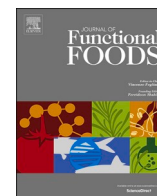




Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Anti-inflammatory and antiviral activities of flavone C-glycosides of *Lophatherum gracile* for COVID-19

Yu-Li Chen^{a,b}, Chun-Yu Chen^{c,d,e}, Kuei-Hung Lai^{f,g,h}, Yu-Chia Chang^{a,b}, Tsong-Long Hwang^{a,b,c,d,i,*}

^a Research Center for Chinese Herbal Medicine, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan 33303, Taiwan

^b Graduate Institute of Health Industry Technology, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan 33303, Taiwan

^c Graduate Institute of Natural Products, College of Medicine, Chang Gung University, Taoyuan 33302, Taiwan

^d Department of Anesthesiology, Chang Gung Memorial Hospital, Taoyuan 33305, Taiwan

^e Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taoyuan 33302, Taiwan

^f PhD Program in Clinical Drug Development of Herbal Medicine, College of Pharmacy, Taipei Medical University, Taipei 11031, Taiwan

^g Graduate Institute of Pharmacognosy, College of Pharmacy, Taipei Medical University, Taipei 11031, Taiwan

^h Traditional Herbal Medicine Research Center, Taipei Medical University Hospital, Taipei 11031, Taiwan

ⁱ Department of Chemical Engineering, Ming Chi University of Technology, New Taipei City 24301, Taiwan

ARTICLE INFO

Keywords:

Lophatherum gracile
Neutrophils
SARS-CoV-2
Flavone C-glycosides
COVID-19

ABSTRACT

Lophatherum gracile (*L. gracile*) has long been used as a functional food and herbal medicine. Previous studies have demonstrated that extracts of *L. gracile* attenuate inflammatory response and inhibit SARS-CoV-2 replication; however, the underlying active constituents have yet to be identified. This study investigated the bioactive components of *L. gracile*. Flavone C-glycosides of *L. gracile* were found to dominate both anti-inflammatory and antiviral effects. A simple chromatography-based method was developed to obtain flavone C-glycoside-enriched extract (FlavoLG) from *L. gracile*. FlavoLG and its major flavone C-glycoside isoorientin were shown to restrict respiratory bursts and the formation of neutrophil extracellular traps in activated human neutrophils. FlavoLG and isoorientin were also shown to inhibit SARS-CoV-2 pseudovirus infection by interfering with the binding of the SARS-CoV-2 spike on ACE2. These results provide scientific evidence indicating the efficacy of *L. gracile* as a potential supplement for treating neutrophil-associated COVID-19.

1. Introduction

Neutrophils are the first line of defense in innate immunity against invading pathogens; however, excessive recruitment and activation of neutrophils can be harmful to human health (Özcan & Boyman, 2022). Respiratory bursts, degranulation, and the formation of neutrophil extracellular traps (NETs) are the main inflammatory responses to the activation of neutrophils. (Özcan et al., 2022). Superoxide anions are a form of reactive oxygen species (ROS) generated via nicotinamide adenine dinucleotide phosphate oxidase (NOX) in activated neutrophils (Feitz et al., 2021). The overwhelming release of ROS from neutrophils

can cause tissue damage as well as enhance immune responses and oxidative stress (Nguyen, Green, & Meccas, 2017). NETs are web-like chromatin structures containing DNA, histones, and various granular proteases that degrade virulence factors and kill pathogens (Arcanjo et al., 2020). ROS production from respiratory burst is the factor driving NOX-dependent NETs (Azzouz, Khan, & Palaniyar, 2021; Feitz et al., 2021). There is compelling evidence indicating that components of NETs induce proinflammatory responses and thereby promote the pathogenesis of diseases, including coronavirus disease 2019 (COVID-19), sepsis, rheumatoid arthritis, cancer, and diabetes (Huang, Hong, Wan, & Zheng, 2022; McKenna et al., 2022; Njeim, Azar, Fares, Azar, Kfoury

Abbreviations: ACE2, angiotensin-converting enzyme 2; COVID-19, coronavirus disease 2019; CB, cytochalasin B; DMSO, dimethyl sulfoxide; fMLF, N-formyl-methionyl-leucyl-phenylalanine; HBSS, Hank's balanced salt solution; HPLC, high-performance liquid chromatography; IC₅₀, half-maximal inhibitory concentration; LDH, lactate dehydrogenase; LG, *Lophatherum gracile*; MRM, multiple reaction monitoring; NETs, neutrophil extracellular traps; O₂^{•−}, superoxide; RBD, receptor-binding domain; ROS, reactive oxygen species; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; UPLC, ultra-performance liquid chromatography.

* Corresponding author at: Graduate Institute of Natural Products, College of Medicine, Chang Gung University, Taoyuan 33302, Taiwan.

E-mail addresses: ylchen03@mail.cgu.edu.tw (Y.-L. Chen), an5376@cgmh.org.tw (C.-Y. Chen), kueihunglai@tmu.edu.tw (K.-H. Lai), ycchang03@mail.cgu.edu.tw (Y.-C. Chang), htl@mail.cgu.edu.tw (T.-L. Hwang).

<https://doi.org/10.1016/j.jff.2023.105407>

Received 3 November 2022; Received in revised form 28 December 2022; Accepted 3 January 2023

Available online 5 January 2023

1756-4646/© 2023 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Kassouf, & Eid, 2020; Özcan et al., 2022).

COVID-19 is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Hong et al., 2022). By August 2022, the COVID-19 pandemic had resulted in nearly 600 million confirmed cases and over 6 million deaths worldwide (<https://coronavirus.jhu.edu/map.html>). Unregulated neutrophilic inflammation contributes to the pathogenesis of COVID-19 and is positively correlated with the severity of the corresponding complications (Amer et al., 2021; He, Qi, Zhang, & Pan, 2022; Pirlog et al., 2022). The excessive release of ROS from neutrophils has been shown to increase oxidative stress, tissue damage, and inflammatory responses in COVID-19 patients (Chiang, Korinek, Cheng, & Hwang, 2020). Oxidative stress has also been shown to promote SARS-CoV-2 replication (Du et al., 2021). Elevated concentrations of NETs in plasma and virus-infected tissues can cause local and systemic tissue damage, help to advance SARS-CoV-2 infection, and promote various COVID-19 complications, including thrombosis, acute respiratory distress syndrome, and multi-system inflammatory disease in children (Arcanjo et al., 2020; Hong et al., 2022; McKenna et al., 2022). Accordingly, neutrophils are considered potential targets in the prevention and treatment of COVID-19 and neutrophil-related disorders (Chiang, Korinek, et al., 2020).

Lophatherum gracile (*L. gracile*) is a perennial plant that has long been used as a functional food and additive in cosmetic products (Lai et al., 2021; Liu, Wang, Ge, Cai, Guo, & Gong, 2022). It has been used to make facial masks, skin cream, and other healthcare products (Lai et al., 2021; Liu et al., 2022). *L. gracile* is the main ingredient of various teas in China, including Guangdong and Wong Lo Kat (He, Tsoi, Li, Yao, & Kurihara, 2011; Liu et al., 2022; Liu et al., 2020). *L. gracile* has a long history in the treatment of fever and inflammatory conditions (Diaz, Jeong, Lee, Khoo, & Koyyalamudi, 2012). Flavonoids and polyphenols are the phytochemicals commonly attributed to the anti-oxidation, anti-inflammation, anti-cancer, and pharmacological bioactivities of *L. Gracile* (Chen et al., 2019; Kim, Im, Gu, & Ma, 2016; Liu et al., 2022; Wang et al., 2012).

The emergence of SARS-CoV-2 variants has decreased the efficacy of vaccines (Callaway, 2022; Luo et al., 2022); however, the combined use of immunomodulators and antiviral agents is expected to have synergistic effects in the treatment of COVID-19 (Wang & Wang, 2022). Recent studies have demonstrated anti-inflammatory and antiviral effects in *L. gracile*. Ethanol extracts of *L. gracile* attenuate superoxide anion generation in stimulated neutrophils (Lai et al., 2021). Water extracts of *L. gracile* inhibit SARS-CoV-2 replication and decrease virus-induced cytotoxicity (Jan et al., 2021). However, the bioactive components in *L. gracile* have yet to be identified. Thus, this study investigated the potential effects of *L. gracile* in the treatment of COVID-19 as well as the bioactive constituents and underlying mechanisms of action.

2. Materials and methods

2.1. Reagents

Cytochalasin B (CB), dextran, dimethyl sulfoxide (DMSO), ethyl acetate, *N*-formyl-methionyl-leucyl-phenylalanine (fMLF), *n*-hexane, methanol, paraformaldehyde, and superoxide dismutase were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hank's balanced salt solution (HBSS), Hoechst 33342, and SYTOX Green were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Trypan blue was purchased from Biological Industries (Beth Haemek, Israel). Ficoll-Paque Plus was procured from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Mouse monoclonal antibodies to neutrophil elastase were purchased from Millipore (MABS46, MA, US), and rabbit polyclonal antibodies to histone H3 were purchased from Abcam (ab5103, Cambridge, UK). Isoorientin, orientin, and cepharanthine were purchased from Sunhank Technology Co., Ltd (Tainan, Taiwan).

2.2. Sample preparation and extraction

Dried *L. gracile* was purchased in January 2021 from Healing Herbar, a distributor of herbal remedies in Taipei, Taiwan. A voucher specimen of plant material (CGU-HTLTTCM-40) was delivered to the herbarium of Chang Gung University (Taoyuan, Taiwan). *L. gracile* (2 g) was cut into 1 cm sections and placed in a Dionex ASE 350 auto-extraction device (Thermo Fisher Scientific, USA). The cells were filled with 60 ml of *n*-hexane, ethyl acetate, methanol, or 50 % methanol. Extraction was performed twice at 50 °C for 15 min. Solvents were removed under vacuum to produce extracts of *L. gracile*, respectively referred to as LG-H, LG-EA, LG-M, or LG-50 M, in accordance with the extraction medium.

2.3. Fractionation of *L. Gracile* extracts using high-performance liquid chromatography (HPLC)

L. gracile methanol extract (LG-M) was dissolved in HPLC-grade methanol (10 mg/ml) and filtered through a 0.45 µm filter before being loaded for HPLC. Fractionation was conducted using a Shimadzu Nexera-I LC-2040C-3D HPLC system (Kyoto, Japan) with a Cosmosil 5C18-MS-II HPLC column (10 × 250 mm; Nacalai Tesque, Inc., Kyoto, Japan). The solvent system consisted of an aqueous solution and methanol with or without 0.1 % formic acid. Methanol gradient elution was performed under the following conditions: 50 % for 0–15 min and 50–100 % for 15–20 min. The flow rate was maintained at 5 ml/min, and the injection volume was 500 µl. Liquid chromatography was performed at 30 °C.

2.4. Analysis of *L. Gracile* extracts by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

Qualitative and quantitative analyses of *L. gracile* extracts were conducted using a Shimadzu Nexera X2 UPLC system (Kyoto, Japan). Liquid chromatography was performed using a YMC-Triart C18 (1.9 µm, 1.2 nm 50 mm × 2.1 mm) column (Kyoto, Japan). The mobile phase consisted of MeCN (A, containing 0.1 % formic acid) and water (W, containing 0.1 % formic acid). Gradient elution was performed using a flow rate of 0.5 ml/min and column temperature of 35 °C as follows: 8 % A for 0–12 min; 8–20 % A for 12–22 min; 20–100 % A for 22–28 min; 100 % A for 28–38 min. Samples were prepared by dissolving *L. gracile* extracts in methanol (2 mg/ml) and then filtered through a 0.45 µm filter. The sample volume was 1 µl per injection. Production scans (in positive and negative modes) and multiple reaction monitoring experiments (MRM) were conducted using a Shimadzu LCMS-8045 mass spectrometry (Kyoto, Japan). The dwell time was 100 msec, and collision energy (CE) was optimized for individual compounds. All acquired MS data were processed using LCMS LabSolutions software (Version 5.93, Shimadzu, Kyoto, Japan).

2.5. GNPS-based molecular networking analysis

MS/MS data collection was conducted using a Waters SYNAPT G2 ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry system (UPLC-QTOF-MS, MA, USA). Liquid chromatography was performed using a C18 column within a Waters Acquity UPLC BEH (100 mm × 2.1 mm, Waters). The mobile phase consisted of A (MeCN, 0.1 % formic acid) and B (ddH₂O, 0.1 % formic acid). Gradient elution was performed using a flow rate of 0.5 ml/min and a consistent oven temperature of 40 °C, as follows: 3–5 % A, 0–2 min; 5–10 % A, 2–4 min; 10 % A, 4–10 min; 10–15 % A, 10–12 min; 15–20 % A, 12–18 min; 20–100 % A, 18–20 min; 100 % A, 20–25 min. Samples (4,000 ppm) were filtered through a 0.45 µm filter, and the injection volume was 4 µl. Non-targeted MS¹ and MS² data with *m/z* 100–2000 were collected (Zhang et al., 2021). MS data obtained via data-dependent acquisition (DDA) were analyzed using Waters MassFragment software (MassLynx4.1, Waters, MA, USA). MS/MS molecular networking data were

generated using a GNPS web-based platform (<https://gnps.ucsd.edu>). MS/MS spectra were filtered to identify the top 5 strongest ion peaks in the ± 50 Da window. A network was created based on linkages between molecular nodes filtered by cosine volume (0.7) and at least four matched peaks. The nodes that appeared in the network were annotated based on the experimental MS² fragmentation of isolates. The molecular network was visualized using Cytoscape 3.8.2 (NRNB, CA, USA).

2.6. Measurement of ¹H nuclear magnetic resonance spectrum

Samples were dissolved in deuterium solvent at 5 mg/ml. Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker AVANCE-400 MHz FT NMR spectrometer (MA, US).

2.7. Preparation of human neutrophils

Blood samples were drawn from healthy human donors (20–30 years old) under the approval and supervision of the Institutional Review Board of Chang Gung Memorial Hospital (202101115A3). Neutrophils were purified in accordance with a standard protocol involving dextran sedimentation, hypotonic lysis of erythrocytes, and Ficoll Hypaque gradient centrifugation, as described previously (Chiang, Cheng, et al., 2020). Isolated human neutrophils were evaluated by trypan blue assay, and > 98 % of living cells were preserved in HBSS (pH 7.4) under ice-cold conditions until use.

2.8. Assessment of superoxide anion generation

Superoxide anion generation was assessed using ferricytochrome c reduction. Briefly, neutrophils (6×10^5 cells/ml) were incubated with ferricytochrome c at 37 °C for 5 min. The cells were then incubated with the *L. gracile* extracts, pure compounds, or DMSO (0.1 %, as control) for 5 min. Neutrophils were treated using CB for 3 min followed by fMLF (0.1 μ M) stimulation. Changes in absorbance with the reduction of ferricytochrome c were monitored continuously at 550 nm using a spectrophotometer (U-3010, Hitachi, Tokyo, Japan).

2.9. Assessment of lactate dehydrogenase (LDH) release

The cytotoxicity of neutrophils was evaluated in a cell-free medium in terms of the percentage of the total LDH release (Lai et al., 2021). Neutrophils (6×10^5 cells/ml) were equilibrated at 37 °C and then incubated for 5 min before treating samples for 15 min, after which the cells were lysed via Triton X-100 (0.1 %) for 30 min. The changes in absorbance at 490 nm were monitored continuously after adding LDH reagent.

2.10. Analysis of total ROS production

Total ROS production by neutrophils was measured using the luminol-amplified chemiluminescence method. Human neutrophils (2×10^6 cells/ml) were pre-mixed with 37.5 μ M luminol and 6 U/ml horseradish peroxidase at 37 °C for 5 min. Cells were then incubated with DMSO or tested agents for 5 min, followed by stimulation with 0.1 μ M fMLF. A 96-well chemiluminometer (Tecan Infinite F200 Pro; Männedorf, Switzerland) was used to detect changes in chemiluminescence.

2.11. Quantification of NETs

Human neutrophils (1×10^6 cells/ml) were resuspended in HBSS with 2.5 μ M SYTOX green and incubated with DMSO or tested agents for 10 min. Cells were stimulated by adding 10 nM phorbol 12-myristate 13-acetate (PMA) for 2 h. The fluorescence intensity was measured by a 96-well chemiluminometer (Tecan Infinite F200 Pro; Männedorf, Switzerland).

2.12. Immunofluorescence staining of NETs

Human neutrophils were incubated on poly-L-lysine-coated glass coverslips at 37 °C for 30 min and then incubated with DMSO or tested agents for 10 min before stimulating the neutrophils through the addition of 10 nM PMA for 2 h. Cells were fixed with 4 % paraformaldehyde for 15 min and then treated with 5 % goat serum buffer for blocking. Samples were incubated with anti-histone H3 (4 μ g/ml) and anti-elastase (5 μ g/ml) antibodies for 30 min and 1 h, followed by Alexa488 or 568-labelled secondary antibodies. After washing with phosphate-buffered saline, cells were stained with Hoechst 33,342 (5 μ g/ml) and ProLong Gold antifade reagent (Invitrogen, CA, USA). Fluorescence images were obtained using a BioTek Cytation 5 cell imaging multimode reader (BioTek, VT, US.).

2.13. Homogeneous time-resolved fluorescence (HTRF) SARS-CoV-2 spike/ACE2 binding assay

SARS-CoV-2 spike/ACE2 binding assays were performed by a HTRF kit (63ADK000CB23PEG, CisBio, Codolet, France). Tag1-SARS-CoV-2 spike protein (5 nM) and Tag2-ACE2 protein (75 nM) were incubated with tested agents for 15 min at room temperature. Pre-mixed anti-Tag1-Eu3+ (HTRF donor) and anti-Tag2-d2 (HTRF acceptor) were added to detect the binding of SARS-CoV-2 spike/ACE2. After incubation for 3 h, the energy transfer of antibody-triggered fluorescent resonance towards the acceptor antibody was detected at 665 nm.

2.14. SARS-CoV-2 pseudovirus neutralization assay

Lentivirus experiments were approved by the Institutional Biosafety Committee of Chang Gung University. SARS-CoV-2 pseudovirus strains (clone name: nCoV-S-Luc-D614G and nCoV-S-Luc-B.1.1.529) were purchased from the RNAi Core Facility at Academia Sinica (Taipei, Taiwan). Equal relative infection units (RIUs) (5×10^3 RIU/well = 0.5 RIU/cell) of the pseudovirus were pretreated with DMSO or tested agents in DMEM at 37 °C for 1 h. Seeded stable hACE-2-overexpressed HEK293T cells (1×10^4 cells/well) were infected with drug-pretreated virus for 1 h and then cultured with fresh DMEM medium for 24 h. The viral infection was determined in terms of luciferase activity measured using a Luciferase Assay System kit (Promega, WIS, USA) and Tecan Infinite F200 Pro (Männedorf, Switzerland) (Ohashi et al., 2021). Cepharanthine was used as positive control.

2.15. WST-1 assay

The cytotoxicity of tested samples in hACE-2-overexpressed HEK293T were evaluated by WST-1 reduction assay. hACE-2-overexpressed HEK293T (1×10^4 cells/well) were preincubated with DMSO or tested agents for 24 h, and then WST-1 reagent (M192427, Sigma-Aldrich, MO, USA) was added. After incubation at 37 °C for 4 h, the changes in absorbance at 405 nm were measured by Multiska GO spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.16. Molecular docking

Ligand-protein interactions were investigated via in silico docking analysis using Discovery Studio 2019 (Biovia, Corp. CA, USA). The 3D structure of isoorientin was downloaded from PubChem (CID 114776). The crystal structure of SARS-CoV-2 spike protein was obtained from the RCSB Protein Data Bank (PDB ID: 6M0J). The initial spike and ligand structures were created using a CHARMM force field and minimized. A sphere radius covering the spike receptor-binding domain was set. Docking poses were generated in accordance with the CDOCKER protocol using Discovery Studio software.

2.17. Statistics

All experiment results were expressed as mean ± S.E.M., and the Student's *t*-test was used for statistical analysis via GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences with *p* values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. *L. Gracile* methanol extract (LG-M) attenuates superoxide anion generation in fMLF-activated human neutrophils

Neutrophilic inflammation is triggered by pathogen-associated molecular patterns, such as fMLF. Excessive superoxide anions produced by activated neutrophils cause tissue damage during inflammation. *L. gracile* is an edible plant used for centuries as a functional food and herbal medicine. Previous studies have shown that *L. Gracile* ethanol extracts attenuate superoxide anion generation and neutrophil activity (Lai et al., 2021). To identify the active constituents, we first sought to

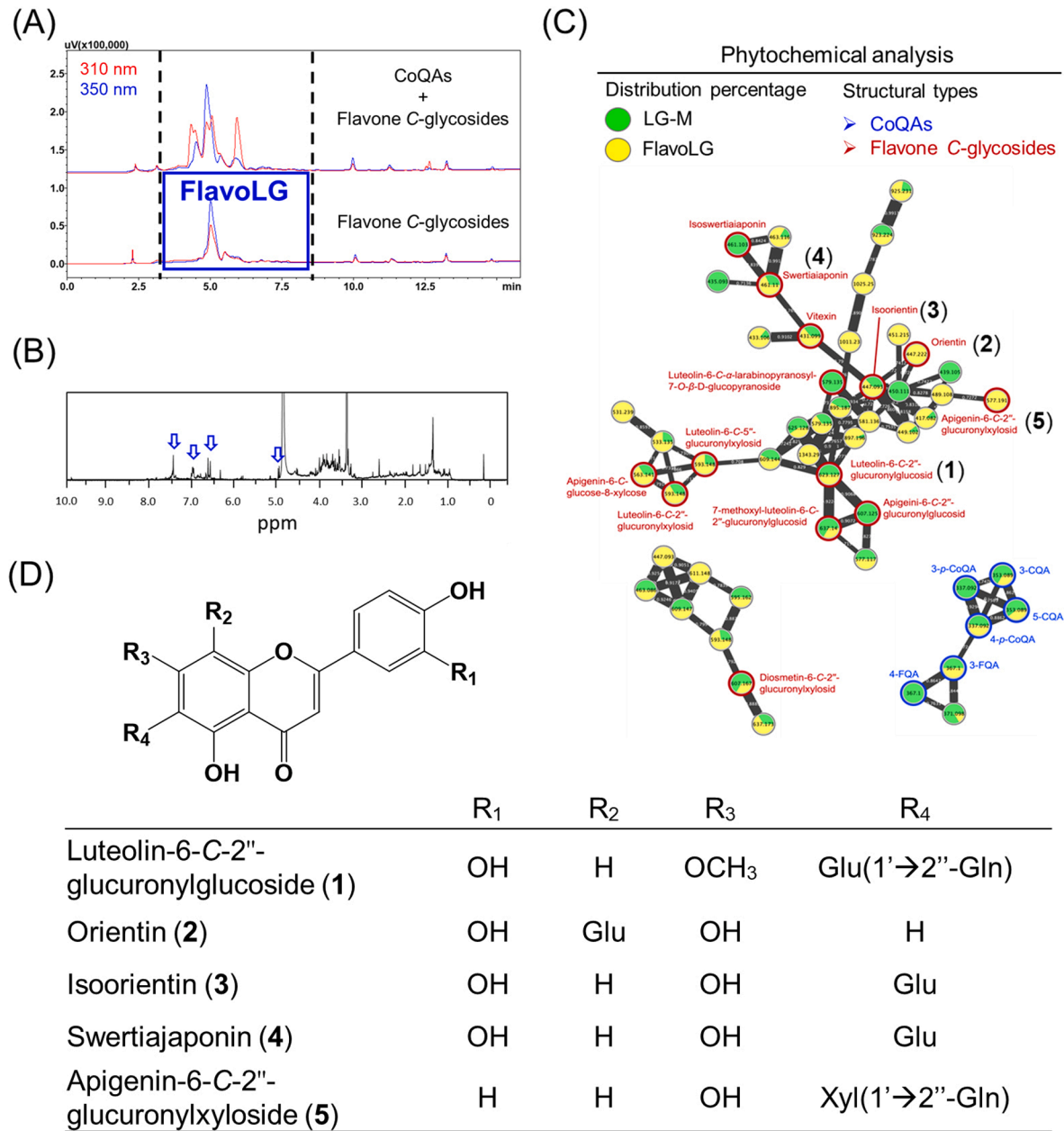


Fig. 1. Structural characteristics of *L. gracile* flavonoid-enriched extract (FlavoLG). (A) Indicated fraction of LG-M was collected in the form of FlavoLG using a liquid chromatography-based method involving methanolic gradient elution without the addition of formic acid. Colored chromatography results revealed UV absorption patterns typical of coumaroylquinic acids (CoQAs) at 310 nm (red) and flavonoids at 350 nm (blue); (B) Blue arrows indicate proton signals indicative of flavone C-glycosides in ¹H NMR spectra, involving protons on C-glycosides and substituted aromatic rings; (C) Combined molecular networks of FlavoLG and LG-M. According to MS² fragmentation analysis, flavone C-glycosides made up the largest cluster. The colored area of each node indicates the relative quantities of given phytochemicals in FlavoLG (yellow) and LG-M (green). Flavone C-glycoside levels were higher in FlavoLG than in LG-M; (D) Structural illustration showing selected flavone C-glycosides in FlavoLG.

clarify the general polar properties of active components. *L. gracile* was extracted using *n*-hexane, ethyl acetate, methanol, or 50 % methanol to create extracts with diverse polar properties. Only the *L. gracile* methanol extract (LG-M) inhibited superoxide anion generation in fMLF-activated human neutrophils (Fig. S1A). Note that LG-M did not affect the viability of neutrophils in an LDH assay, indicating that the observed reduction in superoxide anion production was not due to cytotoxic effects (Fig. S1B).

Polyphenols are phytochemicals commonly identified in *L. gracile* (Liu et al., 2022). To qualitatively analyze the phytochemicals in LG-M, MRM experiments were performed using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (Fu, Wang, Lan, Li, Hashi, & Chen, 2014), which led to the identification of the following flavone C-glycosides: luteolin-6-C-2'-glucuronylglucoside (1), orientin (2), isoorientin (3), swertiajaponin (4), isovitexin (5), and coumaroyl quinic acids (CoQAs): 3-*p*-coumaroyl quinic acid (6), 4-*p*-coumaroyl quinic acid (7), and *o*-coumaroyl quinic acid (8) (Fig. S1C). The fact that all but six of these compounds were detectable in *L. Gracile* ethanol extract suggests that the polar metabolites of *L. Gracile* act as active components against neutrophilic inflammation. These findings also demonstrate that alcohols are suitable solvents for the creation of *L. gracile* extracts optimized for bioactivity.

3.2. Flavone C-glycosides of *L. Gracile* restrict respiratory bursts in stimulated neutrophils

Flavone C-glycosides are a major group of chemical components in *L. gracile*. Isoorientin is generally used in the quality control of *L. gracile* products (Fan, Lee, & Lin, 2015; Wang et al., 2012); however, the roles of flavone C-glycosides in neutrophilic inflammation have seldom been addressed. To elucidate the anti-inflammatory effects of flavone C-glycosides of *L. gracile*, a flavonoid-enriched extract from LG-M (FlavoLG) was produced using a simple process based on liquid chromatography (Fig. 1A). The structural features of FlavoLG were elucidated via NMR and MS²-based molecular networking experiments. The ¹H NMR spectrum presented proton signals characteristic of C-glycosides and substituted aromatic rings (Hao et al., 2016) (Fig. 1B). MS² data revealed that flavone C-glycosides made up the largest molecular cluster. The high yellow-to-green area ratio in the clustering nodes indicates a high concentration of flavone C-glycosides in FlavoLG (Fig. 1C). Isoorientin was then quantified using optimized MRM experiments and used as makers representing flavone C-glycosides in FlavoLG (Fig. S2). The amount of isoorientin in FlavoLG was 5.98 %. These qualitative and quantitative results demonstrated the efficacy of methanolic chromatography in the creation of extracts rich in flavone-C-glycosides from *L. gracile*. Bioassay results revealed that FlavoLG and flavone-C-glycosides (isoorientin and orientin) reduced superoxide anion generation in neutrophils at the concentration without cytotoxicity, as indicated by IC₅₀ values of 7.02 ± 0.56 µg/ml, 6.56 ± 0.17 µM, and 9.57 ± 0.41 µM (Fig. 2 and Fig. S3). Furthermore, FlavoLG, isoorientin, and orientin significantly reduced the production of ROS, as indicated by IC₅₀ values of 0.17 ± 0.01 µg/ml, 0.16 ± 0.00 µM, and 0.15 ± 0.01 µM (Fig. 3). Superoxide anion can be converted to ROS. Our data suggest that FlavoLG and two major flavone C-glycosides, orientin and isoorientin, are more sensitive to inhibit ROS generation (Fig. 3) than superoxide production (Fig. 2), suggesting that FlavoLG, orientin, and isoorientin decrease neutrophil ROS formation via direct scavenging and indirect regulation of cell responses. Indeed, the potent ROS scavenging activity is attributed by the high electron resonance property of phenoxyl substructure in flavonoids (Zhang, et al., 2015). These results indicate that flavone-C-glycosides of *L. gracile* reduce respiratory bursts in activated neutrophils.

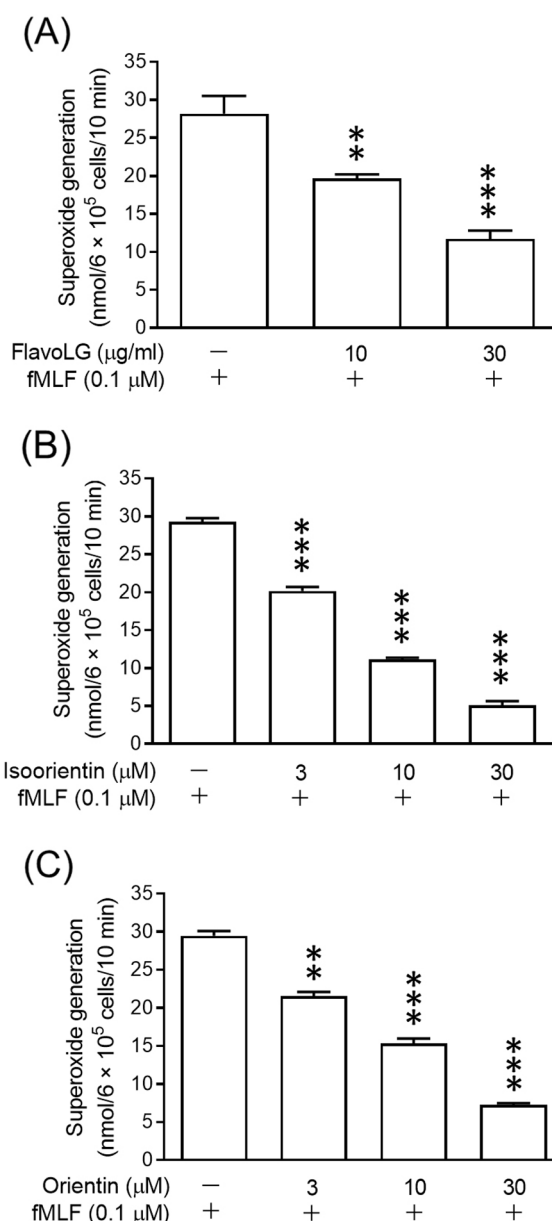


Fig. 2. Flavone C-glycosides reduce superoxide anion generation in fMLF-activated human neutrophils. Human neutrophils were incubated with DMSO (0.1 %), FlavoLG, or flavone C-glycoside compounds for 5 min and then stimulated with or without fMLF/CB for another 10 min. Superoxide anions were detected in terms of ferricytochrome c reduction via spectrophotometric analysis at 550 nm. (A) FlavoLG; (B) Isoorientin; (C) Orientin. All data are expressed as mean ± S.E.M. (n = 3). ** p < 0.01 and *** p < 0.001, compared with the DMSO + fMLF group.

3.3. Flavone C-glycosides of *L. Gracile* inhibit the formation of neutrophil extracellular traps (NETs) induced by phorbol 12-myristate 13-acetate (PMA)

NETs are web-like structures consisting mainly of nuclear chromatin coated with granular proteins of neutrophils, such as neutrophil elastase (McKenna et al., 2022). NETs play an important role in inflammatory and autoimmune disorders, such as rheumatoid arthritis, atherosclerosis, and diabetes (Huang et al., 2022; Mutua & Gershwin, 2021). Excessive NET levels can cause tissue damage and enhance recruitment of immune cells into inflammatory sites (Wang, Du, Hawez, Mörgelin, & Thorlacius, 2019). Inhibiting unregulated NET formation could have beneficial effects in dealing with neutrophil-associated diseases. In this

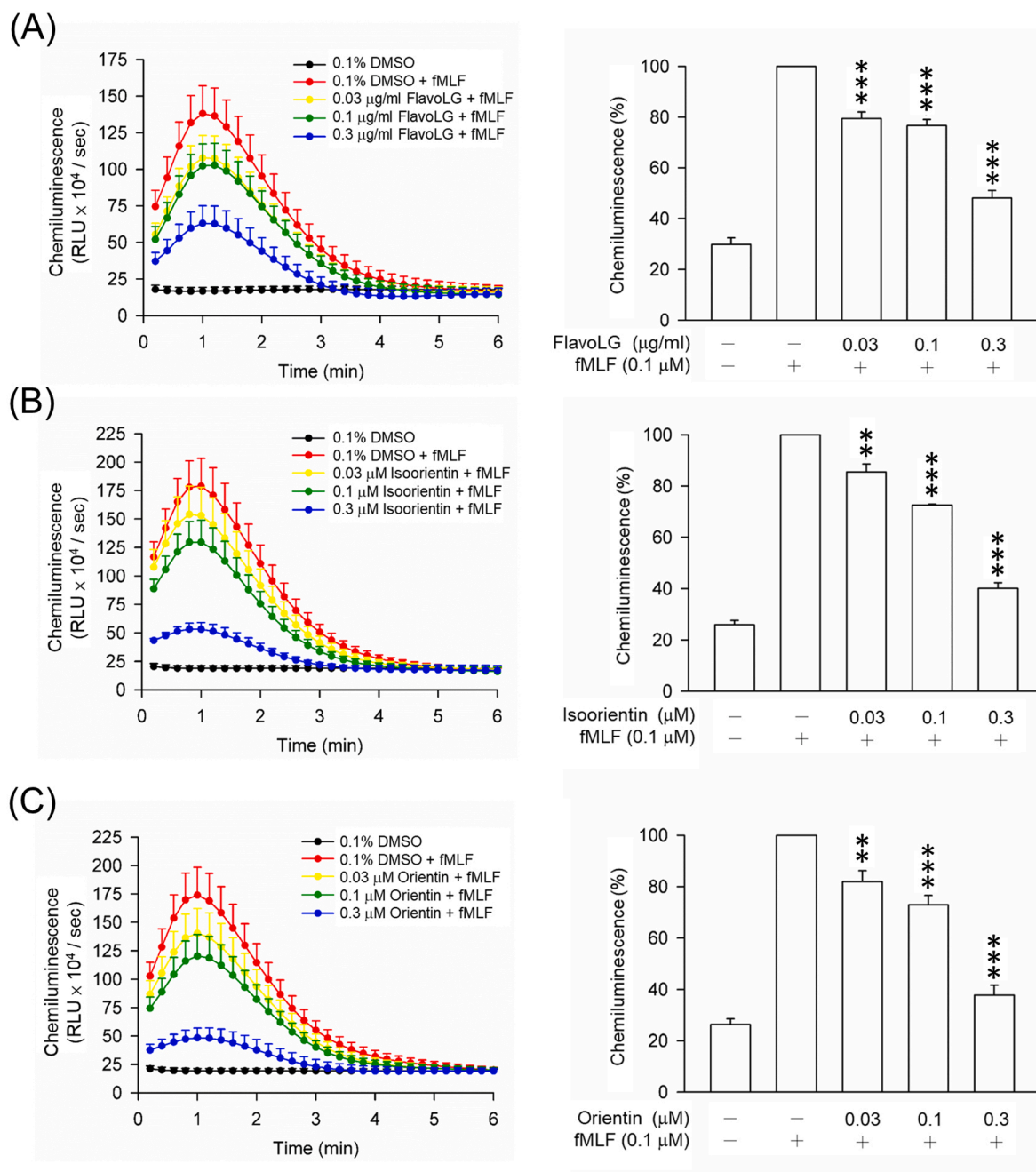


Fig. 3. Flavone C-glycosides of *L. gracile* reduce the generation of reactive oxygen species (ROS) in activated neutrophils. Luminol-incubated neutrophils were treated with DMSO (0.1 %), FlavoloG, or flavone C-glycoside compounds and then activated by fMLF (0.1 μ M). ROS production was detected in terms of changes in chemiluminescence. AUC values are expressed as bars, indicating the mean $\pm$ S.E.M. (n = 4). ** $p < 0.01$ and *** $p < 0.001$, compared with the DMSO + fMLF group.

study, FlavoloG and flavone C-glycosides were found to decrease PMA-induced NET levels (Fig. 4A and 4B) in immunofluorescence staining (Fig. 4C). Superoxide anions and ROS associated with respiratory bursts were shown to play crucial roles in NOX-dependent NETs (Azzouz et al., 2021; Feitz et al., 2021). Our results revealed that the reduction in NET levels by flavone C-glycosides can be attributed to restrictions on respiratory bursts of activated neutrophils.

3.4. Flavone C-glycosides of *L. gracile* decrease SARS-CoV-2 infection by interfering with the binding of SARS-CoV-2 spike and angiotensin-converting enzyme 2 (ACE2)

Flavonoids are considered as safe and promising agents against

COVID-19 for their antioxidant, antiviral, and anti-inflammatory activities (Alzaabi et al., 2022). Several flavones and flavone O-linked glycosides have been demonstrated to inhibit SARS-CoV-2 via targeting Mpro, 3CLpro, or RdRp in *in silico* and *in vitro* studies (Alzaabi et al., 2022; Jantan et al., 2022). Discovering SARS-CoV-2 spike blockers in natural products is a solid strategy for the prevention and/or treatment of SARS-CoV-2 infection (Tomas et al., 2022). Several dietary polyphenols have been shown to affect the interactions between the SARS-CoV-2 spike and ACE2 (Schmidt, Hakeem Said, Ohl, Sharifii, Cotrell, & Kuhnert, 2022). However, the role of flavone C-glycosides in spike/ACE2 interaction is rarely discussed. *L. gracile* has been shown to inhibit SARS-CoV-2 replication and decrease cytotoxicity (Jan et al., 2021); however, the active constituents have yet to be identified. In the current

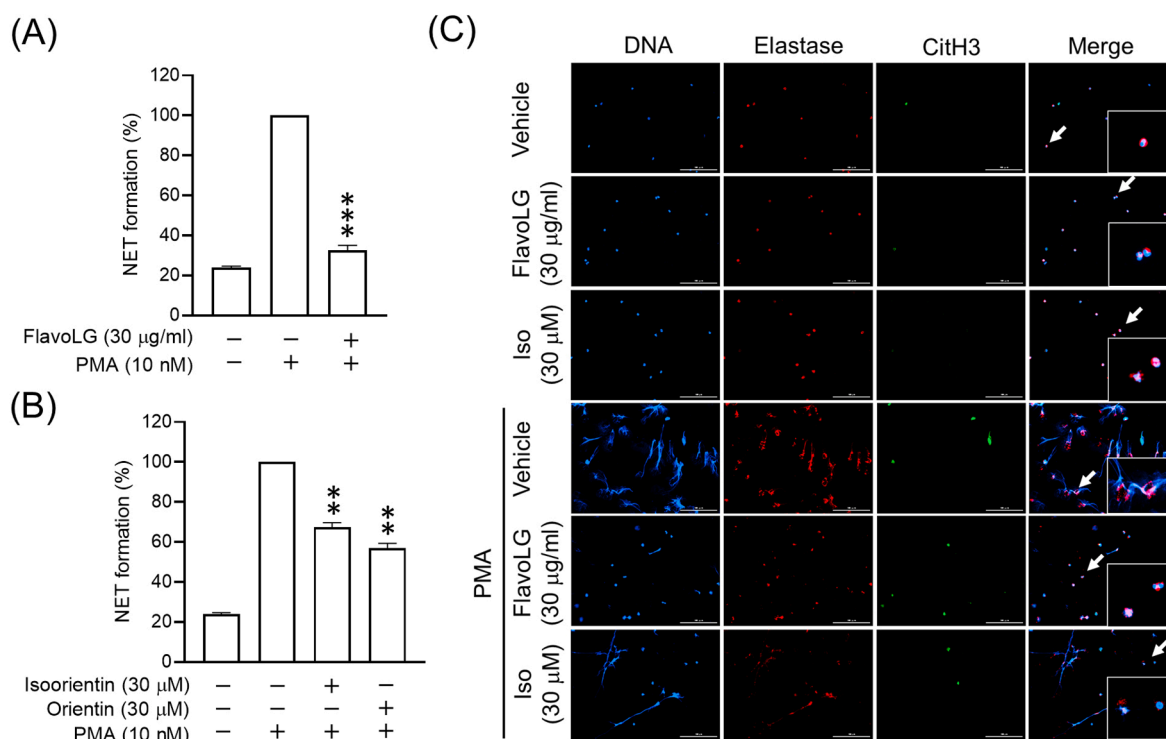


Fig. 4. Flavone C-glycosides of *L. gracile* reduce NET levels in PMA-induced neutrophils. (A–B) Neutrophils were incubated with DMSO (0.1 %), FlavoloG, or flavone C-glycoside compounds and then stimulated with PMA. The amount of free DNA was measured by SYTOX Green; (C) Neutrophils pre-incubated DMSO (0.1 %), FlavoloG (30 µg/ml), or isoorientin (30 µM) were stimulated with 10 nM PMA. Cells were stained with anti-elastase (red), anti-citrullinated histone H3 (green), and Hoechst 33,342 (blue). Scale bar = 100 µm. Data are presented as mean $\pm$ S.E.M. (n = 3). ** $p < 0.01$ and *** $p < 0.001$, compared with the DMSO + PMA group. CitH3: citrullinated histone H3; Iso: isoorientin.

study, FlavoloG and isoorientin inhibited infection of SARS-CoV-2 pseudovirus variants possessing D614G or Omicron spikes into hACE-2-overexpressed HEK293T cells (Fig. 5A) but did not induce cytotoxicity in hACE-2-overexpressed HEK293T cells (Fig. S4). The SARS-CoV-2 spike receptor binding domain (RBD) binds to the ACE2 N-terminal peptidase domain through interactions with key amino acids. Residues Y449 and K417 of spike RBD facilitate ACE2 recognition by forming hydrogen bonds and salt bridges (Lan et al., 2020). Molecular docking analysis was performed to determine whether flavone C-glycosides inhibit viral infection by affecting spike/ACE2 binding. Docking result revealed that isoorientin interacts with Y449, K417, and other amino acids of spike-RBD (CDOCKER energy -27.19 kcal/mol) (Fig. 6). The blockage between SARS-CoV-2 spike and ACE2 was further evaluated using FRET assays. FlavoloG and isoorientin were shown to reduce spike/ACE2 binding in a dose-dependent manner (Fig. 5B). Our data indicated that flavone C-glycosides of *L. gracile* are the antiviral components inhibiting SARS-CoV-2 infection. Note that isoorientin and other flavone C-glycosides of *L. gracile* also inhibit the respiratory syncytial virus (Chen et al., 2019; Wang et al., 2012). The broad antiviral spectrum of *L. gracile* extracts could greatly expand the applicability of this compound in the development of antiviral products. Most flavonoids have low oral bioavailability; therefore the *in vivo* anti-inflammatory effect and anti-SARS-CoV-2 infection activity require further study before clinical application.

4. Conclusions

This study demonstrated that flavone C-glycosides of *L. gracile* are active components against neutrophilic inflammation and SARS-CoV-2 infection. This study also developed a chromatography-based method for the extraction of flavone C-glycosides. These results provide scientific evidence indicating the efficacy of *L. gracile* as a potential supplement for the prevention of COVID-19 and neutrophil-associated

disorders.

The authors confirm that there are no conflicts of interest associated with this study.

Data availability

Data will be made available on request.

Ethics statement

No experiments were performed on animals in this study.

Funding

This work was supported by grants from the Chang Gung Memorial Hospital (CMRPG3K0921, CMRPF1L0071, CMRPF1M0101, CMRPF1M0131, CMRPF1M0091 and CORPF1L0011), the Chang Gung University of Science and Technology (ZRRPF3L0091), and the National Science and Technology Council (108-2320-B-255-003-MY3, 109-2811-B-255-502, 109-2327-B-255-001, 111-2321-B-255-001, 111-2320-B-255-006-MY3, 111-2811-B-255-002, 111-2321-B-182-001).

CRediT authorship contribution statement

Yu-Li Chen: Conceptualization, Methodology, Investigation, Writing – original draft. **Chun-Yu Chen:** Resources, Validation, Funding acquisition. **Kuei-Hung Lai:** Formal analysis, Investigation, Validation. **Yu-Chia Chang:** Formal analysis. **Tsong-Long Hwang:** Conceptualization, Resources, Methodology, Supervision, Funding acquisition, Project administration.

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.

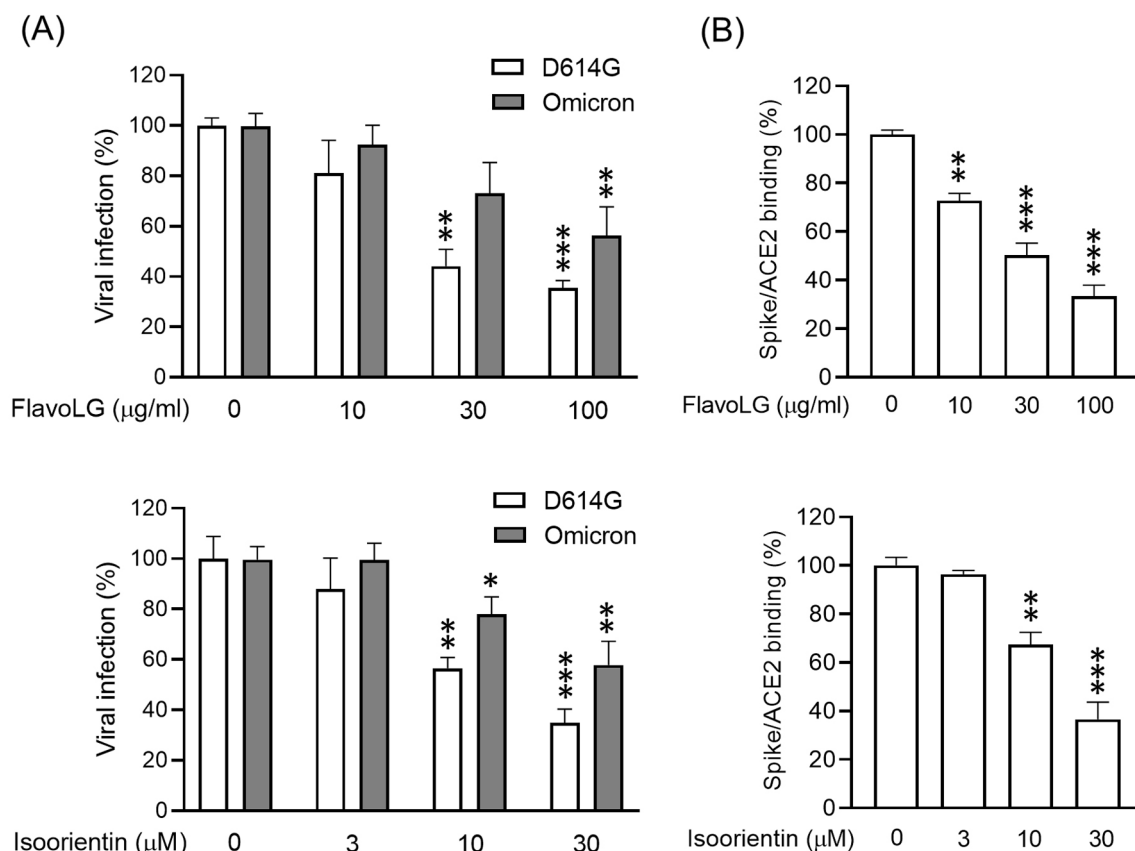


Fig. 5. Flavone C-glycosides of *L. gracile* inhibit SARS-CoV-2 pseudovirus infection by interfering with spike/ACE2 binding. (A) SARS-CoV-2 pseudovirus was pretreated with DMSO (0.1 %) or samples and then infected hACE-2-overexpressed HEK293T for 1 h. The infected cells were washed and incubated with fresh medium until luciferase activity was measured using a Luciferase Assay kit; (B) Tag1-SARS-CoV-2 spike protein and Tag2-ACE2 protein were pretreated with FlavoLG or isoorientin to which anti-tags were added. Spike/ACE2 binding was evaluated by measuring the triggered FRET at 665 nm. The data are presented as mean $\pm$ S.E. M. (n = 3). ** $p < 0.01$ and *** $p < 0.001$, compared with the DMSO group.

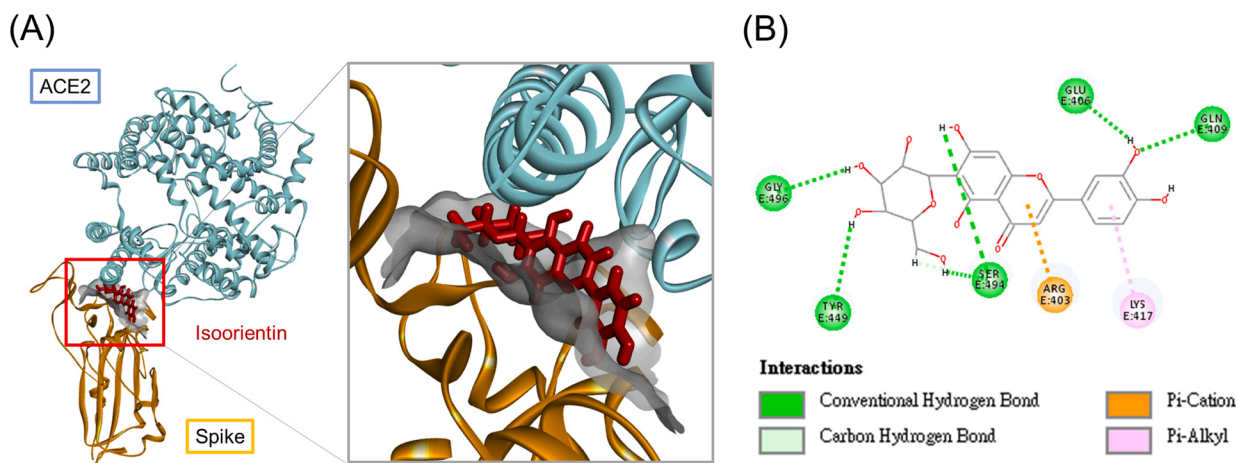


Fig. 6. Molecular docking of isoorientin within the SARS-CoV-2 spike receptor-binding domain (RBD). Isoorientin interacted with Y449, K417, and other amino acids of spike-RBD, which are key residues recognizing the ACE2 receptor: (A) Predicted binding pose of isoorientin; (B) 2D diagram of ligand-protein interaction. Crystal structure of SARS-CoV-2 spike (PDB: 6M0J). Spike protein, isoorientin molecule, spike interface, and ACE2 are respectively shown in orange, red stick, grey, and blue-grey.

Data availability

Data will be made available on request.

Acknowledgments

We would like to thank the National RNAi Core Facility at Academia Sinica in Taiwan for providing the SARS-CoV-2 pseudovirus and related services. We thank Prof. Rei-Lin Kuo (Chang Gung University) for kindly providing hACE-2-overexpressed HEK293T cells.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2023.105407>.

References

- Alzaabi, M. M., Hamdy, R., Ashmawy, N. S., Hamoda, A. M., Alkhayat, F., Khademi, N. N., ... Soliman, S. S. M. (2022). Flavonoids are promising safe therapy against COVID-19. *Phytochemistry Reviews*, 21(1), 291–312. <https://doi.org/10.1007/s11101-021-09759-z>
- Amer, S. A., Albeladi, O. A., Elshabrawy, A. M., Alsharif, N. H., Alnakhli, F. M., Almugathai, A. F., ... Alash, H. (2021). Role of neutrophil to lymphocyte ratio as a prognostic indicator for COVID-19. *Health Science Reports*, 4(4), e442.
- Arcanjo, A., Logullo, J., Menezes, C. C. B., & de Souza Carvalho Giangiarulo, T. C., Dos Reis, M. C., de Castro, G. M. M., Morrot, A. (2020). The emerging role of neutrophil extracellular traps in severe acute respiratory syndrome coronavirus 2 (COVID-19). *Scientific Reports*, 10(1), 19630. <https://doi.org/10.1038/s41598-020-76781-0>
- Azzouz, D., Khan, M. A., & Palaniyar, N. (2021). ROS induces NETosis by oxidizing DNA and initiating DNA repair. *Cell Death Discovery*, 7(1), 113. <https://doi.org/10.1038/s41420-021-00491-3>
- Callaway, E. (2022). What Omicron's BA.4 and BA.5 variants mean for the pandemic. *Nature*, 606(7916), 848–849. <https://doi.org/10.1038/d41586-022-01730-y>
- Chen, L. F., Zhong, Y. L., Luo, D., Liu, Z., Tang, W., Cheng, W., ... Li, M. M. (2019). Antiviral activity of ethanol extract of *Lophatherum gracile* against respiratory syncytial virus infection. *Journal of Ethnopharmacology*, 242, Article 111575. <https://doi.org/10.1016/j.jep.2018.10.036>
- Chiang, C. C., Cheng, W. J., Lin, C. Y., Lai, K. H., Ju, S. C., Lee, C., ... Hwang, T. L. (2020). Kan-Lu-Hsiao-Tu-Tan, a traditional Chinese medicine formula, inhibits human neutrophil activation and ameliorates imiquimod-induced psoriasis-like skin inflammation. *Journal of Ethnopharmacology*, 246, Article 112246. <https://doi.org/10.1016/j.jep.2019.112246>
- Chiang, C. C., Korinek, M., Cheng, W. J., & Hwang, T. L. (2020). Targeting neutrophils to treat acute respiratory distress syndrome in coronavirus disease. *Frontiers in Pharmacology*, 11, Article 572009. <https://doi.org/10.3389/fphar.2020.572009>
- Diaz, P., Jeong, S. C., Lee, S., Khoo, C., & Koyyalamudi, S. R. (2012). Antioxidant and anti-inflammatory activities of selected medicinal plants and fungi containing phenolic and flavonoid compounds. *Chinese Medicine*, 7(1), 26. <https://doi.org/10.1186/1749-8546-7-26>
- Du, L., Xie, Y., Zheng, K., Wang, N., Gao, M., Yu, T., ... Pan, J. A. (2021). Oxidative stress transforms 3CLpro into an insoluble and more active form to promote SARS-CoV-2 replication. *Redox Biology*, 48, Article 102199. <https://doi.org/10.1016/j.redox.2021.102199>
- Fan, J. S., Lee, I. J., & Lin, Y. L. (2015). Flavone glycosides from commercially available *Lophatheri Herba* and their chromatographic fingerprinting and quantitation. *Journal of Food and Drug Analysis*, 23(4), 821–827. <https://doi.org/10.1016/j.jfda.2015.05.003>
- Feitz, W. J. C., Suntharalingham, S., Khan, M., Ortiz-Sandoval, C. G., Palaniyar, N., van den Heuvel, L. P., ... Licht, C. (2021). Shiga toxin 2a induces NETosis via NOX-dependent Pathway. *Biomedicines*, 9(12), Article 20390. <https://doi.org/10.3390/biomedicines9121807>
- Fu, Q., Wang, H., Lan, Y., Li, S., Hashi, Y., & Chen, S. (2014). High-throughput and sensitive screening of compounds with deoxyribonucleic acid-binding activity by a high-performance liquid chromatography-tandem mass spectrometry-fluorescence detection technique using palmatine as a fluorescence probe. *Journal of Chromatography A*, 1323, 123–134. <https://doi.org/10.1016/j.chroma.2013.11.015>
- Hao, B., Caulfield, J. C., Hamilton, M. L., Pickett, J. A., Midega, C. A., Khan, Z. R., ... Hopper, A. M. (2016). Biosynthesis of natural and novel C-glycosylflavones utilising recombinant *Oryza sativa* C-glycosyltransferase (OsCGT) and *Desmodium incanum* root proteins. *Phytochemistry*, 125, 73–87. <https://doi.org/10.1016/j.phytochem.2016.02.013>
- He, R.-R., Tsoi, B., Li, Y.-F., Yao, X.-S., & Kurihara, H. (2011). The anti-stress effects of Guangdong herbal tea on immunocompromise in mice loaded with restraint stress. *Journal of Health Science*, 57(3), 255–263. <https://doi.org/10.1248/jhs.57.255>
- He, X., Qi, S., Zhang, X., & Pan, J. (2022). The relationship between the neutrophil-to-lymphocyte ratio and diabetic retinopathy in adults from the United States: Results from the National Health and nutrition examination survey. *BMC Ophthalmology*, 22(1), 346. <https://doi.org/10.1186/s12886-022-02571-z>
- Hong, W., Yang, J., Zou, J., Bi, Z., He, C., Lei, H., ... Wei, X. (2022). Histones released by NETosis enhance the infectivity of SARS-CoV-2 by bridging the spike protein subunit 2 and sialic acid on host cells. *Cellular & Molecular Immunology*, 19(5), 577–587. <https://doi.org/10.1038/s41423-022-00845-6>
- Huang, J., Hong, W., Wan, M., & Zheng, L. (2022). Molecular mechanisms and therapeutic target of NETosis in diseases. *MedComm*, 3(3), e162.
- Jan, J. T., Cheng, T. R., Juang, Y. P., Ma, H. H., Wu, Y. T., Yang, W. B., ... Wong, C. H. (2021). Identification of existing pharmaceuticals and herbal medicines as inhibitors of SARS-CoV-2 infection. *Proceedings of the National Academy of Sciences of the United States of America*, 118(5), e2021579118. <https://doi.org/10.1073/pnas.2021579118>
- Jantan, I., Arshad, L., Septama, A. W., Haque, M. A., Mohamed-Hussein, Z. A., & Govender, N. T. (2022). Antiviral effects of phytochemicals against severe acute respiratory syndrome coronavirus 2 and their mechanisms of action: A review. *Phytotherapy Research*. <https://doi.org/10.1002/ptr.7671>
- Kim, A., Im, M., Gu, M. J., & Ma, J. Y. (2016). Ethanol extract of *Lophatheri Herba* exhibits anti-cancer activity in human cancer cells by suppression of metastatic and angiogenic potential. *Scientific Reports*, 6, 36277. <https://doi.org/10.1038/srep36277>
- Lai, K. H., Chen, P. J., Chen, C. C., Yang, S. H., El-Shazly, M., Chang, Y. C., ... Hwang, T. L. (2021). *Lophatherum gracile* Brongn. attenuates neutrophilic inflammation through inhibition of JNK and calcium. *Journal of Ethnopharmacology*, 264, Article 113224. <https://doi.org/10.1016/j.jep.2020.113224>
- Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., ... Wang, X. (2020). Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature*, 581(7807), 215–220. <https://doi.org/10.1038/s41586-020-2180-5>
- Liu, X., Wang, Y., Ge, W., Cai, G., Guo, Y., & Gong, J. (2022). Spectrum-effect relationship between ultra-high-performance liquid chromatography fingerprints and antioxidant activities of *Lophatherum gracile* Brongn. *Food Science & Nutrition*, 10(5), 1592–1601. <https://doi.org/10.1002/fsn3.2782>
- Liu, Y., Hu, R., Shen, S., Zhang, Z., Zhang, J., Song, X., & Qiang, S. (2020). Plant diversity in herbal tea and its traditional knowledge in Qingtian County, Zhejiang Province. *China. Plant Diversity*, 42(6), 464–472. <https://doi.org/10.1016/j.pld.2020.12.002>
- Luo, W., Cai, Q., Zhou, Y., Cai, Y., Song, H., Zhang, Y., ... Liao, Y. (2022). Variation of parental feeding practices during the COVID-2019 pandemic: A systematic review. *BioMed Central public health*, 22(1), 1600. <https://doi.org/10.1186/s12889-022-14027-6>
- McKenna, E., Wubben, R., Isaza-Correa, J. M., Melo, A. M., Mhaonaigh, A. U., Conlon, N., ... Molloy, E. J. (2022). Neutrophils in COVID-19: Not innocent bystanders. *Frontiers in Immunology*, 13, Article 864387. <https://doi.org/10.3389/fimmu.2022.864387>
- Mutua, V., & Gershwin, L. J. (2021). A review of neutrophil extracellular traps (NETs) in disease: Potential anti-NETs therapeutics. *Clinical Reviews in Allergy & Immunology*, 61(2), 194–211. <https://doi.org/10.1007/s12016-020-08804-7>
- Nguyen, G. T., Green, E. R., & Mecsas, J. (2017). Neutrophils to the ROScure: Mechanisms of NADPH oxidase activation and bacterial resistance. *Frontiers in Cellular and Infection Microbiology*, 7, 373. <https://doi.org/10.3389/fcimb.2017.00373>
- Njeim, R., Azar, W. S., Fares, A. H., Azar, S. T., Kfoury Kassouf, H., & Eid, A. A. (2020). NETosis contributes to the pathogenesis of diabetes and its complications. *Journal of Molecular Endocrinology*, 65(4), R65–R76. <https://doi.org/10.1530/jme-20-0128>
- Ohashi, H., Watashi, K., Saso, W., Shionoya, K., Iwanami, S., Hirokawa, T., ... Wakita, T. (2021). Potential anti-COVID-19 agents, cepharanthine and nelfinavir, and their usage for combination treatment. *iScience*, 24(4), Article 102367. <https://doi.org/10.1016/j.isci.2021.102367>
- Özcan, A., & Boyman, O. (2022). Mechanisms regulating neutrophil responses in immunity, allergy, and autoimmunity. *Allergy, Online ahead of print*. <https://doi.org/10.1111/all.15505>
- Pirlog, C. F., Cotan, H. T., Parosanu, A., Orlov Slavut, C., Popa, A. M., Iaciu, C., ... Nitipir, C. (2022). Correlation between pretreatment neutrophil-to-lymphocyte ratio and programmed death-ligand 1 expression as prognostic markers in non-small cell lung cancer. *Cureus*, 14(7), e26843.
- Schmidt, D., Hakeem Said, I., Ohl, N., Sharifii, M., Cotrell, P., & Kuhnert, N. (2022). Investigating the interaction between dietary polyphenols, the SARS CoV-2 spike protein and the ACE-2 receptor. *Food & Function*, 13(15), 8038–8046. <https://doi.org/10.1039/d2fo00394e>
- Tomas, M., Capanoglu, E., Bahrami, A., Hosseini, H., Akbari-Alavijeh, S., Shaddel, R., ... Jafari, S. M. (2022). The direct and indirect effects of bioactive compounds against coronavirus. *Food Frontiers*, 3(1), 96–123. <https://doi.org/10.1002/fft2.119>
- Wang, L. G., & Wang, L. (2022). Current strategies in treating cytokine release syndrome triggered by coronavirus SARS-CoV-2. *ImmunoTargets and Therapy*, 11, 23–35. <https://doi.org/10.2147/itt.S360151>
- Wang, Y., Chen, M., Zhang, J., Zhang, X. L., Huang, X. J., Wu, X., ... Ye, W. C. (2012). Flavone C-glycosides from the leaves of *Lophatherum gracile* and their in vitro antiviral activity. *Planta Medica*, 78(1), 46–51. <https://doi.org/10.1055/s-0031-1280128>
- Wang, Y., Du, F., Hawez, A., Mörgelin, M., & Thorlacius, H. (2019). Neutrophil extracellular trap-microparticle complexes trigger neutrophil recruitment via high-mobility group protein 1 (HMGB1)-toll-like receptors (TLR2)/TLR4 signalling. *British Journal of Pharmacology*, 176(17), 3350–3363. <https://doi.org/10.1111/bph.14765>
- Zhang, P., Chun, Z., Shao, Q., Fu, L., Luo, Y., Gu, D., & Chen, R. (2021). Evaluation of the phytochemicals and antioxidant activity of *Lophatherum gracile* Brongn based on chemical fingerprinting by HPLC with electrochemical detection. *Journal of Separation Science*, 44(20), 3777–3788. <https://doi.org/10.1002/jssc.202100318>
- Zhang, Y., De Stefano, R., Robine, M., Butelli, E., Bulling, K., Hill, L., ... Schoonbeek, H. J. (2015). Different reactive oxygen species scavenging properties of flavonoids determine their abilities to extend the shelf life of tomato. *Plant Physiol.*, 169(3), 1568–1583. <https://doi.org/10.1104/pp.15.00346>