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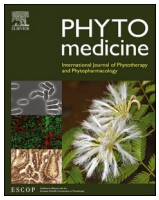

Original Article

Discovery and characterization of the covalent SARS-CoV-2 3CL^{pro} inhibitors from *Ginkgo biloba* extract *via* integrating chemoproteomic and biochemical approaches

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ABSTRACT

Background: The 3C-like proteases (3CL^{pro}s) are cysteine-rich homodimeric proteins and can be covalently modified by numerous natural and synthetic compounds, which in turn, block the proteolytic activity or the formation of enzymatically active dimeric forms. Although herbal medicines have been widely used to treat COVID-19, identification of the key herbal constituents that can covalently modify the 3CL^{pro}s in β-coronaviruses (CoVs) remains a big challenge.

Aims: To construct a comprehensive approach for efficient discovering the covalent SARS-CoV-2 3CL^{pro} inhibitors from herbal medicines. To decipher the key anti-SARS-CoV-2 3CL^{pro} constituents in *Ginkgo biloba* extract 50 (GBE50) and to study their anti-SARS-CoV-2 3CL^{pro} mechanisms.

Methods: SARS-CoV-2 3CL^{pro} inhibition assay including time-dependent inhibition assays and inactivation kinetic analyses were conducted using a fluorescence-based biochemical assay. The constituents in GBE50 were analyzed by UHPLC-Q-Exactive Orbitrap HRMS. The peptides modified by herbal constituents were characterized by using nanoLC-MS/MS.

Results: Following testing the anti-SARS-CoV-2 3CL^{pro} effects of 104 herbal medicines, it was found that *Ginkgo biloba* extract 50 (GBE50) potently inhibited SARS-CoV-2 3CL^{pro} in dose- and time-dependent manners. A total of 38 constituents were identified from GBE50 by UHPLC-Q-Exactive Orbitrap HRMS, while 26 peptides modified by 18 constituents were identified by chemoproteomic profiling. The anti-SARS-CoV-2 3CL^{pro} effects of 18 identified covalent inhibitors were then validated by performing time-dependent inhibition assays. The results clearly demonstrated that most tested constituents showed time-dependent inhibition on SARS-CoV-2 3CL^{pro}, while gallocatechin and sciadopitysin displayed the most potent anti-SARS-CoV-2 3CL^{pro} effects.

Conclusion: Collectively, GBE50 and some constituents in this herbal product could strongly inhibit SARS-CoV-2 3CL^{pro} in dose- and time-dependent manner. Gallocatechin and sciadopitysin were identified as potent SARS-CoV-2 3CL^{pro} inhibitors, which offers promising lead compounds for the development of novel anti-SARS-CoV-2 drugs.

Abbreviations: 3CL^{pro}, 3-chymotrypsin-like protease; COVID-19, coronavirus disease 2019; CoVs, coronaviruses; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetic acid; FASP, filter aided sample preparation; FRET, fluorescence resonance energy transfer; GBE50, *Ginkgo biloba* extract 50; HEPES, 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid; HRMS, high resolution mass spectrometry; IC_{50}, half maximal inhibition concentration; K_I, concentration producing half the maximal inactivation rate; nanoLC-MS/MS, nano liquid chromatography-tandem mass spectrometer; NH_4HCO_3, ammonium bicarbonate; PBS, phosphate buffered saline; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; UHPLC, ultrahigh performance liquid chromatography.

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Introduction

The Coronavirus disease 2019 (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has created a huge threat on the public health and a negative impact on the world economy. Although more than 20 vaccines reached clinical trials, the emergence of viral escape mutants may compromise the efficacy of vaccines. COVID-19 reinfection was also observed in the presence of neutralizing antibodies (Chen et al., 2021a). Therefore, it is urgent and necessary to develop more efficacious therapeutic approaches for combating SARS-CoV-2 or other emerging coronaviruses. To date, the research community worldwide have devoted to developing efficacious medicines *via* targeting some key proteins involved in virus replication and transcription (Yan et al., 2022). Among all validated antiviral targets, the 3C-like proteases (3CL^{pro}s, as termed as the main proteases) have been validated as ideal antiviral targets (Vandyck and Deval, 2021). 3CL^{pro}s are highly conserved cysteine hydrolases distributed in multiple β-coronaviruses (CoVs), and have been validated as ideal targets for developing broad-spectrum anti-CoVs drugs (Hu et al., 2022). Specifically, the 3CL^{pro}s in the two of most serious COVID pandemics in 21st century (SARS-CoV-2 and SARS-CoV) shares highly identical amino acid sequences and extremely closed catalytic cavities, making their inhibitor spectra highly overlapping (Tahir Ul Qamar et al., 2020).

Structurally, 3CL^{pro}s are cysteine-rich homodimeric proteins with several cysteines (such as Cys145 and Cys44) located in the catalytic sites, while some other cysteines (such as Cys156 and Cys300) are essential for the formation of enzymatically active dimeric forms (Ferreira et al., 2021; Kuzikov et al., 2021; Tao et al., 2021; Verma et al., 2020; Xiong et al., 2021b). In this scenario, many efforts have been made to develop new selective covalent inhibitors targeting these key cysteines in 3CL^{pro}. Additionally, the 3CL^{pro}s are non-human proteins, there is no functional protein in the human body that is structurally similar to viral 3CL^{pro}s (Boras et al., 2021). Thus, it is feasible to develop the highly specific inhibitors/inactivators of 3CL^{pro}s to fatally block the catalytic activity of 3CL^{pro}, with little impact on the function of key human proteins.

Over the past few decades, herbs have been frequently utilized to prevent and treat viral influenza and related epidemics (Zhang et al., 2020). There is accumulating evidence that a wide range of herbal constituents could covalently modify the cysteines in 3CL^{pro} and inactivate this antiviral target enzyme (Chen et al., 2021b). Particularly, herbal constituents with some certain structural characteristics, such as *o*-quinone, *p*-quinone, Michael receptor and catechol, have been found to have the potential to covalently bind 3CL^{pro}s. For instance, Xiong et al., 2021b have reported that some catechols (myricetin and its analogs) in *Ampelopsis grossedentata* (Hand.-Mazz.) W. T. Wang (Family: Vitaceae) are readily oxidized to *o*-quinone, which covalently bound to several cysteines of 3CL^{pro}. Moreover, quinones (vitamin K and its derivatives) have also been identified as potent covalent inhibitors against 3CL^{pro} (Wang et al., 2021). In contrast to classical reversible inhibitors, covalent inhibitors against 3CL^{pro}s have some inherent advantages, including potent and broad-spectrum inhibition, as well as long-lasting effect, which are of great interest to medicinal chemists (Baillie, 2016).

Although a variety of natural and synthetic compounds have been reported as covalent inhibitors against 3CL^{pro}s, most of them were discovered through large-scale screening that rely on large collections of pure compounds (Song et al., 2019). It is well-known that herbal medicines are extremely complex mixtures, often containing hundreds of chemically diverse constituents, most of which are present in trace amounts (Shan et al., 2021). Generally, most of trace ingredients in herbs are commercially unavailable and difficult to get the purified compound, which strongly hampers the identification and characterization of active constituents in herbal medicines. Herein, to solve this problem, we established a comprehensive method for highly efficient discovering and characterizing the covalent 3CL^{pro} inhibitors from herbal medicines, *via* integrating a set of analytical techniques including fluorescence-based 3CL^{pro} inhibition assay, mass spectrometry-based global analysis of herbal constituents and chemoproteomic profiling of cysteine-modified peptides (Fig. 1). Following testing the anti-SARS-CoV-2 3CL^{pro} effects of 104 herbal medicines, *Ginkgo biloba* extract 50 (GBE50) was discovered with the most potent anti-SARS-CoV-2 3CL^{pro} effect. More importantly, it is found that GBE50 potently inhibit SARS-CoV-2 3CL^{pro} in time-dependent manner, suggesting the presence of covalent 3CL^{pro} inhibitors in this herbal extract. Encouraged by these findings, we further identify the naturally occurring covalent inhibitors of SARS-CoV-2 3CL^{pro} in GBE50, while the covalently modified sites and inhibitory mechanisms of the newly identified covalent inhibitors were also investigated.

Material and methods

Chemicals and reagents

SARS-CoV-2 3CL^{pro} was expressed and purified as stated previously (Xiong et al., 2021b), SDS-PAGE analysis of the purified 3CL^{pro} was shown in **Fig. S1**. The GBE50 was provided by Shanghai Shangyao Xingling Technology Pharmaceutical Co. Ltd, other herbal products were supplied by Tianjiang Pharmaceutical Co., Ltd. (Jiangsu, China) (Shanghai, China). The purified constituents in GBE50 including **(1)** gallocatechin, **(2)** laricitrin 3-*O*-rutinoside, **(3)** sciadopitysin, **(4)** bilobetin, **(5)** ginkgetin, **(6)** isoginkgetin, **(7)** apigenin, **(8)** luteolin, **(9)** amentoflavone, **(10)** catechin, **(11)** epicatechin, **(12)** manghaslin, **(13)** isorhamnetin-3-*O*-glucoside, **(14)** kaempferol 3-*O*-β-D-glucopyranosyl-(1→2)-α-l-rhamnopyranoside, **(15)** quercetin-3-*O*-(6′′-trans-*p*-coumaroyl-2′-glucosyl) rhamnoside, **(16)** protocatechuic acid, **(17)** isorhamnetin-3-*O*-neohespeidoside, **(18)** nicotiflorin, as well as the positive inhibitor myricetin were ordered from Shanghai Standard Biotech Co. Ltd. (Shanghai, China). The purity of each tested compound is higher than 95%. Iodoacetamide, 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid (HEPES), chymotrypsin and trypsin were provided by Sigma-Aldrich (St. Louis, MO, USA). Fluorescent substrate (Dabcyl-KNSTLQSGLRK-Edans) was obtained from GenScript biology science and technology Co. Ltd. (Nanjing, China), with the purity of 95.8%. Ethylene Diamine Tetraacetic Acid (EDTA) was purchased from Dalian Meilun Biotechnology Co. Ltd. (Dalian, China). LC grade formic acid, acetonitrile, methanol, and dimethyl sulfoxide were purchased from Tedia Company (Fairfield, Iowa, USA). Other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

SARS-CoV-2 3CL^{pro} inhibition assay

The proteolytic activity of SARS-CoV-2 3CL^{pro} was assessed using the fluorescence-based biochemical assay, in which Dabcyl-KNSTLQSGLRK-Edans was used as a fluorescently tagged substrate (Alhadrami et al., 2021). In brief, a mixture (200 μl) contained 10 μl of SARS-CoV-2 3CL^{pro} (2 μg/ml, final concentration), 2 μl of DMSO/inhibitors (GBE50 and other compounds), 10 μl of substrate (Dabcyl-KNSTLQSGLRK-Edans, 20 μM, final concentration) and 178 μl PBS (pH 7.4, 100 mM, 1 mM EDTA). The mixture was preincubated for 63 min or 3 min before adding the substrate and reacting for 22 min at room temperature. After then, a microplate reader (SpectraMax® iD3, Molecular Devices, California, USA) was used to detected fluorescent products, with excitation and emission wavelengths of 340 and 490 nm, respectively.

Comprehensive chemical profiling of GBE50 by UHPLC-Q-Exactive Orbitrap HRMS

Since the constituent of the herbal medicine is extremely complex, we performed a comprehensive profiling of the constituent of GBE50 with the help of UHPLC-Q-Exactive Orbitrap HRMS (Liu et al., 2021). The liquid phase and mass spectrometry conditions were described in

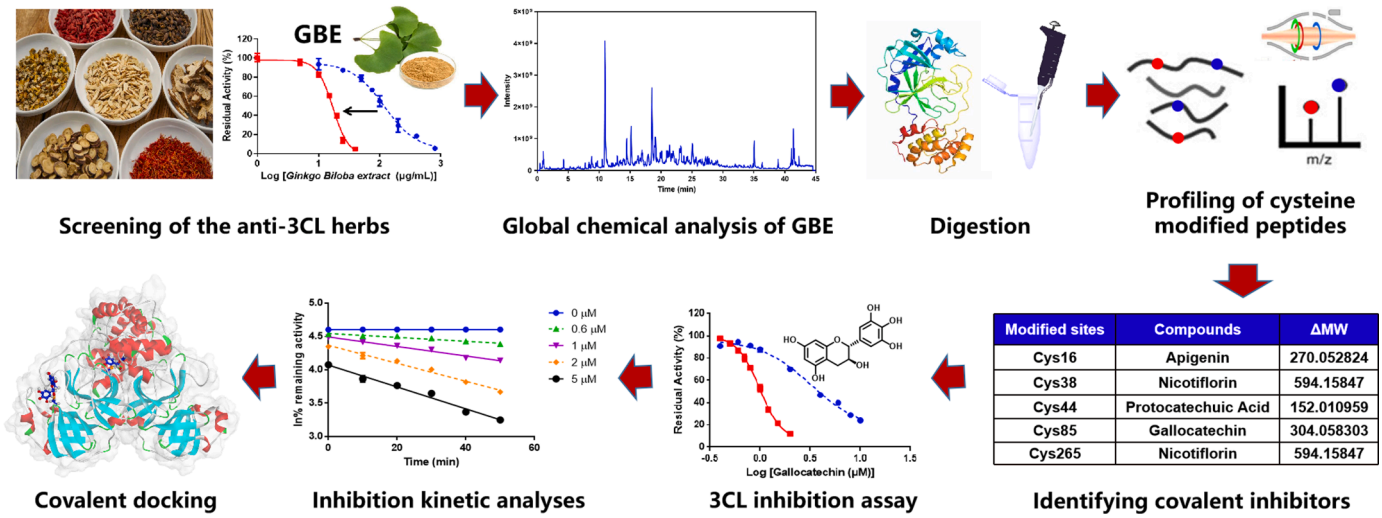

Fig. 1. Schematic illustration of highly efficient discovering SARS-CoV-2 3CL^{pro} covalent inhibitors from herbs.

Supplementary data 2.

Cysteine modification profiling of SARS-CoV-2 3CL^{pro} by GBE50

After a comprehensive analysis of the constituents of GBE50, we meticulously characterized the covalently bound modified peptides using nanoLC-MS/MS, the molecular weights of 38 ingredients were entered to explore which constituents could covalently modify the cysteines on SARS-CoV-2 3CL^{pro} and to clarify their modification sites. First, 30 μM SARS-CoV-2 3CL^{pro} and GBE50 (200 μg/ml, final concentration of) were co-incubated at 37 for 3 h The mixture was transferred to a 10 kDa centrifugal filter tube and washed twice with 200 μl water to remove unbound molecules (Nel et al., 2015). Afterwards, the protein-inhibitor adducts were denatured with 8 M urea for 1 h, reduced with 200 mM (1 mM, final concentration) dithiothreitol for 60 min and alkylated with 500 mM (3 mM, final concentration) iodoacetamide for 30 min in darkness, washed twice with 200 μl water and finally washed with 200 μl 50 mM NH_4HCO_3. Steps aforementioned were centrifuged at 16,000 g for 15 min. Samples were digested with chymotrypsin and trypsin in 100 μl 50 mM ammonium bicarbonate at 37 °C for 16 h at a ratio of 1:50 (w/w) of enzyme to substrate. After digestion, the peptides were collected by centrifugation and desalted with MonoSpin C18 columns (GL Sciences, Tokyo, Japan). Finally, the eluents were vacuum dried and resolved in 0.1% formic acid (40 μl) for nanoLC-MS analysis.

The settings of mass spectrometry and analytical conditions were listed in supplementary file. SARS-CoV-2 3CL^{pro} was set up as protein database, trypsin and chymotrypsin were selected as cleavage enzyme. In addition, carbamidomethylation of cysteine, most constituents of cysteine, oxidation of methionine were set to dynamic modifications. Mass tolerance for precursor ions was less than 10 ppm and the false discovery rates (FDR) of peptide, protein and site were all < 0.01.

Inactivation kinetic analyses

The inactivation kinetics of gallocatechin and sciadopitysin anti-SARS-CoV-2 3CL^{pro} were analyzed similarly to the 3CL^{pro} inhibition assay (Harrelson et al., 2012). 3CL^{pro} was preincubated with different concentrations of gallocatechin and sciadopitysin or DMSO in PBS at 37 °C for 0, 10, 20, 30, 40, 50 and 60 min. Then the generated fluorescent signals were measured as described in section 2.2. The inactivation kinetic curves were plotted against various concentrations with gradient-ascending durations. And the inactivation kinetic parameters were determined using the following equation:

$$K_{obs} = K_{inact} \times I/(I + K_I)$$

here, I is the final concentration of each inhibitor tested, K_I represents the inhibitor concentration required for 50% the maximal rate of inactivation, K_{inact} is the maximal inactivation rate constant, and K_{obs} is the apparent first-order inactivation rate constant.

Covalent docking simulations

The MOE covalent docking module was utilized to perform simulations of covalent docking (MOE, Molecular Operating Environment 2019.01, Chemical Computing Group Inc., Montreal, Canada) (Paul et al., 2022). The crystal structure of SARS-CoV-2 3CL^{pro} (PDB Code: 7NBY) was prepared by the QuickPrep functions including generation of missing secondary structures, optimizing of hydrogen bond network and partial charges. The structures of gallocatechin and sciadopitysin were defined as ligands. Covalent docking simulations were performed according to hereinafter options. According to the exprimental data listed in Table 1, Cys85 and Cys128 of 3CL^{pro} were selected as reactive sites of gallocatechin, while Cys85 and Cys156 of 3CL^{pro} were selected as reactive sites of sciadopitysin. The respective reaction formulas for compounds covalently binding cysteine residue were generated by MarvinSketch and referred in MOE. The docking post-placement manipulated rigid receptor and GBVI/WSA dG scoring methodology. The GBVI/WSA binding free energy calculation in the S field of ligand poses was further analyzed.

Data analysis

Graphpad Prism 7.0 was utilized to obtain the IC_{50} and K_I values. Proteome Discoverer 2.4 coupling with the Sequest HT algorithmwas applied to analyze the MS raw data (Thermo Fisher Scientific).

Results

Discovering the herbal medicines with potent anti-SARS-CoV-2 3CL^{pro} effects

First, we collected a total of 104 herbal products and measured the inhibition effect of their extracts anti-SARS-CoV-2 3CL^{pro} utilizing a FRET-based protease assay as previously reported. Under the same incubation conditions (pH 7.4, 37 °C). GBE50 was found to have the strongest anti-3CL^{pro} activity, with a residual activity of 0.88% at a final concentration of 100 μg/ml (Fig. 2). Achieving IC_{50} values as low as 17.19 ± 1.038 μg/ml after 63 min of preincubation, further inhibition experiments demonstrated that GBE50 inhibited SARS-CoV-2 3CL^{pro} in

Table 1
Identification of the covalent binding sites of the constituents in *Ginkgo biloba* extract 50 with SARS-CoV-2 3CL^{pro}.

Peptides	Modified sites	ΔMW	Molecular formula	Potential herbal constituent
KMAFPSGKVEGC*MVQVTCGTTTLNGLW	Cys16	640.16395	$C_{28}H_{32}O_{17}$	laricitrin 3-*O*-rutinoside
KMAFPSGKVEGC*MVQVTCGTTTLNGLW		624.16904	$C_{28}H_{32}O_{16}$	isorhamnetin-3-*O*-neohespeidoside
KMAFPSGKVEGC*MVQVTCGTTTLNGLW		270.05282	$C_{15}H_{10}O_5$	apigenin
KMAFPSGKVEGC*MVQVTCGTTTLNGLW		538.09000	$C_{30}H_{18}O_{10}$	amentoflavone
KMAFPSGKVEGC*MVQVTCGTTTLNGLW		552.10565	$C_{31}H_{20}O_{10}$	bilobetin
MAFPSGKVEGCMVQVTC*GTTTLNGLW	Cys22	594.15847	$C_{27}H_{30}O_{15}$	nicotiflorin
VEGCMVQVTC*GTTTLNGLWLDDVVY		624.16904	$C_{28}H_{32}O_{16}$	isorhamnetin-3-*O*-neohespeidoside
VEGCMVQVTC*GTTTLNGLWLDDVVYCPR		284.03209	$C_{15}H_8O_6$	luteolin
VEGCMVQVTC*GTTTLNGLWLDDVVY		640.16395	$C_{28}H_{32}O_{17}$	laricitrin 3-*O*-rutinoside
MAFPSGKVEGCMVQVTC*GTTTLNGLW		478.11113	$C_{22}H_{22}O_{12}$	isorhamnetin-3-*O*-glucoside
C*PRHVICTSEDMLNPNYEDLLIR	Cys38	594.15847	$C_{27}H_{30}O_{15}$	nicotiflorin
				kaempferol 3-*O*-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside
CPRHVIC*TSEDMLNPNYEDLLIR	Cys44	152.01096	$C_7H_4O_4$	protocatechuic acid
VIGHSMQNC*VLKLKVDTANPK	Cys85	580.13695	$C_{33}H_{24}O_{10}$	sciadopitysin
VIGHSMQNC*VLKLK		304.05830	$C_{15}H_{12}O_7$	gallocatechin
VIGHSMQNC*VLKLK		288.06339	$C_{15}H_{12}O_6$	catechin
				epicatechin
LVQAGNVQLRVIGHSMQNC*VLK		478.11113	$C_{22}H_{22}O_{12}$	isorhamnetin-3-*O*-glucoside
DC*VSFCYMHHMELPTGVHAGTDLEGNF	Cys156	580.13695	$C_{33}H_{24}O_{10}$	sciadopitysin
QC*AMRPNFTIKGSF	Cys128	754.19564	$C_{33}H_{38}O_{20}$	manghaslin
QC*AMRPNFTIKGSF		754.17452	$C_{36}H_{34}O_{18}$	quercetin-3-*O*-(6′′′-trans-p-coumaroyl-2′′-glucosyl) rhamnoside
QC*AMRPNFTIKGSF		288.06339	$C_{15}H_{12}O_6$	catechin
				epicatechin
QC*AMRPNFTIKGSF		304.05830	$C_{15}H_{12}O_7$	gallocatechin
DCVSFC*YMHHMELPTGVHAGTDLEGNF	Cys160	566.12130	$C_{32}H_{22}O_{10}$	ginkgetin
				isoginkgetin

both time- and dose-dependent manners (Fig. 2)**.** These findings suggest that GBE50 contains naturally covalent inhibitors anti-SARS-CoV-2 3CL^{pro}.

Comprehensive chemical profiling of GBE50 by UHPLC-Q-Exactive Orbitrap HRMS

The complex chemical constituents in GBE50 were comprehensively characterized by UHPLC-Q-Exactive Orbitrap HRMS, their chemical structures were assigned *via* MS^2 spectra of the reference standards, retention time comparison, reference literatures, Pubchem and other databases. As illustrated in **Figs. S2-S40** and **Table S1**, altogether 38 compounds were analyzed in GBE50, including 23 flavonoids, 5 biflavonoids, 4 catechins, 5 terpene lactones, and 1 organic acid. Structurally, some flavonoids (luteolin and apigenin) and catechins (gallocatechin and catechin) carry one or more catechol groups that can be readily oxidized to *o*-quinone, which may covalently bind to several cysteines of SARS-CoV-2. In addition, most flavonoids and biflavonoids bears at least one Michael acceptor moiety, Michael receptor molecules' ability to interact with cysteines related with protein function may make them effective and long-lasting modulators of enzyme activity.

Cysteine modification profiling of SARS-CoV-2 3CL^{pro} modified by GEB50

A chemoproteomic approach was used to identify the cysteine sites covalently modified by GBE50 on SARS-CoV-2 3CL^{pro}. For getting a high peptide coverage, we compare two sample preparation methods, including a commonly used method (acetonitrile precipitation of proteins followed by in-solution enzymatic digestion) and the filter aided sample preparation (FASP). According to **Table S2**, the sequence coverage of target protein peptide map was only 65.69%, while the coverage of cysteine-containing peptide was only 58.33%. The low sequence coverage and poor confidence might be due to high protein losses during sample processing. Proteins might denature during protein precipitation, which is a key drawback because it makes the pellet difficult to re-solubilize. By contrast, peptide coverage of FASP method (the denatured enzyme-inhibitor mixture was prepared followed by enzymatic digestion on the membranes of 10 kDa ultrafiltration tubes) could reach to 85.95%, and more satisfactorily, the coverage of cysteine-containing peptides was up to 91.67%. It is noted that cysteines in the catalytic domain and cysteines essential for the formation of dimeric form were almost all detected.

Afterwards, we systematically characterized the modified peptides in SARS-CoV-2 3CL^{pro} after co-incubation with GBE50. According to Table 1, altogether 26 peptides modified by at least 15 small molecules (15 different molecular weights) were identified. The MS^2 spectra of all modified peptides of 3CL^{pro} were demonstrated in **Figs.S41-S59**. It can be seen from Table 1 that some constituents in GBE50 (such as luteolin and gallocatechin) can bind to the catalytic cysteines (such as Cys22, Cys85), while several constituents (such as sciadopitysin and bilobetin) can bind to the key cysteines at the dimer interface (such as Cys156 and Cys16). Several less important cysteines in SARS-CoV-2 3CL^{pro} (such as Cys128, Cys160) were also found to be modified. Considering the existence of isomers in GBE50 and the fact that some compounds can modify more than one site in target protein, 18 constituents (including two pairs of isomers) with covalent binding potentials to 3CL^{pro} were chosen to subsequent anti-SARS-CoV-2 3CL^{pro} activity analysis.

Anti-3CL^{pro} effects of the GBE50 constituents

To verify the inhibitory potential of the above mentioned 18 ingredients from GBE50 anti-SARS-CoV-2 3CL^{pro}, we collected these compounds and incubated individual compound with the target protein to measure their inhibitory effect using three different inhibitor doses (1, 10 and 100 μM). As shown in Fig. 3, 11 ingredients were found with strong to moderated anti-SARS-CoV-2 3CL^{pro} effect, while 7 ingredients showed weak anti-3CL^{pro} activity. Chemcial structures of 18 ingredients were shown in Fig. 4. Futher investigations showed that the newly identified 11 ingredients from GBE50 show time- and dose- dependent inhibitory effects on SARS-CoV-2 3CL^{pro} (Fig. 5). Of all the ingredients tested, gallocatechin exhibited the strongest anti-3CL^{pro} effect, showing the IC_{50} value of 0.98 μM after 63-min pre-incubation. Several biflavonoids, such as sciadopitysin and ginkgetin, also showed strong anti-3CL^{pro} effects, with the IC_{50} values are less than 10 μM (after 63 min pre-incubation). The IC_{50} values of 11 constituents in GBE50 were listed in Table 2. The IC_{50} value of the positive inhibitor (myricetin) was calculated to be 0.66 μM (Fig. 5). These results clearly demonstrate that most of the identified SARS-CoV-2 3CL^{pro} inhibitors in GBE50 are time-

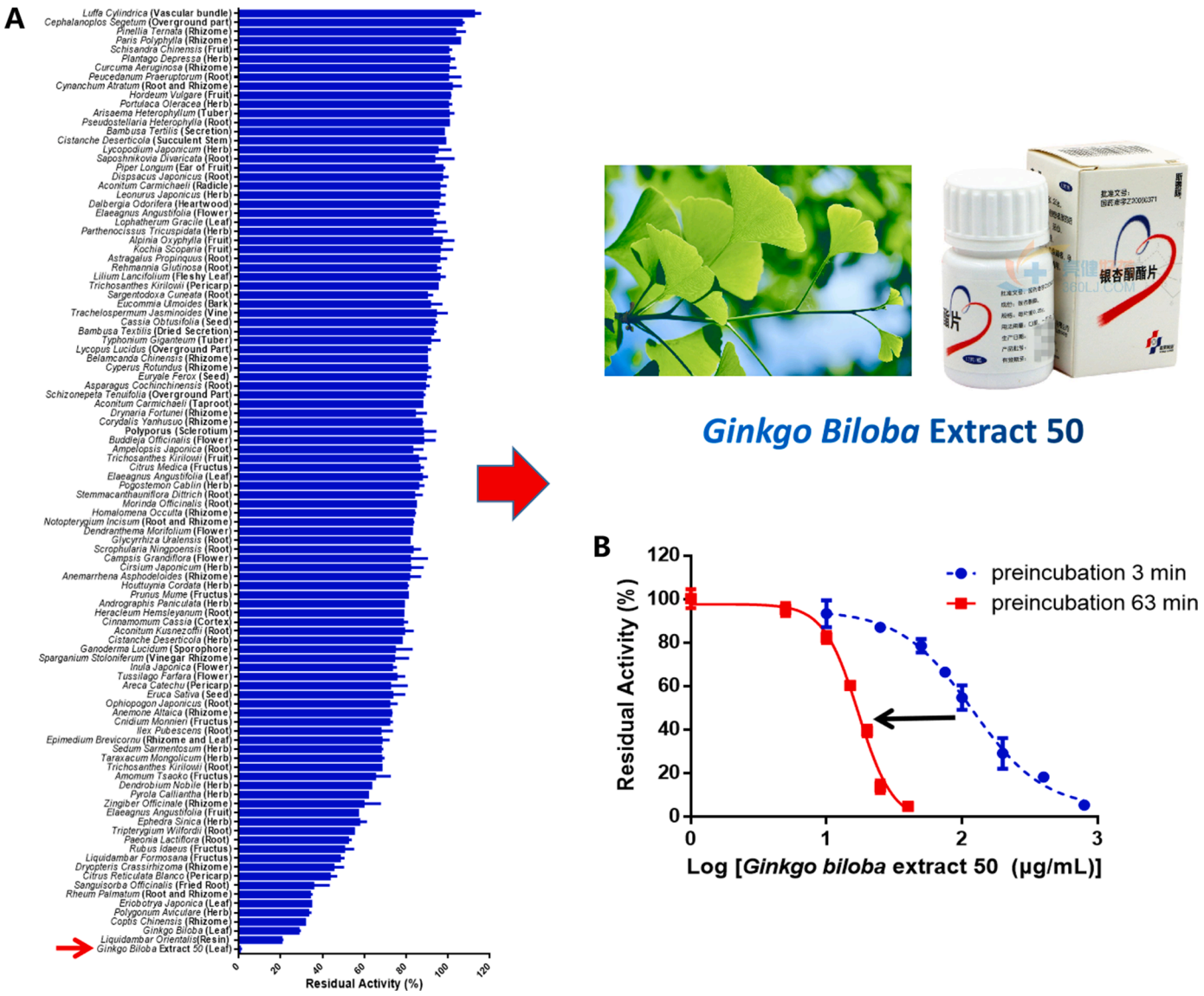

Fig. 2. (A) The inhibitory effects of 104 herbal products (100 μg/ml, final concentration) against SARS-CoV-2 $3CL^{pro}$. (B) The inhibition curves of *Ginkgo biloba* extract 50 against SARS-CoV-2 $3CL^{pro}$ following short (3 min) and long (63 min) incubation time.

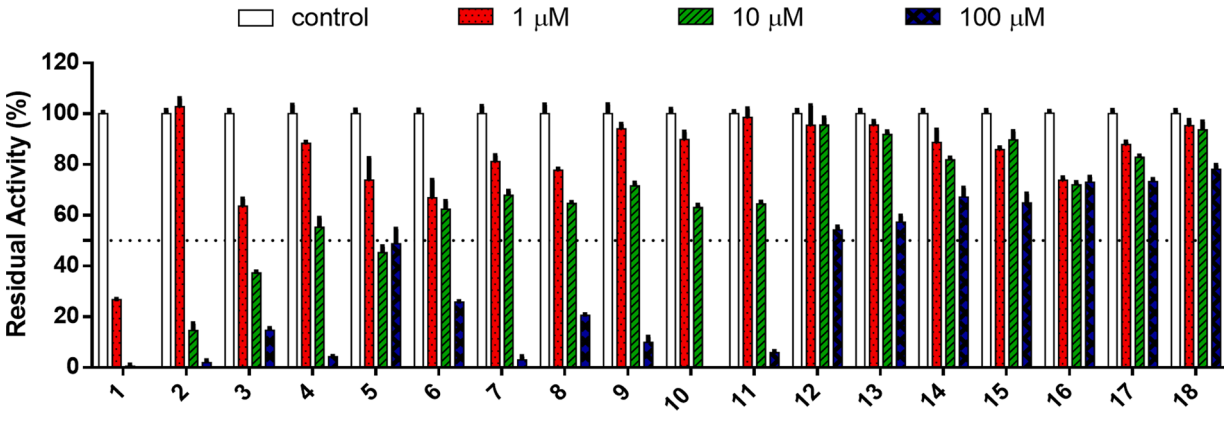

Fig. 3. The anti-SARS-CoV-2 $3CL^{pro}$ effects of 18 constituents from GBE50. The chemical structures and compound names of these constituents were shown in Fig. 4.

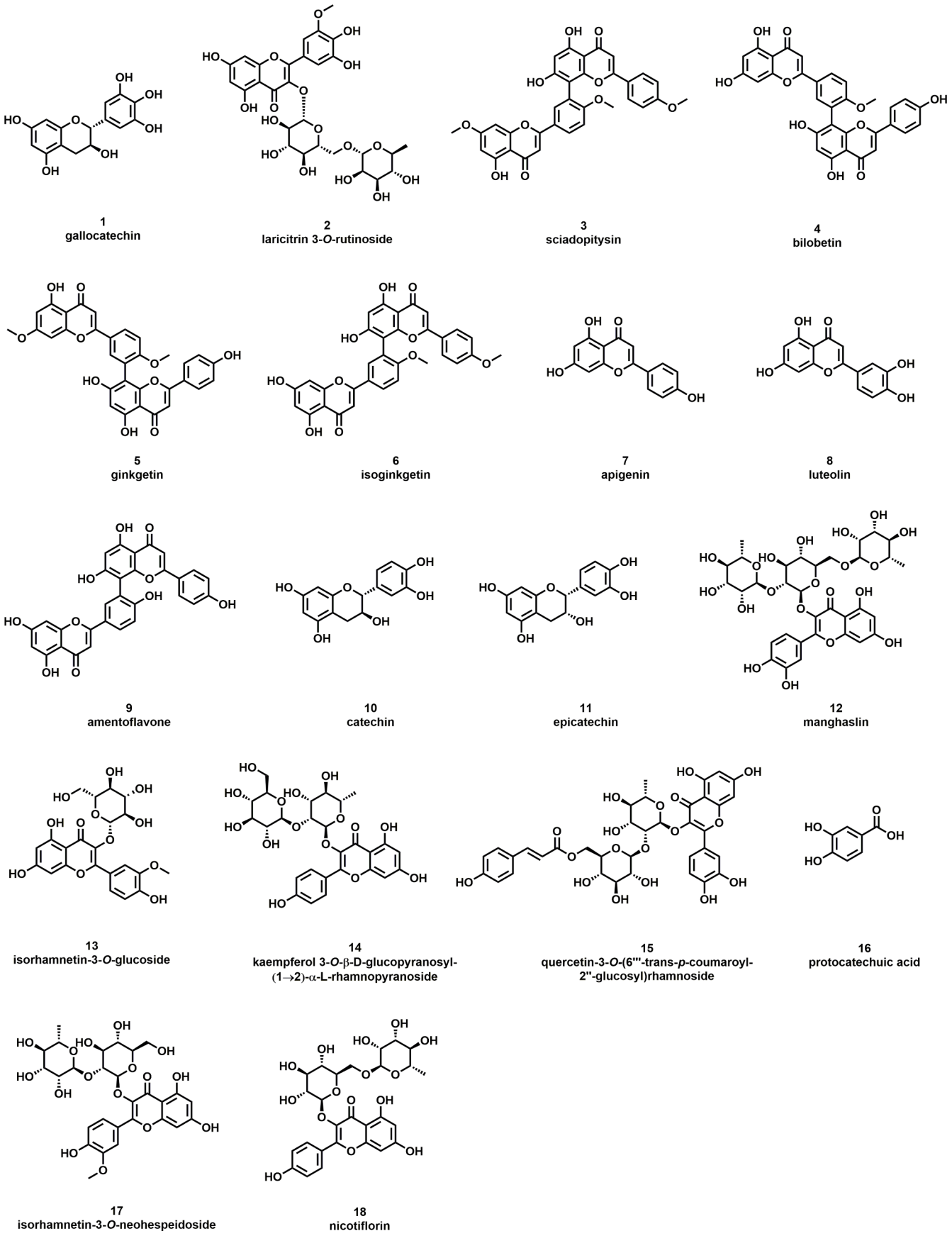

Fig. 4. Structures of the newly identified covalent inhibitors in the *Ginkgo biloba* extract 50.

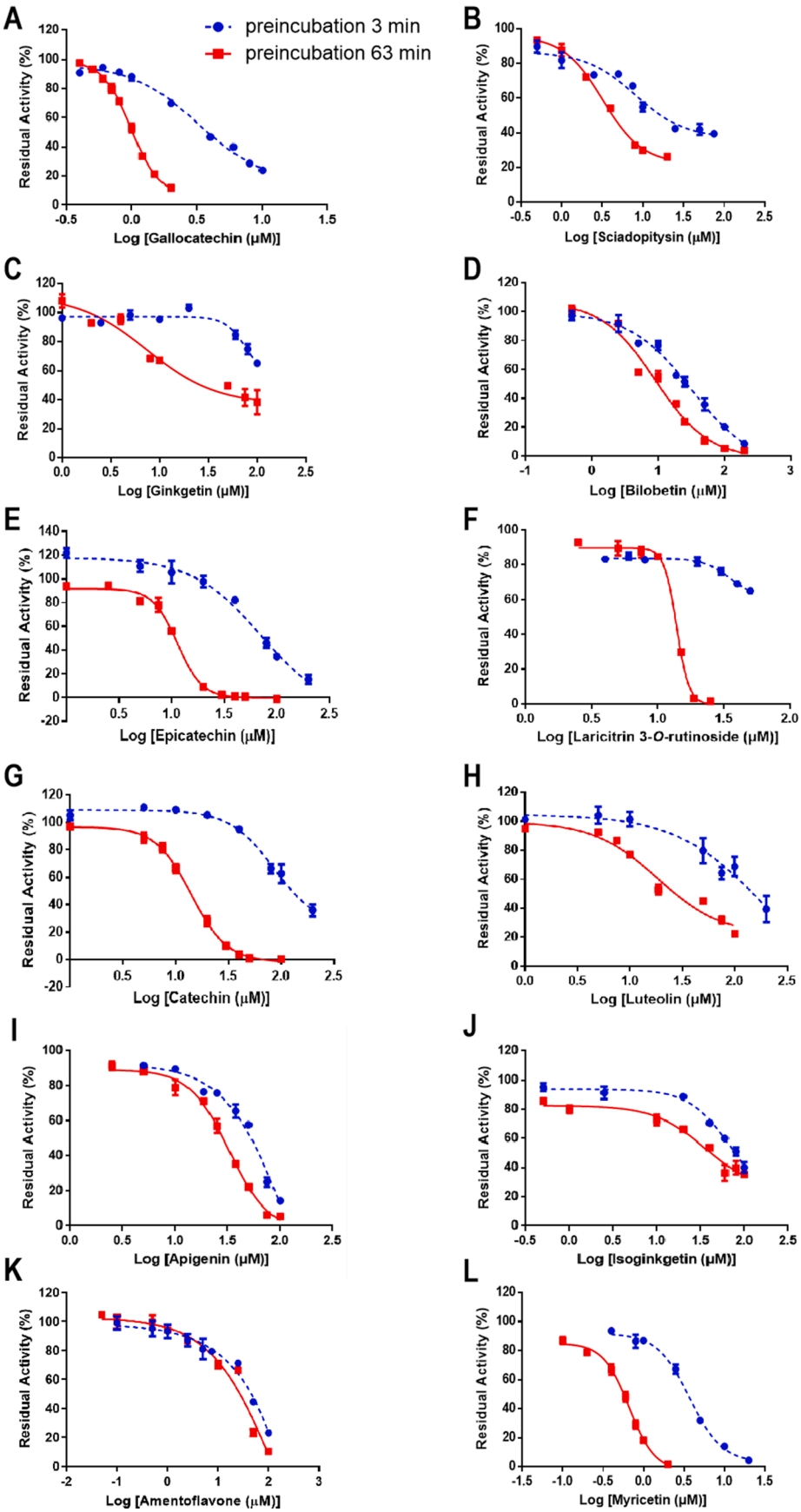

Fig. 5. Dose- and time- dependent inhibition curves of gallocatechin (A), sciadopitysin (B), ginkgetin (C), bilobetin (D), epicatechin (E), laricitrin 3-*O*-rutinoside (F), catechin (G), luteolin (H), apigenin (I), isoginkgetin (J), amentoflavone (K), and the positive inhibitor myricetin (L) against SARS-CoV-2 $3CL^{pro}$. All data are shown as the means ± SD ($n = 3$).

Table 2
IC_{50} values of the natural constituents in *Ginkgo biloba* extract 50 against SARS-CoV-2 $3CL^{pro}$.

No.	Compound	IC_{50} (μM)		Ratio	K_I (μM)	K_{inact} (min^{-1})
		3 min	63 min			
1	gallocatechin	3.23	0.98	3.30	2.52	0.03
2	sciadopitysin	8.14	3.21	2.54	7.59	0.01
3	ginkgetin	76.54	7.42	10.32		
4	bilobetin	32.08	9.06	3.54		
5	epicatechin	70.61	11.35	6.22		
6	laricitrin 3-*O*-rutinoside	35.43	13.86	2.56		
7	catechin	85.87	14.09	6.09		
8	luteolin	194.5	18.40	10.57		
9	apigenin	114.9	31.70	3.62		
10	isoginkgetin	70.82	35.18	2.01		
11	amentoflavone	82.14	42.86	1.92		
12	*myricetin	3.71	0.66	5.62		

* a naturally occurring positive inhibitor of SARS-CoV-2 $3CL^{pro}$.

dependent inhibitors, and in combination with the identified covalently modified peptides, confirming that these ingredients act as covalent inhibitors (rather than reversiable inhibitors) of SARS-CoV-2 $3CL^{pro}$.

Inactivation kinetics of gallocatechin and sciadopitysin against SARS-CoV-2 $3CL^{pro}$

The inactivation effects of gallocatechin and sciadopitysin were further investigated by performing a set of inactivation kinetics. As depicted in Fig. 6, gallocatechin and sciadopitysin could strongly inactivate the catalytic activity of SARS-CoV-2 $3CL^{pro}$ in both dose- and time-dependent manners. The K_I values for gallocatechin and sciadopitysin were determined as 2.52 and 7.59 μM, respectively, while the K_{inact} values for gallocatechin and sciadopitysin were calculated to be 0.03 and 0.01 min^{-1}, respectively. These findings suggest that gallocatechin and sciadopitysin are two potent and naturally occurring covalent inhibitors against SARS-CoV-2 $3CL^{pro}$.

Covalent docking simulations

To further explore the covalent binding mechanisms of gallocatechin and sciadopitysin to SARS-CoV-2 $3CL^{pro}$, covalent docking simulations were conducted. The docking poses with the lowest S score of gallocatechin and sciadopitysin were chosen for receptor-ligand interaction analysis. As shown in Fig. 7, gallocatechin covalently bound to Cys85 and Cys128, and sciadopitysin covalently bound to Cys85 and Cys156 of $3CL^{pro}$. At site of Cys85, Asp187 and Arg40 were consistent interactive residues with gallocatechin and sciadopitysin through charge interactions (Pi-Cation or Pi-Anion) and hydrogen bonding. For gallocatechin at the Cys85 site, hydrophobic interactions (Pi-Sigma, Alkyl and Pi-Alkyl) aimed to immobilize the ligand on the protein surface. Sciadopitysin forms two additional hydrogen bonds with residues around Cys85 (Asn180 and Pro184). Cys128 also combined with gallocatechin firmly, as gallocatechin formed attractive Pi-Cation with Lys100, Pi-Alkyl with Cys156 and hydrogen bond with Tyr101. Whereas at Cys156 site only one additional hydrogen bond connected Glu288 with sciadopitysin (**Fig. S60**). These observations from covalent docking simulations suggest that gallocatechin and sciadopitysin can interact with several major residues on SARS-CoV-2 $3CL^{pro}$ after covalently modify this key anti-viral target. Meanwhile, the covalent docking simulations of myricetin into SARS-CoV-2 $3CL^{pro}$ was also conducted and the results were compared with the crystal $3CL^{pro}$-myricetin structure 7B3E (Kuzikov et al., 2021). As shown in **Fig. S61**, the predicted docking pose of myricetin by MOE was highly overlapped with the reported experimental conformation, suggesting that MOE covalent docking was a reliable tool for studying the interactions between

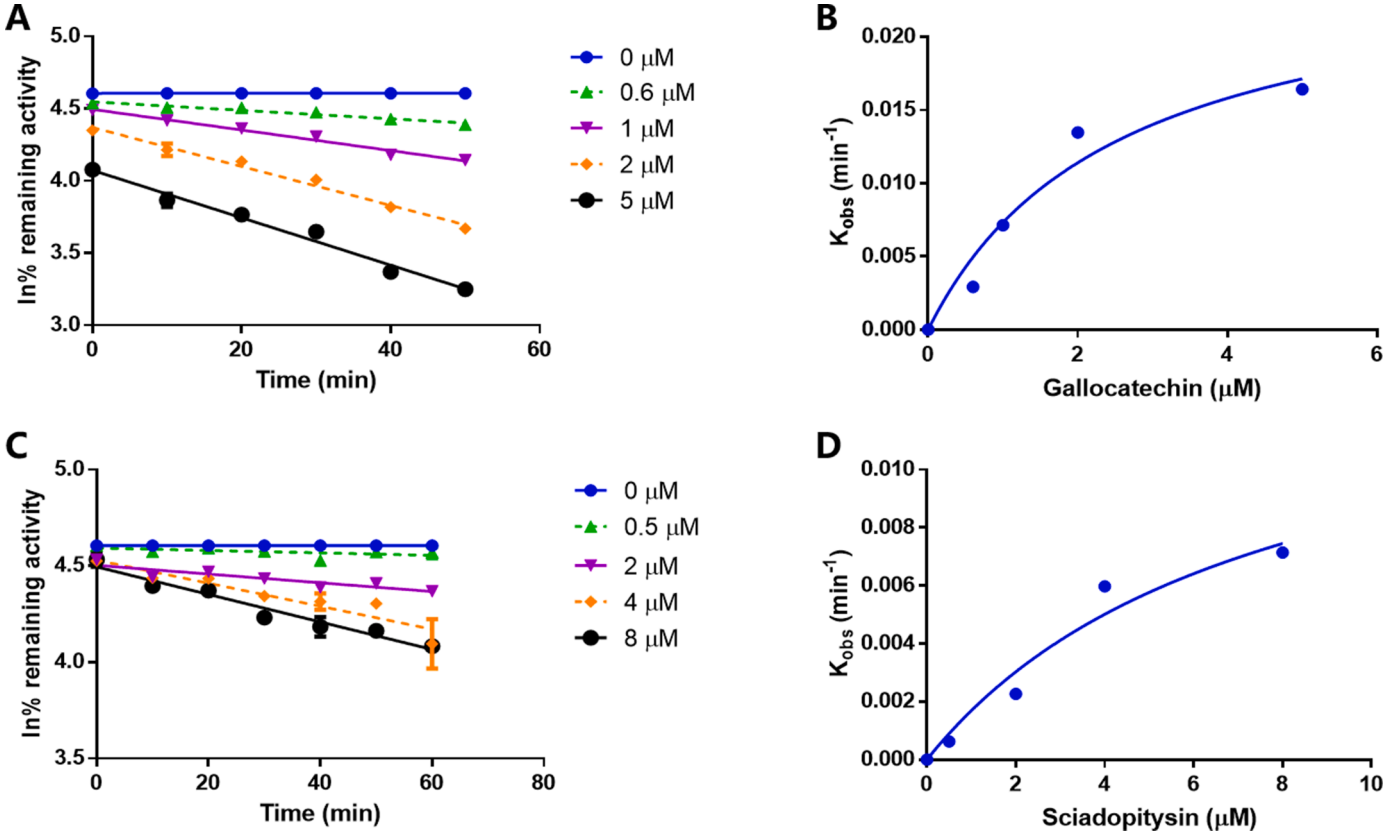

Fig. 6. The inactivation plots of gallocatechin and sciadopitysin against SARS-CoV-2 $3CL^{pro}$.

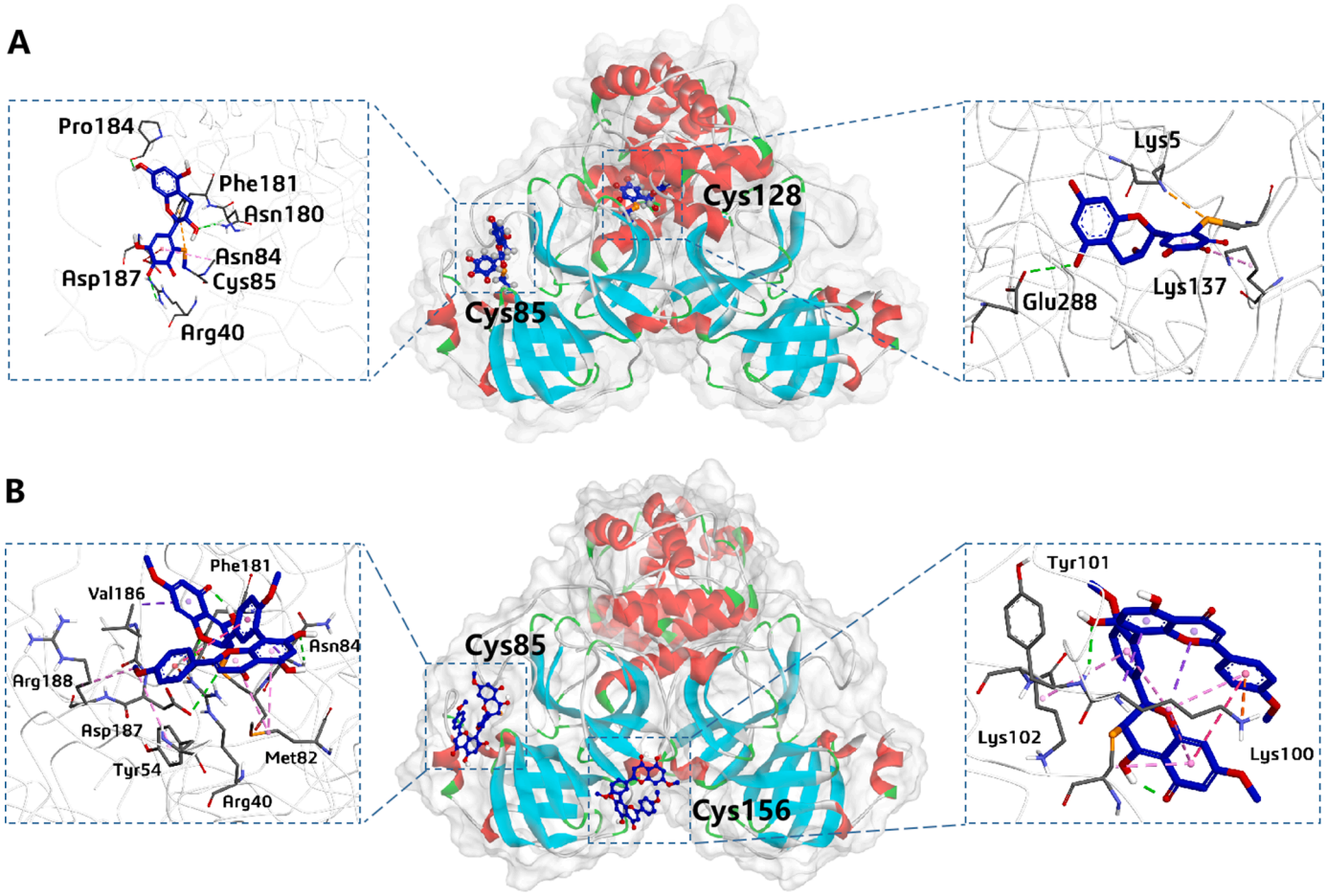

Fig. 7. A) The covalent binding poses of gallocatechin on Cys85 and Cys128 in SARS-CoV-2 3CL^{pro} (middle). Global view of gallocatechin interactions (sides). B) The covalent binding poses of sciadopitysin on Cys85 and Cys156 in SARS-CoV-2 3CL^{pro} (middle). Global view of sciadopitysin interactions (sides). The protein structures were shown in secondary structures. Helices are red, beta sheets are cyan, turns are green, and coils are white. Conventional hydrogen bonds are shown in green. Carbon hydrogen bonds are shown in light green. Pi-Sulfur is shown in orange. Pi-Pi T-shaped is shown in magenta. Pi-sigma is shown in purple. Alkyl and Pi-Alkyl are shown in pink.

small-molecule ligand and SARS-CoV-2 3CL^{pro}.

Discussion

The ongoing outbreak of COVID-19 has brought a significantly negative impact on public health, the global economy and social stability. Among all the validated therapeutic targets against COVID-19, SARS-CoV-2 3CL^{pro} has been validated as a key anti-COVID-19 target, primarily because the essential role of this protease in viral replication. Although the herbal medicines have been frequently used for treating viral diseases (including inflenza and COVID-19) (Adhikari et al., 2021), deciphering the key constituents in herbal medicines contributing to their antiviral effects is also a big challenge. To address this issue, this study aimed to construct a practical platform for highly efficient discovery of the SARS-CoV-2 3CL^{pro} covalent inhibitors in herbs. Considering that covalent inhibitors can modify some important cysteines on SARS-CoV-2 3CL^{pro}, a novel comprehensive approach for highly efficient discovery of the 3CL^{pro} covalent inhibitors from herbal medicines were proposed, *via* integrating a panel of state-of-the-art techniques including fluorescence-based 3CL^{pro} inhibition assay, mass spectrometry-based global analysis of herbal medicines, and chemoproteomic profiling of cysteine-modified peptides.

The newly proposed platform for highly efficient discovery of the SARS-CoV-2 3CL^{pro} covalent inhibitors from herbal medicines includes at least three key steps and involves multiple key points. Firstly, fluorescence-based 3CL^{pro} inhibition assay should be used for large-scale screening of the herbal medicines with significant time-dependent inhibition potentials (IC_{50} fold-shift > 2.0). Secondly, a comprehensive chemical analysis of the target herb should be performed to get the accurate chemical information of all constituents in the herb of interest. Meanwhile, the major fragment ions and the predominant fragmentation pathways of the herbal constituents should also be elucidated. Thirdly, considering the complexity of herbal medicines, the purified target protein should be used for chemoproteomic profiling of the cysteine-modified peptides. This step is a key step to identify the constituents that can covalently modify target enzyme. To get the information of the modify peptides as completely as possible, the peptide coverage should be as high as possible (more preferably at 100%). The platform is applicable to other cysteine-rich proteases.

According to the above key steps, this study successfully found a herbal medicine (GBE50) with significant time-dependent inhibition potential and identified the SARS-CoV-2 3CL^{pro} covalent inhibitors from this herbal medicine. Following high-throughput screening of anti-SARS-CoV-2 3CL^{pro} effects of over one hundred herbal products, GBE50 was discovered with the strongest anti-SARS-CoV-2 3CL^{pro} effect, and its inhibitory activity was enhanced significantly after long pre-incubation (the IC_{50} value was decreased about 6.9 folds). In this case, a comprehensive chemical analysis of GBE50 was constructed through high-resolution mass spectrometry, while a total of 38 constituents were identified from GBE50. Next, 26 peptides modified by 18 constituents in

GBE50 were identified by a chemoproteomic approach. These 18 constituents include three classes of natural products (namely flavonoids, biflavonoids and catechins), while the terpene lactones in GBE50 are incapable to covalently modify this anti-viral target. Afterwards, 18 ingredients were tested for their ability to inhibit SARS-CoV-2 $3CL^{pro}$, while the results showed that flavonoids, biflavonoids and catechins showed moderate to strong anti-$3CL^{pro}$ effects. Considering that the terpene lactones are major constituents in GBE50, we also tested their anti-SARS-CoV-2 $3CL^{pro}$ effects. The results amply supported that these compounds did not inhibit SARS-CoV-2 $3CL^{pro}$ ($IC_{50} > 100$ μM). These findings are consistent with the previously reported results that terpene lactones hardly inhibit SARS-CoV-2 $3CL^{pro}$ (Xiong et al., 2021a). All evidence strongly suggest that the newly adapted strategy allows for the rapid discovery of the categories of major ingredients from herbal medicines that can covalently modify SARS-CoV-2 $3CL^{pro}$.

Among all identified naturally occurring covalent inhibitors in GBE50, gallocatechin and sciadopitysin demonstrated the most potent anti-SARS-CoV-2 $3CL^{pro}$ effects. It has been reported that gallocatechin and sciadopitysin show a wide range of biological activities including anti-inflammatory, antioxidant, antiviral, anti-tumor and anti-coagulation activities (Li et al., 2022; Zuo et al., 2021), these two agents may bring beneficial effects to the COVID-19 patients. In the future, gallocatechin and sciadopitysin could be used as lead compounds for the development of novel anti-CoV drugs *via* targeting both $3CL^{pro}$ and other targets (such as anti-inflammatory or anticoagulation target). As shown in Fig. 7 and S60, the catecholic moiety in gallocatechin is an essential moiety for SARS-CoV-2 $3CL^{pro}$ inhibition, owing to this moiety could create strong interactions with this protease. By contrast, other phenolic groups of gallocatechin could not create strong interactions with this protease, indicating that the phenolic groups at A ring of phenolic groups could be further modified to create more hydrophobic interactions with the surrounding hydrophobic amino acids aiming to develop more potent SARS-CoV-2 $3CL^{pro}$ inhibitors. Similarly, the hydroxyl groups of sciadopitysin did not interact strongly with SARS-CoV-2 $3CL^{pro}$. These groups could also be modified to create more hydrophobic interactions with the surrounding hydrophobic amino acids. Considering that the biflavones are now available from total synthesis (Ndoile and van Heerden, 2013), the medicinal chemists can synthesize more biflavones with high structural diversity for comprehensive studies on structure-$3CL^{pro}$ inhibition relationships in the future.

The newly adapted strategy authenticated the SARS-CoV-2 $3CL^{pro}$ covalent inhibitors from herbal medicines and characterized the covalently modified sites mainly by comprehensive profiling the modified peptides *via* searching the adducted peptides (the molecular weights of the herbal constituents were added in the peptides), thus the isomers with the same molecular weight presented in one herbal medicine should be further verified one by one using the reference standards. Interestingly, in this study, two isomers distributed in GBE50 (ginkgetin and isoginkgetin) were discovered to have differential SARS-CoV-2 $3CL^{pro}$ inhibitory effects. Ginkgetin was found as a potent $3CL^{pro}$ inhibitor ($IC_{50} = 7.42$ μM), while isoginkgetin was a moderate $3CL^{pro}$ inhibitor ($IC_{50} = 35.18$ μM). In order to accurate identify the covalently modified sites of ginkgetin and isoginkgetin, these two compounds were individually co-incubated with SARS-CoV-2 $3CL^{pro}$ under physiological conditions and the modified peptides were assayed one by one. The results clearly demonstrated that ginkgetin could covalently modify Cys38 (a key catalytic cysteine in the catalytic pocket on SARS-CoV-2 $3CL^{pro}$), while isoginkgetin could not modify Cys38 but this agent could covalently modify Cys117 of $3CL^{pro}$ that brought little effect on SARS-CoV-2 $3CL^{pro}$ activity. These observations further explained why ginkgetin displayed more potent anti-SARS-CoV-2 $3CL^{pro}$ effect than that of isoginkgetin.

As a marketed herbal medicine, GBE50 has been approved by the FDA as a new class II Chinese medicine and has been broadly utilized to treat cardiovascular and cerebrovascular diseases in more than 100 countries (Yang et al., 2022). Extensive investigations on GBE50 have found that GBE50 contains numerous pharmacological activities (such as antioxidantive, anti-inflammatory and antiviral activities) and good safety profiles (Xiong et al., 2021a). In this study, GBE50 was also found with potent inactivation effect of SARS-CoV-2 $3CL^{pro}$, while multiple ingredients from GBE50 were identified naturally occurring $3CL^{pro}$ covalent inhibitors. According to these results, GBE50 might be helpful in the prevention and treatment of COVID-19. Up to now, a few synthetic SARS-CoV-2 $3CL^{pro}$ covalent inhibitors have been approved by the FDA as novel anti-COVID-19 drugs (Lobo-Galo et al., 2021). We noticed that the modified sites of some constituents in GBE50 are different from that of the synthetic anti-COVID-19 agents. Therefore, it is conceivable that the combination use of GBE50 and synthetic drugs may have synergistic effects on the inactivation of SARS-CoV-2 $3CL^{pro}$. Currently, there are both covalent inhibitors and non-covalent inhibitors of SARS-CoV-2 $3CL^{pro}$ have been reported, while their inhibitory mechanisms are totally different. When the two inhibitors are used together, the non-covalent inhibitor may flip the conformation of $3CL^{pro}$ after binding on this protease. In some cases, several internal cysteines of $3CL^{pro}$ will be exposed, which may facilitate the covalent modifications by the covalent inhibitors. In future, the combination use of the covalent inhibitors and non-covalent inhibitors against SARS-CoV-2 $3CL^{pro}$ should be investigated in depth. On the other hand, the COVID-19 patients are often prone to thrombosis and inflammation (Manjunath and Thimmulappa, 2022), the excellent anti-inflammatory and anti-coagulant activity of GBE50 will be very beneficial for the COVID-19 patients to combat COVID-19 related disorders. In the future, the *in vivo* effects and the clinical effectiveness of the combination (GBE50 and western anti-COVID-19 agents) against COVID-19 needs further in-depth investigations.

Conclusion

In summary, a new strategy for efficient discovering the covalent inhibitors of SARS-CoV-2 $3CL^{pro}$ from herbal medicines was proposed *via* integrating fluorescence-based $3CL^{pro}$ inhibition assay, mass spectrometry-based chemical analysis of herbs, and chemoproteomic profiling of cysteine modified peptides. Fluorescence-based high-throughput screening against SARS-CoV-2 $3CL^{pro}$ effects of 104 herbal products showed that GBE50 displayed the most potent anti-SARS-CoV-2 $3CL^{pro}$ effect, showing an IC_{50} value of 17.19 μg/ml after 63 min preincubation. UHPLC-Q-HRMS based chemical profiling of GBE50 revealed that a total of 38 ingredients were distributed in GBE50, while chemoproteomic profiling of cysteine-modified peptides showed that 18 ingredients in GBE50 could covalently modify SARS-CoV-2 $3CL^{pro}$. The anti-SARS-CoV-2 $3CL^{pro}$ assay showed that 11 out of 18 ingredients were identified as strong to moderate SARS-CoV-2 $3CL^{pro}$ inhibitors. Within all tested ingredients, gallocatechin and sciadopitysin showed the most potent anti-SARS-CoV-2 $3CL^{pro}$ effects, with the IC_{50} values of 0.98 μM and 3.21 μM, respectively. All these findings provide strong evidence in support of the anti-CoV effect of GBE50. Most importantly, the newly proposed strategy can be readily adopted to discover more efficacious covalent inhibitors against other cystein-rich target proteins.

CRediT authorship contribution statement

Ya-Ni Zhang: Methodology, Data curation, Writing – original draft. **Guang-Hao Zhu:** Software, Visualization. **Wei Liu:** Methodology, Data curation. **Yuan Xiong:** Methodology, Data curation. **Qing Hu:** Methodology. **Xiao-Yu Zhuang:** Writing – review & editing. **Gui-Hua Jia:** Methodology. **Wei-Dong Zhang:** Project administration. **Guang-Bo Ge:** Supervision, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2023.154796.

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