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Discovery of naturally occurring inhibitors against SARS-CoV-2 3CL^{pro} from *Ginkgo biloba* leaves *via* large-scale screening

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ABSTRACT

3-Chymotrypsin-like protease (3CL^{pro}) is a virally encoded main proteinase that is pivotal for the viral replication across a broad spectrum of coronaviruses. This study aims to discover the naturally occurring SARS-CoV-2 3CL^{pro} inhibitors from herbal constituents, as well as to investigate the inhibitory mechanism of the newly identified efficacious SARS-CoV-2 3CL^{pro} inhibitors. Following screening of the inhibitory potentials of eighty herbal products against SARS-CoV-2 3CL^{pro}, *Ginkgo biloba* leaves extract (GBLE) was found with the most potent SARS-CoV-2 3CL^{pro} inhibition activity (IC_{50} = 6.68 μg/mL). Inhibition assays demonstrated that the ginkgolic acids (GAs) and the bioflavones isolated from GBLE displayed relatively strong SARS-CoV-2 3CL^{pro} inhibition activities (IC_{50} < 10 μM). Among all tested constituents, GA C15:0, GA C17:1 and sciadopitysin displayed potent 3CL^{pro} inhibition activities, with IC_{50} values of less than 2 μM. Further inhibition kinetic studies and docking simulations clearly demonstrated that two GAs and sciadopitysin strongly inhibit SARS-CoV-2 3CL^{pro} *via* a reversible and mixed inhibition manner. Collectively, this study found that both GBLE and the major constituents in this herbal product exhibit strong SARS-CoV-2 3CL^{pro} inhibition activities, which offer several promising leading compounds for developing novel anti-COVID-19 medications *via* targeting on 3CL^{pro}.

1. Introduction

The pandemic of coronavirus disease 2019 (COVID-19), an unprecedented disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is sweeping expeditiously all over the world [1,2]. Owing to the high infectivity and lack of efficacious drugs, COVID-19 is putting unprecedented pressures to public health, economic development, and global safety [3]. As to April 12th, 2021, COVID-19 has infected over 136.5 million people and has killed more than 2.9 million people worldwide [4]. Currently, scientists around the world are trying to find efficacious therapeutics for combating COVID-19, *via* targeting on several validated therapeutic targets [5]. Among all identified therapeutic targets for treating COVID-19, the conserved 3-chymotrypsin-like protease (3CL^{pro}), a key enzyme responsible for coronavirus replication, has been validated as a pivotal therapeutic target for fighting this pandemic [6]. 3CL^{pro} is a virally encoded main proteinase that implicated in the maturation of the functional proteases by cleaving the viral polyproteins [7,8], dysfunction or strong inhibition on 3CL^{pro} will block the replication of multiple coronaviruses.

The crucial role of 3CL^{pro} in coronavirus replication has aroused great interests in discovery of efficacious 3CL^{pro} inhibitors as the leading compounds for the development of novel antiviral agents. Although a wide range of phytochemicals and synthetic molecules have been reported with anti-3CL^{pro} activity [9,10], the efficacious SARS-CoV-2 3CL^{pro} inhibitors with high potency and favorable safety profiles are rarely reported. Thus, it is an urgent need for discovering more efficacious SARS-CoV-2 3CL^{pro} inhibitors to develop novel antiviral agents to fight COVID-19. It is well-known that the phytochemicals from medicinal plants remain one of the major sources for discovery of the leading compounds for drug development [11], while some natural compounds (such as flavonoids [12], triterpenes [13], and phenolic compounds [14]) have been reported with 3CL^{pro} inhibition activity, encouraging us to find more potent SARS-CoV-2 3CL^{pro} inhibitors from herbal medicines. For this purpose, a large-scale screening campaign was performed

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by us, in which a total of 80 herbal products were collected and the inhibition potentials of these herbal products against SARS-CoV-2 $3CL^{pro}$ were assayed *via* a biochemical approach. After screening, we noticed that the *Ginkgo biloba* leaves extract (GBLE) displayed the most potent SARS-CoV-2 $3CL^{pro}$ inhibition activity (IC_{50} = 6.68 μg/mL), which encouraged us to further characterize the key constituents in *Ginkgo biloba* leaves responsible for SARS-CoV-2 $3CL^{pro}$ inhibition.

This study intended to assay the inhibition potentials for the major phytochemicals isolated from GBLE, as well as to explore the inhibitory mechanisms of the newly identified SARS-CoV-2 $3CL^{pro}$ inhibitors from this herbal product. For these purposes, a total of twenty natural products isolated from *Ginkgo biloba* leaves were collected, while the inhibitory potentials of these compounds against SARS-CoV-2 $3CL^{pro}$ were carefully assayed. Among all tested natural compounds, the bioflavones and ginkgolic acids (GAs) were identified as strong SARS-CoV-2 $3CL^{pro}$ inhibitors (IC_{50} < 10 μM). To further reveal the inhibitory mechanisms of the newly identified anti-SARS-CoV-2 $3CL^{pro}$ molecules, inhibition kinetic analyses and docking simulations were performed. All these studies are helpful for deciphering the key active ingredients in GBLE responsible for SARS-CoV-2 $3CL^{pro}$ inhibition, which will facilitate the development of novel anti-COVID-19 agents deriving from natural products.

2. Experimental

2.1. Chemicals, reagents, and herbal products

Dithiothreitol (DTT), imidazole, phenylmethylsulfonyl fluoride (PMSF), sodium chloride (NaCl), hydrochloric acid (HCl) and lysozyme were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Super Nuclease was purchased from Sino Biological Inc. (Beijing, China). Plasmid was customized from GENEWIZ, Inc. (Beijing, China). *Escherichia coli* (*E. coli*) BL21 (DE3) was gained from Shanghai Weidi Biotechnology Co., Ltd. (Shanghai, China). Tris-Base was gained from Amresco (USA). 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid (HEPES) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Seventy-nine herbal products were provided by Tianjiang Pharmaceutical Co., Ltd. (Jiangsu, China). The GBLE was prepared as depicted in a previous report [15]. Bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, amentoflavone, ginkgetin, bilobetin, isoginkgetin, sciadopitysin, kaempferol, quercetin, quercitrin, apigenin, isorhamnetin, genkwanin, luteolin were ordered from Chengdu Preferred Biological Technology Co., Ltd. (Chengdu, China). Ginkgolic acids (C13:0, C15:0, C15:1, and C17:1) were gained from National Institutes for Food and Drug Control (China). Ebselen were provided from TCI (Shanghai, China), as the positive inhibitor for SARS-CoV-2 $3CL^{pro}$ [16]. The purities of all tested natural compounds were higher than 96%. The stock solution of each compound (100 mM) was prepared in DMSO and stored at 4 °C. Ethylene Diamine Tetraacetic Acid (EDTA) was obtained from Dalian Meilun Biotechnology Co. LTD. (Dalian, China). Dabcyl-KNSTLQSGLRKE-Edans (fluorescent substrate) was provided by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd. (Shanghai, China), with the purity of 99%. The stock solution of this fluorescent substrate was prepared by Millipore water and stored at 4 °C. The buffer was prepared using Millipore water (Millipore, Bedford, USA) and stored at 4 °C for further use. HPLC grade DMSO (Tedia, USA) was used throughout.

2.2. Expression and purification of SARS-CoV-2 $3CL^{pro}$

A Smt3-SARS-CoV-2 $3CL^{pro}$ fusion construct was *Escherichia coli* (*E. coli*) codon optimized and cloned into the pET29a (+) vector [17,18]. The $3CL^{pro}$ cleavage site (SAVLQS↓GFRK) located at the N-terminus of SARS-CoV-2 $3CL^{pro}$. The C-terminus of SARS-CoV-2 $3CL^{pro}$ was followed by a modified HRV 3C protease cleavage site (SGVTFQ↓GP) and a His6-tag. The expression of SARS-CoV-2 $3CL^{pro}$ was induced in autoinduction method in *E. coli* stain BL21 (DE3) at 18 °C. After 36 h of induction, cells were harvested by centrifugation. Cell pellet was resuspended in lysis buffer (pH 8.0, 25 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, 0.1 mg/mL lysozyme, 25 U/mL SuperNuclease) and incubated for 30 min at room temperature. Cells were lysed by sonication on ice, then cell debris was removed by centrifugation at 18000 rpm at 4 °C for 30 min. The supernatant was collected and purified by Ni-NTA agarose. The SARS-CoV-2 $3CL^{pro}$ was purified in further on a Superdex 200 10/300 GL column in protein storage buffer (pH 7.4, 25 mM HEPES, 150 mM NaCl, 1 mM DTT).

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

Dabcyl-KNSTLQ↓SGLRKE-Edans was a fluorescence quenching peptide with 12-amino acid based on the fluorescence resonance energy transfer (FRET), which would be recognized and cleaved by $3CL^{pro}$ (the cleavage site: ↓) [19]. SARS-CoV-2 $3CL^{pro}$ inhibition assay was performed in a 96-well plate filled with the reaction mixture (100 μL, final volume). Initially, the analytes, SARS-CoV-2 $3CL^{pro}$ (4 μg/mL, final concentration), 100 mM PBS (pH 7.4, 1 mM EDTA) were incubation at 37 °C for 40 min. The hydrolytic reaction was initiated by addition of the substrate Dabcyl-KNSTLQSGLRKE-Edans (20 μM, final concentration). The hydrolytic product emits strong fluorescence signals around 490 nm, which can be recorded by a microplate reader (SpectraMax® iD3, Molecular Devices, Austria).

2.4. Determination of inhibition constants of GA C15:0, GA C17:1, and sciadopitysin

The inhibition kinetics were performed a set of inhibition assay, in which increasing concentrations of each inhibitor (GA C15:0, GA C17:1 or sciadopitysin) and various concentrations of Dabcyl-KNSTLQSGLRKE-Edans were used. The data were analyzed according to the equations of various inhibition kinetic modes (competitive mode, non-competitive mode, and mixed mode), while the second plot of Lineweaver-Burk plots slopes were used to determine the inhibition constant (K_i) values, as previously reported [20,21].

2.5. Molecular docking simulations

The structure of SARS-CoV-2 $3CL^{pro}$ was downloaded from the Protein Data Bank (PDB Code: 6XHU) [22]. AutoDockTools 1.5.6 was used for the preparation of input files of SARS-CoV-2 $3CL^{pro}$ and the ligands by adding and merging non-polar hydrogen atoms and adjusting charges. Druggable cavities of SARS-CoV-2 $3CL^{pro}$ were recommended by CavityPlus [23]. The implementation of docking simulations was using AutoDock Vina (1.1.2). The grid boxes were placed at the catalytic binding pockets and recommended cavities. The output files were imported into Discovery Studio Visualizer 2020 software in order to check ligand-receptor interactions.

2.6. Statistical analysis

The IC_{50} values and the K_i values were evaluated by nonlinear regression using GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, USA).

3. Results and discussion

3.1. Screening of the inhibition potentials of 80 herbs against SARS-CoV-2 $3CL^{pro}$

Firstly, the inhibition potentials of 80 herbal products (100 μg/mL, final concentration) on SARS-CoV-2 $3CL^{pro}$ were screened by using Dabcyl-KNSTLQSGLRKE-Edans as a FRET-based substrate. As depicted in Fig. 1, among all tested herbal products, GBLE displayed the most

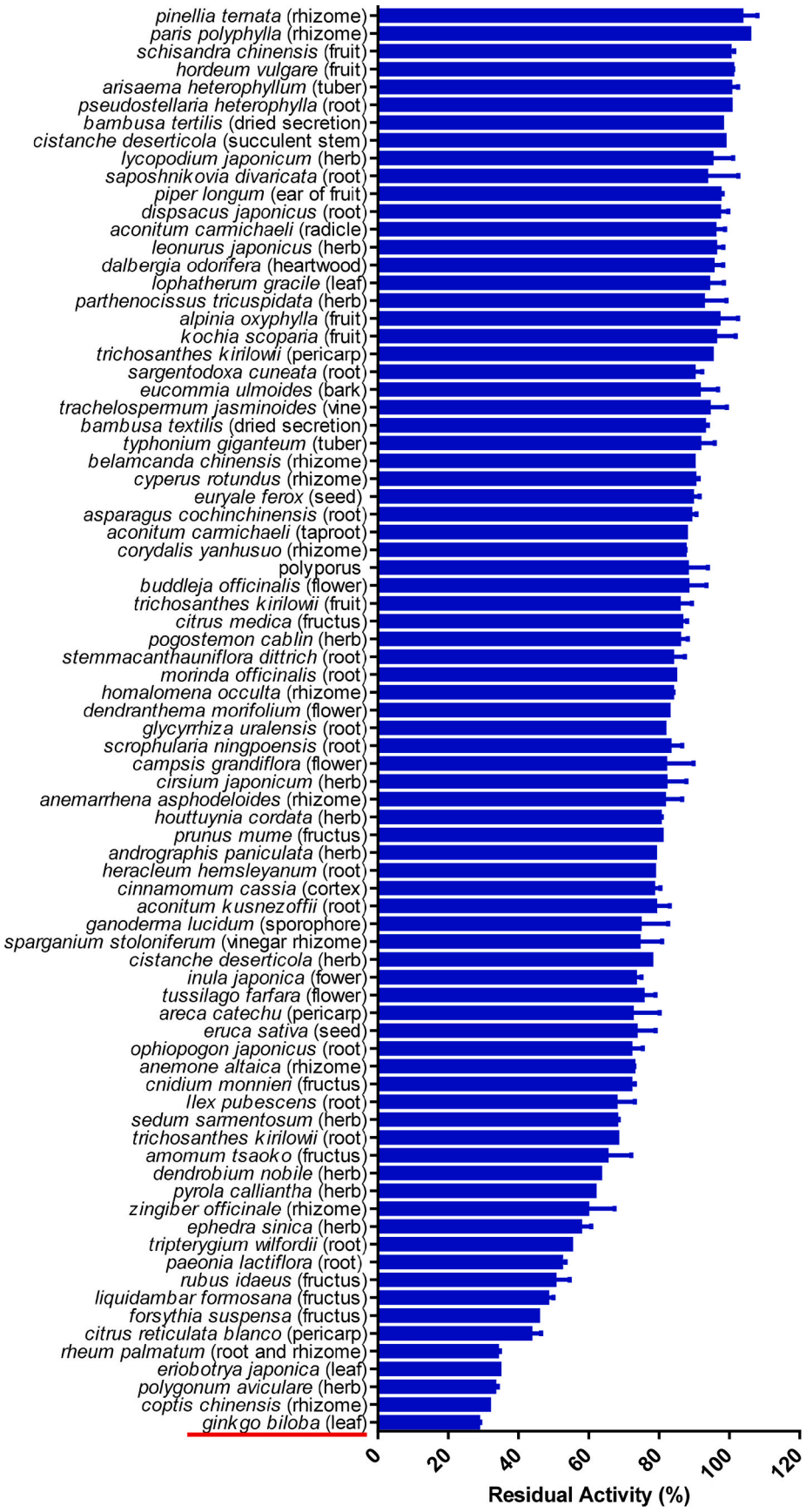

Fig. 1. The inhibitory effects of eighty herbal products (100 μg/mL, final concentration) against SARS-CoV-2 $3CL^{pro}$.

potent 3CL^{pro} inhibition activity, and this herbal product could inhibit nearly 70 % hydrolytic activity of SARS-CoV-2 3CL^{pro} at 100 μg/mL. To further quantify the inhibitory activity of GBLE, the dose-inhibition curve of GBLE on SARS-CoV-2 3CL^{pro} was plotted using increasing dosages (from 1.25 to 100 μg/mL). As shown in Fig. 2, GBLE dose-dependently inhibited the target enzyme, with the calculated IC_{50} value of 6.68 μg/mL. This finding suggests that GBLE contains naturally occurring inhibitors against SARS-CoV-2 3CL^{pro}.

3.2. Inhibition of SARS-CoV-2 3CL^{pro} by the constituents in GBLE

Next, twenty known major constituents in GBLE were collected, including the bioflavones (amentoflavone, ginkgetin, bilobetin, isoginkgetin, sciadopitysin), flavonoids (kaempferol, quercetin, apigenin, isorhamnetin, genkwanin, luteolin, quercetrin), the terpene lactones (bilobalide, ginkgolide A, ginkgolide B, ginkgolide C), as well as GAs (C13:0, C15:0, C15:1, and C17:1) [24]. To discover SARS-CoV-2 3CL^{pro} inhibitors in an efficient way, three inhibitor concentrations (1 μM, 10 μM and 100 μM) of each tested compound were used for screening of the SARS-CoV-2 3CL^{pro} inhibition activity (Fig. 3). Among all tested constituents isolated from GBLE, two flavonoids (kaempferol, quercetrin) and the terpene lactones displayed extremely weak inhibition on SARS-CoV-2 3CL^{pro} ($IC_{50} > 100$ μM). By contrast, the bioflavones and the GAs exhibited relatively strong inhibitory effects on SARS-CoV-2 3CL^{pro} ($IC_{50} < 10$ μM). Meanwhile, five flavonoids (genkwanin, quercetin, isorhamnetin, luteolin, apigenin) in GBLE showed moderate inhibition on SARS-CoV-2 3CL^{pro}, with the IC_{50} values ranged from 10 μM to 100 μM. The dose-inhibition curves of these phytochemicals isolated from GBLE were depicted in Fig. 4 and Fig.S5 while their IC_{50} values were listed in Table 1. It is obvious from Table 1 that the GAs and a bioflavone (sciadopitysin) display strong SARS-CoV-2 3CL^{pro} inhibition activities, while the IC_{50} values are lower than that of the positive inhibitor (ebselen).

3.3. Inhibition kinetics of three newly identified SARS-CoV-2 3CL^{pro} inhibitors

To further investigate the inhibitory mechanisms of the newly identified 3CL^{pro} inhibitors in GBLE, three potent SARS-CoV-2 3CL^{pro} inhibitors (GA C15:0, GA C17:1 and sciadopitysin) isolated from GBLE were selected for inhibition kinetic studies. As shown in Fig. 5, both inhibition kinetic plots and Lineweaver-Burk plots of these three agents against SARS-CoV-2 3CL^{pro} clearly demonstrated that two GAs (C15:0 & C17:1) and sciadopitysin strongly inhibited SARS-CoV-2 3CL^{pro} *via* a mixed-inhibition manner, with K_i values of 0.73 μM, 1.02 μM and 2.96 μM for GA C15:0, GA C17:1, and sciadopitysin, respectively. These results suggest that the GAs and sciadopitysin act as mixed-type inhibitors against SARS-CoV-2 3CL^{pro}, implying that these agents may bind on this key target enzyme at both catalytic site and another non-catalytic site.

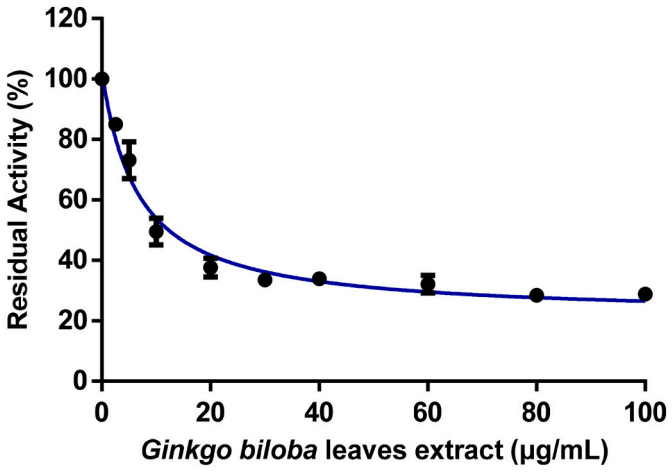

Fig. 2. The dose-inhibition curve of the *Ginkgo biloba* leaves extract against SARS-CoV-2 3CL^{pro}.

3.4. Molecular docking simulations

To gain deeper insights into the inhibitory mechanisms of the GAs and sciadopitysin against SARS-CoV-2 3CL^{pro}, a dimer crystal structure of SARS-CoV-2 3CL^{pro} was used for docking simulations [22]. Firstly, the possible ligand-binding pockets in SARS-CoV-2 3CL^{pro} were assessed by utilizing the ligandability and druggability scores calculated by CavityPlus. As shown in Fig. 6 and Table S1, three druggable pockets (the V site located at the dimer interface, and two catalytic sites of both chain A and chain B) were identified as possible ligand-binding pockets of this key enzyme. Docking simulations demonstrated that GA C15:0, GA C17:1 and sciadopitysin could be well-docked into both the V site and the catalytic sites of SARS-CoV-2 3CL^{pro} (Fig. 7), while the predicted binding energies of three compounds binding on either the V site or the catalytic site were listed in Table S2.

The key interactions between three newly identified 3CL^{pro} inhibitors and SARS-CoV-2 3CL^{pro} were also analyzed. As shown in Fig. S7 and Fig. S8, the GAs (including C15:0 and C17:1) created strong interactions with some residuals surrounding on the V site mainly on hydrophobic interactions and hydrogen bonding. Meanwhile, these two hydrophobic compounds could also occupy the catalytic site of SARS-CoV-2 3CL^{pro} *via* hydrophobic interactions and hydrogen bond interactions, as well as the Pi-sulfur interaction with the key catalytic residue (Cys145). By contrast, sciadopitysin interacted with the residuals surrounding on both the V site and the catalytic site mainly on hydrophobic interactions and hydrogen bonding (Fig. S9). Notably, the phenolic groups of sciadopitysin at both the C-19 and the C-21 sites played crucial roles in binding on SARS-CoV-2 3CL^{pro}, owing to that the C-19 and the C-21 phenolic groups could create strong interactions with Asp216 and Asn119 for the catalytic site and the V site, respectively.

Currently, there are limited therapeutic options available to treat COVID-19 in clinical settings. To fight against COVID-19 effectively, the medicinal chemists have made great endeavors to discover efficacious agents for blocking replication of COVID-19 virus *via* targeting on several validated therapeutic targets [6]. Among all identified therapeutic targets for treating COVID-19, 3CL^{pro} is a promising therapeutic target owing to its indispensable role in the replication of this new coronavirus [25]. Until now, the efficacious 3CL^{pro} inhibitors with high potency and favorable safety profiles are rarely reported. Thus, there is an urgent need for discovering more efficacious 3CL^{pro} inhibitors for the development of therapeutic agents for combating COVID-19. To find naturally occurring 3CL^{pro} inhibitors, a screening campaign was performed to discover the herbal medicine(s) with anti-SARS-CoV-2 3CL^{pro} activity. Within all tested eighty herbal products, GBLE exhibited the most potent SARS-CoV-2 3CL^{pro} inhibition activity, with the IC_{50} value of 6.68 μg/mL. This finding encouraged us to identify the key bioactive constituents in GBLE responsible for SARS-CoV-2 3CL^{pro} inhibition, as well as to investigate the inhibitory mechanisms of these newly identified naturally occurring 3CL^{pro} inhibitors.

As a popular herb used in both western and eastern countries, *Ginkgo biloba* leaves have been widely applied for preventing and treating a variety of human diseases, including cardiovascular disorders, pulmonary disease, and central nervous system diseases [26–28]. Increasing evidence has indicated that some major constituents (such as bioflavones and GAs) in GBLE exhibited broad antiviral activities against a wide range of DNA (such as human Cytomegalovirus) and RNA (such as SARS-CoV, Ebola virus and human immunodeficiency virus) viruses [12,29–31]. However, the anti-SARS-CoV-2 effects of GBLE have not been reported yet. In this study, our findings revealed that both GBLE and several phytochemicals in this herbal extract (such as the bioflavones and GAs) could strongly inhibit the hydrolytic activity of

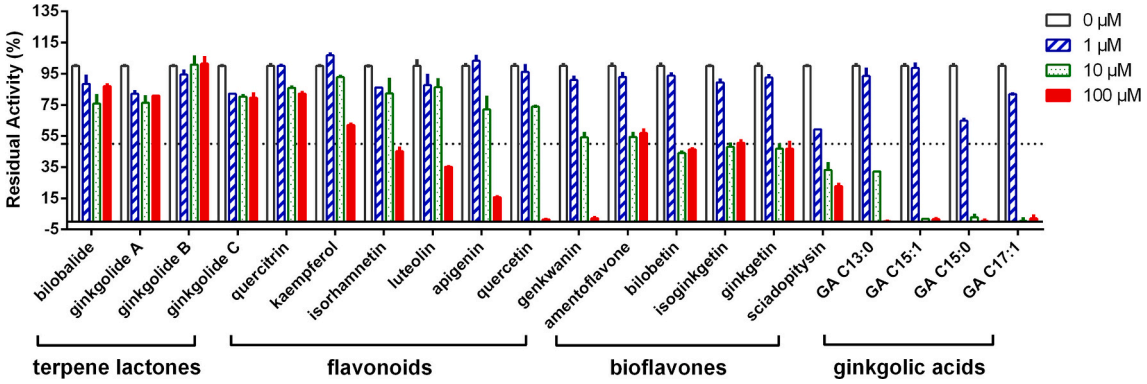

Fig. 3. Inhibitory effects of chemical constituents in *Ginkgo biloba* leaves (1 μM, 10 μM, 100 μM, final concentration) against SARS-CoV-2 $3CL^{pro}$.

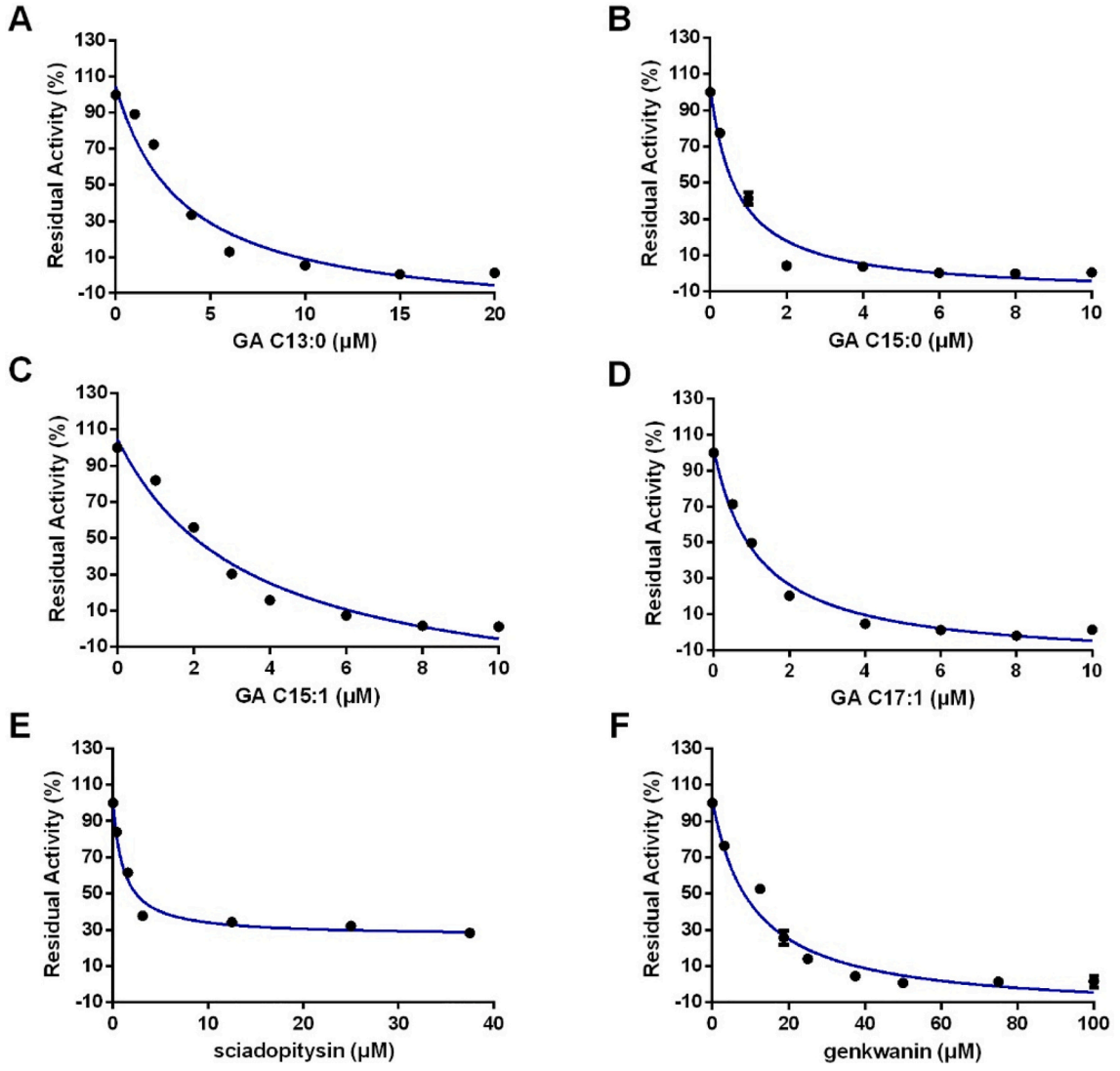

Fig. 4. Dose-inhibition curves of GA C13:0 (A), GA C15:0 (B), GA C15:1 (C), GA C17:1 (D), sciadopitysin (E), genkwanin (F) against SARS-CoV-2 $3CL^{pro}$.

$3CL^{pro}$, suggesting that GBLE and its major phytochemicals might block the replication of SARS-CoV-2 *via* targeting this pivotal enzyme. In addition to the anti-$3CL^{pro}$ activity, previous studies have revealed that GBLE constituents (such as the bioflavones) could modulate the blood coagulation and ameliorates inflammation both *in vitro* and *in vivo* [28,32–34]. It is well-known that COVID-19 is a complex, multi-organ and heterogeneous illness, the severe disease cases are frequently accompanied by the hypercoagulable inflammatory state [35,36]. As one of the most popular herbal medicines, *Ginkgo biloba* leave extract possess a variety of beneficial effects, such as anti-coagulate, anti-inflammatory, anti-oxidative lowing blood pressure, anti-obesity, and other biological activities [26,32,37–39]. Thus, it is easily conceivable

Table 1
Inhibition parameters the constituents in *Ginkgo biloba* leaves against SARS-CoV-2 $3CL^{pro}$.

No.	Phytochemical Class	Compound	MW	IC_{50} (μM)	K_i (μM)	Inhibition mode	Goodness of fit (R^2)
1	Terpene lactones	bilobalide	326.30	> 100	–	–	
2		ginkgolide A	408.40	> 100	–	–	
3		ginkgolide B	424.40	> 100	–	–	
4		ginkgolide C	440.40	> 100	–	–	
5	Flavonoids	quercitrin	448.38	> 100	–	–	
6		kaempferol	286.24	> 100	–	–	
7		genkwanin	284.27	10.62 ± 1.89	–	–	
8		quercetin	302.24	12.65 ± 2.37	–	–	
9		isorhamnetin	316.26	31.59 ± 16.60	–	–	
10		luteolin	286.24	74.86 ± 18.15	–	–	
11		apigenin	270.24	84.94 ± 18.69	–	–	
12	Bioflavones	sciadopitysin	580.54	1.09 ± 0.19	2.96	Mixed	0.97
13		ginkgetin	566.51	2.98 ± 0.86	–	–	
14		isoginkgetin	566.51	2.33 ± 0.46	–	–	
15		amentoflavone	538.46	8.65 ± 3.72	–	–	
16		bilobetin	552.48	11.19 ± 2.41	–	–	
17	Ginkgolic acids	GA C17:1	374.57	1.19 ± 0.15	1.02	Mixed	0.99
18		GA C15:0	348.53	0.70 ± 0.13	0.73	Mixed	0.97
19		GA C15:1	346.51	3.45 ± 0.78	–	–	
20		GA C13:0	320.47	3.57 ± 0.90	–	–	
21	Positive inhibitor	ebselen[a]	274.18	2.61	–	–	

[a] Positive inhibitor for SARS-CoV-2 $3CL^{pro}$.

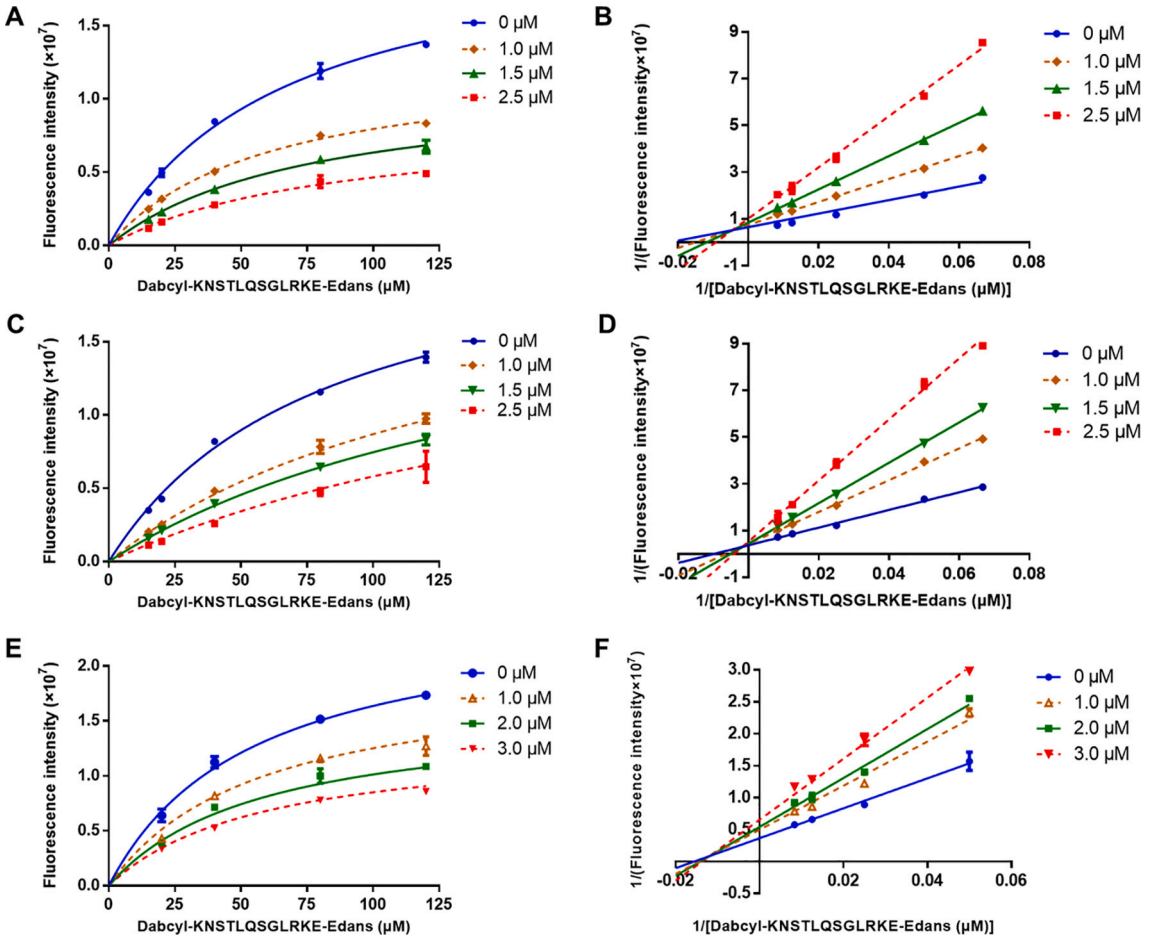

Fig. 5. Inhibition kinetic plots (left) and the Lineweaver-Burk plots (right) of GA C15:0 (A, B), GA C17:1 (C, D) and sciadopitysin (E, F) against SARS-CoV-2 $3CL^{pro}$.

that GBLE may bring significant benefits to the COVID-19 patients with certain underlying medical conditions (such as cardiovascular disease, stroke, high blood pressure, diabetes, and obesity), by alleviating the major symptoms of both COVID-19 and other underlying health problems.

It is well-known that the bioflavones are the major class of bioactive constitutes in GBLE. Previous studies have reported that the bioflavones isolated from GBLE and other medicinal plants exhibited a wide range of beneficial effects, including anti-thrombin, anti-oxidative, anti-inflammatory, and antiviral activities [28,31,32,40]. It also has been

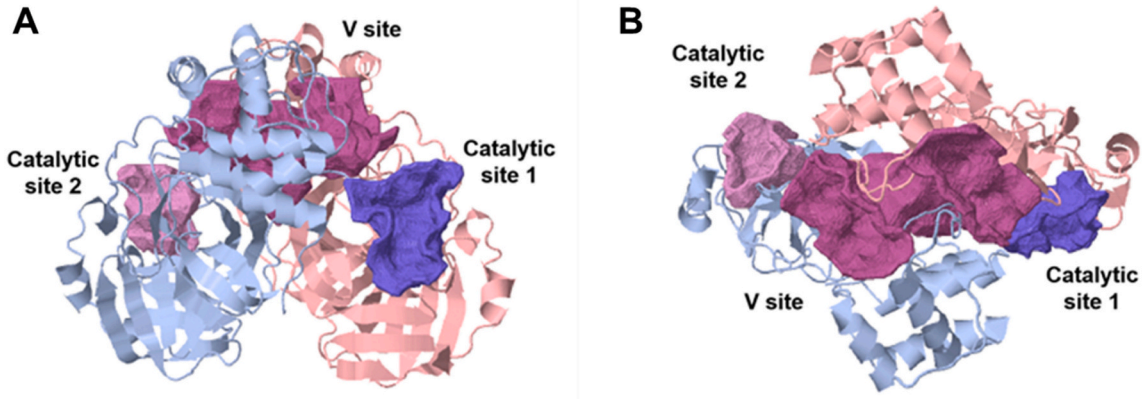

Fig. 6. Three potential combined pockets of SARS-CoV-2 3CL^{pro} were predicted with CavityPlus. A) Front view; B) Top view.

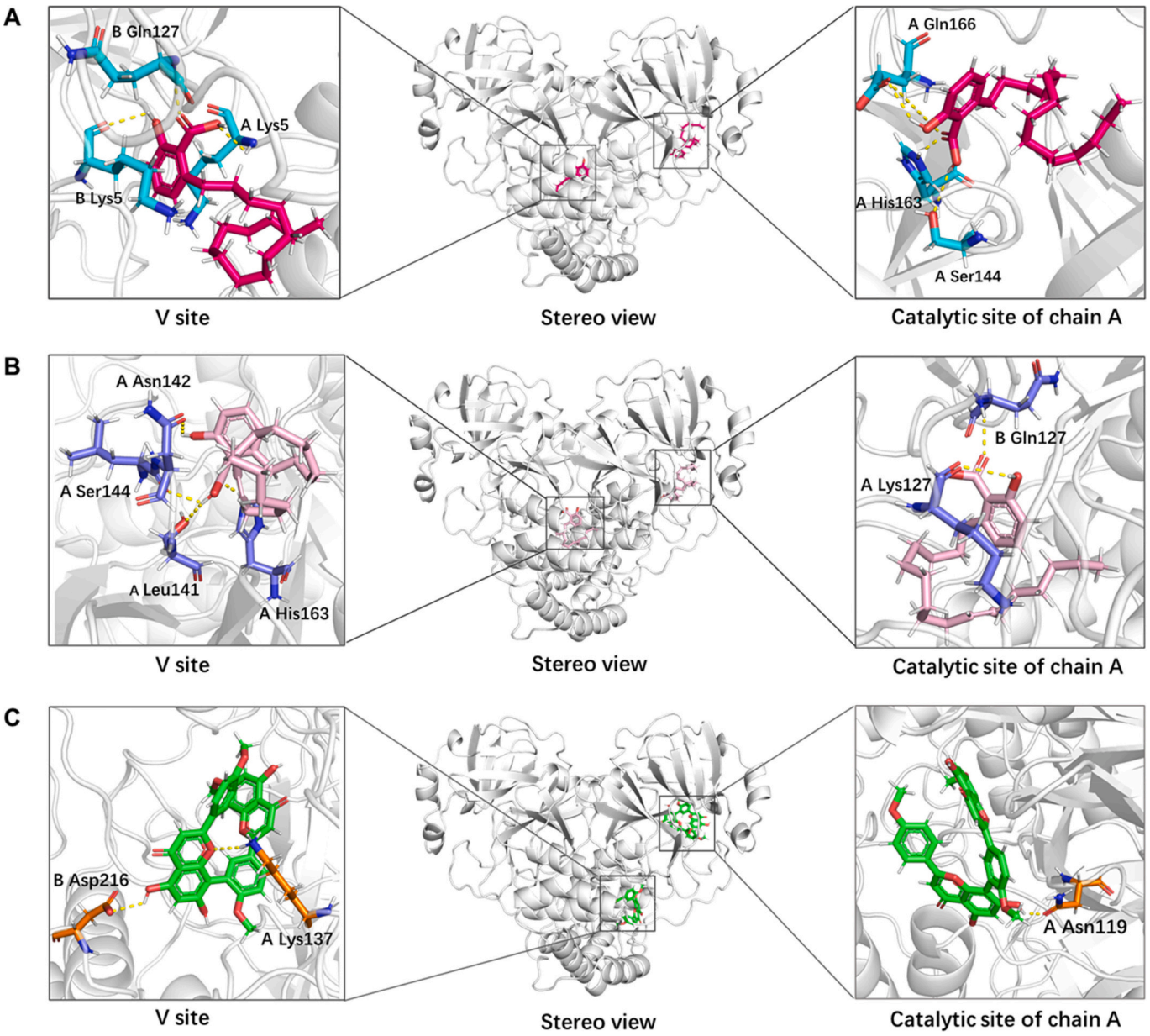

Fig. 7. The stereo view of the crystal structure SARS-CoV-2 3CL^{pro} (PDB Code: 6XHU), and the binding sites for GA C15:0 (A, red) GA C17:1 (B, light pink) and sciadopitysin (C, green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reported that the bioflavones displayed strong SARS-CoV $3CL^{pro}$ inhibition potency [31]. This study found that the bioflavones isolated from GBLE displayed strong inhibition against SARS-CoV-2 $3CL^{pro}$ potency. These findings suggested that the natural bioflavones could act as promising leading compounds for developing anti-COVID-19 or the broad-spectrum anti-CoVs agents. Nevertheless, most naturally occurring bioflavones are found with poor membrane permeability, poor solubility, and extremely poor oral bioavailability [32]. To develop more efficacious orally administrated $3CL^{pro}$ inhibitors, the natural bioflavones should be extensively modified to simultaneously improve the inhibition potency and the drug-likeness properties. Another strategy is to develop the nasal administration systems for delivery of the bioactive bioflavones to lung, for blocking replication of COVID-19 virus at this target organ and then alleviating the major symptoms of COVID-19. In addition to bioflavones, GAs in GBLE were also found with strong SARS-CoV-2 $3CL^{pro}$ inhibition activities ($IC_{50} < 5$ μM). Accumulating evidence has demonstrated that GAs possess a wide range of antiviral effects *via* disturbing the viral replication [29,30,41]. Generally, the levels of toxic ginkgolic acids in marketed *Ginkgo biloba* products are strictly limited lowering than 5 ppm, owing to that these agents could cause severe allergic reactions [42]. In these cases, GAs is not recommended as the orally administrated agents for treating COVID-19, but these abandoned ingredients could be used as the disinfection or cleaning products for external use. Structurally, the GAs isolated from GBLE bear a hydrophobic long chain and these compounds could be easily modified as the surfactants. In the future, new antiviral surfactants could be synthesized and developed using GAs as the starting materials, which also can be used in combination with other marketed disinfectants for the prevention and disinfection of broad spectrum of coronaviruses *in vitro*.

4. Conclusion

In summary, this study revealed the key ingredients in *Ginkgo biloba* leaves extract (GBLE) responsible for SARS-CoV-2 $3CL^{pro}$ inhibition and investigated the inhibitory effects of the newly identified SARS-CoV-2 $3CL^{pro}$ inhibitors isolated from GBLE. Following assessing the inhibitory potentials of twenty phytochemicals isolated from GBLE against SARS-CoV-2 $3CL^{pro}$, the bioflavones and the GAs were found with efficacious SARS-CoV-2 $3CL^{pro}$ inhibition activities ($IC_{50} < 10$ μM). Among all tested phytochemicals in GBLE, two GAs (GA C15:0, GA C17:1) and a bioflavone (sciadopitysin) displayed the most potent SARS-CoV-2 $3CL^{pro}$ inhibition activities. Inhibition kinetic analyses demonstrated these three newly identified $3CL^{pro}$ inhibitors strongly inhibit SARS-CoV-2 $3CL^{pro}$ in a reversible and mixed-inhibition manner, with K_i values of 0.73 μM, 1.02 μM and 2.96 μM, for GA C15:0, GA C17:1, and sciadopitysin respectively. Docking simulations showed that two GAs and sciadopitysin could be well-docked into two druggable pockets (the V site and the catalytic sites) of SARS-CoV-2 $3CL^{pro}$, which agreed well with the mixed-inhibition modes of these three agents. Collectively, our findings revealed the key constituents in GBLE responsible for SARS-CoV-2 $3CL^{pro}$ inhibition, which offered several promising lead compounds for developing novel $3CL^{pro}$ inhibitors to treat COVID-19 or other coronavirus.

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Declaration of Competing Interest

No competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2021.104909.

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