Inhibitory effects of senkyuchachosan on SARS-CoV-2 papain-like protease activity *in vitro*

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Abstract

Papain-like protease (PLpro) enzyme plays a vital role in viral replication as it breaks down polyproteins and disrupts the host's immune response. There are few reports on Kampo formulas that focus on PLpro activity. In this study, we evaluated the inhibitory effects of senkyuchachosan, a traditional Japanese medicine, on PLpro of SARS-CoV-2, the virus responsible for causing COVID-19. We purified the PLpro enzyme and conducted *in vitro* enzymatic assays using specific substrates. Among the nine crude drugs present in senkyuchachosan, four (Cyperi Rhizoma, Schizonepetae Spica, Menthae Herba, and Camelliae sinensis Folium [CsF]) strongly inhibited PLpro activity. CsF, derived from *Camellia sinensis* (green tea), contains polyphenols, including catechins and tannins. To confirm that the PLpro inhibitory effects of senkyuchachosan predominantly stem from tannins, the tannins were removed from the decoction using polyvinylpolypyrrolidone (PVPP). The inhibitory effect of senkyuchachosan on PLpro activity was reduced by the removal of PVPP. In addition, the tannin fraction obtained from the CsF extracts showed significant PLpro inhibitory effects. These findings lay the groundwork for the potential development of therapeutic agents that target SARS-CoV-2 infection by intervening in proteolytic cleavage of the virus.

Keywords: SARS-CoV-2, papain-like protease, catechin, Kampo, senkyuchachosan

Introduction

COVID-19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has emerged as one of the most significant global pandemics in recent years [1]. According to the World Health Organization, SARS-CoV-2 has infected over 760 million individuals worldwide, resulting in 6, 954, 336 deaths as of August 11, 2023 [2]. The incidence of SARS-CoV-2 is currently declining owing to ongoing efforts in many countries to develop a vaccine to prevent SARS-CoV-2 infection. Among the strategies employed to treat COVID-19, antiviral compounds designed to target RNA-dependent RNA polymerase and 3-chymotrypsin-like protease (3CLpro or Mpro) of SARS-CoV-2 have been widely utilized [3]. Additionally, there is active research and development in the pursuit of therapeutic agents that target virus-derived molecules.

The viral genome encodes four structural proteins (S, spike; E, envelope; M, membrane; and N, nucleocapsid), 16 non-structural proteins (Nsps), including coronavirus main protease (3CLpro or Mpro), papain-like protease (PLpro), and nine accessory proteins [4]. 3CLpro and PLpro are responsible for processing polyproteins pp1a and pp1ab into mature Nsps [5, 6]. 3CLpro is autocleaved from polyproteins and further cleaves the polyprotein to release Nsp4–Nsp16 and plays a pivotal role in viral replication and transcription [7, 8]. PLpro cleaves viral polyproteins to form Nsp1, Nsp2, and Nsp3, which are required for correcting viral replication [9]. PLpro specifically cleaves polyproteins containing the consensus sequence LXGG↓X [10]. Moreover, SARS-CoV-2 PLpro acts as a deISGylation enzyme that evades the host innate response. Type I interferon (IFN-I)-mediated immune responses are essential for host defense against pathogens, including SARS-CoV-2 [11]. Efficient induction of IFN-α/β signaling and the

consequent induction of IFN-stimulating genes (ISGs) play crucial roles in the control and resolution of SARS-CoV-2 infection [12]. Upregulated ISG-15 conjugates with host signaling molecules, including JAK, STAT, and IRF-3, through ISGylation to mediate antiviral immunity [13–15]. SARS-CoV-2 PLpro mediates the deISGylation of ISG-15 to host signaling molecules, leading to inhibition of the host antiviral innate immune response [16–18]. Thus, PLpro is a promising therapeutic target because of its crucial role in cleaving and disrupting host responses.

Kampo medicine is a traditional Japanese medicine and consists of crude drugs including herbs, insects, minerals, and fungi. Numerous herbal formulas have been used to alleviate the respiratory symptoms associated with the common cold. For example, the therapeutic effects of Kampo formulas on influenza viruses and SARS-CoV-2 have been documented through clinical [19–21] and *in vitro* studies [22, 23]. Senkyuchachosan is a mixture of nine crude drugs, including Camelliae sinensis folium (CsF), and is used to address headaches associated with early cold symptoms, migraines, and health issues in women [23, 24]. A recent study showed that senkyuchachosan has a potential suppressive effect on SARS-CoV-2 infections [23]. Catechins are the major components of CsF tannins and are mostly composed of (+)-epicatechin, (-)-epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG). The leaves of *Camellia sinensis* are known as green tea, and a catechin mixture from green tea has been reported to inhibit SARS-CoV-2 infection in a dose-dependent manner [25]. Green tea extract and EGCG are the major catechins that inhibit SARS-CoV-2 3CLpro [26, 27]. *Camellia sinensis* catechins also inhibit 3CLpro both intracellularly and extracellularly [28]. *Camellia sinensis* catechins have PLpro inhibitory activity both *in vitro* and in computational analysis [29, 30].

However, there are crucial differences in the heating conditions between the consumption of tea and Kampo decoction. Previous reports have indicated that heating affects the extraction rate of catechins and tannins [31]. Although the 3CLpro inhibitory activities of crude drugs have been evaluated [32], there are few reports on the PLpro activities of crude drugs or Kampo formulas.

In this study, we focused on the PLpro inhibitory activity of nine crude drugs, which are components of senkyuchachosan. We showed that CsF and its tannin fraction exhibited inhibitory activities against PLpro.

Materials and methods

Kampo extracts and reagents

The crude drugs used in this study were procured from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan) and Uchida Wakan-yaku Co., Ltd. (Tokyo, Japan) (Table 1). These met the grade standards of the Japanese Pharmacopia 18th Edition or were non-pharmacological crude drugs. To obtain powdered extracts of each product, the following amounts were used: Cyperi Rhizoma (CyR; 4.0 g), Cnidii Rhizoma (ChR; 3.0 g), Notopterygii Rhizoma (NoR; 2.0 g), Schizonepetae Spica (ScS; 2.0 g), Menthae Herba (MeH; 2.0 g), Angelicae Dahuricae Radix (AnDR; 2.0 g), Saposhnikoviae Radix (SaR; 2.0 g), Glycyrrhizae Radix (GlR; 1.5 g), and CsF (1.5 g). Senkyuchachosan was prepared using the above composition according to a previously reported method [23]. Senkyuchachosan (CsF-) was prepared from eight crude drugs, excluding CsF. These ingredients were boiled in 500 ml of distilled water for 60 min, followed by centrifugation at 3,000 × *g* for 10 min and subsequent filtration. The resulting dried powdered extracts

were obtained by lyophilizing the decoctions. These extracts were then suspended in 10% DMSO (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) and stored at -20 °C until further use.

For the Kampo formula, tannins were eliminated using polyvinylpolypyrrolidone (PVPP) [33, 34]. Senkyuchachosan decoction was combined with 2% w/v PVPP (Nacalai Tesque, Kyoto, Japan), stirred for 30 min, and subsequently filtered. The resulting dried powdered extracts were obtained by lyophilizing the decoctions.

(-)-EGCG was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The overall catechin content of the samples was determined as EGCG using a colorimetric method involving ferrous tartrate according to a previous report [35]. To obtain the tannin fraction, CsF extracts were further fractionated using a silica column, as previously reported [36]. The CsF extracts (100 mg) were applied to a column (2 ×40 cm) packed with silica gel in 5% MeOH/$CHCl_3$. Elution was performed with a stepwise gradient of 5%, 8%, 12%, 15%, 50%, and 75% MeOH/$CHCl_3$, and, finally MeOH. The fractions were collected in 100 ml aliquots. The 15%, 50%, 75%, and 100% methanol-fractionated samples were combined and defined as the tannin fraction. The solvent of each fraction was removed by evaporation and prepared in 10% DMSO at 10 mg/ml for use in the PLpro assay.

Expression and purification of SARS-CoV-2 Plpro

The SARS-CoV-2 PLpro gene, corresponding to the amino acid sequence 746-1064 of Nsp3 (NCBI reference sequence: YP_009725299), was optimized for bacterial expression through codon optimization. A coding vector (pGEX-6p-1-SARS-CoV-2-

Plpro) containing an N-terminal GST tag was designed and obtained from GenScript (NJ). The plasmid was transfected into BL21 (DE3) cells for protein expression analysis. *Escherichia coli* cultures were grown overnight at 37 °C to serve as initial cultures. Subsequently, large-scale cultures were cultivated at 37 °C until reaching an O.D. of 0.6. Expression was induced by adding 1 mM IPTG, followed by incubation at 16 °C and 95 rpm for 20 h. *E. coli* was then centrifuged at 3,000 × *g* for 10 min at 4 °C, washed with PBS, and the resulting pellet was resuspended in lysis buffer (25 mM Tris-HCl, pH 7.5, containing 0.5% Triton X-100). Homogenization was achieved by sonication, and the resulting lysate was further processed by centrifugation at 13,000 × *g* for 10 min at 4 °C. The supernatant was purified using GST-accept (Nacalai) and ultrafiltered with 25 mM Tris-HCl (pH 7.5). The eluted GST-Plpro was further treated with PreScission Protease (Cytiva, Tokyo, Japan) and purified using GST-accept. Quantification of purified Plpro was performed using the BCA assay and 12% SDS-PAGE separation, followed by Coomassie brilliant blue staining. The proteolytic activity of PLpro was evaluated *in vitro*.

In vitro PLpro activity

An *in vitro* fluorogenic assay for measuring proteolytic activity was conducted as described previously, with slight modifications [37]. The fluorogenic substrates Z-Arg-Leu-Arg-Gly-Gly-MCA and Ac-Thz-Tle-Leu-Gln-MCA, designed for SARS-CoV/SARS-CoV-2 PLpro or 3CLpro cleavage sites in the virus, were sourced from Peptide Institute, Inc. (Osaka, Japan). The fluorogenic substrates were dissolved in 10% DMSO. The reaction mixture comprised of sample (10 µl), PLpro enzyme (75 pmol/ml) (20 µl), and assay buffer (25 mM Tris-HCl, pH 7.5, 10 mM DTT, 150 mM NaCl) (60 µl).

After pre-incubation at 37 °C for 5 min, 10 µl of 0.5 mM fluorogenic substrate was added. The proteolytic activity was evaluated by measuring fluorescence at excitation and emission wavelengths of 380 and 460 nm, respectively, every 3 min for 30 min using a Synergy H1 instrument (Agilent Technologies, Santa Clara, CA). GRL-0617 (Cayman Chemical, MI) was used as the Plpro inhibitor [37]. The experiments were conducted in triplicate for reproducibility. Statistical analysis was performed using one-way analysis of variance with Dunnett's test. Statistical significance was set at $p < 0.05$.

Results

To evaluate the inhibitory effects of senkyuchachosan on SARS-CoV-2 PLpro, we expressed and purified SARS-CoV-2 PLpro (Fig. 1). The SARS-CoV-2 PLpro plasmid was introduced into *E. coli* to express the recombinant proteins. PLpro was isolated and purified by affinity chromatography (Fig. 1a). Using this enzyme, we conducted *in vitro* PLpro assays with fluorogenic substrates. Z-Arg-Leu-Arg-Gly-Gly-MCA, a substrate specific to SARS-CoV-2 PLpro, exhibited selective cleavage (Fig. 1b). Conversely, Ac-Thz-Tle-Leu-Gln-MCA, which is indicative of SARS-CoV-2 3CLpro, showed no cleavage. Subsequently, we applied the established procedure to examine the inhibitory effects of the nine crude senkyuchachosan extracts (Fig. 2a). Out of the nine crude drugs, CyR (59.6 ± 2.9%), ScS (50.0 ± 0.5%), MeH (61.1 ± 7.2%), and CsF (64.3 ± 0.0%) significantly inhibited PLpro activity at a concentration of 200 µg/ml (Fig. 2a). Conversely, ChR (4.7 ± 2.1%), NoR (27.0 ± 4.6%), AnDR (16.6 ± 4.9%), SaR (5.1 ± 0.6%), and GlR (-3.0 ± 0.1%) displayed minimal to no PLpro inhibitory activity. In

addition, four crude drugs (CyR, MeH, ScS, and CsF) showed PLpro inhibitory effects in a concentration-dependent manner (Fig. 2b).

To examine the PLpro inhibitory activity of senkyuchachosan and the contribution of CsF to senkyuchachosan, we prepared senkyuchachosan decoctions with and without CsF (senkyuchachosan CsF[-]). Senkyuchachosan CsF(-) decoctions were composed of eight crude drugs, excluding CsF. Using lyophilized extracts, we evaluated the PLpro inhibitory activity of senkyuchachosan (Table 2). Senkyuchachosan showed higher PLpro inhibitory activity than senkyuchachosan (CsF-) at three concentrations (1000, 500, and 250 μg/ml). Although the tannin content of senkyuchachosan was 122 μg per decoction volume (ml), the total catechin content of senkyuchachosan (CsF-) was 28.0 μg/ml. These results indicated that CsF was responsible for the tannin content and PLpro inhibitory activity of senkyuchachosan. We further removed tannins from the senkyuchachosan decoction by adsorption onto PVPP [34]. PVPP selectively absorbs phenolic compounds, such as tannins, by forming hydrogen bonds between the hydroxyphenolic and amide groups of PVPP [34]. After treatment with PVPP, the tannin content of the decoction was 13.3 μg/ml and the PLpro inhibitory activity significantly decreased (Table 2). These results indicated that the tannin content of senkyuchachosan was responsible for its PLpro inhibitory activities. We separated the tannin fraction from the CsF extract and evaluated its PLpro inhibitory activity. The tannin fraction showed strong inhibitory effects, with an IC_{50} value of 8.9 ± 0.5 μg/ml. These results indicated that the tannins of CsF contributed to the PLpro inhibitory activity.

Discussion

PLpro is highly conserved among coronaviruses. The amino acid sequence of SARS-CoV-2 PLpro is 98% identical to the related proteases from bat coronaviruses BANAL-52 and RaTG13 and 83 % identical to SARS-CoV [38, 39]. In contrast, the amino acid sequence of SARS-CoV-2 PLpro is only 29% identical and 51% similar to that of MERS-CoV PLpro [40]. SARS-CoV and SARS-CoV-2 PLpro are closely related homologs, but differ significantly from MERS-CoV PLpro. However, the X-ray crystal structures of PLpro from SARS-CoV-2, SARS-CoV, and MERS-CoV demonstrate structural similarities and high conservation [40]. These insights indicate that SARS-CoV-2 PLpro inhibitors could provide useful therapeutic drug repositioning for not only the current SARS-CoV-2 but also possible next coronaviruses.

There are few reports on Kampo formulas that focus on PLpro activity. Because PLpro is related to viral replication and the innate immune response, its inhibitory activity promises to improve the efficacy of Kampo medicines against SARS-CoV-2. Because senkyuchachosan is used to treat innate cold symptoms, it has been reported that the Kampo formula inhibits SARS-CoV-2 infection *in vitro*, we focused on senkyuchachosan and evaluated its PLpro inhibitory activities. Of the nine crude drugs, four (CyR, ScS, MeH, and CsF) showed inhibitory effects compared with the other crude drugs. In this study, we demonstrated that CsF and its tannins exhibit strong PLpro inhibitory activities. A previous report indicated that ECG and 50% ethanol extracts of *Camellia sinensis* inhibited PLpro activity [41]. One of our novel point in this study was to evaluate PLpro inhibitory activities using samples extracted with hot water, whose extraction procedure was the same as that used for the Kampo decoction. The tea-related infection-preventive effects on COVID-19 are well documented. In addition, differences in COVID-19

morbidity and mortality rates between countries with high and low green tea consumption were observed until December 6, 2022 [42]. EGCG inhibits the SARS-CoV-2 proteolytic activities of 3CLpro [43, 44] and PLpro [17, 45] required for SARS-CoV-2 replication. Therefore, the Kampo formula of senkyuchachosan with CsF is expected to inhibit SARS-CoV-2 replication by targeting the virus-derived proteolytic cleavage. Interestingly, CyR, ScS, and MeH inhibited PLpro and the inhibitory activities of these crude drugs were almost equal to those of CsF. CyR is a rhizome of *Cyperus rotundus* Linne, which belongs to the Cyperaceae family. ScS and MeH, which belong to the Lamiaceae family, represent *Schizonepeta tenuifolia* Briquet and *Mentha arvensis* Linné var. piperascens, respectively[46–48]. Of the nine crude drugs of senkyuchachosan, CyR, ScS, and MeH contained essential oils. Plants, including Lamiaceae perilla and sage, inhibit SARS-CoV-2 infection and SARS-CoV-2 replication in Vero E6 and Caco-2 cells [49]. These effects were considered to be caused by the synergistic effects of caffeic acid and monoterpenoids, such as perillaldehyde. Because CyR, ScS, and MeH contain caffeic acids and several monoterpenoids as essential oil components, they may have potential inhibitory activities against SARS-CoV-2 infection. Compared with senkyuchachosan, the PLpro inhibitory activity of senkyuchachosan (CsF-) decreased but was not completely abolished. In addition, PVPP-treated senkyuchachosan reduced the PLpro inhibitory effect compared with senkyuchachosan without PVPP treatment and senkyuchachosan (CsF-). It has been reported that PVPP absorbs not only tannins but also phenolic compounds [50]. Previous reports have indicated that phenolic compounds from natural products inhibit PLpro activity in the deISGylation assay and exhibit anti-SARS-CoV-2 activity in Vero cells [51]. Thus, phenolic compounds derived from CyR, ScS,

and MeH may be involved in inhibiting the PLpro activity. Further studies are required to elucidate the inhibitory mechanisms of these crude drugs on PLpro activity.

Conclusion

We demonstrated the inhibitory effects of senkyuchachosan on PLpro. Using an established *in vitro* PLpro proteolytic assay, crude drugs with strong PLpro inhibitory effects were evaluated. Of the nine crude drugs, four had PLpro inhibitory effects compared with the other five crude drugs. Among the four active crude drugs, CsF, derived from *Camellia sinensis* leaves and its tannin fraction, showed strong PLpro inhibitory activities. These results provide a basis for identifying therapeutic targets of SARS-CoV-2.

Acknowledgments

Declarations

Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

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References

1. Kumar A, Singh R, Kaur J, et al Wuhan to World: The COVID-19 Pandemic. https://doi.org/10.3389/fcimb.2021.596201
2. Coronavirus disease (COVID-19). https://www.who.int/emergencies/diseases/novel-coronavirus-2019. Accessed 27 Jul 2023
3. Sarangi MK, Padhi S, Dheeman S, et al (2022) Diagnosis, prevention, and treatment of coronavirus disease: a review. Expert Rev Anti Infect Ther 20:243-266. https://doi.org/10.1080/14787210.2021.1944103
4. Gordon DE, Jang GM, Bouhaddou M, et al (2020) A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. Nature 583:459-468. https://doi.org/10.1038/S41586-020-2286-9
5. Freitas BT, Durie IA, Murray J, et al (2020) Characterization and Noncovalent Inhibition of the Deubiquitinase and deISGylase Activity of SARS-CoV-2 Papain-Like Protease. ACS Infect Dis 6:2099-2109. https://doi.org/10.1021/ACSINFECDIS.0C00168
6. Wu C, Liu Y, Yang Y, et al (2020) Analysis of therapeutic targets for SARS-CoV-2 and discovery of potential drugs by computational

methods. Acta Pharm Sin B 10:766–788. https://doi.org/10.1016/J.APSB.2020.02.008

7. Lobo-Galo N, Terrazas-López M, Martínez-Martínez A, Díaz-Sánchez ÁG (2021) FDA-approved thiol-reacting drugs that potentially bind into the SARS-CoV-2 main protease, essential for viral replication. J Biomol Struct Dyn 39:3419–3427. https://doi.org/10.1080/07391102.2020.1764393

8. Zhang L, Lin D, Sun X, et al (2020) Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors. Science 368:409–412. https://doi.org/10.1126/SCIENCE.ABB3405

9. Lin MH, Moses DC, Hsieh CH, et al (2018) Disulfiram can inhibit MERS and SARS coronavirus papain-like proteases via different modes. Antiviral Res 150:155–163. https://doi.org/10.1016/J.ANTIVIRAL.2017.12.015

10. Arya R, Prashar V, Kumar M (2022) Evaluating Stability and Activity of SARS-CoV-2 PLpro for High-throughput Screening of Inhibitors. Mol Biotechnol 64:. https://doi.org/10.1007/S12033-021-00383-Y

11. Diamond MS, Kanneganti TD (2022) Innate immunity: the first line of defense against SARS-CoV-2. Nat Immunol 23:165–176. https://doi.org/10.1038/S41590-021-01091-0

12. Lee JS, Shin EC (2020) The type I interferon response in COVID-19: implications for treatment. Nat Rev Immunol 20:585–586. https://doi.org/10.1038/S41577-020-00429-3

13. McClain CB, Vabret N (2020) SARS-CoV-2: the many pros of targeting PLpro. Signal Transduct Target Ther 5:. https://doi.org/10.1038/S41392-020-00335-Z

14. Ratia K, Kilianski A, Baez-Santos YM, et al (2014) Structural Basis for the Ubiquitin-Linkage Specificity and deISGylating activity of SARS-CoV papain-like protease. PLoS Pathog 10:. https://doi.org/10.1371/JOURNAL.PPAT.1004113

15. Jeon YJ, Yoo HM, Chung CH (2010) ISG15 and immune diseases. Biochim Biophys Acta 1802:485–496. https://doi.org/10.1016/J.BBADIS.2010.02.006

16. Klemm T, Ebert G, Calleja DJ, et al (2020) Mechanism and inhibition of the papain-like protease, PLpro, of SARS-CoV-2. EMBO J 39:. https://doi.org/10.15252/EMBJ.2020106275

17. Kawall A, Lewis DSM, Sharma A, et al (2023) Inhibitory effect of phytochemicals towards SARS-CoV-2 papain like protease (PLpro) proteolytic and deubiquitinase activity. Front Chem 10:. https://doi.org/10.3389/FCHEM.2022.1100460

18. Shin D, Mukherjee R, Grewe D, et al (2020) Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity. Nature 587:657–662. https://doi.org/10.1038/S41586-020-2601-5

19. Kuchta K, Cameron S, Lee M, et al (2022) Which East Asian herbal medicines can decrease viral infections? Phytochem Rev 21:219–237. https://doi.org/10.1007/S11101-021-09756-2

20. Yoshino T, Arita R, Horiba Y, Watanabe K (2019) The use of maoto (Ma-Huang-Tang), a traditional Japanese Kampo medicine, to alleviate flu symptoms: a systematic review and meta-analysis. BMC Complement Altern Med 19:. https://doi.org/10.1186/S12906-019-2474-Z

21. Ogawa-Ochiai K, Ishikawa H, Nishimura H, et al (2022) Clinical and epidemiological features of healthcare workers after a coronavirus disease 2019 cluster infection in Japan and the effects of Kampo formulas-Hochuekkito and Kakkonto: A retrospective cohort study. Medicine 101:E29748. https://doi.org/10.1097/MD.0000000000029748

22. Masui S, Nabeshima S, Ajisaka K, et al (2017) Maoto, a Traditional Japanese Herbal Medicine, Inhibits Uncoating of Influenza Virus. Evid Based Complement Alternat Med 2017:. https://doi.org/10.1155/2017/1062065

23. Kakimoto M, Nomura T, Nazmul T, et al (2022) In vitro Suppression of SARS-CoV-2 Infection by Existing Kampo Formulas and Crude Constituent Drugs Used for Treatment of Common Cold Respiratory Symptoms. Front Pharmacol 13:. https://doi.org/10.3389/FPHAR.2022.804103

24. Ishida K, Sato H (2006) Kampo medicines as alternatives for treatment of migraine: six case studies. Complement Ther Clin Pract 12:276–280. https://doi.org/10.1016/J.CTCP.2006.07.002

25. Nishimura H, Okamoto M, Dapat I, et al (2021) Inactivation of SARS-CoV-2 by Catechins from Green Tea. Jpn J Infect Dis 74:421–423. https://doi.org/10.7883/YOKEN.JJID.2020.902

26. Upadhyay S, Tripathi PK, Singh M, et al (2020) Evaluation of medicinal herbs as a potential therapeutic option against SARS-CoV-2 targeting its main protease. Phytother Res 34:3411–3419. https://doi.org/10.1002/PTR.6802

27. Jang M, Park YI, Cha YE, et al (2020) Tea Polyphenols EGCG and Theaflavin Inhibit the Activity of SARS-CoV-2 3CL-Protease In Vitro. Evid Based Complement Alternat Med 2020:. https://doi.org/10.1155/2020/5630838

28. Liu SY, Wang W, Ke JP, et al (2022) Discovery of Camellia sinensis catechins as SARS-CoV-2 3CL protease inhibitors through molecular docking, intra and extra cellular assays. Phytomedicine 96:. https://doi.org/10.1016/J.PHYMED.2021.153853

29. Gogoi B, Chowdhury P, Goswami N, et al (2021) Identification of potential plant-based inhibitor against viral proteases of SARS-CoV-2 through molecular docking, MM-PBSA binding energy calculations and molecular dynamics simulation. Mol Divers 25:1963–1977. https://doi.org/10.1007/S11030-021-10211-9

30. Kawall A, Lewis DSM, Sharma A, et al (2023) Inhibitory effect of phytochemicals towards SARS-CoV-2 papain like protease (PLpro) proteolytic and deubiquitinase activity. Front Chem 10:. https://doi.org/10.3389/FCHEM.2022.1100460

31. Latos-Brozio M, Masek A (2020) Natural Polymeric Compound Based on High Thermal Stability Catechin from Green Tea. Biomolecules 2020, Vol 10, Page 1191 10:1191. https://doi.org/10.3390/BIOM10081191

32. Tsai KC, Huang YC, Liaw CC, et al (2021) A traditional Chinese medicine formula NRICM101 to target COVID-19 through multiple pathways: A bedside-to-bench study. Biomed Pharmacother 133:. https://doi.org/10.1016/J.BIOPHA.2020.111037

33. Yamauchi Y, Nakamura A, Kitai M, et al (2007) Improved sample pre-treatment for determination of caffeine in tea using a cartridge filled with polyvinylpolypyrroridone (PVPP). Chem Pharm Bull (Tokyo) 55:1393-1396. https://doi.org/10.1248/CPB.55.1393

34. Mitchell AE, Hong YJ, May JC, et al (2005) A Comparison of Polyvinylpolypyrrolidone (PVPP), Silica Xerogel and a Polyvinylpyrrolidone (PVP)-Silica Co-Product for Their Ability to Remove Polyphenols from Beer. Journal of the Institute of Brewing 111:20-25. https://doi.org/10.1002/J.2050-0416.2005.TB00644.X

35. Goto I, Saga S, Ichitani M, et al (2023) Investigation of Components in Roasted Green Tea That Inhibit Streptococcus mutans Biofilm Formation. Foods 12:2502. https://doi.org/10.3390/FOODS12132502

396 36. Wall ME, Wani MC, Brown DM, et al (1996) Effect of tannins on
397 screening of plant extracts for enzyme inhibitory activity and
398 techniques for their removal. Phytomedicine 3:281–285.
399 https://doi.org/10.1016/S0944-7113(96)80067-5
400 37. Fu Z, Huang B, Tang J, et al (2021) The complex structure of GRL0617
401 and SARS-CoV-2 PLpro reveals a hot spot for antiviral drug discovery.
402 Nat Commun 12:. https://doi.org/10.1038/S41467-020-20718-8
403 38. Rota PA, Oberste MS, Monroe SS, et al (2003) Characterization of a
404 novel coronavirus associated with severe acute respiratory syndrome.
405 Science 300:1394–1399. https://doi.org/10.1126/SCIENCE.1085952
406 39. Temmam S, Vongphayloth K, Baquero E, et al (2022) Bat coronaviruses
407 related to SARS-CoV-2 and infectious for human cells. Nature 2022
408 604:7905 604:330–336. https://doi.org/10.1038/s41586-022-04532-4
409 40. Ullrich S, Nitsche C (2022) SARS-CoV-2 Papain-Like Protease:
410 Structure, Function and Inhibition. ChemBioChem 23:e202200327.
411 https://doi.org/10.1002/CBIC.202200327
412 41. Montone CM, Aita SE, Arnoldi A, et al (2021) Characterization of the
413 Trans-Epithelial Transport of Green Tea (C. sinensis) Catechin
414 Extracts with In Vitro Inhibitory Effect against the SARS-CoV-2
415 Papain-like Protease Activity. Molecules 26:.
416 https://doi.org/10.3390/MOLECULES26216744

42. Storozhuk M, Lee S, Lee JI, Park J (2023) Green Tea Consumption and the COVID-19 Omicron Pandemic Era: Pharmacology and Epidemiology. Life 13:852. https://doi.org/10.3390/LIFE13030852/S1

43. Du A, Zheng R, Disoma C, et al (2021) Epigallocatechin-3-gallate, an active ingredient of Traditional Chinese Medicines, inhibits the 3CLpro activity of SARS-CoV-2. Int J Biol Macromol 176:1–12. https://doi.org/10.1016/J.IJBIOMAC.2021.02.012

44. Du A, Zheng R, Disoma C, et al (2021) Epigallocatechin-3-gallate, an active ingredient of Traditional Chinese Medicines, inhibits the 3CLpro activity of SARS-CoV-2. Int J Biol Macromol 176:1–12. https://doi.org/10.1016/J.IJBIOMAC.2021.02.012

45. Chourasia M, Koppula PR, Battu A, et al (2021) EGCG, a Green Tea Catechin, as a Potential Therapeutic Agent for Symptomatic and Asymptomatic SARS-CoV-2 Infection. Molecules 26:. https://doi.org/10.3390/MOLECULES26051200

46. Wang F, Zhang S, Zhang J, Yuan F (2022) Systematic review of ethnomedicine, phytochemistry, and pharmacology of Cyperi Rhizoma. Front Pharmacol 13:. https://doi.org/10.3389/FPHAR.2022.965902

47. Zhao X, Zhou M (2022) Review on Chemical Constituents of Schizonepeta tenuifolia Briq. and Their Pharmacological Effects. Molecules 27:. https://doi.org/10.3390/MOLECULES27165249

48. Masumoto N, Ito M (2023) Genetic identification of the original plant species for Mentha Herb listed in the Japanese Pharmacopoeia and

analyses of their essential oil composition. J Nat Med 77:489–495. https://doi.org/10.1007/S11418-023-01690-1

49. Le-Trilling VTK, Mennerich D, Schuler C, et al (2022) Identification of herbal teas and their compounds eliciting antiviral activity against SARS-CoV-2 in vitro. BMC Biol 20:. https://doi.org/10.1186/S12915-022-01468-Z

50. Durán-Lara EF, López-Cortés XA, Castro RI, et al (2015) Experimental and theoretical binding affinity between polyvinylpolypyrrolidone and selected phenolic compounds from food matrices. Food Chem 168:464–470. https://doi.org/10.1016/J.FOODCHEM.2014.07.048

51. Srinivasan V, Brognaro H, Prabhu PR, et al (2022) Antiviral activity of natural phenolic compounds in complex at an allosteric site of SARS-CoV-2 papain-like protease. Communications Biology 2022 5:1 5:1–12. https://doi.org/10.1038/s42003-022-03737-7

456 **Table 1 Crude drug composition of senkyuchachosan used in this study**

Latin name (crude drug)	weight ratio (%)	Company
CYPERI RHIZOMA	20.0	Tochimoto Tenkaido Co., Ltd.
CNIDII RHIZOMA	15.0	Tochimoto Tenkaido Co., Ltd.
NOTOPTERYGII RHIZOMA	10.0	Tochimoto Tenkaido Co., Ltd.
SCHIZONEPETAE SPICA	10.0	Tochimoto Tenkaido Co., Ltd.
MENTHAE HERBA	10.0	Tochimoto Tenkaido Co., Ltd.
ANGELICAE DAHURICAE RADIX	10.0	Tochimoto Tenkaido Co., Ltd.
SAPOSHNIKOVIAE RADIX	10.0	Tochimoto Tenkaido Co., Ltd.
GLYCYRRHIZAE RADIX	7.5	Uchida Wakanyaku Co., Ltd.
CAMELLIAE SINENSIS FOLIUM	7.5	Uchida Wakanyaku Co., Ltd

457

458 **Table 2 Tannin contents and PLpro inhibitory activity of senkyuchachosan**

	Tannin contents / decoctions (μg/ml)	PLpro inhibitory rate (%)		
		1000 μg/ml	500 μg/ml	250 μg/ml
Senkyuchachosan	122	57.4 ± 1.7	32.6 ± 1.6	14.9 ± 2.4
Senkyuchachosan CsF (-)	28.0	27.9 ± 4.5	17.6 ± 2.4	4.3 ± 1.2
Senkyuchachosan (PVPP treatment)	13.3	5.1 ± 1.7	1.7 ± 0.6	1.1 ± 2.4

459

460 **Figure legends**

461 **Fig. 1 Fluorogenic *in vitro* activity assay for SARS-CoV-2 PLpro.**

462 (a) PLpro was expressed in *E. coli* and purified using affinity chromatography. The total
463 lysate and purified PLpro were separated by 12% SDS-PAGE and stained with CBB. (b)
464 PLpro activity was measured using an *in vitro* assay. To evaluate the specificity of PLpro,
465 two fluorogenic substrates for PLpro (Z-Arg-Leu-Arg-Gly-Gly-MCA) and 3CLpro (Ac-
466 Thz-Tle-Leu-Gln-MCA) were used. An increase in time-dependent fluorescence was
467 observed when the PLpro substrate was used. When GRL-0617, a PLpro inhibitor, was
468 added, the increase in fluorescence due to PLpro activity was inhibited

469

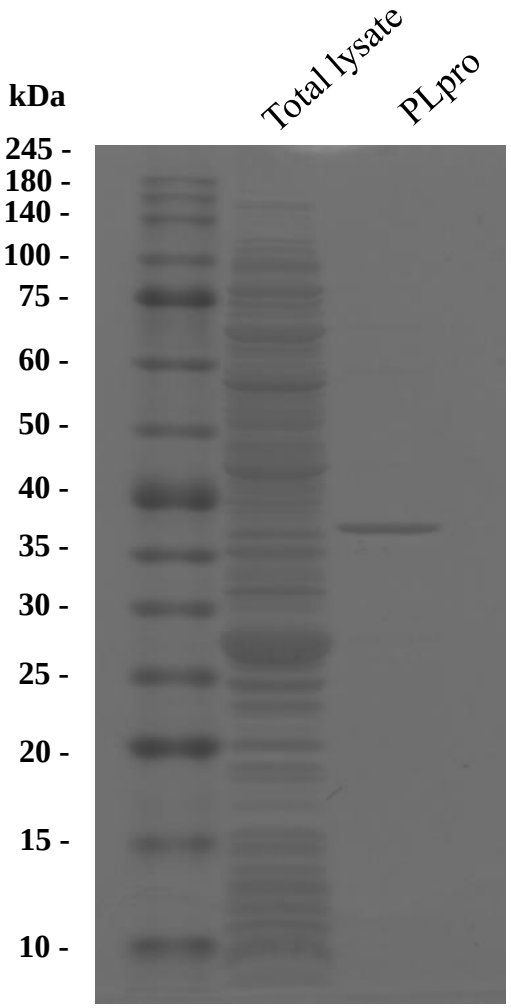
470 **Fig. 2 Inhibitory effects of the crude drug component of senkyuchachosan formula**
471 **on PLpro activity.** (a) PLpro inhibitory effects of nine crude drug extracts (final
472 concentration: 200 μg/ml). Values represent the mean ± SD of three independent
473 experiments ($n = 3$). Statistical significance was represented as $^{*}p < 0.05$, $^{**}p < 0.01$, and
474 $^{***}p < 0.001$ compared with the control using ANOVA and post-hoc Dunnett's tests. (b)
475 PLpro activity was evaluated in the presence of crude drugs at various concentrations
476 (500, 250, 125, and 62.5 μg/ml). Cyperi Rhizoma, Schizonepetae Spica, Menthae Herba,
477 and Camellia sinensis folium inhibited PLpro activities in a dose-dependent manner.

478

479 **Fig. 3 PLpro inhibitory activity of CsF-derived tannins.** CsF extracts were
480 fractionated using the tannin fraction. The PLpro inhibitory activity (IC_{50}) of the tannin
481 fraction was determined using dose-response curves ($n = 3$)

Fig. 1

(a)


kDa
Total lysate
PLpro
245 -
180 -
140 -
100 -
75 -
60 -
50 -
40 -
35 -
30 -
25 -
20 -
15 -
10 -

(b)

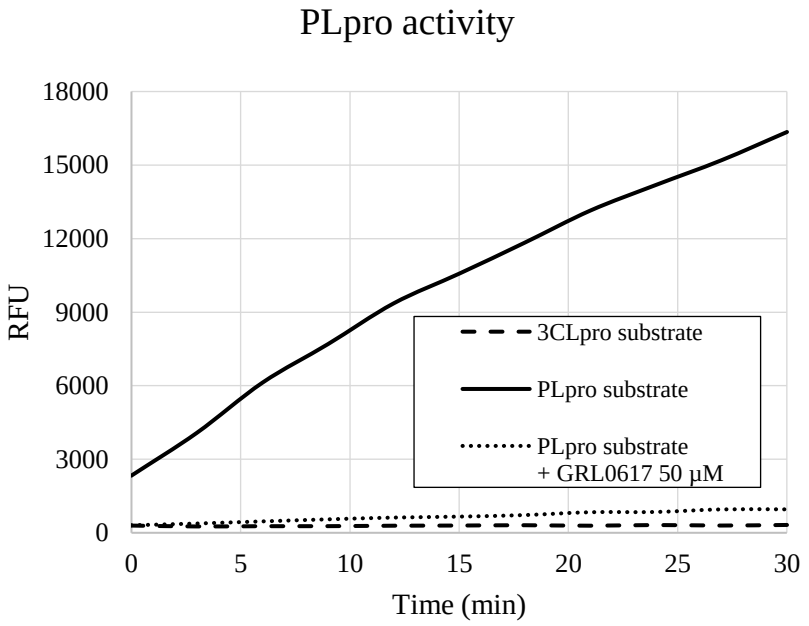
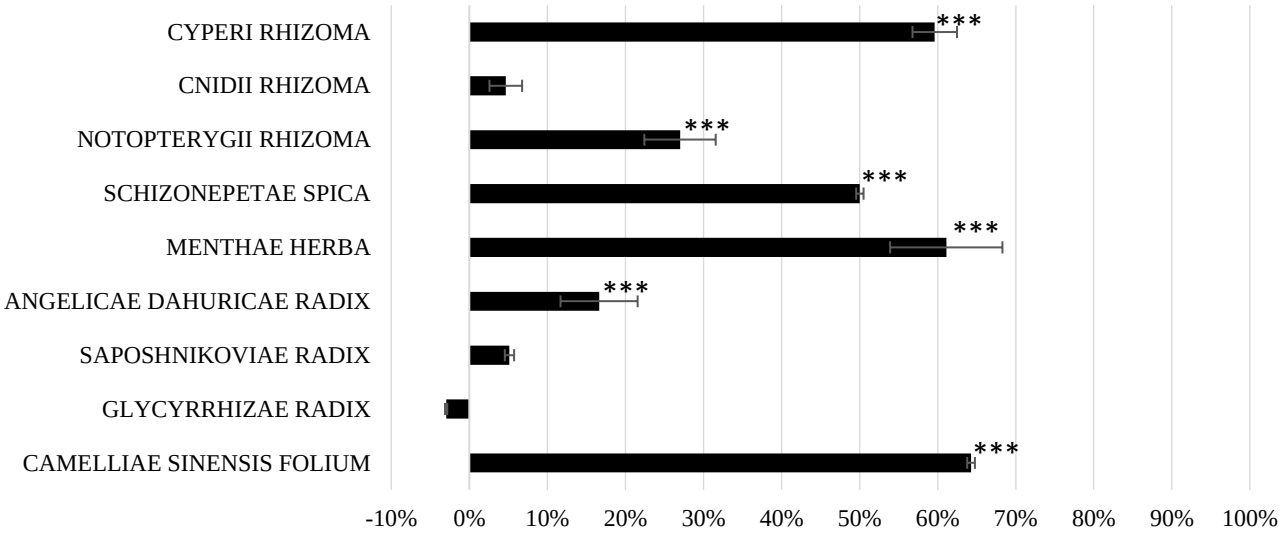

PLpro activity
RFU
18000
15000
12000
9000
6000
3000
0
0
5
10
15
20
25
30
Time (min)
3CLpro substrate
PLpro substrate
PLpro substrate
+ GRL0617 50 µM

Fig. 2

(a)


CYPERI RHIZOMA
CNIDII RHIZOMA
NOTOPTERYGII RHIZOMA
SCHIZONEPETAE SPICA
MENTHAE HERBA
ANGELICAE DAHURICAE RADIX
SAPOSHNIKOVIAE RADIX
GLYCYRRHIZAE RADIX
CAMELLIAE SINENSIS FOLIUM
***
-10% 0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

(b)

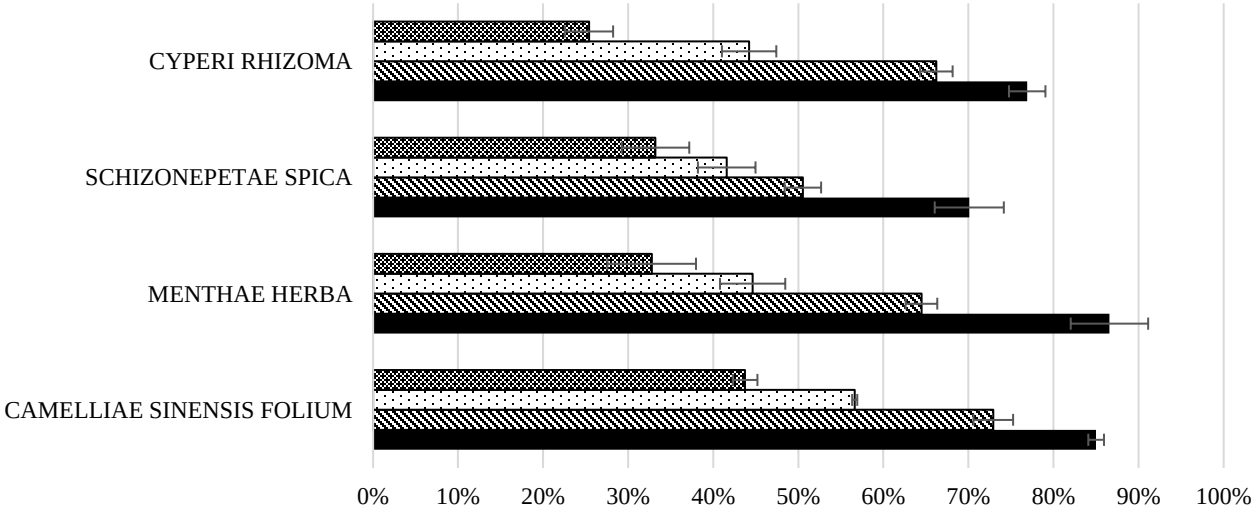
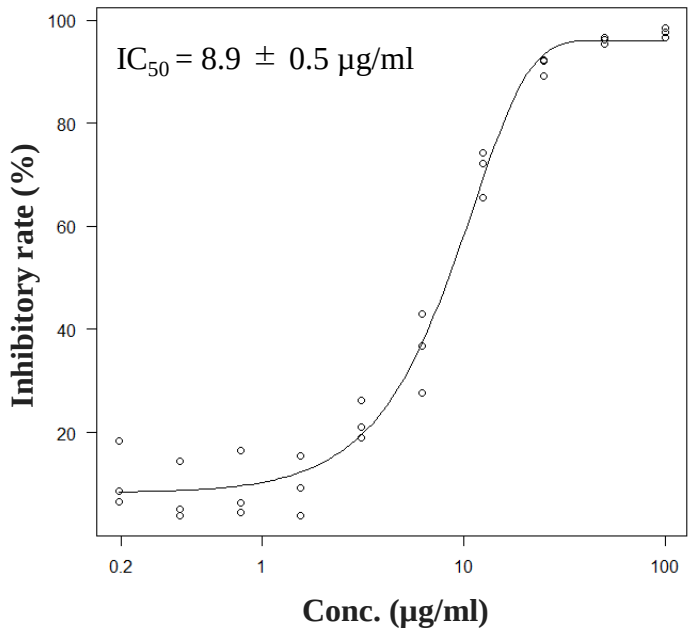

CYPERI RHIZOMA
SCHIZONEPETAE SPICA
MENTHAE HERBA
CAMELLIAE SINENSIS FOLIUM
0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

Fig. 3


IC50 = 8.9 ± 0.5 µg/ml
Inhibitory rate (%)
100
80
60
40
20
0.2
1
10
100
Conc. (µg/ml)