 times at 10,000 rpm for another 10 minutes at 25°C to release the potential bioactive components from the enzyme-ligand complexes. Eventually, those released filtrates were lyophilized and reconstituted with 50 µL MeOH, and later HPLC-UV/ESI-MS/MS technology was applied to analyze these samples.

HPLC-UV/ESI-MS/MS analysis

The HPLC-UV/ESI-MS/MS analysis was executed using a Thermo Access 600 HPLC system connected with a TSQ Quantum Access MAX mass spectrometer. A Waters Symmetry RP-C18 column with a guard column at 30 °C was used for the HPLC analysis at a wavelength of 292 nm with the flow rate at 0.8 mL/min, and the mobile phases were composed of 0.1% formic acid-H₂O (A) and acetonitrile (ACN, B), and

the optimized HPLC elution conditions were set as follows: 0-40 minutes, 5%-95% B. The mass spectrometer collocated with electrospray ionization (ESI) was carried out in the positive ion mode to generate multifarious fragment ions. Briefly, the optimized instrument parameters were practiced as followed: the mass range was scanned from 150 to 1100 (m/z) in the full-scan mode; the drying gas flow rate was set as 6.0 L/min; the capillary temperature and vaporizer temperature were regulated to 250 °C and 350 °C, respectively; the spray voltage, the cone voltage energy and the collision energy were adjusted to 3000 V, 40 V and 20 eV, respectively; the aux gas pressure and the sheath gas pressure were modulated to 10 psi and 40 psi, respectively. Meanwhile, the Thermo Xcalibur ChemStation software (Thermo Fisher Scientific) was devoted to the acquirement and analysis of all the above data.

***In vitro* anti-proliferative assays of samples**

Non-small lung cancer cells (A549) and colon cancer cells (HT-29) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution under 5% CO₂ at 37°C in a humidified incubator. The cell viability was measured by sulforhodamine B (SRB) cell proliferation and cytotoxicity detection kits as described previously [27]. In brief, these two cells suspension (1.0×10^4 cells) were seeded in 96-well plates and incubated for 24 h. Then, those tested samples (100 mM) dissolved in the dimethyl sulfoxide (DMSO) were added and treated into the two cells. Thereinto, dimethyl sulfoxide (DMSO) was taken as the blank control, etoposide and 5-fluorouracil (5-FU) were treated as the positive controls. The optical density (OD) value of each well was measured at 540 nm with a microplate reader. The inhibition rate was calculated by the following equation:

$$\text{Inhibition rate (\%)} = [(OD_1 - OD_2) / OD_1] \times 100\%$$

OD₁ and OD₂ represent the absorbance of the blank control and positive control or samples, respectively. Additionally, all groups were tested in triplicate, and these results were computed by the GraphPad Prism 8 software.

***In vitro* COX-2 inhibitory assays of samples**

In vitro COX-2 inhibitory assays of samples were implemented by using COX-2 (human) inhibitor screening kits in the light of the manufacturer's instructions and our previous publication [28]. Briefly, the tested sample solution was firstly diluted with DMSO into a series of various concentration solutions. COX-2 cofactor working solution (50×), COX-2 substrate (50×) and COX-2 probe (50×) were prepared by diluting 10 times with COX-2 assay buffer, respectively. Then, 10 μL COX-2 cofactor working solution, 10 μL COX-2 working solution, 10 μL tested sample solution (0.625-20 mM), and 150 μL Tris-HCl buffer (pH = 7.9) were appended, mixed and incubated in a 96-well black plate for 10 minutes at 37 °C. In addition, 10 μL COX-2 working solution was replaced by the same volume of COX-2 assay buffer as the blank control group. It was also substituted for the equal amount of DMSO as the 100% enzyme activity control group. After that, 10 μL COX-2 probe and 10 μL COX-2 substrate were rapidly added into each well, and incubated in the darkness for 5 min at 37 °C. Finally, the fluorescence measurement was conducted by the microplate reader, in which the

excitation and emission wavelengths were monitored at 560 nm and 590 nm, respectively. Indomethacin was used as the positive control. The COX-2 inhibitory activity percentage of each sample was computed referring to the following calculation equation:

$$\text{Inhibition rate (\%)} = [(\text{RFU}_{100\% \text{enzyme}} - \text{RFU}_{\text{sample}}) / (\text{RFU}_{100\% \text{enzyme}} - \text{RFU}_{\text{blank}})] \times 100\%$$

where can be recorded as RFU_{blank} (relative fluorescence unit of the blank control group), RFU_{100%enzyme} (relative fluorescence unit of 100% enzyme activity control group), and RFU_{sample} (relative fluorescence unit of the tested sample), respectively. Additionally, the data results were measured in three parallels and presented as means ± standard deviation (SD). The half maximal inhibitory concentration (IC₅₀) value stood for the optimal sample concentration when the COX-2 activity was inhibited by 50%, and was calculated by the GraphPad Prism 8 software.

Results

Analysis of the extracts from *S. hexandrum* before and after ultrafiltration with HPLC-UV/ESI-MS/MS analysis

Lignans and flavonoids are the two primary ingredients from *S. hexandrum* [3]. Prior to the ultrafiltration screening, the extracts from *S. hexandrum* were firstly subjected to HPLC-UV/ESI-MS/MS analysis in the positive ion mode, and each chromatographic peak was detected and tentatively identified in accordance with the retention time (Rt), UV spectra, protonated molecular fragment information ([M + H]⁺), characteristic fragment information, the corresponding standards and literature data. After the ultrafiltration screening of the extracts above with Topo I, Topo II, COX-2 and ACE2, the resulting samples were analyzed under the same LC-MS conditions. As a result, the chemical structures of 10 potential ligands binding to Topo I, Topo II, COX-2 and ACE2 were identified and summarized at great length in Table 1 and Fig. 1.

Table 1

Enrichment factors and LC-ESI-MS/MS data obtained from AUF screening of *S. hexandrum* with Topo I, Topo II, COX-2 and ACE2.

Compound	Rt (min)	EF (%)				[M + H] ⁺	Characteristic fragment (<i>m/z</i>)	Identification
		Topo I	Topo II	COX-2	ACE2			
1	9.430	0.31	1.22	–	0.29	342	296, 279, 264, 236	Isocorydine ^b
2	13.329	1.85	0.68	0.20	0.07	611	611, 303	Rutin ^a
3	14.091	2.41	4.11	0.07	2.49	465	465, 303, 285	Quercetin 3- <i>O</i> -glucoside ^a
4	15.142	4.83	–	0.39	1.01	449	449, 287	Kaempferol 3- <i>O</i> -glucoside ^a
5	17.753	0.78	1.66	1.47	4.65	493	493, 397, 331, 317, 137	Unknown
6	18.588	3.98	–	0.70	0.47	397	397, 313, 282, 247, 229	β -Apopicropodophyllin ^b
7	19.737	1.24	13.90	0.33	–	303	303, 285, 229, 165, 153	Quercetin ^a
8	20.490	4.50	3.33	1.07	5.89	317	317, 302	Isorhamnetin ^a
9	22.297	0.51	7.06	1.58	0.43	287	287, 241, 213, 165, 153	Kaempferol ^a
10	22.911	7.03	–	1.35	1.49	415	397, 313, 282, 247, 229	Podophyllotoxin ^a
^a Compared with the corresponding standard; ^b Identified based on the published literature.								

Among the potential bioactive components, the [M + H]⁺ ion at *m/z* 342 was considered as the molecular ion of compound 1, revealing the molecular formula of C₂₀H₂₃NO₄. The MS/MS fragment ions comprised *m/z* 296 [M + H-CH₃-OCH₃]⁺, *m/z* 279 [M + H-(CH₃)₂NH-H₂O]⁺, *m/z* 264 [M + H-(CH₃)₂-OCH₃]⁺ and *m/z* 236 [M + H-(CH₃)₂-OCH₃-CO]⁺. By comparing its MS/MS data with previous literature, compound 1 was recognized as isocorydine (341 Da) [29]. Compound 2 yielded the [M + H]⁺ ion at *m/z* 611 in the full scan mode. It produced characteristic fragment ions at *m/z* 303 that stemmed from the consecutive elimination of molecular 146 Da and 162 Da (rhamnosylglucoside). As regards compound 7, interestingly, the MS/MS spectra demonstrated the semblable result as compound 2, indicating the similar basic skeleton of flavonoids. Compound 7 exhibited the [M + H]⁺ ion at *m/z* 303. By comparing the Rt, the [M + H]⁺ ion and the MS/MS behaviors of corresponding reference standards, compounds 2 and 7 were successfully characterized as

quercetin 3-rutinaside (rutin, calculated for $C_{27}H_{30}O_{16}$, 610 Da) and quercetin (calculated for $C_{15}H_{10}O_7$, 302 Da), respectively. And the MS/MS spectra of these two components were correspondent with the discussion in the known literature [30]. Concerning compound 3, the $[M + H]^+$ ion was detected at m/z 465 in the full scan MS, and the molecular formula was deduced as $C_{21}H_{20}O_{12}$. In addition, the neutral loss of a hexose moiety at m/z 162 generated the aglycon ion $[M + H-Glu]^+$ at m/z 303. By comparison with the R_t and MS/MS information of the standard, compound 3 was thus identified as quercetin 3-*O*-glucoside (isoquercitrin, 464 Da). As for compound 4, the $[M + H]^+$ ion was produced at m/z 449 and the molecular formula was regarded as $C_{21}H_{20}O_{11}$. Typically, the aglycone ion at m/z 287 was formed by the neutral loss of a hexose moiety at m/z 287, tentatively inferred as kaempferol monoglycoside. By comparing the MS/MS data with the related reference standard, compound 4 was characterized as kaempferol 3-*O*-glucoside (astragalin, 448 Da), and the mass spectra were consistent with previously reported literature [26].

Compounds 6 and 10 presented the $[M + H]^+$ ions at m/z 397 and m/z 415, and their molecular formulas were presumed to be $C_{22}H_{20}O_7$ and $C_{22}H_{22}O_8$, respectively. Furthermore, these two components possessed similar fragmentation pathways and MS/MS fragment information such as the ions at m/z 397, m/z 313 and m/z 282, derived from the loss of a water molecular moiety ($[M + H-H_2O]^+$), the characteristic retro Diels-Alder (RDA) cleavage ($[M + H-H_2O-C_4H_4O_2]^+$) and the successive neutral loss of a methoxy moiety ($[M + H-H_2O-C_4H_4O_2-OCH_3]^+$), respectively. Moreover, the neutral losses of the $C_6H_3(OCH_3)_3$ moiety and a molecular of water moiety acquired the fragment ions at m/z 247 ($[M + H-C_6H_3(OCH_3)_3]^+$) and m/z 229 ($[M + H-C_6H_3(OCH_3)_3-H_2O]^+$). Therefore, compounds 6 and 10 were further identified as β -apopicropodophyllin (396 Da) and podophyllotoxin (414 Da) in comparison to ESI-MS/MS spectra and characterized fragmentation pathways, combined with previous literature and the corresponding reference standards, respectively.

Compounds 8 and 9 exhibited the $[M + H]^+$ ions at m/z 317 and m/z 287, respectively. By comparing the ESI-MS/MS information of the existent reference standards and related literature reports [26], compounds 8 and 9 were unambiguously suggested as isorhamnetin (calculated for $C_{16}H_{12}O_7$, 316 Da) and kaempferol (calculated for $C_{15}H_{10}O_6$, 286 Da).

Screening of *S. hexandrum* for Topo I, Topo II, COX-2 and ACE2 ligands

Most medicinal plants have the characteristic of multiple drug targets for the treatment of various diseases. In order to further clarify the mechanism of action for medicinal plants, it is the first task to screen and identify active ingredients. Therefore, it is necessary to develop a fast, simple and effective approach for targeted screening of active components so as to associate the chemical ingredients and certain pharmacological activities. In addition, there are few reports on multi-targeted screening to date, which cannot meet the growing demand for multi-targeted screening of medicinal plant extracts. It is essential to accelerate the screening of multi-targeted medicinal plant activity for the discovery and development of new drugs. In this study, AUF-HPLC/MS with the characteristics of simplicity, efficiency and sensitivity was developed to screen Topo I, Topo II, COX-2 and ACE2 ligands from *S. hexandrum*, respectively. The variation in peak area for screened constituents could reflect a specific binding affinity between the activated and

denatured enzymes before and after ultrafiltration. The enrichment factor (EF) represented the capacity for those ligands binding to target enzymes, and the calculation was shown as below:

$$EF (\%) = (A_a - A_b) / A \times 100\%$$

where A_a , A_b and A represent peak areas of each chromatographic peak from the extract of *S. hexandrum* upon ultrafiltration with activated, denatured and without target enzymes (Topo I, Topo II, COX-2 and ACE2), respectively [31]. If the peak area from the activated group was greater than that of the corresponding inactivated group, those constituents were tentatively speculated as potentially bioactive ligands for target enzymes.

As illustrated in Figs. 2, 10, 7, 9 and 9 components from the extract of *S. hexandrum* exhibited specific bindings to Topo I, Topo II, COX-2 and ACE2 after AUF screening assay, which were deduced as potential ligands for Topo I, Topo II, COX-2 and ACE2, respectively. And the EF values of each component were shown in Table 1. The difference in the EF values of each compound indicated those potential ligands could specifically and differentially bind to the four target enzymes. That is, they possessed distinct and complicate interactions with the target enzymes. It also implied that different chemical components in the extracts could exert distinct but synchronous pharmaceutical effects in the pharmaceutical use as shown in Table 1.

In vitro anti-proliferative and COX-2 inhibitory assays of bioactive ligands screened

In order to explore the correlation between potential bioactive phytochemicals and pharmacological effects, anti-proliferative and COX-2 inhibitory assays *in vitro* were carried out so as to detect and validate their inhibitory effects of several bioactive ligands screened out targeting Topo and COX-2. For *in vitro* anti-proliferative assay, compounds 7, 9, 10 and 8 with relatively higher EF values exerted strong inhibitory activities (63.24%, 60.48%, 60.70%, and 32.76%, respectively) on A549 cell at the concentration of 100 μ M, and were comparable with the positive controls of etoposide and 5-FU (71.13% and 50.44%, respectively). Moreover, compounds 10 and 7 also showed remarkable inhibitory effects (46.36% and 40.47%, respectively) on HT-29 cell at the concentration of 100 μ M, whereas etoposide and 5-FU were 32.24% and 10.95%, respectively. With regard to *in vitro* COX-2 inhibitory assays, compared with the positive control of indomethacin at 0.73 ± 0.07 μ M, compounds 9 and 10 with higher EF values showed significant inhibitory effects with IC_{50} value at 0.36 ± 0.02 μ M and 10.49 ± 0.61 μ M, respectively. Hence, it was well worth fishing out and identifying potential bioactive ligands from *S. hexandrum* combining its empirical applications.

Molecular docking simulation

Molecular docking studies were implemented to further simulate the target proteins and several representative compounds with the highest EF values. We applied the AutoDockTools 1.5.6 and Discovery Studio 4.5 Client software with the three-dimensional crystal structures of Topo I, Topo II, COX-2 and ACE2 in

this work, respectively. During this simulation procedure, the grid box dimensions and the centroid coordinate for molecular docking of the macromolecular target proteins were shown in Table 2. The molecular docking results of several representative compounds and positive drugs against Topo I, Topo II, COX-2 or ACE2 were also displayed in Fig. 4 and Table 3.

Table 2

Dimensions and centroid coordinates of grid box the macromolecular target proteins in the molecular docking analysis.

Protein targets	PDB ID	Dimension of the grid box (npts)	Center grid box (xyz coordinates)	Grid box spacing (angstrom)
Topo I	1T8I	60 · 60 · 60	21.474, -2.226, 27.863	0.375
Topo II	3QX3	60 · 60 · 60	33.026, 95.765, 51.567	0.375
COX-2	1CX2	60 · 60 · 60	24.263, 21.528, 16.497	0.375
ACE2	1R42	60 · 60 · 60	52.874, 68.399, 33.501	0.375

Table 3

The molecular docking results of several representative compounds screened from *S. hexandrum* and positive drugs against Topo I, Topo II, COX-2 or ACE2.

No.	Phytochemicals	Drug targets	BE (kcal/mol)	IC ₅₀ (μM)	H-bond atoms
1	Podophyllotoxin	Topo I	-6.32	23.25	Dc112, Lys425 and Met428
2	Quercetin	Topo II	-6.99	7.53	Dg13, Dt9, Gln778 and Asp479
3	Kaempferol	COX-2	-7.22	5.10	Tyr355, Gln192 and Gly526
4	Isorhamnetin	ACE2	-5.72	63.95	Glu375 and Ala348
5	Camptothecin ^a	Topo I	-7.57	2.85	Da113, Dc112, Glu356, Lys425
6	5-FU ^b	Topo I	-3.70	1950.00	Met428, Tyr426
7	Etoposide ^c	Topo II	-7.62	2.59	Da12, Gln778
8	Indomethacin ^d	COX-2	-9.18	0.19	Glu524
9	MLN-4760 ^e	ACE2	-4.27	738.62	Glu375, Glu402, Thr371
a, b, c, d, e positive control.					

As shown in Table 3, podophyllotoxin exhibited a higher affinity to Topo I as its binding energy (BE) and the theoretical IC₅₀ values were – 6.32 kcal/mol and 23.25 μM, lower than the positive control 5-FU (-3.70

kcal/mol and 1.95 mM) and slightly higher than Topo I inhibitor camptothecin (-7.57 kcal/mol and 2.85 μ M). Considering Topo II, quercetin with a larger EF value displayed a strong affinity to Topo II with the BE of -6.99 kcal/mol and the theoretical IC₅₀ value of 7.53 μ M, which was comparable to Topo II inhibitor etoposide (-7.62 kcal/mol and 2.59 μ M). In addition, kaempferol exhibited a higher affinity to COX-2, and its BE and the theoretical IC₅₀ values were calculated as -7.22 kcal/mol and 5.10 μ M, which was not much different from the positive control indomethacin (-9.18 kcal/mol and 186.44 nM). With regard to ACE2, isorhamnetin was discovered with a high binding affinity of -5.72 kcal/mol and the theoretical IC₅₀ value of 63.95 μ M, lower than that of ACE2 inhibitor MLN-4760 (-4.27 kcal/mol and 738.62 μ M). Above all, molecular docking analysis indicated that some bioactive ligands screened with the largest EF values were provided with relatively lower binding energies and inhibitory effects compared with the positive controls or the same group combining other bioactivity assays *in vitro* such as anti-proliferative and COX-2 inhibitory assays. The docking results were consistent with the ultrafiltration screening and *in vitro* bioactivity verification results, further confirming the feasibility of the molecular docking approach.

Discussion

Ultrafiltration analysis of Topo I, Topo II, COX-2 and ACE2 ligands and the *in vitro* bioactive validation

Based on the findings in Table 1, the intrinsic and intricate correlations between bioactive constituents and multiple targets as well as the pharmacological effects could be partially explored, and the mechanism of action for *S. hexandrum* could also be further deciphered especially on how to exert its traditional curative effect on inflammatory, cancer and viral diseases.

Among these compounds screened out, compounds 7, 8, 9, and 10 exhibited relatively higher affinity to Topo I or Topo II, which was speculated that these components may target one or two enzymes so as to exert potential anti-proliferative effects. *In vitro* anti-proliferative assays displayed that these four compounds with higher EF values presented stronger inhibitory effects compared with etoposide and 5-FU. These results indicated that these active components screened out may act on Topo I or Topo II to exert potential anti-proliferative effects. With respect to COX-2 inhibitory assays *in vitro*, compounds 9 and 10 with higher EF values exerted favorable inhibitory activities comparable to indomethacin. These compounds were preliminarily inferred to be the potential anti-inflammatory ingredient group. Concerning the anti-viral effect on ACE2, compound 8 with the highest EF value exhibited high affinity with ACE2, which may be speculated that this compound can be provided with anti-viral activity.

On the one hand, these potential active constituents screened out with AUF-LC/MS were further verified to possess anti-proliferative and anti-inflammatory effects through *in vitro* inhibitory assays. On the other hand, the chemical constituents with larger EF values are tightly bound to target enzymes, and they could be maintained during the interaction with target enzymes. Owing to numerous and diversified active ligands screened out and identified, several active components may be able to act on one or more target enzymes. Meanwhile, it was observed that there existed synergistic effects among these potential bioactive constituents, conjointly exerting various pharmacological activities. It was found that compound 8 could

potently action all four drug targets to exert anti-proliferative, anti-inflammatory and anti-viral activities; while some compounds exhibited obviously special preference for certain drug targets, like compound 7 for Top I and Top II, compound 9 for Topo II and COX-2 as well as compound 10 for Top I and COX-2. More importantly, this newly integrative strategy combining four drug targets with UF-LC/MS could be used to construct a multi-component and multi-target network based upon experimental evidences as shown in Fig. 3, which could bring insight into the mechanism of action regarding the empirical use of *S. hexandrum* as a traditional medicine, and promote more new methods to be developed for a better understanding of other traditional herbal medicines.

Molecular docking analysis of representative compounds

Molecular docking is an essential approach to study and predict the interactions between receptors and ligands, further exploring the possible mechanism of bioactive ligands against the target enzymes involved in multiple pharmacological effects. Podophyllotoxin, quercetin, kaempferol and isorhamnetin exhibited the highest specific EF values of 7.03, 13.90, 1.58 and 5.89, indicating their stronger affinities to Topo I, Topo II, COX-2 and ACE2 than that of others, respectively. The molecular docking assay further threw great light on the interactions between these bioactive ligands and Topo I, Topo II, COX-2 or ACE2, respectively.

Among these components, podophyllotoxin was observed to shape three conventional hydrogen bonds (H-bonds) with amino acid residues Dc112, Lys425 and Met428; pi-alkyl interaction with residue Lys425; and van der Waals interaction with residues Da113, Dc8, Dt9, Dt10, Lys436, Ile424, Ile427, Ala351 and Tyr426. As an anti-tumor lignan ingredient, podophyllotoxin can effectively inhibit the herpes virus, the mitosis at metaphase, and also inhibit the division or proliferation of tumor cells [32]. Quercetin was found to form four conventional H-bonds with residues Dg13, Dt9, Gln778 and Asp479, part of which interacted with etoposide as well. Additionally, there existed pi-lone pair interaction with residue Da12; and other non-covalent bond interactions such as van der Waals interaction with residues Dg10 and Leu502. Studies have shown that quercetin possessed the greatest potential for interacting with Topo II to cause DNA damage in cells, and kaempferol as well as isorhamnetin with relatively high EF values was found to induce DNA damage in human hepatic cells [33]. Furthermore, the docking simulation results uncovered that three conventional H-bonds were formed between the hydroxyl group of kaempferol and residues Tyr355, Gln192 as well as Gly526 of COX-2, part of which interacted with celecoxib as well [34]. Kaempferol also revealed other interaction forces such as pi-cation interaction with residue Arg513; pi-alkyl interaction with residue Leu352; pi-sigma interaction with residues Ser353 and Val523; and van der Waals interaction with residues Ala516, Ala527, Arg120, His90, Ile517, Leu384, Met522, Phe381, Phe518, Phe529, Ser530 and Tyr385. Moreover, isorhamnetin exerted a relatively strong affinity to ACE2 and was observed to form the conventional H-bonds with residues Glu375 and Ala348 to enhance their affinity against ACE2. There also existed in other important driven forces such as van der Waals force (interaction with residues Asp382, Arg518, Glu398, Gly395, Gly399, Thr347 and Zn804), hydrophobic (interaction with residues His378 and His401) and electrostatic (interaction with residues Arg514 and Glu402) effects in the processes of molecular docking analysis. In addition, it has been reported that isorhamnetin can be used as potential 3CL pro inhibitors to target PIK3CG and E2F1, and then inhibit the replication of SARS-CoV-2 through the PI3K-Akt signaling

pathway, and play the anti-viral effect to treat lung injury in COVID-19 by acting on CASP3, CCL2, IL6 or other targets through IL-17 or HIF-1 signaling pathways [35]. In general, these representative components in *S. hexandrum* exhibited the potential to be developed into the lead candidates responsible for its empirical anti-proliferative, anti-inflammatory or anti-viral effects.

Conclusions

S. hexandrum, as a traditional herbal medicine, has long suffered from a lack of experimental evidence regarding the material base and the mechanism of actions regarding its empirical use for the treatment of various diseases due to its complicated and diversified chemical components and intricate efficacy. To overcome this tough challenge, the integrative strategy combining four drug targets closely correlated to empirical application of *S. hexandrum* and affinity ultrafiltration LC-MS in the present study showed very promising potential for the quick screening and identifying its multiple bioactive components corresponding to the four respective drug targets selected. Based on these direct experimental evidences, we inferred and constructed for the first time a ligand-target network among its multi-components and respective multi-targets, which could be very conducive to unveil the underlying mechanism of the empirical traditional applications of *S. hexandrum*. Next, the screening results above were further verified using other *in vitro* bioactivity assays and molecular docking analysis, which confirmed that the integrative strategy could offer direct and reliable experimental evidences for the empirical applications of *S. hexandrum*. More strikingly, some bioactive compounds corresponding to the therapeutic drug targets obtained in this work were even better than those positive drug controls. Herein, we showcased a quick and reliable experimental strategy for uncovering the underlying mechanism of the empirical applications of *S. hexandrum*, and could provide valuable information for a better understanding of the main therapeutic targets and therapeutic roles of other traditional herbal medicines.

Abbreviations

ACE2, angiotensin-converting enzyme II; AUF, affinity ultrafiltration; BE, binding energy; COX-2, cyclooxygenase-2; EF, enrichment factor; ESI, electrospray ionization; 5-FU, 5-fluorouracil; H-bond, hydrogen bond; HPLC, high performance liquid chromatography; IC₅₀, the half maximal inhibitory concentration; MS, mass spectrometry; RAS, renin-angiotensin system; Rt, retention time; SD, standard deviation; SRB, sulforhodamine B; Topo I, DNA topoisomerase I; Topo II, DNA topoisomerase II.

Declarations

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Authors' contributions

Mingquan Guo conceived of, designed and supervised the whole study. Huixia Feng, Guilin Chen, and Yongli Zhang performed the experiments, analyzed the data and wrote the manuscript. All the authors have read and approved the final manuscript.

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Availability of data and materials

All data are fully available without restriction.

Ethics approval and consent to participate

No applicable.

Consent for publication

No applicable.

Competing interests

The authors declare that they have no known competing financial interest.

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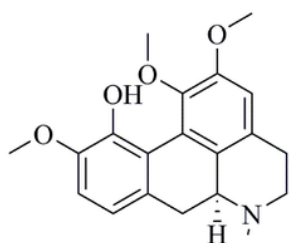
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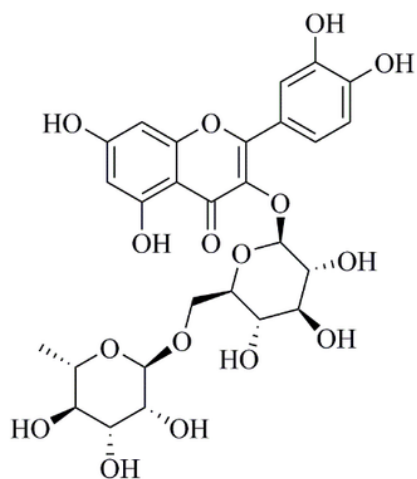
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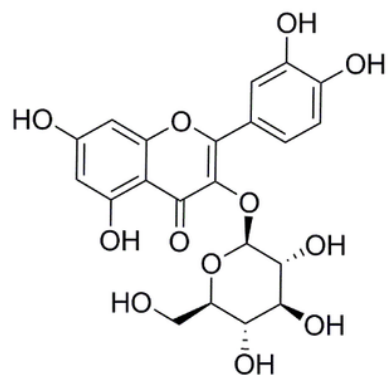
Figures



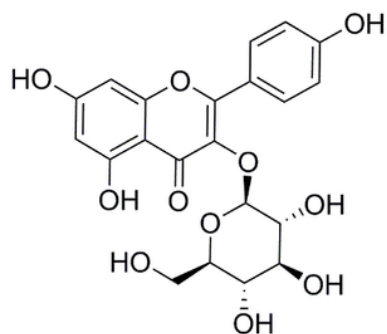
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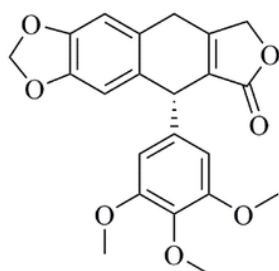
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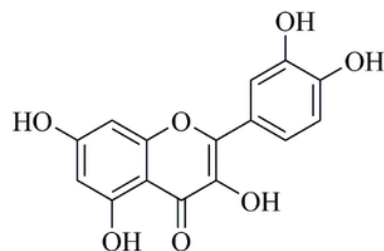
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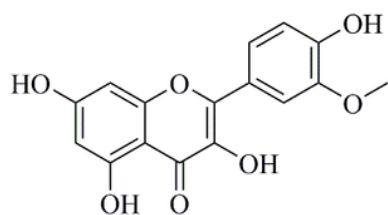
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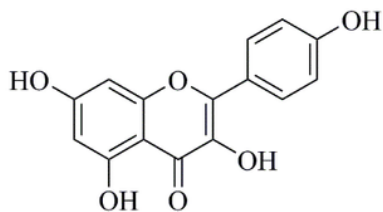
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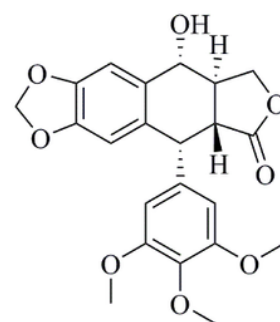
Compound 7



Compound 8



Compound 9



Compound 10

Figure 1

Chemical structures of ten potential bioactive ligands from *S. hexandrum*.

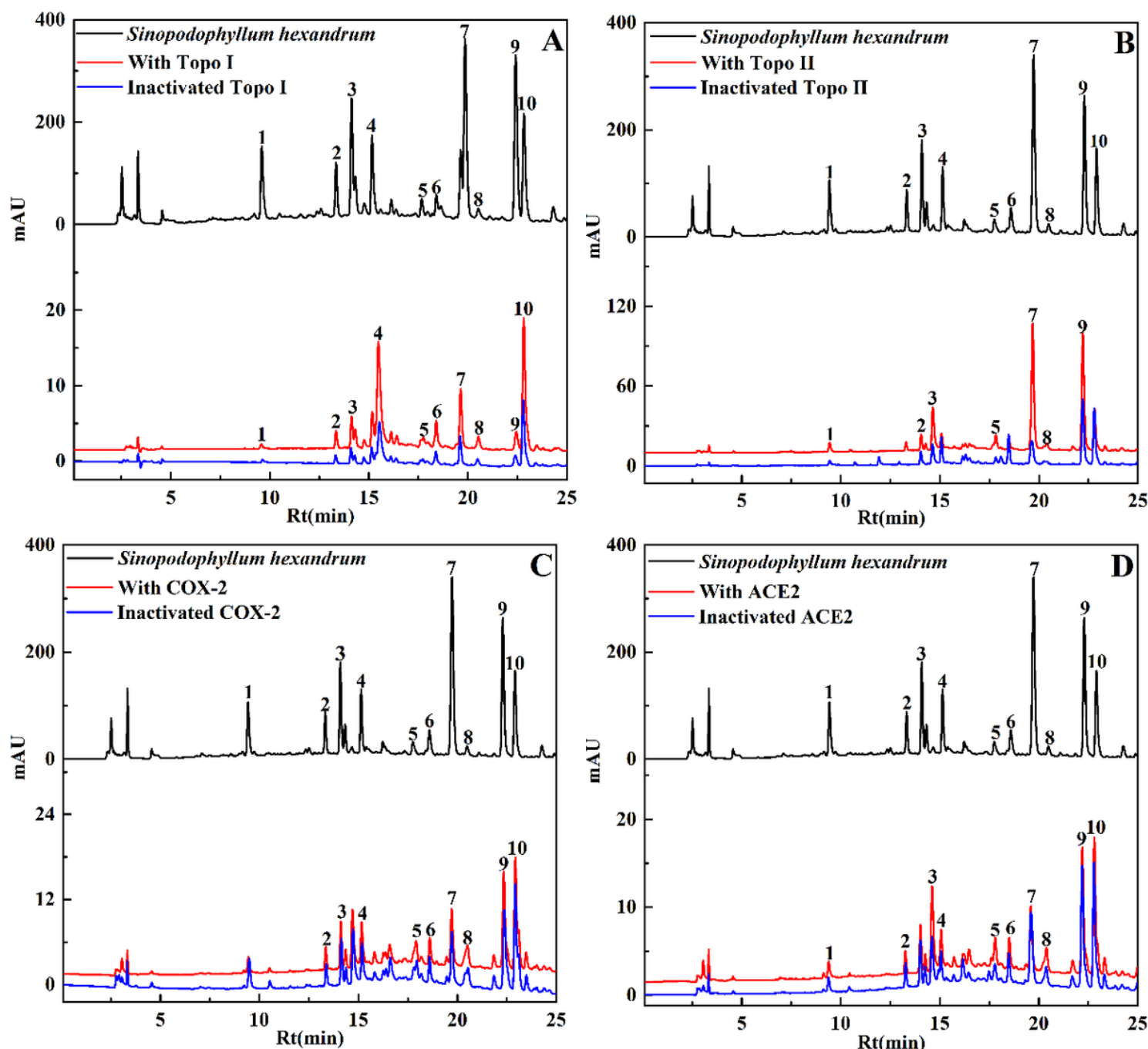


Figure 2

The HPLC chromatograms for screening of Topo I (A), Topo II (B), COX-2 (C) and ACE2 (D) ligands in the crude extract of *S. hexandrum* after ultrafiltration. The black solid line represents the HPLC profile of *S. hexandrum* without enzymes; the red and blue lines denote the HPLC profile of *S. hexandrum* mixed with activated and denatured Topo I, Topo II, COX-2 and ACE2, respectively.

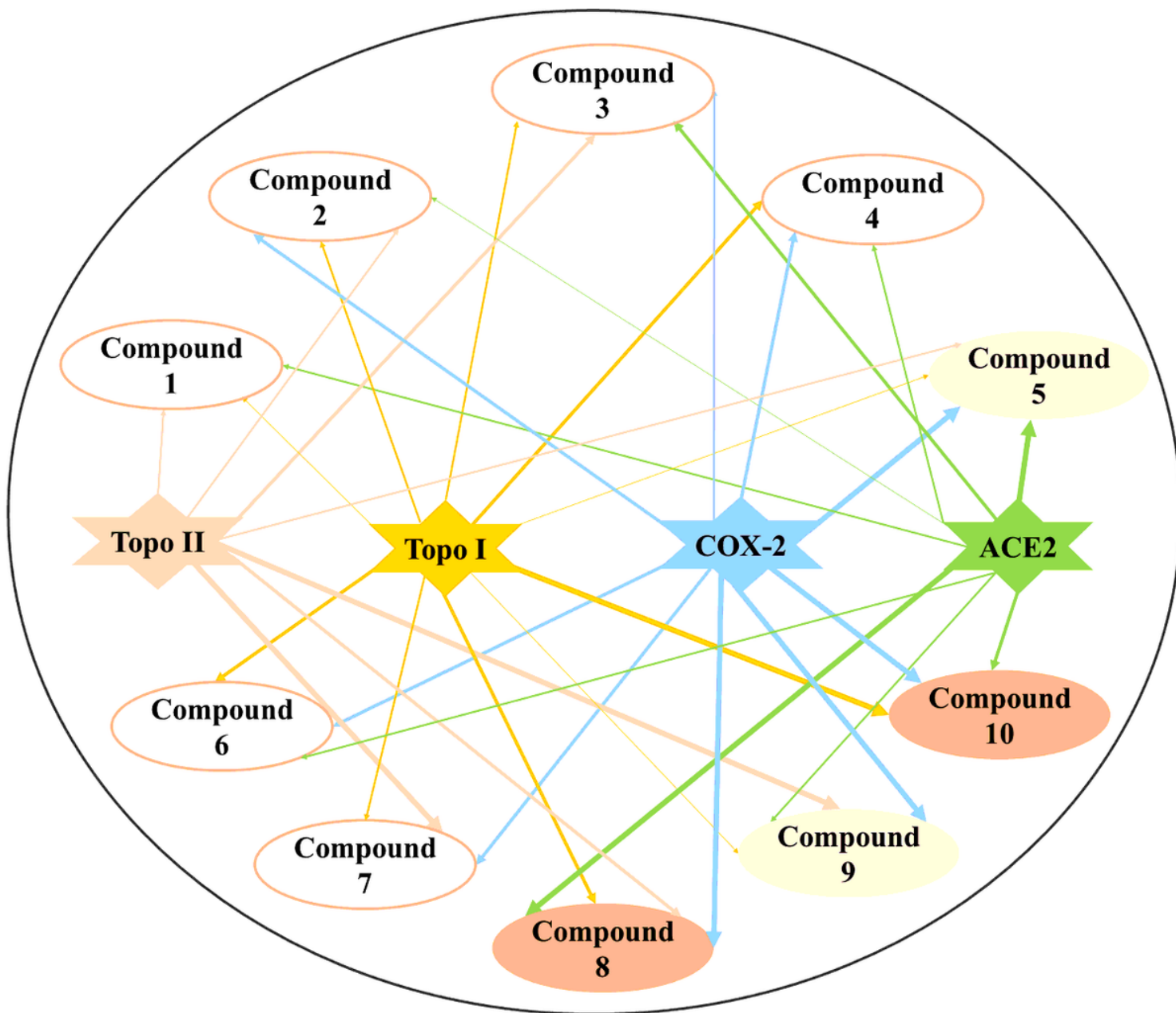


Figure 3

The intricate multi-component and multi-target network was constructed based on potential active components screened out, and four multiple drug targets (Topo I, Topo II, COX-2 and ACE2). The line thicknesses roughly imply the strength of binding or acting on multiple drug targets.

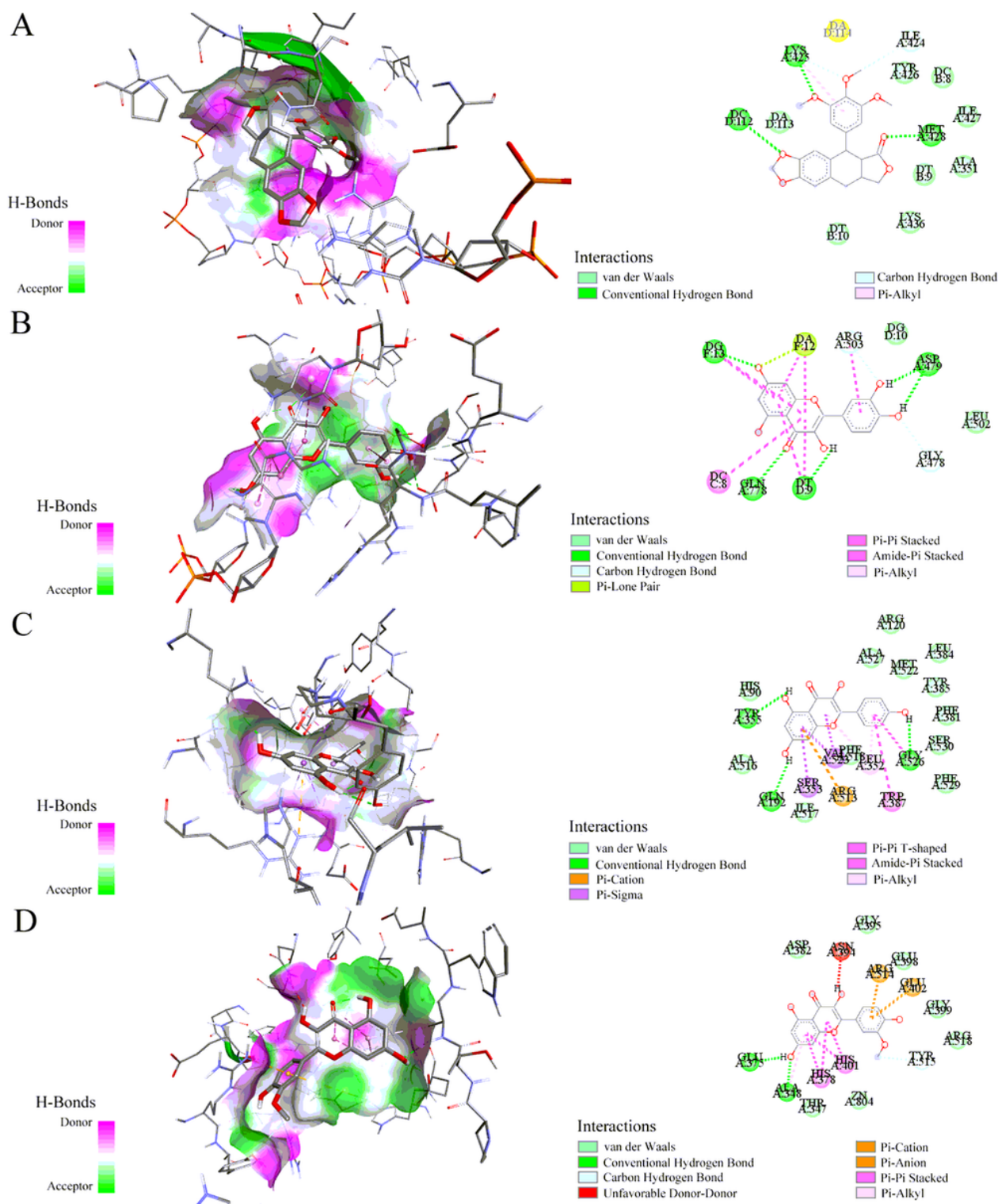


Figure 4

The 2D or 3D ligand-to-target interactions between podophyllotoxin (A), quercetin (B), kaempferol (C) or isorhamnetin (D) and Topo I, Topo II, COX-2 or ACE2, respectively.