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Highly efficient discovery of the covalent M^{pro} inhibitors from crude Pu-erh tea by integrating biochemical and chemoproteomic approaches

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ABSTRACT: The main proteases (M^{pro}s) are hydrolases playing essential roles in the replication of β-coronaviruses including SARS-CoV-2. Herein, a highly efficient strategy was developed for discovering the M^{pro} inactivators from crude plant extract integrating target-based biochemical assay and chemoproteomic approaches. Firstly, Pu-erh tea was found to potently suppress SARS-CoV-2 M^{pro} in a time-dependent manner. Next, global chemical analysis coupling with peptide-modification profiling were used to identify the cysteine-modified constituents in Pu-erh tea. The results suggested that seven constituents in Pu-erh tea could modify SARS-CoV-2 M^{pro}, which turned out that epigallocatechin, gallocatechin and gallic acid were the most efficacious M^{pro} inactivators. Further investigations demonstrated that epigallocatechin and gallocatechin could inactivate SARS-CoV-2 M^{pro} *via* blocking the formation of the homodimers. Collectively, this work proposed a novel and practical strategy for highly efficient discovery of time-dependent inhibitors of SARS-CoV-2 M^{pro} from plant extracts, while three constituents in Pu-erh tea have emerged as robust SARS-CoV-2 M^{pro} inactivators.

Keywords: M^{pro}; Plant extract; Pu-erh tea; Global chemical analysis; Cysteine-modification profiling

1. Introduction

The worldwide COVID-19 pandemic, stemming from the highly infectious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has caused millions of deaths and generated high healthcare resource utilization, as well as shocking cost in both medical test and health care [1]. Fever, cough and fatigue are the most frequently observed clinical symptoms in COVID-19 patients, which have significantly decreased the quality of life among the COVID-19 patients [2]. For elderly patients or the COVID-19 patients with chronic diseases, it is particularly important to use efficacious and safe therapies to prevent or halt the deterioration of their quality of life [3,4]. Over the past three years, plant-based products have been frequently used as alternative therapies for attenuating the main clinical symptoms of COVID-19, while parts of therapies have gained significant attention due to their impressive health-promoting effects and high safety profiles [5-8]. Compared to the western medicines and some marketed Chinese patent medicines, plant extracts are readily

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available to the general public, convenient to use, highly safe, and do not necessarily require professional guidance from pharmacists or healthcare practitioners [9-11]. Notably, some commonly used edible plant in families (such as senna, licorice, ginger, rosemary, and *Lonicerae japonicae flos*), have now been widely used for attenuating the main clinical symptoms of COVID-19 in most of Chinese families [8,12-15]. Increasing studies have demonstrated that parts of natural constituents in plant extracts exhibit various health-promoting effects including anti-oxidative, anti-inflammatory, anti-thrombotic and anti-viral effects, which inspired us to further decipher the anti-SARS-CoV-2 substances in plant extracts, as well as to uncover their anti-SARS-CoV-2 mechanisms [16-19].

Plant extracts have a long history of utilization in the treatment of epidemics [20]. Unlike Western medicines (often consist of a single active ingredient), plant extracts are composed of a diverse array of natural compounds [5,21,22]. SARS-CoV-2 main protease (i.e., SARS-CoV-2 M^{pro}) is one of the most promising targets during the replication of this β-coronavirus [23]. The M^{pro}s, alternatively known as 3-chymotrypsin-like proteases ($3CL^{pro}$s), are a group of cysteine hydrolases that exhibit high conservation, cleaving viral polyproteins into individual functional proteins, thus playing essential roles in the replication of multiple β-coronaviruses [24-27]. Currently, researchers identified considerable number of compounds as potent inhibitors of this key protease, and some of these compounds exhibited potent anti-M^{pro} activity at the nanomolar level [28]. Additionally, most of the reported M^{pro} peptidomimetic inhibitors were structure-based covalent inhibitors that modified Cys145, including compounds like GC376, GC373, 15l, 1e, 5h and azanitrile 8 [29-34]. Non-peptidomimetic inhibitors with covalent binding properties usually contained Michael receptors, α,β-unsaturated carbonyl groups, and other cysteine-reactive functional groups that can covalently bind to critical residues like Cys145 [35]. Ebselen and its derivatives effectively functioned in open-formed intermediates to bind the thiol, and were synthesized as inhibitors of SARS-CoV-2 M^{pro} [36]. Another compound, GRL-0920, which contained a carbonyl-indole group to bind cysteine of enzyme, was identified as a covalent inhibitor that bound to Cys145 of M^{pro} [37]. Extensive investigations have also identified a range of natural-derived compounds as M^{pro} inhibitors, parts of these compounds could strongly block the enzymatic activity of M^{pro} *in vitro*. Numerous structurally diverse natural compounds and their derivatives, including flavonoids, phenolic acids, tannins, and quinones, exhibited anti-SARS-CoV-2 M^{pro} effects, with some identified as covalent inhibitors of M^{pro} [38,39]. For example, baicalin, quercetin, resveratrol, and puerarin were identified as strong inhibitors to moderate M^{pro} *in vitro*, while some of them could suppress SARS-CoV-2 replication in cells [40-43]. Natural-derived covalent M^{pro} inhibitors mainly showed superior activity to non-covalent ones [44]. Among all them, myricetin was one of the most potent natural-derived M^{pro} inactivator (IC_{50}=1.21 μmol/L), which showed significant time-dependent inhibition effect [38]. Covalent inhibitors have demonstrated long-lasting and highly effective inhibitory effects against SARS-CoV-2 M^{pro}, suggesting a promising strategy for the development of anti-COVID-19 agents [45].

Herein, to highly efficiently identify the covalent M^{pro} inhibitors from plant extracts, an integrated strategy was proposed by integrating fluorescence-based M^{pro} inhibition assay and chemoproteomic

approaches **(Fig. 1)** [38,39]. Large-scale screening of the anti-M^{pro} potentials of plant extracts found that the crude extract of Pu-erh exhibited potent inhibitory activity to M^{pro}, while the anti-M^{pro} effect of Pu-erh tea was time-dependent and such inhibition was sensitive to dithiothreitol (DTT). After then, chemical constituents in Pu-erh tea were globally analyzed utilizing UHPLC-HRMS, while the cysteine-modified peptides of M^{pro} were also comprehensively profiled to efficiently identify the constituents in Pu-erh tea that are capable of modifying the cysteine residues of M^{pro}. Consequently, seven constituents in Pu-erh tea were identified as covalent agents to modify the cysteine residues of M^{pro}, while epigallocatechin (EGC), gallocatechin (GC), and gallic acid (GA) were identified as three strong M^{pro} inactivators in Pu-erh tea. After then, a set of assays including inactivation kinetics, binding kinetics and oligomerization state analysis were used to uncover the inactivation mechanisms of these newly identified M^{pro} inactivators [46-49].

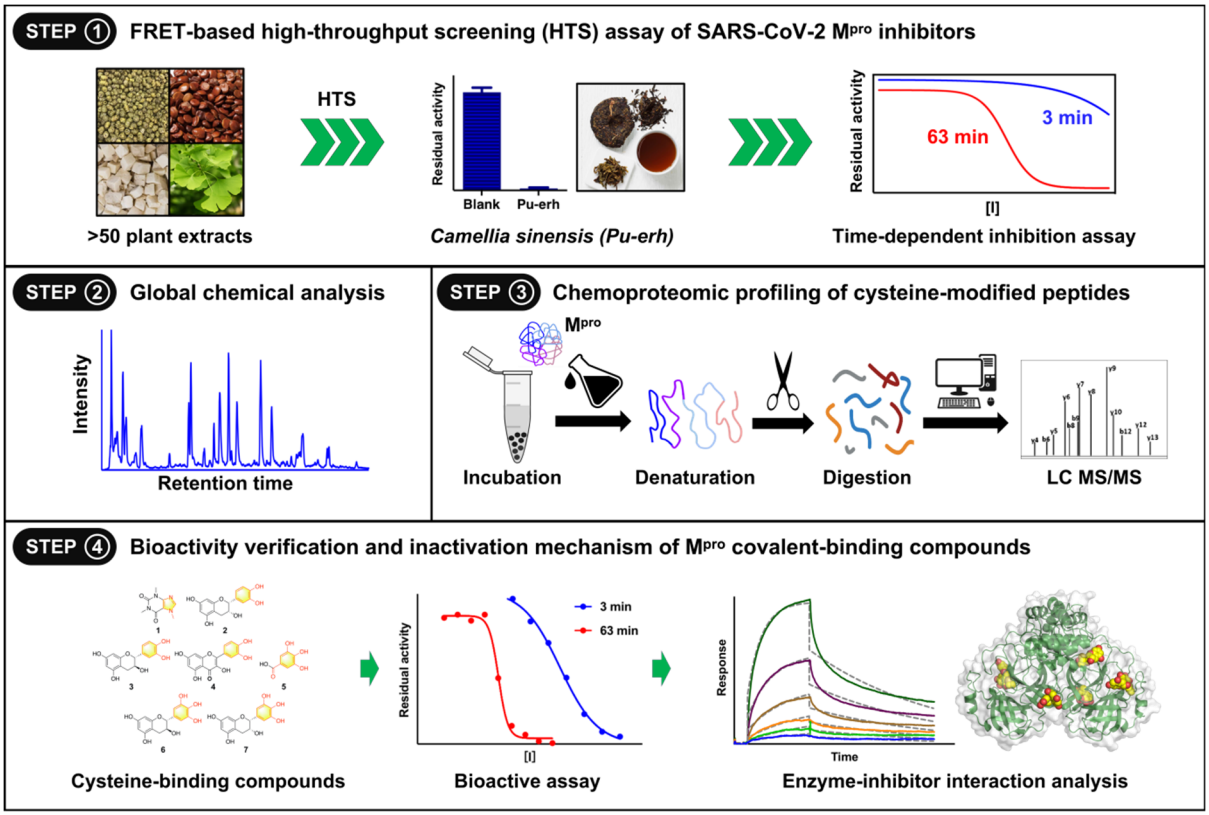

Fig. 1. Schematic illustration of highly efficient discovery of time-dependent SARS-CoV-2 M^{pro} inhibitors from plant extracts.

2. Material and methods

2.1 Chemicals and reagents

Fifty plant extracts were provided by Tianjiang Pharmaceutical Co., Ltd. (Jiangsu, China). Pu-erh tea was from Yunnan Tianshili Dipoer Biological Tea Group Co., Ltd. (Yunnan, China). M^{pro} inhibitor dihydromyricetin was from Dalian Meilun Biotech Co., Ltd. (Dalian, China). Standards of constituents in Pu-erh tea referred in this paper were all purchased from Baoji Herbest Bio-Tech Co., Ltd. (Yunnan, China). Recombinant SARS-CoV-2 M^{pro} was produced in-house using our established protocols **(Fig. S1)** [39]. The fluorescent substrate of M^{pro} (Dabcyl-KNSTLQSGLRKE-Edans, DKE) was customized by Nanjingjinsirui Science & Technology Biology Corp. (Jiangsu, China). HPLC grade methanol, acetonitrile, dimethyl

sulfoxide (DMSO), and formic acid were from Tedia Company, Inc. (Fairfield, USA). High-purity water was provided through the Millipore water purification system (Millipore, Bedford, USA).

2.2 M^{pro} inhibition assays

To evaluate the inhibition activity of M^{pro}, we employed a pre-established fluorescence resonance energy transfer (FRET)-based assay for enzyme inhibition [39]. The assay was based on reliable positive control, nirmatrelvir. The hydrolytic rates of DKE (20 μmol/L) were continuously measured within a 100 μL reaction mixture, which contained M^{pro} (4 μg/mL) and each tested plant extract or compound. The fluorescent signals (excitation: 340 nm, emission: 490 nm) were recorded using a microplate reader (SpectraMax® iD3, Molecular Devices, Austria).

2.3 Global chemical analysis of Pu-erh tea

The chemical constituents of Pu-erh tea were comprehensively analyzed by UHPLC-Q-Exactive Orbitrap HRMS using a previously established protocol [46]. Mobile phases, linear gradients, and MS conditions were provided in the supplementary information..

2.4 Chemoproteomic profiling of M^{pro} peptides modified by Pu-erh tea

The modified peptides of M^{pro} by Pu-erh tea were carefully characterized utilizing UHPLC-Q-Exactive Orbitrap HRMS as the previously reported protocol [46]. Peptide samples preparation, mobile phases, linear gradients, MS conditions, and data analysis were provided in the supplementary information.

2.5 Inactivation kinetic analyses

The inactivation kinetics of analytes against M^{pro} were analyzed accord to the previous article [38]. The hydrolytic rates of DKE (20 μmol/L) were monitored in a 100 μL reaction mixture, which contained M^{pro} (4 μg/mL) and each tested compound. The inactivation kinetic curves were fitted to a standard kinetic expression (Michaelis-Menten) to obtain the kinetic parameters:

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

2.6 Surface plasmon resonance (SPR) binding assays

The binding affinity of analytes onto M^{pro} were evaluated using SPR on Biacore T200 instruments (Cytiva, Sweden) [47]. In brief, M^{pro} was covalently attached to the CM5 chip *via* amine coupling at a flow rate of 10 μL/min in 10 mmol/L sodium acetate buffer (pH 5.5). The chip surface was activated with 50 mmol/L N-hydroxysuccinimide (NHS) and 200 mmol/L 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). M^{pro} was diluted to 30 μg/mL, and then injected to attach a density of 13396.5 RU. The chip was then treated with 1 M ethanolamine (pH 8.5) as a quenching agent. Analytes of varying quantities were injected into the flow system for 90-s association and 180-s dissociation. Subsequently, background subtractions and solvent adjustments were performed. All assays were performed in running buffer (PBS, 0.05% Tween-20, 1% DMSO, pH 7.4).

2.7 Covalent docking simulations

MOE 2019.01 (Chemical Computing Group Inc., Montreal, Canada) was utilized to perform covalent docking simulations. The structure of M^{pro} (PDB Code: 6XHU) was downloaded from RCSB Protein Data Bank (PDB) [50]. Water molecules were removed manually from 6XHU. The cleaned protein structure was processed by the QuickPrep function of MOE, including correcting number of hydrogens, charging the system, and generating missing secondary structures. The structures of ligands were downloaded from PubChem. The reactive sites of ligands were accord to the experimental results of cysteine modification profiling. The reaction formula for the pyrogallol-cysteine covalent modification was done by MarvinSketch software 20.8.0 (ChemAxon, Budapest) as reported previously [51]. The docking and scoring methodology was set as default. Subsequent analysis was focused on the docking poses with the lowest binding free energy.

2.8 Data analysis

Raw MS files were analyzed in Proteome Discoverer version 2.4 (Thermo Scientific, Waltham, MA) with Sequest HT algorithm, converted to mgf format by MSconvert software and output MS^2 spectra by pLabel v2.4.1 software. IC_{50} and K_I values were fitted with GraphPad Prism software 9.5.1 (GraphPad Software, La Jolla, CA). The SPR binding affinity was fitted to a Langmuir 1:1 binding model by Biacore Evaluation software (Cytiva, Sweden).

3. Results

3.1 Discovery of Pu-erh tea as potent anti-M^{pro} plant extract

First of all, the anti-M^{pro} effects of over fifty edible plant extracts (100 μg/mL) were tested under identical conditions. The IC_{50} value of positive control nirmatrelvir was (33.89 ± 2.02) nmol/L **(Fig. S2A)**. Among all tested plant extracts, the leaf extract of *Camellia sinensis* (Pu-erh) exhibited the strongest anti-M^{pro} effect, the crude Pu-erh tea (100 μg/mL) could inactivate SARS-CoV-2 M^{pro} near completely, with the residual activity of 2.1% **(Fig. 2A, Table S1)**. The inhibition curves of Pu-erh tea against M^{pro} were then plotted to determine its anti-M^{pro} activity. As depicted in **Fig. 2B**, the Pu-erh tea dose-dependently inhibited SARS-CoV-2 M^{pro} (IC_{50} = (1.57 ± 0.05) μg/mL, following 63 min preincubation). It was also found that the Pu-erh tea showed significant time-dependent inhibition (TDI) effect against SARS-CoV-2 M^{pro}. These data indicated that Pu-erh tea was a potent anti-M^{pro} extract, implying that this tea extract contained time-dependent inhibitors of M^{pro}.

3.2 Characterization of cysteine-modified compounds (CMCs) in Pu-erh tea

Next, the key anti-M^{pro} constituents in Pu-erh tea were identified utilizing a suite of assays. Firstly, the influence of DTT on the anti-M^{pro} effect of Pu-erh tea was tested. The result showed that the anti-M^{pro} effect of Pu-erh tea could be totally reversed by DTT (1 mmol/L) **(Fig. S2B)**, suggesting that Pu-erh tea exerted its anti-M^{pro} activity predominantly *via* modifying the cysteine residues of this viral enzyme. After then, the chemical constituents in Pu-erh tea were identified comprehensively by using UHPLC-Q-Exactive Orbitrap HRMS. As shown in **Fig. 2C, 2D** and **Table S2**, twenty-four components were tentatively identified in both negative and positive ion modes, on the basis of the retention times (RT), MS^2 spectra of the standards or

referred to the MS^2 spectra reported previously [52,53]. The MS^2 spectra of all identified constituents were shown in **Fig. S3-S26.** As shown in **Table S2**, two alkaloids, one tea-specific amino acid, fourteen flavonoids, and seven organic acids were identified from Pu-erh tea. Notably, some constituents in Pu-erh tea (e.g., gallic acid and chlorogenic acid) bear one or more Michael acceptors, which may covalently bind to the cysteine residues on M^{pro} [54].

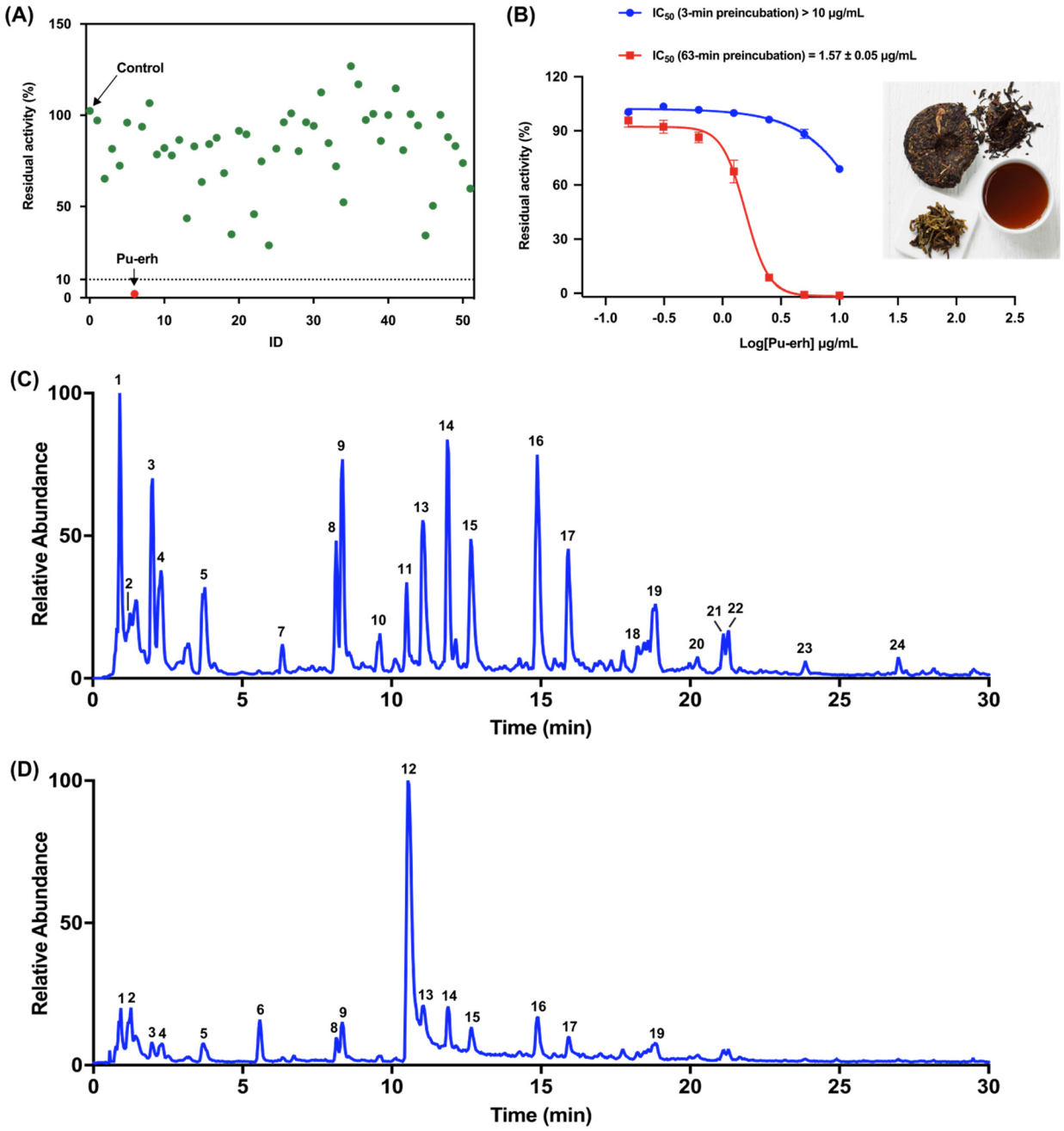

Fig. 2. (A) The anti-SARS-CoV-2 M^{pro} potentials of over fifty plant extracts. The final concentration of each extract was 100 μg/mL. *Camellia sinensis*, known as Pu-erh tea, totally inactivated the enzymatic activity of M^{pro}. (B) The dose-inhibition curves of Pu-erh extract against SARS-CoV-2 M^{pro} at different pre-incubation times (3-min and 63-min). IC_{50} values were fitted as mean ± S.E.M. (N = 3). Global chemical analysis of Pu-erh. (C, D) Total ionic chromatograms analyzed by UHPLC-Q-Exactive Orbitrap HRMS in negative (C) and positive (D) ion modes.

To discover the covalent agents of M^{pro} from the Pu-erh tea in a more efficient way, a chemoproteomic approach was constructed and then used to characterize the constituents modified peptides of M^{pro}. As listed in **Table 1**, the modified peptides of M^{pro} were characterized comprehensively, while at least seven CMCs

including caffeine, epicatechin, catechin, quercetin, GA, GC, and EGC, were found with the cysteine-modifying ability of SARS-CoV-2 M^{pro}. The MS^2 spectra of all detected peptides with cysteine modifications were shown in **Fig. S27-S37**. The modified cysteines located at the catalytic dyad (Cys145), surface of the catalytic pocket (Cys22, Cys38, and Cys44), dimer interface (Cys117 and Cys300), and minor peripheral surface of M^{pro} (Cys156 and Cys265). These findings clearly demonstrated that some constituents in Pu-erh tea could covalently modify several key cysteine residues of SARS-CoV-2 M^{pro}, implying that these CMCs acted as the primary anti-M^{pro} constituents in Pu-erh tea.

Table 1 Characterization of the CMCs in Pu-erh by UPLC-Q Exactive Orbitrap-MS/MS.

Peptides	Modified site	M.F.	ΔMW	CMCs
VEGCMVQVTC*GTTTLNGLWLDDVVY	Cys22	$C_{15}H_{10}O_7$	302.04265	Quercetin
VEGCMVQVTCGTTTLNGLWLDDVVYC*PR	Cys38	$C_8H_{10}N_4O_2$	194.08038	Caffeine
HVIC*TSEDMLNPNYEDLLIR	Cys44	$C_7H_4O_5$	168.00587	GA
HVIC*TSEDMLNPNYEDLLIR		$C_{15}H_{12}O_7$	304.05830	EGC, GC
HVIC*TSEDMLNPNYEDLLIR		$C_{15}H_{12}O_6$	288.06339	Epicatechin, catechin
SVLAC*Y	Cys117	$C_{15}H_{12}O_7$	304.05830	EGC, GC
GSFLNGSC*GSVGF	Cys145	$C_{15}H_{12}O_7$	304.05830	EGC, GC
NIDYDC*VSF	Cys156	$C_{15}H_{12}O_7$	304.05830	EGC, GC
NYEPLTQDHVDILGPLSAQTGIAVLDMC*ASLK	Cys265	$C_{15}H_{12}O_6$	288.06339	Epicatechin, catechin
DVVRQC*SGVTFQ	Cys300	$C_{15}H_8O_7$	300.02700	Quercetin
QC*SGVTF		$C_{15}H_{12}O_7$	304.05830	EGC, GC

3.3 Anti-M^{pro} effects of CMCs isolated from Pu-erh tea

Subsequently, the anti-M^{pro} efficacy of seven identified CMCs presented in Pu-erh tea was assessed at two doses (1 and 10 μmol/L). As shown in **Fig. 3A and 3B**, three CMCs in Pu-erh tea (including EGC, GC, and GA) could totally inactivated the hydrolytic activities of M^{pro} at 10 μmol/L, but their inactivation effects could be totally reversed by DTT (1 mmol/L) **(Fig. S38)**, indicating that these three compounds inactivated SARS-CoV-2 M^{pro} mainly *via* modifying the cysteine residues of this viral enzyme. The dose-dependent inhibition curves of EGC, GC, and GA were then plotted using increasing inhibitor concentrations, to determine their IC_{50} values. As shown in **Fig. 3C-E**, EGC, GC, and GA dose-dependently inactivated SARS-CoV-2 M^{pro}, IC_{50} of (0.63 ± 0.03) μmol/L, (0.90 ± 0.05) μmol/L, and (2.60 ± 0.25) μmol/L, respectively **(Table 2)**. It also should be noted that pre-incubation of these three compounds could dramatically improve their anti-M^{pro} efficacy **(Fig. 3C-E)**, as the ratio of IC_{50} (3-min preincubation / 63-min preincubation) of these three compounds was over 10. These findings suggested that three compounds showed significant TDI effects against M^{pro}. The standard curves of EGC, GC, and GA were analyzed, and their content in the Pu-erh product were respectively measured to be 6.09 mg/g, 8.06 mg/g, and 14.89 mg/g based on UHPLC-Q-Exactive Orbitrap HRMS **(Table 2 and Fig. S51)**. Meanwhile, the dose- and time-dependent inhibitory effects of dihydromyricetin (a previously reported natural-derived covalent SARS-CoV-2 M^{pro} inhibitor) were also tested, showing an IC_{50} value of (1.32 ± 0.06) μmol/L (after 63-min pre-incubation) **(Fig. 3F and Table 2)**. These findings demonstrated that EGC, GC, and GA showed strong time-dependent

inhibition against SARS-CoV-2 M^{pro}, suggesting that these three compounds might be key anti-M^{pro} constituents in Pu-erh tea.

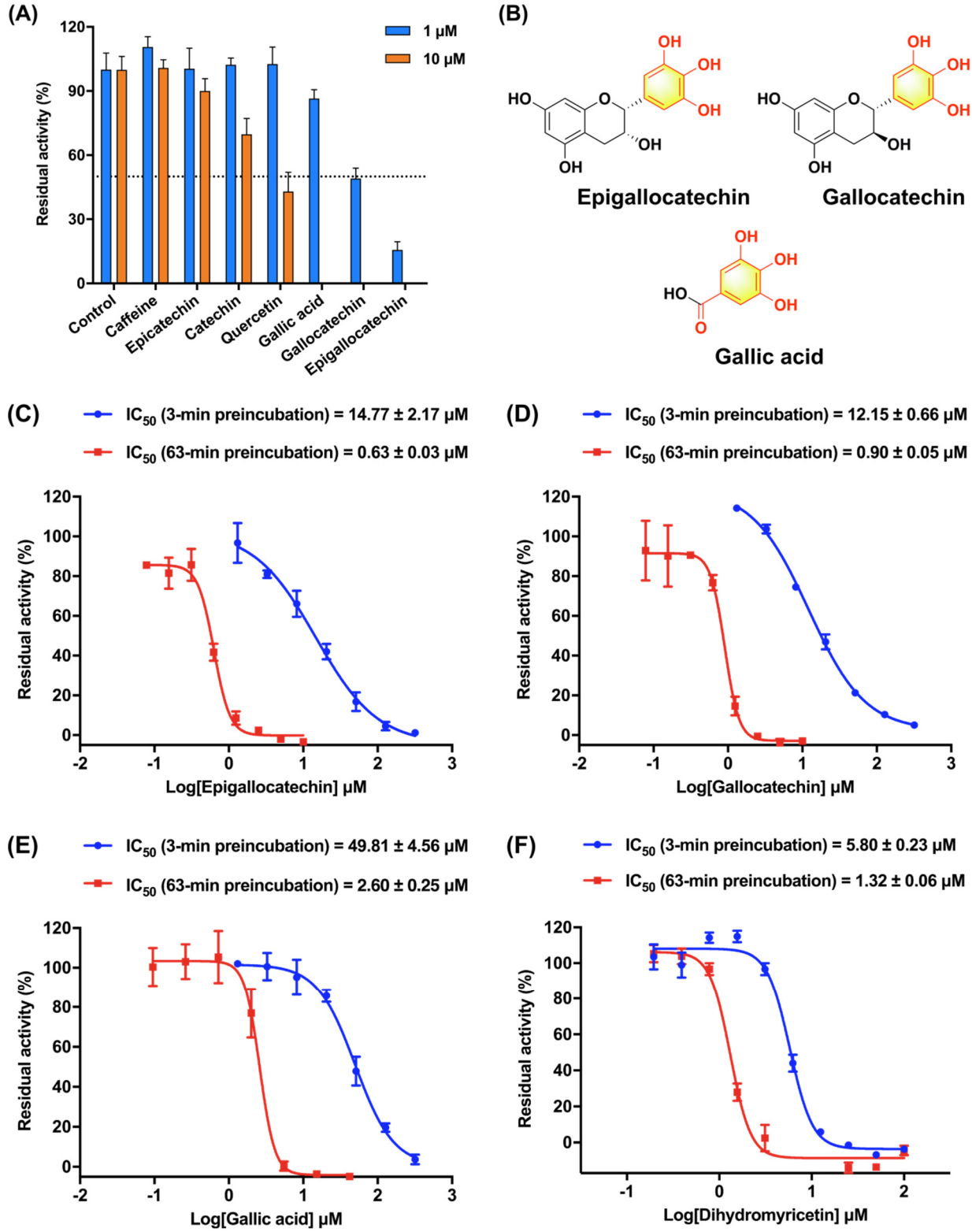

Fig. 3. The anti-SARS-CoV-2 M^{pro} effects of the newly identified covalent constituents in Pu-erh. (A) Bioactivity tests of seven M^{pro} cysteine-modified compounds (CMCs) in Pu-erh at two doses (1 μmol/L and 10 μmol/L). The residual activities were expressed as mean ± SD (N = 3). (B) Structures of the top three anti-M^{pro} CMCs with reactive groups highlighted. (C-F) The dose-inhibition curves of EGC (C), GC (D), GA (E), and the positive M^{pro} inhibitor dihydromyricetin (F) against SARS-CoV-2 M^{pro} following pre-incubation for 3 min (blue line) or 63 min (red line). IC_{50} values were fitted as mean ± S.E.M. (N = 3).

Table 2 The inactivation parameters and the content analysis of the three identified covalent SARS-CoV-2 M^{pro} inhibitors in Pu-erh.

Compound	MW	Content (mg/g)	IC_{50} (μmol/L)*		Ratio (3/63 min)	K_I (μmol/L)	k_{inact} (min^{-1})	R^2
			3 min	63 min				
Epigallocatechin	306.27	6.09	14.77 ± 2.17	0.63 ± 0.03	23.44	5.650	0.1084	0.9981
Gallocatechin	306.27	8.06	12.15 ± 0.66	0.90 ± 0.05	13.50	7.780	0.1130	0.9984
Gallic acid	170.12	14.89	49.81 ± 4.56	2.60 ± 0.25	19.16	8.009	0.0699	0.9997
Dihydromyricetin	320.25	--	5.80 ± 0.23	1.32 ± 0.06	4.39	--	--	--

*The IC_{50} values were determined at various conditions (with 3 min or 63 min pre-incubation time).

3.4 Cysteine modification profiling of EGC, GC, and GA on M^{pro}

To uncover the inactivation mechanisms of EGC, GC, and GA against M^{pro}, each compound (100 μmol/L) was incubated with SARS-CoV-2 M^{pro} under physiological conditions, while the peptides modified by these natural compounds were comprehensively characterized. The coverage of the protein-peptide maps were 98.37% for both EGC and GC samples, and 92.48% for GA sample. The cysteine-containing peptides were completely covered by scanning in all samples. **Fig. S39-S47** displayed the MS^2 spectra of the modified peptides of M^{pro}. As listed in **Table S3**, EGC could modify five cysteines on M^{pro} (Cys16, 38, 85, 128, and 265) **(Fig. S48)**, GC modified three cysteines (Cys85, 128, and 265) **(Fig. S48B)**, while only one modified cysteine (Cys44) was detected following GA treatment. The modifications on Cys38 and Cys44 were more likely to disrupt the substrate binding of SARS-CoV-2 M^{pro}, as these two cysteines were located at the surface of the catalytic pocket [55]. Moreover, Cys128 was a cysteine clamped at the inter-molecule surface of two monomeric M^{pro}, thus the modification of Cys128 might halt the monomeric M^{pro} assembling into dimer [56]. By contrast, Cys16, Cys85, and Cys265 were virtually situated on the peripheral surface of SARS-CoV-2 M^{pro}, and barely solid evidence could prove that they were responsible to maintain the enzymatic activity of SARS-CoV-2 M^{pro} [57]. These findings may partially explain why EGC displayed the most potent anti-M^{pro} effect, also confirmed that EGC, GC, and GA inactivated SARS-CoV-2 M^{pro} mainly *via* modifying some key cysteine residues in this viral enzyme.

3.5 Inactivation and binding kinetics of EGC, GC, and GA to M^{pro}

To further explore the action mechanisms of EGC, GC, and GA against M^{pro}, their inactivation kinetics and binding kinetics towards SARS-CoV-2 M^{pro} were analyzed **(Fig. 4A and 4B, Fig. S49A)**. As depicted in **Fig. S50**, EGC, GC, and GA dose- and time-dependently inactivated SARS-CoV-2 M^{pro}**,** K_I values of 5.650 μmol/L, 7.780 μmol/L, and 8.009 μmol/L, respectively. The k_{inact} values of these three compounds were also determined as 0.1084 min^{-1} (EGC), 0.1130 min^{-1} (GC), and 0.0699 min^{-1} (GA), suggesting that EGC and GC were more efficacious SARS-CoV-2 M^{pro} inactivators when compared with GA **(Table 2)**. The SPR results showed that EGC, GC, and GA could dose-dependently bind to the immobilized M^{pro}, and the binding modes of these three compounds were followed fast-binding and slow-dissociation kinetics. Similarly, EGC and GC exhibited higher binding rate constant than GA, k_{on} of 3514 Ms^{-1} (EGC), 4010 Ms^{-1} (GC), and 1922 Ms^{-1} (GA) **(Fig. 4C and 4D, Fig. S49B)**, indicating that EGC and GC exhibited faster binding ability to M^{pro} than GA.

These findings emphasized that EGC and GC acted as more efficacious inactivators and faster binders to M^{pro} than GA, which partially explained the superior inactivation ability of EGC and GC against M^{pro}.

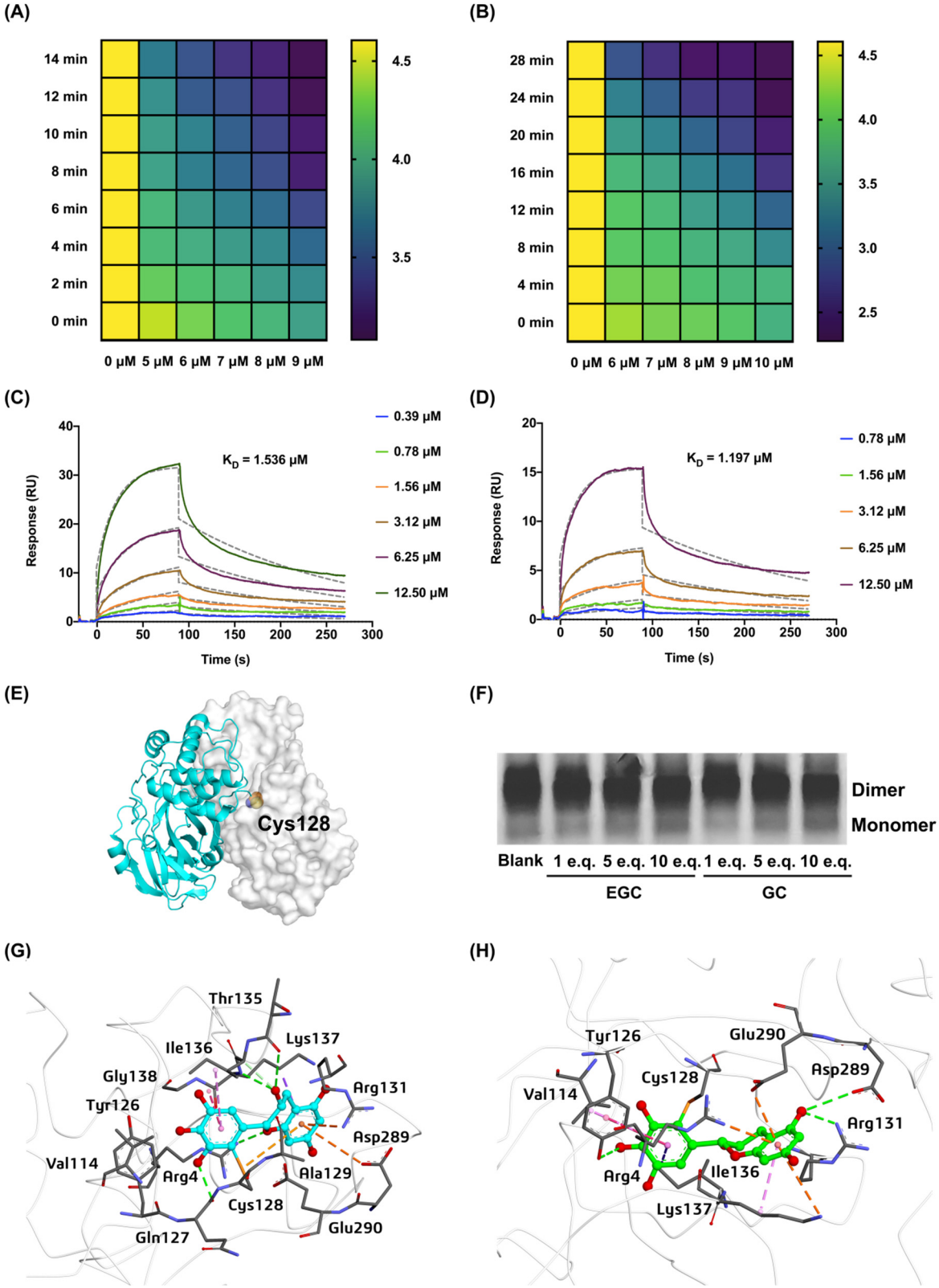

Fig. 4. (A, B) Inactivation kinetics of EGC (A) and GC (B) were plotted as enzymatic residual activity *v.s.* gradient preincubation time and doses of inhibitors in heatmaps. (C, D) Binding kinetics of EGC (C) and GC (D) to M^{pro} were plotted as SPR *v.s.* gradient doses of inhibitors, respectively. (E) The structure of SARS-CoV-2 M^{pro} dimer (PDB code: 6XHU). Cys128 was highlighted in sphere type. (F) Native-PAGE analysis of M^{pro} incubated (63 min) with different equivalent EGC or GC. (G, H) 3D enzyme-inhibitor interaction analysis of EGC (G) and GC (H) modified at Cys128 of SARS-CoV-2 M^{pro}, respectively. The interaction types were illustrated in **Fig. S52**.

3.6 Inactivation mechanisms of EGC and GC to M^{pro}

We further disclosed the superior inactivation mechanisms of EGC and GC against M^{pro} at a microscopic level. As both EGC and GC could modify Cys128, while this cysteine was located at the dimer interface of M^{pro} **(Fig. 4E)**, modification on this cysteine may strongly block the formation of catalytic active dimer. In this case, the oligomerization states of M^{pro} (10 μg) following EGC or GC treatment (incubated for 1 h within 10 μL) were assayed by Native-PAGE analysis [49]. As shown in **Fig. 4F**, the formation of M^{pro} monomer virtually increased after incubated with EGC or GC. The covalent docking simulations found that EGC and GC could form a set of hydrogen bonding, hydrophobic interactions and electrostatic interactions with the interface residues of M^{pro} **(Fig. S52)**. Most importantly, the modification at Cys128 of either EGC or GC could disrupt Arg4 through hydrogen bond or Pi-Cation interaction **(Fig. 4G and 4H)**. As the salt bridge interactions of Arg4-Glu290 played significant role in the dimerization of M^{pro}, we believed that the perturbations of EGC and GC to the key Arg4 would largely accelerate the dissociation of dimer, and disrupted the association of monomer [55-57]. These findings clearly demonstrated that EGC and GC could block the the hydrolytic activity of M^{pro} by a unique de-dimerization mechanism.

4. Discussion

The global pandemic COVID-19 has resulted in millions of deaths and affected cases [1]. To fight this deadly outbreak, scientists have been tirelessly searching for potential anti-SARS-CoV-2 agents to treat COVID-19 or alleviate its complications [8,20,40,57,58]. Among all validated therapeutic targets, the main protease (M^{pro}) has attracted significant research attention due to its pivotal role in viral replication, similarity to other beta-coronaviruses like SARS-CoV M^{pro} (96% sequence identity), as well as lacking of analogs in humans [23,55]. Over the past three years, a variety of anti-SARS-CoV-2 M^{pro} agents have been identified using various approaches, while some natural compounds with covalent binding ability on M^{pro} has stood out for their superior inhibitory activities [38,51,59,60]. For instance, myricetin, dihydromyricetin, and iso-dihydromyricetin were found to covalently bind on the catalytic Cys145 of SARS-CoV-2 M^{pro} [51]. Notably, some naturally occurring compounds isolated from edible plants or fruits contain cysteine-reactive motifs (such as catechol, pyrogallol, and Micheal receptor), indicating that some plant extracts hold promise as sources of anti-M^{pro} agents [51,61].

Plant extracts are commonly used as food additives, beverages, and nonprescription therapies, while their bioactive compounds are widely accepted in the market for their safety and effectiveness [62-64]. However, identifying the key bioactive compounds from plant extracts is challenging due to their extremely complex chemical compositions, with numerous components in uncertain amounts [65,66]. The commonly used phytochemisty methods for identifying the key biologically active natural compounds always involve isolation and purification of various chemically diverse molecules, but this process is time-consuming and labor-intensive, which strongly hampers the rapid identification of the key active compounds from plant extracts [67,68].

To overcome these challenges, we have established a highly efficient platform for rapid discovering covalent M^{pro} inhibitors from crude plant extracts. This process integrates FRET-based enzymatic inhibition screening, global chemical analysis of plant extract, and characterization of modified peptides using mass spectrometry-based chemoproteomics. In this study, we firstly assessed the time-dependent inhibition of plant extracts against M^{pro}, which is the distinctive feature of the covalent inhibitors [69]. To comprehensively identify the phytochemicals present in the plant extracts, we assigned the chemical structures of constituents by matching the retention times and MS^2 spectra data using the commercially available standards or comparing the MS^2 spectra with the known natural compounds in databases or previously published literature. Meanwhile, the mass spectrometry-based chemoproteomic assay was opitimized to gain a high coverage of cysteine-containing peptides when characterizing the modified peptides of M^{pro}, as it enables us to comprehensively identify cysteine-modified compounds (CMCs) with potential anti-M^{pro} properties [70]. In our study, we screened over fifty edible plants and the results showed that Pu-erh tea exhibited potent anti-M^{pro} activity with significant time-dependent inhibition. Pu-erh tea is a rich source of bioactive compounds, and it likely contains numerous components that may contribute to its effectiveness against M^{pro}. Previous research has identified several constituents in Pu-erh tea as inhibitors of M^{pro}, including chlorogenic acid, ellagic acid, epigallocatechin gallate, kaempferol, kaempferol-3-*O*-rutinoside, quercetin, quercitrin, and quinic acid [39,71]. However, most of these constituents exhibited relatively weak anti-M^{pro} activity when tested without incubation, with inhibitory activity typically falling below 70% at a concentration of 10 μmol/L. Notably, even after a 40-minute incubation with 10 μmol/L quercitrin, SARS-CoV-2 M^{pro} retained over 75% enzymatic activity [39]. Similarly, in our study, Pu-erh tea showed limited inhibitory activity after a 3-minute incubation. Therefore, the potent anti-M^{pro} activity of Pu-erh tea may be attributed to other bioactive constituents or a time-dependent mechanism.

Using high-resolution mass spectrometry, we carefully identified twenty-four components from Pu-erh tea and discovered at least seven compounds capable of modifying cysteines in SARS-CoV-2 M^{pro} with 100% coverage of cysteine-containing peptides. Among these, three compounds (EGC, GC, and GA) were demonstrated to have potent anti-M^{pro} effects. EGC, GC, and GA interact with key cysteines on M^{pro} through cysteine-reactive motifs (such as pyrogallol or Michael receptor), resulting in potent inhibitory activities against M^{pro}. These reactive motifs are abundant not only in tea products but also in various other edible plants and plant-based foods, suggesting their practical applicability across different sources [61,72]. Notably, we also found that the covalent binding sites and inhibitory mechanisms of three key constituents in Pu-erh tea components are much different from the marketed anti-M^{pro} agents, indicating that the combination use of Pu-erh tea with the marketed anti-M^{pro} agents may get more efficacious anti-M^{pro} effects [73]. Covalent inhibitors such as nirmatrelvir, N3, and α-ketoamides mainly target Cys145 as their primary binding site [57,74,75]. Natural compounds such as myricetin also create covalent connections with Cys145, which is supported by X-ray crystal structures of the M^{pro}-myricetin complex [51]. However, in our cysteine modification profiling, we discovered other covalent binding sites. For example, our previous research found

that myricetin also interacted with Cys300 of M^{pro}, which was known to be an allosteric site for M^{pro} [38,56]. Additionally, sciadopitysin, an inhibitor derived from *Ginkgo biloba*, attached to Cys85 and Cys156 of M^{pro} [46]. Beyond its anti-M^{pro} potential, Pu-erh tea also offers multiple beneficial effects to humans, including its hypotensive, anti-inflammatory, neuroprotective, hepatoprotective, lipid-lowering, and antiviral properties [76-81]. In future, more combination therapeutics of Pu-erh tea with the Western medicines against COVID-19 or other β-coronaviruses caused diseases should be investigated both i*n vitro* and *in vivo*, which may develop more practical and efficacious anti-viral strategies for the treatment of β-coronaviruses caused diseases.

5. Conclusion

In summary, this study proposed a new strategy to efficiently discover M^{pro} inactivators from plant extracts by integrating biochemical and chemoproteomic approaches. A commonly used tea (Pu-erh) was identified as a potent anti-M^{pro} extract among all tested plant extracts. The crude extract of Pu-erh could potently inhibit SARS-CoV-2 M^{pro} in dose- and time-dependent manners. Subsequent investigations showed that at least seven constituents in Pu-erh tea including caffeine, epicatechin, catechin, quercetin, GA, GC, and EGC, could covalently modify SARS-CoV-2 M^{pro}, while epigallocatechin (EGC), gallocatechin (GC) and gallic acid (GA) were identified as the top three most efficacious M^{pro} inhibitors. The cysteine-modification profiling assays, inactivation and binding kinetics of three newly identified M^{pro} inactivators were then carefully investigated. It is also found that EGC and GC could covalently modify M^{pro} and then inactivated this key viral enzyme by a unique de-dimerization mechanism. All these findings proposed a novel and practical strategy for highly efficient discovery of SARS-CoV-2 M^{pro} inactivators from plant extract, while three newly identified M^{pro} inhibitors from Pu-erh tea provide powerful evidence to support the anti-coronavirus effects of this plant extract.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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