



Research article

Inhibition of novel severe acute respiratory syndrome coronavirus type 2 M^{pro} by sugarcane polyphenol compound

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Abstract

Importance of the work: There have been limited studies on high-yield polyphenol sources from agroindustry by-products for cost-effective novel severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) antivirals.

Objectives: To isolate polyphenols from molasses and bagasse, to test their inhibition of SARS-CoV-2 M^{pro} and to develop cost-effective antiseptic products.

Materials and Methods: Polyphenols from molasses and bagasse were purified and analyzed using spectrometry and high-performance liquid chromatography with diode-array detection (HPLC-DAD). Extracts were tested for SARS-CoV-2 M^{pro} inhibition, with the concentrations determined required to inhibit SARS-CoV-2 M^{pro} activity by 50% (IC₅₀). Each candidate fraction was analyzed using mass spectrometry and HPLC-DAD. Molecular docking of 12 molecules against SARS-CoV-2 M^{pro} was conducted using the GOLD software.

Results: The polyphenol crude extract achieved 100% inhibition of SARS-CoV-2 M^{pro} at 100 µg/mL, while fractions 1, 3 and 5 achieved inhibition values (mean ± SE) of 84.15 ± 3.57%, 46.25 ± 13.54% and 29.54 ± 6.14% inhibition, respectively. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) identified key compounds in fraction 1, including linarin, which produced strong binding in molecular docking studies. Linarin formed hydrogen bonds with key residues in SARS-CoV-2 M^{pro} and demonstrated significant inhibitory activity with a (mean ± SE) IC₅₀ value of 89.98 ± 10.15 µM. Based on these results, linarin, a naturally occurring flavonoid, could serve as a promising inhibitor of SARS-CoV-2 M^{pro}.

Main finding: Polyphenol extracts showed substantial SARS-CoV-2 M^{pro} inhibition, with fraction 1 containing linarin, which had strong binding and inhibitory activity (IC₅₀ of 89.98 ± 10.15 µM). Linarin could be a promising natural inhibitor against SARS-CoV-2 M^{pro}.

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Introduction

COVID-19 is a disease caused by a coronavirus called novel severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2). The World Health Organization first learned of this new virus on 31 December 2019. SARS-CoV-2 can be transmitted easily via contact, saliva, sputum and droplets from patients. Common symptoms range from chills, sore throat and cough to severe symptoms including high fever, trouble breathing, pneumonia and sepsis or even death. The consequences from the COVID-19 outbreak changed people's lifestyles, resulting in people attaching importance to cleanliness and the use of products to increase prevention measures and to inhibit pathogenesis, as highlighted on the weblink covid19.who.int (Lai et al., 2020).

The infection initiates at the cell surface when the trimeric S protein on the viral envelope recognizes a cellular receptor; angiotensin-converting enzyme 2 (ACE2; Mittal et al., 2020; Shpakov, 2020). After entry to the host cell cytoplasm, the viral genome directly functions as mRNA translated into two open reading frames 1a and 1b to polyproteins. Cleavage of the polyproteins into 16 non-structural proteins, which are functional proteins depend on two types of proteases: consisting of a papain-like cysteine protease (PL^{pro}; Uma Sankar Gorla and Rao, 2020; Yan and Wu, 2020) and a chymotrypsin-like cysteine protease known as 3C-like protease (3CL^{pro}, also called the main protease; M^{pro}) (Liang, 2006; Santana-Vieira et al., 2014). The proteolytic cleavage at 3 sites by PL^{pro} and no less than 11 sites on the large polyprotein by M^{pro}, results in the development of viral enzymes involved in the replication-transcription complex that plays a crucial role in initiating replication of the virus in host cells (Lv et al., 2021; Zhao et al., 2022). Therefore, with more cleavage sites, M^{pro} is one of the best targets for antiviral drug discovery.

Polyphenols are natural polymers capable of destroying bacteria, fungi and viruses with specific and different mechanisms depending on the nature of the active ingredient and the target pathogen. For example, numerous studies have reported on the diverse roles of plant polyphenols on human health ranging from being antioxidant, anti-inflammatory, antitumor and antimicrobial agents (Yan et al., 2020; Niu et al., 2023) to antiviral agents, and reporting the positive effects of polyphenol compounds against the SARS-CoV-2 M^{pro} such as epigallocatechin gallate, ellagic acid, curcumin, resveratrol, quercetin (Wang et al., 2022) and rutin, isoquercitrin (quercetin-3-O-glycoside), kaempferol, dihydrokaempferol,

dihydromyricetin, myricetin and quercetin (Hoffmann et al., 2020). Most studies have focused on the interaction between polyphenols and SARS-CoV-2 M^{pro} using a combination of *in-vitro* and *in-silico* approaches to develop novel anti-COVID-19 agents derived from natural polyphenols. However, only a small amount of active ingredients can be extracted from the plant, resulting in high costs. Acquiring active ingredients from sources with high amounts would be an ideal solution such as from a by-product of the sugar processing industry (Lemes et al., 2021).

For these purposes, a polyphenol compound was isolated from a by-product of the sugar processing industry and investigated for inhibitory potential against SARS-CoV-2 M^{pro}. The current study aimed to select a polyphenol compound, as a by-product of the polyphenols from molasses and bagasse for evaluation of its inhibitory activity against SARS-CoV-2 M^{pro} using a combination of *in-vitro* and *in-silico* approaches for the development of antiseptic products in the future.

Materials and Methods

Materials

The molasses and bagasse were obtained from the Mitr Phol Group, Thailand. Polyphenols from the molasses were extracted using solvent extraction (water and ethanol), while the polyphenols from the bagasse were extracted using alkalis, with the alkalis being removed using an electrolytic process (electrodialysis) from the lignin extract. The total phenolic compounds extracted from the molasses with resin were purified using dynamic axial chromatography. The total phenolic and flavonoid contents were analyzed quantitatively based on spectrometry and the compound profiling was performed initially using dynamic axial compression preparative high-performance liquid chromatography (DAC preparative HPLC), followed by identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS). All crude extracts and fractions were prepared by the Herb and Bioactive Compounds Technology Laboratory, Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Kasetsart University, Bangkok, Thailand.

Novel severe acute respiratory syndrome coronavirus type 2 M^{pro} inhibition assay

Initially, crude extract and each fraction of the polyphenols

from the molasses and bagasse were screened for inhibitory activity against SARS-CoV-2 M^{pro}, which was produced in-house at 0.8 μ M, using the synthetic fluorescence resonance energy transfer (FRET) substrate Dabcyl-Lys-Thr-Ser-Ala-Val-Leu-Gln-Ser-Gly-Phe-Arg-Lys-Met-Glu (Edans)-NH₂ (Genscript USA Inc.; Piscataway, NJ, USA). The screening and dose-response studies, to determine the concentrations required to inhibit SARS-CoV-2 M^{pro} activity by 50% (IC₅₀) followed Panwar et al. (2022), Seetaha et al. (2022), Choowongkamon et al. (2023) and Yang et al. (2023). The screening process involved several steps. First, the stock solutions of synthetic FRET substrate, known drugs and the sample were prepared in dimethyl sulfoxide (DMSO) before dilution in assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 4 mM dithiothreitol and 0.4 mM ethylenediaminetetraacetic acid) with the final concentration of DMSO being less than 1% (volume per volume) in the reaction. Second, 0.8 μ M of SARS-CoV-2 M^{pro} was incubated with the different samples at 100 μ g/mL for 10 min at room temperature with an assay buffer in black 384-well plates. After that, the reaction was initiated by adding a fluorogenic substrate at a final concentration of 40 μ M; the fluorescence signals were recorded every 1 min for 30 min using a fluorescence microplate reader (Infinite F200 Pro; Tecan; Männedorf, Switzerland) at an excitation of 340 nm and an emission of 430 nm. The inhibitory effects of the crude extract and each fraction of the polyphenols from the molasses and bagasse were investigated by comparing the percentage of relative inhibition with lopinavir and ritonavir as commercial drugs for HIV-1 protease inhibition, which were used as reference compounds due to their known inhibitory activity against SARS-CoV-2 M^{pro}, thereby allowing benchmarking of the activity of the natural extracts. The percentage of relative inhibition was calculated based on the initial velocity (V₀) using Equation 1:

Percentage of relative inhibition =

$$\frac{\{[(V_0\text{Enzyme control} - V_0\text{Blank control}) - (V_0\text{Sample control} - V_0\text{Blank control})] / (V_0\text{Enzyme control} - V_0\text{Blank control})\}}{\times 100} \quad (1)$$

The same steps were used to determine the concentrations of the selected fraction of polyphenol extract required to inhibit 50% of the SARS-CoV-2 M^{pro} activity (IC₅₀), according to Seetaha et al., 2022. The IC₅₀ values were calculated by plotting the percentage of inhibition against various concentrations of protease inhibitors using a dose-response curve developed with the GraphPad Prism 6 software (GraphPad Software, Inc.; San

Diego, CA, USA).

Phytochemical profiling and qualitative metabolite analysis

Initially, fraction 1 was analyzed using a Q-Exactive HF-X Orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA, USA). Fraction 1 was dissolved in 50% methanol to 500 parts per million. Then, 2 μ L of the sample was separated on a Hypersil GOLD TM Vanquish C18 column (2.1 $\times$ 100 mm, 1.9 μ m, Thermo Scientific, Waltham, MA, USA) equipped with a guard column at 40°C, using a flow rate of 0.4 mL/min. The mobile phase comprised 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a gradient starting from 5% B, held for 4 min, before raising to 100% B over 14 min. Then, the column was flushed with 100% B for 4 min, reduced to 5% B over 1 min, before returning to the starting condition until 25 min. MS acquisition was performed via a heated electrospray ionization source, negative and positive ions were detected using the full scan MS¹/data-dependent MS² (dd-MS²) mode, with the parameter settings: spray voltage 3.5kV (positive) and 2.5kV (negative), sheath gas 45 atomic units (AU), auxiliary gas 10 AU, sweep gas 2 AU, capillary temperature 320°C, probe heater temperature 400°C, full scan MS¹ resolution 120,000, data-dependent MS² resolution 30,000, scan range 100–1,500 m/z, automatic gain control (AGC) target 3 $\times$ 10⁶, max injection time (IT) 100 ms, stepped normalized collision energy (NCE) 20 eV, 30 eV and 40 eV. Identified active compounds from fraction 1 were processed by comparison of their acquired files using the Compound Discoverer software 3.1 (Thermo Fisher Scientific; Waltham, MA, USA) including the Mzcloud (<https://www.mzcloud.org>), Mzvault (<https://documents.thermofisher.com/TFS-Assets/CMD/manuals/man-xcali-97970-mzvault-user-manxcali97970-en.pdf>) and Chemspider databases (<http://www.chemspider.com>).

Quantitative analysis of linarin in fraction 1

Quantitative analysis of linarin (ChemFaces; Wuhan, People's Republic of China) in fraction 1 was performed using HPLC-DAD on a Primaide unit equipped with a Primaide 1430 diode array detector (Hitachi; Tokyo, Japan). Linarin was dissolved in absolute ethanol, passed through 0.20 μ m polytetrafluoroethylene (PTFE) membrane filters (Whatman Syringe Filter 25 mm, 0.2 μ m) and separated on an Agilent Varian Polaris C18-Ether 5U 250 $\times$ 4.6 mm column (Varian; Palo Alto, CA, USA) with mobile phase A being 0.1%

trifluoroacetic acid (TFA) in ultrapure water and mobile phase C being 0.1% TFA in acetonitrile. The gradient elution was used under the conditions: 0–7 min, 5–60% C; 7–14 min, 60–84% C; and 14–25 min, 5% C. The flow rate of the mobile phase was set to 0.8 mL/min and the injection volume was 10 μ L. The detection wavelengths for linarin compounds were set at 220 nm, 254 nm, 331 nm and 360 nm. The linarin calibration concentrations were 0.01, 0.1 and 1 mg/mL (coefficient of determination = 1). Fraction 1 compounds were identified by comparing retention times with the linarin standard and quantitative analysis of the linarin compound. Each sample was injected in triplicate on the same day. The method of precision was evaluated using intra-day tests to calculate SD values (Hwang et al., 2016).

Molecular docking

The three-dimensional structure of the SARS-CoV-2 (COVID-19) main protease (M^{pro}) in complex with the inhibitor UAW246 was downloaded from the Protein Data Bank (PDB Code: 6XBG; Sacco et al., 2020). The 12 molecules of interest were lopinavir (CID: 92727), ritonavir (CID: 392622), linarin (CID: 5317025), sakuranin (CID: 73607), adenosine (CID:60691), khellin (CID: 3828), α,α -trehalose (CID:91696341), 1,4-dihydroxy-2-naphthoic acid (CID:671), gluconic acid (CID: 10690), 4-oxo-4,5,6,7-tetrahydro-1-benzofuran-3-carboxylic acid (CID:713937), D-(-)-quinic acid (CID:6508) and D-glucopyranuronic acid (CID:94715). They were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) in structure-data file format (SDF) format. The current study utilized the GOLD CCDC version 2022.3.0 docking software for structure-based molecular docking. Initially, the protein structure of SARS-CoV-2 M^{pro} was prepared by removing all water molecules and adding hydrogen atoms using the protein preparation wizard window.

Subsequently, the compounds of interest were investigated as ligands for docking. The docking site was defined as the center of the reference ligand (UAW246) in the PDB structure of the SARS-CoV-2 with inhibitor UAW246 complex and the docking cavity extended within an 8.0 Å radius around it.

In the docking simulations, the protein structure was fixed, while the structure of ligands was freely rotated to identify a hit compound that could tightly bind within the binding pocket of the protein target. The number of genetic algorithm runs was set to 15 per molecule and the Goldscore fitness score was used as the default parameter for performing protein-ligand docking. The analysis of docking results was performed using

Pymol (Yuan et al., 2016) and Discovery Studio visualizer (v21.1.0.20298; <https://www.3ds.com/products-services/biovia/>).

Results

Inhibition of main protease of novel severe acute respiratory syndrome coronavirus type 2 by polyphenol crude extract

The screening effects were determined based on the SARS-CoV-2 M^{pro} inhibition activity of the polyphenol crude extract and each fraction of crude extract using a FRET-based protease inhibition assay. The inhibitory activities and the dose-dependent inhibition of each fraction were compared to lopinavir and ritonavir, which are two antiviral drugs approved drugs used for the treatment of COVID-19 patients in the early stages by the United States Food and Drug Administration (Magro et al., 2021; Citarella et al., 2021). The findings (Table 1) demonstrated that the polyphenol crude extract completely inhibited M^{pro} activity (100% inhibition) at 100 μ g/mL. In contrast, fractions 1, 3 and 5 had substantially lower inhibitory activities of $84.15 \pm 3.57\%$, $46.25 \pm 13.54\%$ and $29.54 \pm 6.14\%$, respectively. All results were compared to lopinavir and ritonavir, which exhibited inhibition percentages of $63.36 \pm 2.48\%$ and $58.56 \pm 2.90\%$, respectively (Table 1). Notably, fraction 1 had the highest inhibitory activity among the tested fractions, exceeding the inhibitory effects of both ritonavir and lopinavir (Fig. 1). Therefore, to identify which compounds in fraction 1 inhibited the main protease of SARS-CoV-2, the phytochemicals in fraction 1 were identified using LC-MS/MS. In addition, to examining the inhibitory effect of the candidate inhibitors, the IC_{50} values were determined of all compounds for comparison.

Phytochemical profiling and qualitative metabolite analysis of fraction 1

Table 1 Percentage (mean $\pm$ SE) of main SARS-CoV-2 protease inhibition of lopinavir, ritonavir, polyphenol crude extract and fractions 1, 3 and 5

Sample	Relative inhibition (%)
Lopinavir	63.36 $\pm$ 2.48
Ritonavir	58.56 $\pm$ 2.90
Crude extract	100.00
Fraction 1	84.15 $\pm$ 3.57
Fraction 3	46.25 $\pm$ 13.54
Fraction 5	29.54 $\pm$ 6.14

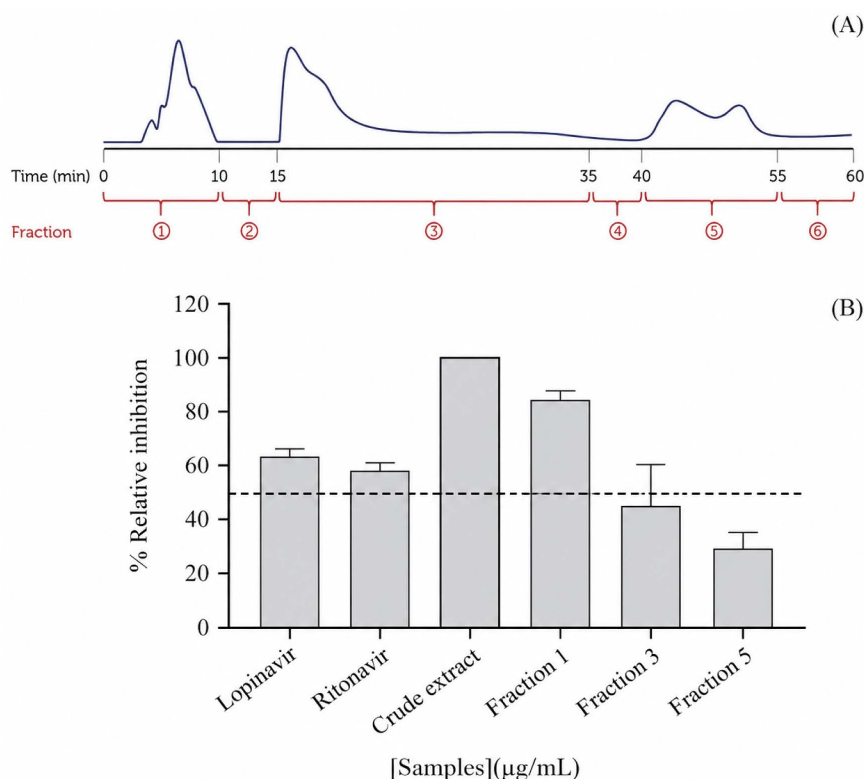


Fig. 1 (A) Dynamic axial compression preparative high-performance liquid chromatography (DAC preparative HPLC) of extract monitored at 270 nm; (B) relative inhibition of main protease of novel severe acute respiratory syndrome coronavirus type 2 with 100 µg/mL of lopinavir, ritonavir, crude extract and fractions 1, 3 and 5, where error bar = SD

Fraction 1, isolated from the crude extract, exhibited $84.15 \pm 3.57\%$ inhibition of SARS-CoV-2 M^{pro} activity at a concentration of 100 µg/mL. Fraction 1 was dissolved in 50% methanol and its mass ionization signals were detected using full scan MS1 and data-dependent MS² (dd-MS²) modes. The resulting data were analyzed with Compound Discoverer software and compared against the MzCloud, MzVault and ChemSpider databases, revealing similarity indices in the range of 65–100%. The result from LC-MS/MS presents the 10 major components in fraction 1 that were selected from 120 compounds based on the LC-MS/MS results: adenosine, quinic acid, α,α -trehalose, gluconic acid, linarin, khellin, 4-Oxo-4,5,6,7-tetrahydro-1-benzofuran-3-carboxylic acid, 1,4-dihydroxy-2-naphthoic acid, sakuranin and D-glucopyranuronic acid, respectively (Table 2). The presence of these annotated compounds was confirmed and validated using their respective accurate mass value, experimental and calculated m/z , molecular formula and matches in well-known databases, corresponding to the molecular weights shown in Table 2. Based on these results, the identified polyphenolic compounds included quinic acid, linarin and sakuranin. Further

analysis confirmed that linarin (Mottaghipisheh et al., 2021) and sakuranin (Hasegawa and Shirato, 1955) are flavonoids within the polyphenol class.

Molecular docking simulations

Structure-based ligand docking against SARS-CoV-2 M^{pro} was studied using molecular docking techniques to assess the binding affinity between ligands and the crystal structure of M^{pro} . The potential ligand-binding pockets in SARS-CoV-2 M^{pro} were assessed using the fitness score calculated using the GOLD Score. Table 3 shows the ranking of the binding scores of each compound based on their binding strength, with higher values indicating stronger binding. Notably, the highest-scoring compound in the dataset was linarin (80.77). The control compounds scores for the two standard inhibitors and one reference compound were ritonavir (110.48), lopinavir (92.77) and the peptide-like inhibitor UAW (94.65), respectively. Upon analyzing the binding pose with the highest score for linarin, visualized using Discovery Studio visualizer program as shown in Fig. 2B, it was evident that linarin formed three hydrogen

Table 2 List of high-abundance phytochemicals detected in fraction 1

List	Compound	Phytochemical class	Formula	Molecular weight (Da)	Peak area (a.u.)
1	Adenosine	Nucleoside	C ₁₀ H ₁₃ N ₅ O ₄	267.09	9.68×10 ⁸
2	Quinic acid	Cyclitol	C ₇ H ₁₂ O ₆	192.06	2.19 ×10 ⁸
3	α,α-Trehalose	Disaccharide	C ₁₂ H ₂₂ O ₁₁	342.11	1.38 ×10 ⁸
4	Gluconic acid	Sugar acid	C ₆ H ₁₂ O ₇	196.06	4.11×10 ⁷
5	Linarin	Flavonoid	C ₂₈ H ₃₂ O ₁₄	592.18	2.71×10 ⁷
6	Khellin	Furanochrome	C ₁₄ H ₁₂ O ₅	260.07	4.02×10 ⁶
7	4-Oxo-4,5,6,7-tetrahydro-1-benzofuran-3-carboxylic acid	Benzofurans	C ₉ H ₈ O ₄	180.04	3.70×10 ⁶
8	1,4-Dihydroxy-2-naphthoic acid	Naphthoic acid	C ₁₁ H ₈ O ₄	204.04	2.83×10 ⁶
9	Sakuranin	Flavonoid	C ₂₂ H ₂₄ O ₁₀	448.14	3.46×10 ⁷
10	D-Glucopyranuronic acid	D-glucopyranuronic acid	C ₆ H ₁₀ O ₇	194.04	1.01×10 ⁷

bonds with Leu141, Ser144 and His164 through oxygen atom in its hydroxyl and glycosidic groups. Additionally, nonpolar interactions involved the flavone moiety group with Cys145, His163 and Met165 (π -alkyl) as shown in Figs. 2A and 2B. The predicted complex 3D structures and their 2D schematic representations of all compounds are illustrated in the Supplementary Information (Fig. S1)

Quantitative analysis of linarin in fraction 1

The contents of linarin in fraction 1 were determined based on HPLC-DAD and the results are shown in the chromatogram

of the linarin standard (Fig. 3) that was detected at a wavelength of 360 nm. The fraction 1 retention times were compared to the linarin standard; the linarin contents were calculated based on the linear equation for the linarin standard. The linarin concentration in fraction 1 was 23.49 ± 0.17 $\mu\text{g/mL}$.

Determination of concentrations required to inhibit novel severe acute respiratory syndrome coronavirus type 2 M^{pro} activity by 50%

A general FRET-based assay method was used to independently confirm the inhibitory activity of linarin against

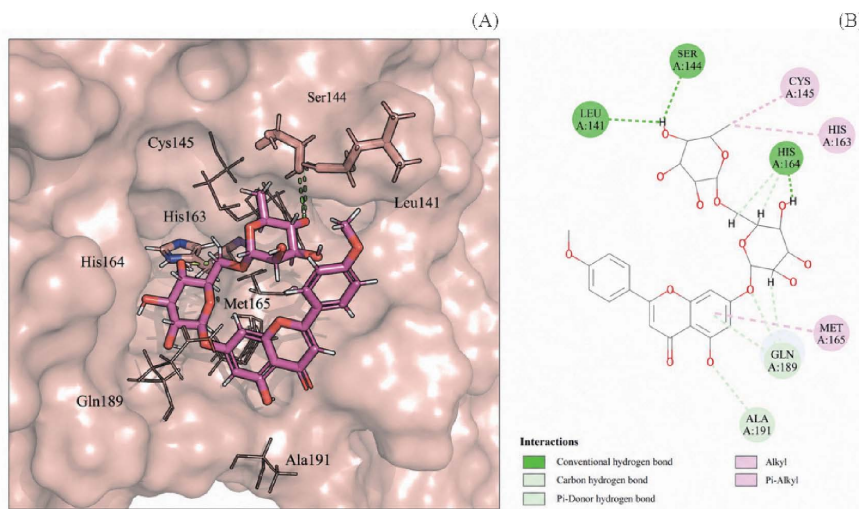


Fig. 2 Crystal structure of novel severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) M^{pro} in complex with linarin, showing an overview of structure of linarin-bound M^{pro} (PDB code: 6XBG): (A) three-dimensional interactions between linarin compound (purple) are bound to the binding pocket of SARS-CoV-2 M^{pro} (pink), with hydrogen bond interactions represented by green dashed lines; (B) two-dimensional interactions between linarin and surrounding residues of SARS-CoV-2 M^{pro} generate using PyMOL program (Yuan et al., 2016) and Discovery Studio Visualizer software (v21.1.0.20298; <https://www.3ds.com/products-services/biovia/>).

Table 3 Binding score of molecules of interest with novel severe acute respiratory syndrome coronavirus type 2 M^{pro} determined based on their molecular docking results

Ranking	Compound name	GOLD docking score
1	Ritonavir	110.48
2	Reference ligand (UAW246)	94.65
3	Lopinavir	92.77
4	Linarin	80.77
5	Sakuranin	76.61
6	Adenosine	56.50
7	Khellin	52.56
8	α,α -Trehalose	51.03
9	1,4-Dihydroxy-2-naphthoic acid	46.70
10	Gluconic acid	42.80
11	4-Oxo-4,5,6,7-tetrahydro-1-benzofuran-3-carboxylic acid	41.62
12	D-(-)-Quinic acid	40.95
13	D-Glucopyranuronic acid	39.40

the SARS-CoV-2 M^{pro}. Lopinavir and ritonavir were included in Table 4 as reference HIV protease inhibitors that have been previously reported to exhibit inhibitory activity against SARS-CoV-2 M^{pro}. Their IC₅₀ values were determined under the same assay conditions and serve as comparative standards for evaluating the potency of the test samples. Our previous study reported the IC₅₀ values for lopinavir and ritonavir as 77.03±8.51 µg/mL (122.50 µM) and 23.39±2.52 µg/mL

(32.44 µM), respectively (Seetaha et al., 2022). Fraction 1 was identified as an inhibitor of SARS-CoV-2 M^{pro}, with an IC₅₀ value of 5.59±1.42 µg/mL (Fig. 4A), while linarin exhibited an IC₅₀ value of 89.98±10.15 µM (Fig. 4B and Table 4), which is higher than that of lopinavir. Although linarin shows lower medicinal efficacy, its natural origin suggests it may be safer to use as a natural therapeutic agent. These results indicate that linarin is a potential candidate inhibitor of the SARS-CoV-2 M^{pro}.

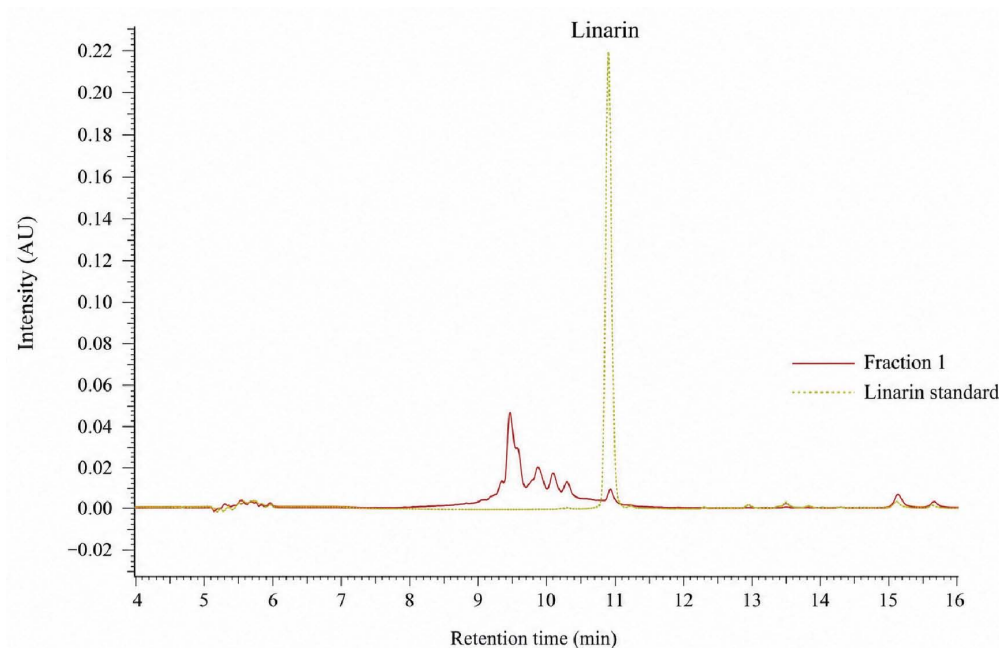
Discussion

At present, finding highly effective antiviral drugs for COVID-19 remains a major challenge. SARS-CoV-2 M^{pro}, which mediates proteolytic cleavage of viral polyproteins essential for replication, has been recognized widely as a

Table 4 Concentrations (mean ± SD) required to inhibit novel severe acute respiratory syndrome coronavirus type 2 M^{pro} activity by 50% (IC₅₀)

Sample	IC ₅₀ (µg/mL)	IC ₅₀ (µM)
Lopinavir	77.03±8.51	122.50±13.54
Ritonavir	23.39±2.52	32.44±3.49
Fraction 1	5.59±1.42	-
Linarin	53.27±6.01	89.98±10.15

IC₅₀ values calculated based on molecular weight of each compound.

**Fig. 3** Chromatographic profiles of linarin standard (1 mg/mL) and fraction 1

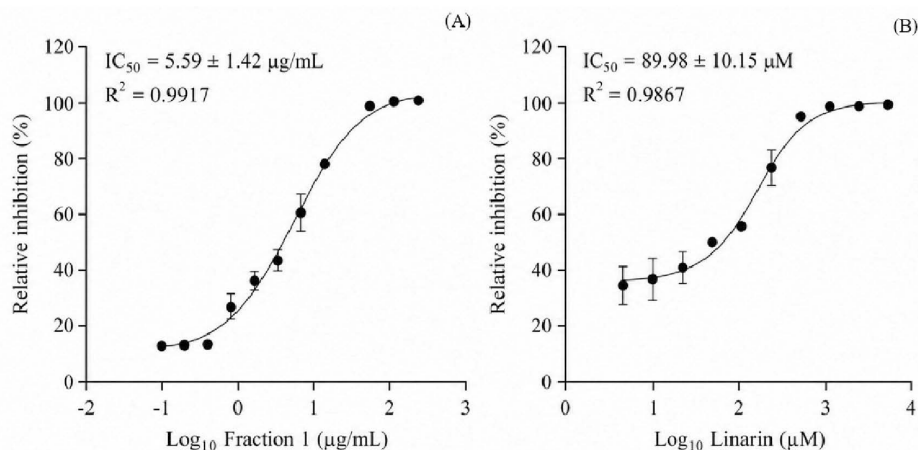


Fig 4. Concentrations required to inhibit novel severe acute respiratory syndrome coronavirus type 2 M^{pro} activity by 50% (IC₅₀) of: (A) fraction 1; (B) linarin, where error bars = $\pm$ SD and R² = coefficient of determination

promising therapeutic target (Xiong et al., 2021). Several studies have reported different high throughput screening technologies for the examination of new drugs or inhibitors to treat COVID-19 through inhibition of SARS-CoV-2 M^{pro}, including the BPL 3CL protease (SARS-CoV-2) assay kit for an enzyme-linked immunosorbent assay (Pitsillou et al., 2020) and FRET-based protease assay (Ma et al., 2020; Su et al., 2020; Zhu et al., 2020).

Polyphenols are a large family of naturally occurring phenols and are bioactive compounds found in fruits, vegetables and herbs that have been studied extensively for their medicinal benefits, including anti-bacterial, anti-cancer, anti-inflammatory, anti-diabetes and antiviral properties (Liu et al., 2008). Polyphenols have been divided into many classes such as lignans, phenolic alcohols, stilbenes, phenolic acids and flavonoids (Moga et al., 2016). The current test successfully purified recombinant SARS-CoV-2 M^{pro} that could be produced in-house, which could be used in the laboratory for M^{pro} inhibitor screening with Dabcyl-Lys-Thr-Ser-Ala-Val-Leu-Gln-Ser-Gly-Phe-Arg-Lys-Met-Glu (Edans)-NH₂ as its commercially available substrate, using a combination of *in-vitro* and *in-silico* approaches. Initially, the polyphenol extract from molasses and bagasse was screened, with positive effects against the SARS-CoV-2 M^{pro}.

Based on the current results, some polyphenol compounds were identified in the polyphenol crude extracts using mass spectrometry, consisting of the phenolic acids class (quinic acid; Slatnar et al., 2014) and the flavonoid class (linarin, sakuranin). These compounds were further evaluated for their relative binding potential to the active site of SARS-CoV-2

M^{pro} using molecular docking analysis (Rubio-Martinez et al., 2021). The docking analysis suggested that linarin and sakuranin exhibited favorable predicted interaction patterns with M^{pro}, primarily through hydrogen bonding with amino acid residues within the binding pocket. Among the tested polyphenols, linarin achieved a relatively high GOLD docking score against SARS-CoV-2 M^{pro}, ranking below the reference inhibitors ritonavir and lopinavir. The predicted binding pose of linarin involved hydrogen bond interactions with key residues of M^{pro}, consistent with another docking-based report (Mohamad et al., 2021). Linarin (acaciin, acacetin-7-O-rutinoside; Li et al., 2008) a glycosylated flavonoid, has the potential to be antioxidant, anti-apoptosis, anti-inflammatory, anti-Alzheimer (Fan et al., 2008) and anti-viral (Zarenezhad et al., 2021).

The current study tested the crude polyphenols from molasses and bagasse before purification and fractionation against recombinant SARS-CoV-2 M^{pro}. The crude extract exhibited complete inhibition of recombinant SARS-CoV-2 M^{pro}, prompting further fractionation into three products. Among them, fraction 1 had the strongest activity, with 84.15 $\pm$ 3.57% inhibition at 100 µg/mL. Linarin was identified as one of the major polyphenolic constituents in fraction 1 based on the HPLC-DAD and LC-MS/MS analyses. Linarin inhibited SARS-CoV-2 M^{pro} with an IC₅₀ value of 89.98 $\pm$ 10.15 µM, supporting its potential role as a candidate M^{pro} inhibitor. The markedly lower IC₅₀ of fraction 1 compared to pure linarin suggests possible synergistic contributions from other co-occurring compounds. Thus, linarin is likely not the main active constituent but one of multiple components contributing to the

overall effect. Docking-based binding mode analysis indicated that linarin engages residues surrounding the M^{pro} active site through a combination of hydrogen bonding and hydrophobic contacts, with a relatively higher GOLD docking score than the other polyphenolic compounds evaluated in the current study. These interactions were mainly hydrophobic and hydrogen bonds, confirming the strong interactions between linarin and the main protease of SARS-CoV-2. Overall, the combined enzymatic inhibition results and docking-based interaction analysis support linarin as a putative inhibitor of SARS-CoV-2 M^{pro}. In addition to linarin, the LC-MS/MS analysis of fraction 1 revealed several other abundant phytochemicals, such as flavonoid glycosides (e.g., vicianin-2, isoschaftoside, schaftoside), phenolic acids and derivatives (trans-caffeic acid, caffeic acid 3-glucoside, D-(−)-quinic acid), as well as merimepodib, triptolide and others, as detailed in Table S1. Although direct inhibitory activity against SARS-CoV-2 M^{pro} was not assessed in the current study, several of these compounds belong to chemical classes that have been associated with antiviral or protease-inhibitory activity. For example, vicianin-2 and isoschaftoside have been identified as potential inhibitors of SARS-CoV-2 3CL^{pro}, supported by experimental validation through *in vitro* enzymatic inhibition assays reporting IC₅₀ values and further confirmed by affinity measurements (Kd) determined based on surface plasmon resonance (Liao et al., 2021). Merimepodib has been described as a broad-spectrum antiviral agent with activity against multiple viruses, including Zika virus and foot-and-mouth disease virus and has also been investigated in the context of COVID-19 treatment in a Phase 2 clinical trial (Tong et al., 2018; Li et al., 2019; ClinicalTrials.gov., 2020; Ruckmani et al., 2020). Triptolide, a diterpenoid epoxide derived from *Tripterygium wilfordii*, exhibits potent antiviral and immunomodulatory activity against SARS-CoV-2 by inhibiting viral entry and disrupting the viral spike protein-ACE2 receptor interaction (Kousar et al., 2025). Schaftoside has been shown, through both molecular docking and *in vitro* inhibition assays, to bind SARS-CoV-2 3CL^{pro} and PL^{pro} and exert immunomodulatory and anti-inflammatory effects relevant to COVID-19 therapy (Yi et al., 2022). Other detected compounds, such as adenosine, gluconic acid and trehalose, have not yet been associated with M^{pro} inhibition. Further studies are warranted to elucidate the individual and combinatorial contributions of these metabolites.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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