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Orally delivered perilla (Perilla frutescens) leaf extract effectively inhibits SARS-CoV-2 infection in a Syrian hamster model

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Abstract

On analyzing the results of cell-based assays, we have previously shown that perilla (Perilla frutescens) leaf extract (PLE), a food supplement and orally deliverable traditional Chinese medicine approved by the Taiwan Food and Drug Administration, effectively inhibits SARS-CoV-2 by directly targeting virions. PLE was also found to modulate virus-induced cytokine expression levels. In this study, we explored the anti-SARS-CoV-2 activity of PLE in a hamster model by examining viral loads and virus-induced immunopathology in lung tissues. Experimental animals were intranasally challenged with different SARS-CoV-2 doses. Jugular blood samples and lung tissue specimens were obtained in the acute disease stage (3–4 post-infection days). As expected, SARS-CoV-2 induced lung inflammation and hemorrhagic effusions in the alveoli and perivascular areas; additionally, it increased the expression of several immune markers including lung Ki67-positive cells, Iba-1-positive macrophages, and myeloperoxidase-positive neutrophils. Virus-induced lung alterations were significantly attenuated by orally administered PLE. In addition, pretreatment of hamsters with PLE significantly reduced viral loads and immune marker expression. A purified active fraction of PLE was found to confer higher antiviral protection. Notably, PLE prevented SARS-CoV-2-induced increase in serum markers of liver and kidney function as well as the decrease in serum high-density lipoprotein and total cholesterol levels in a dose-dependent fashion. Differently from lung pathology, monitoring of serum biomarkers in Syrian hamsters may allow a more humane assessment of the novel drugs with potential anti-SARS-CoV-2 activity. Our results expand prior research by confirming that PLE may exert an in vivo therapeutic activity against SARS-CoV-2 by attenuating viral loads and lung tissue inflammation, which may pave the way for future clinical applications.

Keywords: COVID-19, Hamster, Herbal medicine, Perilla frutescens, SARS-CoV-2, Serum biomarkers

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1. Introduction

While first-generation vaccines are generally sufficient to elicit a robust immunological response against severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), a subgroup of fully vaccinated individuals remains at risk of breakthrough infections during different waves of the pandemic driven by novel variants of concern. SARS-CoV-2 enters human cells by utilizing the angiotensin-converting enzyme 2 (ACE2) receptor expressed in various tissues (e.g., lung, heart, kidney, gastrointestinal tract, and liver). Additionally, elevated serum concentrations of proinflammatory molecules are hallmarks of the cytokine storm occurring in severe Coronavirus Disease 2019 (COVID-19) [1]. A clinical trial has shown that the viral polymerase inhibitor remdesivir may shorten the duration of hospital stay in patients with mild disease [2]. Another study indicated that the viral 3C-like inhibitor PF-07321332 in combination with ritonavir (commercial name: Paxlovid) may reduce hospitalizations by 89% when given in the first few days after disease inception [3]. However, the appearance of novel variants of concerns and the adverse effects of ritonavir have prompted intense research on other antiviral compounds with novel mechanisms of action to tackle new pandemic waves in countries characterized by vaccine shortage. In the quest to address this unmet medical need, animal models can be useful to predict the effectiveness of novel antiviral compounds before their progression to clinical trials. While murine models have been largely popular in antiviral research, mice are not naturally infected with SARS-CoV-2; therefore, experimental manipulations aimed at either virus adaptation or at inducing exogenous expression of the human ACE2 receptor are required. However, the induction of human ACE2 expression has yielded inconsistent findings with aberrant tissue distribution in different ACE2 transgenic models, making the results difficult to interpret [4]. In contrast, hamsters are naturally susceptible to SARS-CoV-2 and, upon infection, they can develop certain key features of COVID-19 (e.g., pneumonia and release of proinflammatory cytokines) [5–7]. Notably, preclinical research with hamster models — involving testing of antiviral drugs, monoclonal antibodies, and vaccines — has yielded results that were consistent with subsequent clinical data [8–11].

Perilla (Perilla frutescens) leaves have been extensively used in folk Asian medicine to rectify stomach function, discharge heat, and improve healthy qi. Additionally, they have enjoyed large popularity for ethnopharmacological uses in Europe. Using in vitro experiments, we have previously shown that perilla leaf extract (PLE) exerts virucidal activity against SARS-CoV-2 in Vero E6 and Calu3 cells — which was noticeably accompanied by an immunomodulatory effect [12]. A synergistic antiviral activity when PLE was combined with remdesivir was also observed. In this study, we expanded our prior research and tested whether PLE can exert in vivo anti-SARS-CoV-2 activity in the hamster animal model. Because clinical manifestations of the disease in animals are frequently subtle and difficult to chart without resorting to pathological examinations, we also assessed whether serum biomarker-guided disease monitoring may foster our ability to investigate the effects of compounds with purported anti-SARS-CoV-2 activity — including PLE.

2. Materials and methods

2.1. Cell cultures and viral infections

African green monkey kidney epithelial cells (Vero E6) were obtained from ATCC (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium (DMEM; Gibco; Gaithersburg, MD, USA) supplemented with 10% (w/v) fetal bovine serum (FBS). The prototype SARS-CoV-2 strain CGU4 (Taiwan/CGMH-CGU-01/2020; GISAID identifier: EPI_ISL_411915; NCBI accession number: MT192759) was kindly provided by the Taiwan CDC and propagated in Vero E6 cells [12]. Sanger sequencing was used to confirm the genomic sequence. All SARS-CoV-2 experiments were carried out in either biosafety level (BSL)-3 or −4 certified laboratories.

2.2. Ethical statement

All animal procedures complied with the ARRIVE guidelines (www.arriveguidelines.org/) and were reviewed and approved by the National Defense Medical Center Animal Care and Use Committee (approval numbers: AN-109-02 and AN-109-41).

2.3. Syrian hamster experiments

The hamster model of SARS-CoV-2 infection has been previously described in detail [6,7]. Briefly, Syrian hamsters were purchased from the National Laboratory Animal Center (Taipei, Taiwan).
Fig. 1. PLE alleviates SARS-CoV-2-induced body weight loss and viral protein expression in hamsters which had undergone a high-dose SARS-CoV-2 challenge. (A) Summary of the experimental design. Hamsters were intranasally infected with SARS-CoV-2 (5.73 × 10^5 pfu). Each animal in the low-dose group (LDV) received 80 mg PLE/kg/day in drinking water from the day before infection (−1 dpi) until 6 dpi. The high-dose group (HDV) received a single intraperitoneal daily dose of PLE (90 mg/kg/day) as well as oral PLE (80 mg/kg/day) in drinking water, totaling 170 (90 mg + 80 mg) mg/kg/day. Animals (n = 3 per group) were euthanized on day 3 or day 6 and jugular blood was collected for biochemical analyses. (B) Changes in body weight following SARS-CoV-2 infection were recorded on a daily basis; the body weight measured on the day of infection was arbitrarily set to 1. (C) Western blot analysis of NP protein expression in lung tissues collected at 3 dpi. Lysates obtained from lobe 2 were prepared in RIPA buffer and subjected to 8% SDS-PAGE (quantity of lysate per lane: 50 μg) using specific antibodies raised against NP and GAPDH. The intensity of the NP protein bands was quantified after normalization to GAPDH expression. (D) At 3 and 6 dpi, immunohistochemistry was applied to investigate NP expression levels in lung tissues of hamsters treated with PLE. The ratio of positive NP expression — calculated as the number of NP-positive cells divided by the total number of cells — was analyzed using an Aperio Imagescope v12.3. (B–C) Data are expressed as means ± standard errors of the mean. Statistical significance was determined by a two-tailed Student’s t-test. ns, not significant; **p < 0.01.
Fig. 1. (Continued)
and fed a standard low-fat polysaccharide-rich chow diet (LabDiet 5053; Purina, St Louis, MO, USA). Intranasal inoculation of SARS-CoV-2 (strain CGU4) was performed under anesthesia (Zoletil® 40 mg/kg plus xylazine 4 mg/kg). The antiviral activity of PLE was assessed through the following parameters: daily changes in body weight, serum biochemistry, histopathology, immunopathology, and virus quantification in the lungs. Lung lobe #2 was used for qPCR, western blotting, and titer determination, whereas histopathology was carried out on lobe #5. A high-dose SARS-CoV-2 challenge was applied to mimic severe disease in humans (Fig. 1A). To this aim, 8-9-week-old hamsters were intranasally infected (0 day postinfection [dpi]) with SARS-CoV-2 (5.73 × 10⁵ plaque-forming units [pfu] in 50 μL PBS). Two different PLE doses (low versus high dose) were administered from the day before infection (−1 dpi) until 6 dpi. Each hamster in the low-dose group (LDV) received 80 mg PLE/kg/day in drinking water – based on a reported daily consumption of 10 mL drinking water per animal [13] – ad libitum and refreshed on day 3. The high-dose group (HDV) received a single intraperitoneal daily dose of PLE (90 mg/kg/day) as well as oral PLE (80 mg/kg/day) in drinking water, totaling 170 (90 mg + 80 mg) mg/kg/day. The amount of PLE delivered to hamsters was calculated based on an EC₅₀ value of 0.125 mg/mL derived from cell-based assays [12]. Because each hamster weighed ~100 g and had a total blood volume of 8 mL, animals in the LDV and HDV arms received 8 × EC₅₀ (80 mg/kg/day) and 17 × EC₅₀ (170 mg/kg/day) of PLE, respectively. Hamsters were euthanized on day 3 or day 6 by Zoletil® overdosing, and lung and jugular blood samples were collected for analyses. In separated experiments, a low-dose SARS-CoV-2 challenge (350 pfu in 50 μL PBS) was applied to mimic mild disease in humans. Two different PLE preparations obtained as previously described [12] were given to experimental hamsters. PLE (2.5 × EC₅₀) and its enriched fraction were administered at a dose of 12.5 mg/kg/day b.i.d. (time interval: 12 h) for four consecutive days. Animals were sacrificed for sample collection at 4 dpi (Fig. 3A).

2.4. Sample preparation for plaque assay and western blotting

Lung tissues were homogenized in DMEM using the bead-beating technology (Precellys Lysing kits; Bertin Technologies, Aix-en-Provence, France). Following centrifugation, supernatants were stored in small aliquots. Viral titers were determined using a plaque assay following a previously described methodology [12]. For western analysis, thawed supernatants were mixed with equal amounts of RIPA buffer containing lysozyme and proteinase inhibitors. The protein content was measured with a protein assay kit (Bio-Rad, Richmond, CA, USA). Equal amounts of cellular proteins were separated by SDS-PAGE and subsequently transferred onto polyvinylidene fluoride membranes. Subsequently, the membranes were immunoblotted with specific primary antibodies and subsequently exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies. The Immobilon Western HRP Chemiluminescence Substrate (Millipore, Burlington, MA, USA) was used for blot development. Mouse monoclonal anti-nucleocapsid (GTX632269, 1:2000 dilution) and anti-GAPDH (GTX627408, 1:5000 dilution) antibodies were obtained from Genetex (Hsinchu, Taiwan).

2.5. RNA extraction and qRT-PCR analysis

Lung tissues were stored overnight in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C, homogenized using the bead-beating technology, and total RNA was subsequently extracted using the TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. After removal of genomic DNA with DNase (Promega, Madison, WI, USA), RNA samples were extracted with the phenol/chloroform method followed by ethanol precipitation. Equal amounts of total extracted RNA were subsequently reverse transcribed with the M-MLV reverse transcriptase system (Thermo Fisher Scientific) using random primers. Subsequently, qRT-PCR was performed on a QuantStudio3 RT-qPCR system (Applied Biosystems, Foster City, CA, USA) using the following primers specific for the E gene: forward 5'-ACA GGT ACG TTA ATA GTT AAT AGC GTG A-3' and reverse 5'-ATA TTG TCG TTG GAC TAC GCA CAC A-3'. The primers for hamster beta-actin, which served as internal control, were as follows: forward 5'-ACTG CCG CAT CCT CCT CCT-3' and reverse 5'-TGAG TCC CCA ATG GTG ATG AC-3'. The relative mRNA expression was calculated with the 2⁻ΔΔCt method.
Fig. 2. PLE attenuates SARS-CoV-2-induced immune cell infiltrates and alterations in serum biochemical markers. (A) Lung tissues from hamsters subjected to a high-dose SARS-CoV-2 challenge were collected at 3 dpi (a) and 6 dpi (b). Immunohistochemistry was applied to investigate the expression rates of the proteins of interest using an Aperio Imagescope v12.3. (B) Biochemical analyses of serum samples collected from jugular veins were performed on an autoanalyzer. Data are expressed as means ± standard errors of the mean. Statistical significance was determined by a two-tailed Student’s t-test. *p < 0.05, **p < 0.01, and ***p < 0.005.
2.6. Lung tissue immunohistochemistry

Lung tissue immunohistochemistry (IHC) was performed by an independent company (BioTools Co., Ltd; New Taipei City, Taiwan). Briefly, antigen retrieval was carried out by incubating tissues for 20 min in citrate buffer followed by bovine serum albumin (BSA) blocking. Primary antibodies raised
### A. Table

|         | Water | Original PLE | Enrich PLE | SARS-CoV-2 |
|---------|-------|--------------|------------|------------|
| VC      | +     | +            | +          |            |
| ODV     | +     | +            |            |            |
| EDV     |       | +            | +          |            |

### B. Western Blot

| Protein | VC | ODV | EDV |
|---------|----|-----|-----|
| NP      | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| GAPDH   | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |

### C. Viral RNA

![Image](image7.png)

### D. Titer

![Image](image8.png)

### E. Immunohistochemistry

![Image](image9.png)
against SARS-CoV-2 nucleoprotein (NP; Genetex; product number: GTX135357; 1:200 dilution), ionized calcium-binding adaptor protein-1 (Iba-1; Genetex; 1:100 dilution), Ki-67 (Santa Cruz Biotechnology, Dallas, TX, USA; product number: sc-23900; 1:50 dilution), Mx1 (Santa Cruz Biotechnology; product number: sc-365436; 1:50 dilution), myeloperoxidase (MPO; Santa Cruz Biotechnology; product number: sc-35516; 1:50 dilution), and viral titers were determined using Vero E6 cells (D). Immunohistochemistry revealed a significant reduction of NP expression in PLE-treated animals. The number of NP-positive cells identified on lung sections was normalized to the total number of cells using an Aperio Imagescope v12.3. Data are expressed as means ± standard errors of the mean. Statistical significance was determined by a one-tailed Student’s t-test. *p < 0.05 and ***p < 0.005.

2.8. Serum biochemistry

Biochemical analyses of serum samples collected from the jugular veins were performed by the National Applied Research Laboratories (Taipei, Taiwan) on an autoanalyzer (Hitachi 7080; Hitachi Tokyo, Japan). The following panels were assayed: 1) liver function (aspartate aminotransferase [AST], alanine aminotransferase [ALT], γ-glutamyl transferase [γ-GT], albumin [ALB], total bilirubin [T-BIL], alkaline phosphatase [ALP], total protein [TP]); 2) renal function (creatinine [CREA], blood urea nitrogen [BUN]); 3) lipid metabolism (high-density lipoprotein cholesterol [HDL-CHO], low-density lipoprotein cholesterol [LDL], total cholesterol [T-CHO], triglycerides [TG]); 4) electrolytes: magnesium [Mg], calcium [Ca], phosphorus [P]); and 5) glucose and other metabolic parameters (glucose [GLU], creatine phosphokinase [CPK], uric acid [UA], lactate dehydrogenase [LDH]).

2.9. Statistical analysis

Continuous data are expressed as means ± standard errors of the mean and comparisons were performed with two-tailed Student’s t-test (Figs. 1 and 2; Supplementary Fig. S2). A one-tailed Student’s t-test was subsequently used to test differences between PLE-treated and mock-infected control (virus control [VC]) group; Figs. 3–5). p values < 0.05 were considered to reflect statistically significant differences.

3. Results

3.1. PLE attenuates body weight loss and reduces viral protein expression in hamsters exposed to a high-dose SARS-CoV-2 challenge

We have previously shown that PLE exerts an in vitro virucidal activity against SARS-CoV-2 in Vero
E6 cells [22]. To test whether the same anti-SARS-CoV-2 effect could be elicited in vivo, hamsters underwent a high-dose intranasal SARS-CoV-2 challenge (strain CGU4, 5.73 × 10⁵ pfu) one day after oral PLE administration – either with or without a concomitant intraperitoneal injection (Fig. 1A). Two different PLE dosages were tested and three hamsters in each dose group were sacrificed at 3 and 6 dpi. On the day before infection, the LDV group received PLE in drinking water (80 mg/kg/day per hamster) to mimic oral PLE uptake. In addition to PLE in drinking water, the HDV group received a single daily intraperitoneal PLE injection (90 mg/kg/day). Hamsters in the negative control (NC) group did not receive PLE and were not experimentally infected with SARS-CoV-2. While animals in the NC
group gained weight over a 6-day interval, hamsters in the VC group experienced weight loss after SARS-CoV-2 infection (Fig. 1B) [5–7]. Similar to the VC groups, animals in the HDV group started losing weight from 1 dpi. However, differently from the former, a gradual recovery was evident from 3 to 6 dpi in the latter group (Fig. 1B). No protection against weight loss was observed in the LDV group. Compared with the VC group at 3 dpi, animals in the HDV group — but not those in the LDV group —
showed a lower level of NP expression in the lung (Fig. 1C). At 6 dpi, the amount of NP expression in all groups was below the limit of detection of western blotting (data not shown).

Immunohistochemistry carried out at 3 dpi revealed that — differently from the NC group — animals in the VC group showed an abundant expression of NP in lung tissues. However, NP expression levels declined significantly at 6 dpi — a finding in accordance with previous observations [1,5,7] on peak viral replication occurring at ~3 dpi, followed by a steep decline at barely detectable levels (Fig. 1D). A quantitative analysis of NP expression levels revealed a significantly higher abundance ($p < 0.05$) in the VC group compared with the NC group; a similar albeit not significant trend was observed for MPO and CD3 (panel a; Fig. 1D). At 3 dpi and compared with the VC group, the number of NP-positive cells did not differ significantly in the LDV and HDV groups. However, PLE promoted a substantial dose-dependent inhibition of NP expression at 6 dpi (bottom panels, Fig. 1D). Collectively, these data indicate that PLE — at the dose applied in the HDV group — tends to down-regulate the lung cellular expression of the NP viral protein, although not significantly so (Fig. 1C and D).

3.2. PLE attenuates inflammation and reduced alterations in serum biomarkers in hamsters exposed to a high-dose SARS-CoV-2 challenge

The severity of SARS-CoV-2-induced pneumonia was paralleled by an increased expression of immune cell markers — including Iba-1-positive macrophages, CD3-positive T lymphocytes, and MPO-positive neutrophils (Fig. 2A; Fig. 5.1). Immunohistochemical expression of both Iba-1 and Mx1 was significantly higher in the VC group compared with the NC group; a similar — albeit not significant — trend was observed for MPO and CD3 (panel a;
Fig. 2A). In addition, SARS-CoV-2 infection induced cell proliferation as attested by an increased expression of Ki-67 (panel a; Fig. 2A). Interestingly, these immunohistochemical findings were attenuated by treatment in LDV and/or HDV groups, the only exception being CD3-positive T lymphocytes at 3 and 6 dpi (panels a and b, Fig. 2A and Fig. S1). Collectively, these data indicate that PLE attenuates SARS-CoV-2-induced lung inflammation by exerting a significant immunomodulatory activity (Fig. 2Aa and Fig. S1) [12]. The severity of COVID-19 has been associated with alterations in serum biomarkers – including AST, ALT, γ-GT, BUN, HDL-CHO, and T-CHO [15–18]. At 3 dpi, we found that – compared with the VC group – both the HDV and LDV groups showed less marked increases in serum AST, ALT, γ-GT, and BUN levels, whereas no effect on CREA was observed (Fig. 2B and Fig. S2). On the one hand, these results suggest that PLE does not cause hepatic or renal toxicity; on the other hand, they also indicate that it can exert hepatoprotective effects in SARS-CoV-2-infected hamsters [19,20]. Interestingly, serum levels of HDL-CHO and T-CHO were reduced following SARS-CoV-2 infection – possibly as a consequence of virus-induced hepatic damage. Both of these effects were attenuated by PLE (panel c; Fig. 2B).

3.3. PLE is more effective in hamsters exposed to a low-dose SARS-CoV-2 challenge

To mimic a mild human infection and to test the potential effectiveness of oral PLE, hamsters underwent a low-dose intranasal SARS-CoV-2 challenge (350 pfu; 100 ID50) following a procedure similar to that implemented for the testing of the orally bioavailable prodrug molnupiravir (MK-4482) in hamsters [10]. To this aim, animals received oral PLE at a dose of 12.5 mg/kg b.i.d. (~2.5 × EC50, Fig. 3A) in two different forms, i.e., 1) the original perilla water extract (ODV) and 2) the partially purified active (enriched) fraction (EDV). The latter had a 2-fold higher anti-SARS-CoV-2 activity in Vero E6 cells compared with the former (data not shown). Following sacrifice at 4 dpi, Western blot analysis revealed that EDV inhibited lung NP expression more effectively than ODV (Fig. 3B). Both EDV and ODV groups showed suppression of viral RNA expression and viral titers based on the results of qPCR and plaque assay, respectively (Fig. 3C-D). Additionally, IHC revealed that hamsters in the VC group had a markedly high lung NP expression – which was significantly attenuated in the EDV and ODV groups (Fig. 3E).
On examining H&E-stained lung tissues harvested at 4 dpi, hamsters in the VC group showed mixed alveolar, interstitial, peribronchial, and perivascular inflammatory infiltrates consisting of neutrophils, lymphocytes, and histiocytes (row a, Fig. 4A). Vasculitis in VC animals was characterized by fibrinoid degeneration and infiltration by neutrophils and lymphocytes (arrows; Fig. 4A). Compared with the VC group, hamsters in the EDV and ODV groups were characterized by less severe inflammatory damage and lower amounts of alveolar and perivascular hemorrhage and edema (Fig. 4A and analysis in panels a–c of Fig. 4B). However, the lowest degrees of hemorrhage, edema, vasculitis, epithelial degeneration, and alveolar wall necrosis were observed in the EDV group (panels a–f, Fig. 4B) as shown by the sum of histological scores (panel g, Fig. 4B). We subsequently applied IHC to investigate the expression of inflammatory markers in lung tissues (Fig. 5; Fig. S3). The results consistently revealed reduced expression levels of Iba-1, MPO, and Ki-67 in the ODV group and, more markedly, in the EDV group (Fig. 4). Collectively, these results indicate that PLE inhibits SARS-CoV-2-induced inflammation (panels a–c, Fig. 5). However, treatment with PLE did not appreciably change both CD3-positive T cells (Fig. 2A; panel d, Fig. 5) and the expression of the interferon-inducible gene Mx1 — although a transient increase of these variables was observed during the course of the experiment (panel e, Fig. 5).

4. Discussion

An orally available antiviral drug with preventive and therapeutic potential is expected to enrich our armamentarium in the battle against the ongoing COVID-19 pandemic. We have previously demonstrated that PLE — an orally deliverable traditional Chinese medicine approved by the Taiwan Food and Drug Administration — not only inhibits SARS-CoV-2 by directly targeting the virions but also hampers the exaggerated cytokine response in Calu-3 cells [12]. In this proof-of-concept animal study, we investigated whether the hamster may represent a suitable model for human COVID-19 and investigated whether PLE may exert an in vivo immunomodulatory and therapeutic activity. The summary of our main findings is shown in Fig. 6: PLE attenuates SARS-CoV-2-induced lung injury and biochemical alterations in the hamster model. Hamsters are known to express the ACE2 receptor [1,6,10] and — following SARS-CoV-2 infection — may develop symptoms resembling those of human COVID-19 [5,7,21]. In addition, and similarly to the human receptor, hamster ACE2 has higher affinity for SARS-CoV-2 than SARS-CoV-1 [21]. Previous research has focused on the hamster as an animal platform for vaccine development and the testing of antiviral agents against SARS-CoV-2 [8–11]. In the current study, we demonstrated that infected Syrian hamsters develop mixed alveolar, interstitial, peribronchial, and perivascular inflammatory infiltrates (Fig. 4A) — a finding in agreement with previous observations from patients with COVID-19 [22–24]. Other lesions similar to those reported in humans include alveolar wall necrosis, vasculitis, as well as hemorrhage and edema of the alveoli and perivascular areas (Fig. 4). Interestingly, we found that orally delivered PLE attenuated the severity of these alterations in our experimental model — especially when the enriched fraction was administered (Fig. 4). Collectively, these data indicate that the hamster model is suitable to shed light on the mechanistic underpinnings of novel anti-SARS-CoV-2 drugs.

Liver biochemical abnormalities have been extensively documented in patients with COVID-19 — especially in presence of severe disease [25–27]. Notably, hepatotoxicity has also been reported in COVID-19 patients who had been treated with remdesivir or lopinavir-ritonavir [25,28]. Another study demonstrated that patients with COVID-19 patients without pre-existing liver disease who required intensive care had more severe virus-induced liver damage than those who did not [29]. Additionally, more pronounced increases in AST and ALT have been reported in severe COVID-19 compared with mild disease [15,16,30]. Interestingly, there is also evidence that SARS-CoV-2 can exert direct hepatotoxic damage [27].

Another notable finding from our study is that SARS-CoV-2-infected hamsters displayed increased BUN and lower levels of HDL-CHO and T-CHO (Fig. 2B) — a finding in accordance with published clinical studies in patients with COVID-19 [17,18]. While both HDL-CHO and T-CHO may play a role in SARS-CoV-2 entry into host cells [31,32], their levels tend to decrease over the course of infection either because of their consumption during viral invasion [18] or virus-induced hepatocyte dysfunction that impairs normal lipid metabolism. As for renal function, an increased BUN — which may result from renal damage related to virus-induced aberrant release of proinflammatory cytokines — has been identified as being an adverse prognostic factor in terms of in-hospital mortality [17,33]. This is, to our knowledge, the first animal study to investigate the biochemical indices of liver (AST, ALT, γ-GT) and renal (BUN) function in hamsters experimentally infected with SARS-CoV-2.
Alterations in these biochemical markers might not only reflect the direct pathogenic effects of the virus but also the potential hepatic and renal toxicities of antiviral drugs. In this scenario, the combined monitoring of body weight changes and alterations in biochemical markers following SARS-CoV-2 infection may allow conducting more humane research in the hamster model, without resorting to animal sacrifice.

PLE has a multifaceted therapeutic potential that includes anti-oxidant properties [34,35]. Interestingly, a previous study in a rodent model demonstrated that PLE can alleviate hydroperoxide-induced oxidative hepatotoxicity—suggesting that the extract may protect the liver against drug-induced damage [20]. On analyzing the results of cell-based assays, we have previously shown that PLE and remdesivir may exert synergistic therapeutic effects [12]; however, remdesivir has the potential to induce liver injury [25,28]. An interesting implication of our data is that PLE, given in combination with other anti-SARS-CoV-2 drugs, might not only show pharmacological synergy but also concomitantly reduce the risk of drug-induced hepatotoxicity.

PLE may also have prophylactic applications against COVID-19 based on its virucidal activity against virus particles [16]. In the current study, we found that PLE-mediated inhibition of NP expression in hamster lung tissues was more pronounced following a low-dose SARS-CoV-2 challenge (Figs. 3–5). Consequently, the oral administration of PLE may be a viable strategy both for preventing SARS-CoV-2 infections and for tackling the disease at an early stage. Additionally, PLE appears to be well-tolerated and is “Generally Recognized as Safe” (GRAS) by the Taiwan FDA [36]. In this scenario, PLE may have applications in the risk management of the COVID-19 pandemic during the progressive reopening of international flights. For example, PLE may be given on arrival spots in destinations where quarantine is mandatory before entering a country. Other potential applications of oral PLE may include disease prevention in COVID-19 patients’ households and out-of-hospital treatment of mild or asymptomatic cases. Pending confirmation in phase 3 clinical trials, the use of PLE may have important public health implications during the ongoing pandemic, especially in low-income countries where antibody cocktail treatments might not be invariably affordable.

5. Conclusions

Our current data indicate that the Syrian hamster is a suitable animal model that recapitulates the main features of human COVID-19—both in terms of lung pathology and alterations of biological markers. Collectively, our findings suggest that this animal platform may have valuable applications for testing novel herbal medicines against SARS-CoV-2 infection. Finally, our results expand prior research by confirming that PLE may exert an in vivo immunomodulatory and therapeutic activity.

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Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix

| 3 dpi | lba1 | MPO | Ki-67 | Mx1 | CD3 |
|-------|------|-----|-------|-----|-----|
| NC    | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) | ![Image](image5) |
| VC    | ![Image](image6) | ![Image](image7) | ![Image](image8) | ![Image](image9) | ![Image](image10) |
| SARS-CoV-2 | ![Image](image11) | ![Image](image12) | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| LD-V  | ![Image](image16) | ![Image](image17) | ![Image](image18) | ![Image](image19) | ![Image](image20) |
| HD-V  | ![Image](image21) | ![Image](image22) | ![Image](image23) | ![Image](image24) | ![Image](image25) |

| 6 dpi | lba1 | MPO | Ki-67 | Mx1 | CD3 |
|-------|------|-----|-------|-----|-----|
| VC    | ![Image](image26) | ![Image](image27) | ![Image](image28) | ![Image](image29) | ![Image](image30) |
| SARS-CoV-2 | ![Image](image31) | ![Image](image32) | ![Image](image33) | ![Image](image34) | ![Image](image35) |
| LD-V  | ![Image](image36) | ![Image](image37) | ![Image](image38) | ![Image](image39) | ![Image](image40) |
| HD-V  | ![Image](image41) | ![Image](image42) | ![Image](image43) | ![Image](image44) | ![Image](image45) |

Fig. S1. PLE attenuates SARS-CoV-2-induced immune cell infiltrates in the lung. Lung tissues from hamsters were exposed to different doses of PLE following SARS-CoV-2 challenges. Cells were subsequently collected at 3 and 6 dpi and stained with different immune cell markers. Immunohistochemical images were subsequently analyzed to assess immune cell infiltration (Fig. 2A).
Fig. S2. PLE attenuates SARS-CoV-2-induced changes in serum biochemical markers. Serum samples were collected from hamsters treated with different doses of PLE and experimentally exposed to SARS-CoV-2. Different biochemical markers of virus-induced organ damage were quantified. Data are grouped according to the biomarker function and presented as dot charts (Fig. 2B and Fig. S2).
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