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Polygooni multiflori radix extracts inhibit SARS-CoV-2 pseudovirus entry in HEK293T cells and zebrafish larvae

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ARTICLE INFO

Keywords:
SARS-CoV-2
Traditional Chinese Medicine
Polygoni Multiflori Radix
S-protein ELISA
3CL protease
Pseudovirus tests

ABSTRACT

Background: Globally, COVID-19 has caused millions of deaths and led to unprecedented socioeconomic damage. There is therefore, in addition to vaccination, an urgent need to develop complementary effective treatments and/or protective and preventative therapies against this deadly disease.

Methods: Here, a multi-component testing platform was established to screen a library of herbal extracts from traditional Chinese medicine (TCM), to identify potent herbal extracts/phytochemicals as possible therapeutics for COVID-19. We utilized assays for spike protein (S-protein) binding to angiotensin-converting enzyme II (ACE2); the enzymatic inhibition of 3CL protease; and entry of the SARS-CoV-2 pseudovirus into cultured HEK293T cells and zebrafish larvae.

Results: Over a thousand herbal extracts were screened and approximately 20 positive hits were identified. Among these, we found that the water and ethanol extracts of Polygoni Multiflori Radix (PMR) significantly inhibited S-protein binding to ACE2, 3CL protease activity, and viral entry into the cell and fish models. The water extract was more effective than the ethanol extract, with IC\textsubscript{50} values of 25 to 500 \(\mu\)g/ml. In addition, the polysaccharide-depleted fraction of the former, and epigallocatechin gallate (EGCG) which was found in both extracts, displayed significant antiviral activity.

Conclusions: Our results indicate that the water and ethanol extracts of PMR have an inhibitory effect on SARS-CoV-2 pseudovirus host-cell entry. Furthermore, EGCG might be an active component of PMR, which blocks SARS-CoV-2 entry to cells. Taken together, our findings suggest that PMR might be considered as a potential treatment for COVID-19.

Introduction

As of December 2021, the COVID-19 pandemic (caused by variants of SARS-CoV-2) has resulted in more than 270 million confirmed cases and caused over 5 million deaths since its outbreak in December 2019. In India, the largest number of daily confirmed cases has reached 400,000 (World Health Organization, 2021, https://covid19.who.int/, accessed on 15/12/2021). These figures clearly indicate the highly contagious nature of the virus, which has caused a public health emergency, as well as unprecedented damage to the global economy. Despite various COVID-19 vaccines having been approved for public use, which are effective in preventing the most life-threatening symptoms, 100%
protection cannot be guaranteed especially with the emergence of new viral variants. In addition, in some cases the vaccines themselves have been reported to have a variety of side effects. As such, there is still an urgent need for the development of potent non-vaccine-based therapies against this deadly disease (Liu and Wang et al., 2021; Mulligan et al., 2020).

Since the start of the pandemic, much has been discovered about the route of entry and infection of SARS-CoV-2 virus. Both endosomal and non-endosomal pathways are reported to be involved in the viral entry mechanism, although the former is considered the main route. During infection by SARS-CoV-2, the spike (S) protein of the virus recognises angiotensin-converting enzyme 2 (ACE2) on the host cell surface, and this triggers viral entry via endocytosis (Shang et al., 2020; Wang et al., 2020). Once inside the host cells, non-structural proteins, such as 3CL protease, enable viral replication and proliferation, leading to the widespread pathogenic damage of characteristic of this disease (Bristow et al., 2021). In view of this entry and replication mechanism, several proteins (including ACE2, S-protein and 3CL protease) have been identified as potential drug targets for the prevention and/or treatment of COVID-19. As an anti-COVID-19 strategy, small molecules that bind one or more of these proteins, inhibit the route of SARS-CoV-2 entry into cells, and/or disrupt the downstream biological activities of the virus should be identified (Prasansuklab et al., 2021).

In China, traditional Chinese medicine (TCM) has been reported to have good efficacy in the clinical treatment of viral infections. Indeed, in 2003, when SARS-CoV-1 began to threaten public health, several TCM products were identified to effectively relieve the symptoms, reduce the number of fatalities, and prevent the course of the disease (Yang et al., 2020). Today in China, more than ten prescriptions containing TCM have been recommended by clinics for the treatment of mild and moderate cases of COVID-19. Their promising efficacy has inspired the search for other novel TCMs as potential treatments for this disease (Wang et al., 2021).

Here, we established a screening platform for anti-SARS-CoV-2 infection by testing the ability of an extensive library of TCM herbs/ single molecules to inhibit the S-protein-ACE2 interaction, 3CL protease activity, and/or viral entry into cells and zebrafish (Danio rerio) larvae. From the 1,000 herbal extracts screened, > 20 positive hits were identified. Among these, the water and ethanol extracts of Polygonum Multiflori Radix (PMR) yielded significant inhibition of SARS-CoV-2 host-cell entry. PMR is a well-established TCM, which has previously been utilized for the treatment of various diseases, including cancer, liver cirrhosis and hair loss. The major phytoc hemicals in PMR are stilbenes, quinones, and flavonoids (Ho et al., 2007; Lee et al., 2015). Here, we demonstrate that the water and ethanol extracts of PMR are non-toxic, and they significantly inhibit the S-protein/ACE2 interaction, 3CL protease activity, and viral entry into both HEK293T cells and zebrafish larvae. As such, they are strong contenders for a more in-depth analysis prior to utilizing the live virus in mammalian models, with a view for potential drug development and/or clinical application.

Materials and methods

Cell culture

HEK293T cells (American Type Culture Collection, Manassas, VA, USA) were maintained in high glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA; herein called culture medium) at 37 °C in an incubator with water-saturated atmosphere and 5% CO2. Fresh culture medium was supplied every other day. HEK293T cells overexpressing hACE2 (human ACE2) were prepared by transfection with the pcDNA3.1-HACE2 plasmid (Addgene, Watertown, MA, USA). The cell viability was determined as described previously (Wu et al., 2019).

Herbal extract preparation

PMR powder (10.0 g), grounded from roots of Polygonum multiforum Thunb., was placed in a 250-ml round-bottomed flask and dissolved in 100 ml 90% ethanol or distilled water. The solutions were refluxed for 1 h before being filtered through a paper filter (Advantec, Tokyo, Japan). These were then evaporated to dryness with a rotary evaporator to yield final ethanol (PMR EtOH) and water (PMR water) extracts of 2.05 g and 4.16 g, respectively. To fractionate the PMR water, 0.64 g of this extract was dissolved in 10 ml water and then added to 40 ml EtOH. The mixture was stored at 4 °C for 16 h, then centrifuged at 7,000 rpm for 15 min and filtered through a paper filter (Advantec). The precipitate was washed twice with 20 ml water and dried to yield a polysaccharide-enriched fraction (PS deplete) of ~32 g, whereas the filtrate was evaporated to dryness with a rotary evaporator to yield a polysaccharide-depleted fraction (PS enrich) of ~0.23 g.

Carbohydrate determination

In accordance with a well-established methodology of colourimetry (HKCMS: Hong Kong Chinese Medicine Medica Standards, 2008), PS deplete or PS enrich (40 mg) was dissolved in 50 ml boiling water in a 50-ml volumetric flask. Three ml of this solution was added to another 50-ml volumetric flask and diluted 10 times. Two ml of this solution was then added to a 10-ml tube and 6 ml of antrane sulphuric acid solution was added. The mixture was cooled on ice for 15 min and then the absorbance was measured at 625 nm.

HPLC analysis

HPLC detection and calculation were conducted according to methods developed by the HKCMS utilising PMR EtOH (1 mg/ml), PMR water (1 mg/ml), PS enrich (1 mg/ml), PS deplete (1 mg/ml), 2,3,5,4′-tetrahydrosphstigeline-2-O-β-D-glucoside (THSG, 0.1 mg/ml), emodin (0.1 mg/ml), physcion (0.1 mg/ml) and EGCG (0.1 mg/ml) (all at purity > 95% from Chengdu Must, Chengdu, China). The HPLC gradient comprised 8% acetonitrile for 0-5 min, 8-20% acetonitrile for 5-15 min, 20% acetonitrile for 15-35 min, and 20-30% acetonitrile for 35-45 min. The detection wavelength was 230 nm.

SARS-CoV-2 pseudotyped-virus production

HEK293T cells at 80% confluence were transfected with components of SARS-Related Coronavirus 2, Wuhan-Hu-1 Spike-Pseudotyped Lentiviral Kit (NR-52948; BEI Resources, Bethesda, MD, USA), including SARS-CoV-2 Spike Glycoprotein (NR-52514), lentiviral backbone expressing Luciferase and ZsGreen (NR-52516), and helper plasmids (NR-52517, NR-52518, NR-52519) using Lipofectamine™ 3000 (Thermo Fisher Scientific) or JetPRIME (Polyplus, Shanghai, China) transfection reagent, according to the manufacturers’ instructions. After 72 h, the SARS-CoV-2 pseudotyped-virus particles (hereafter called pseudovirus) were collected and passed through a 0.45 μm filter. In most HEK293T cell-based experiments, the pseudovirus was used directly. For the zebrafish experiments, the pseudovirus was subsequently purified using polyethylene glycol (PEG; hereafter called PEG-pseudovirus; Lo and Yee, 2007), and the culture medium was replaced with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 pH 7.4). The pseudovirus and PEG-pseudovirus were stored at -80 °C until required.

Inhibiting SARS-CoV-2 pseudovirus entry in HEK293T cells

ACE2-overexpressing HEK293T cells were seeded into 48-well plates, after which 400 μl culture medium containing SARS-CoV-2 pseudovirus (100 μl) and PMR EtOH or PMR water (at various concentrations) were added, and they were incubated at 37 °C for 24 h. This
medium was then replaced with fresh medium, and the cells allowed to recover for 48 h. The cells were washed with PBS just before the luciferase assay. PMR\textsubscript{EtOH}, PMR\textsubscript{water}, PS\textsubscript{srcrich} and PS\textsubscript{septelate} were tested at final concentrations of 1, 10, 25, 50, and 100 µg/ml, whereas EGC\textsubscript{G} was tested at 10, 25, 50, 75 and 100 µM. An anti-SARS-CoV-2 neutralizing antibody (at 1 µg/ml; A19215, ABCionl, Woburn, MA, USA) was used as a positive control, whereas a solvent blank without the pseudovirus was used as a negative control. The inhibition percentage was determined according to the luciferase activity normalized to the luciferase activity without PMR treatment.

Luciferase assay

The luciferase assay was conducted as previously described (Wu \textit{et al.}, 2019). The percentage inhibition of each sample was calculated as follows: Inhibition rate = ((Luciferase activity of the solvent blank – Luciferase activity of the sample) / (Luciferase activity of the solvent blank – Luciferase activity of group without pseudovirus)) × 100%.

Screening spike protein inhibitors

Spike protein inhibition was analysed with SARS-CoV-2 Spike-ACE2 Binding Assay kit (Immunodiagnostics Ltd. Hong Kong, China) according to the manufacturer’s instructions. The reaction was terminated by adding 2 M H\textsubscript{2}SO\textsubscript{4}, and a FlexStation 3 microplate reader (Molecular Devices, San Jose, CA, USA) was used to quantify the data. The percentage of inhibition was calculated as follows: Percentage of inhibition = (P\textsubscript{Avg} – S\textsubscript{Avg}) / P\textsubscript{Avg} × 100%, where P\textsubscript{Avg} and S\textsubscript{Avg} are the mean OD values of the positive control and test sample, respectively.

Screening 3CL protease inhibitors

Extracts were tested for binding 3CL protease on a fluorogenic substrate with the SensoLyte SARS-CoV-2 3CL Protease Activity assay kit (AnaSpec, San Jose). The 3CL protease bound with the substrate emitted fluorescence at 460 nm when using a 360 nm excitation wavelength. The percentage of inhibition was calculated as follows: Percentage of inhibition = (P\textsubscript{Avg} – S\textsubscript{Avg}) / P\textsubscript{Avg} × 100%, where P\textsubscript{Avg} and S\textsubscript{Avg} are the mean fluorescence of the positive control and test sample, respectively, subtracted from the mean fluorescence of the blank.

Inhibiting pseudovirus entry in zebrafish

Zebrafish larvae at 3 days post-fertilization (dpf) were treated for 6 h with Danieau’s solution (Westerfield, 2000) ± 125 µg/ml PMR\textsubscript{water}, or with Danieau’s solution containing 0.1% DMSO ± 30 µg/ml PMR\textsubscript{EtOH} in a 24-well plate (SPL Life Sciences, Gyeonggi-do, Korea). Each experiment was conducted using 10 embryos per treatment group, and repeated n = 4. The larvae were then transferred to 96-well plates, one larva per well, containing the respective treatment solution plus 8 µl PEG-pseudovirus, incubated at 28 °C for 72 h, until they reached ~ 6.25 dpf. The larvae from each treatment group were pooled and washed with Milli-Q water for 6 × 20 min with gentle agitation prior to RNA extraction.

RNA extraction, reverse transcription, and RT-PCR

RNA extraction and reverse transcription were conducted as described (Relu \textit{et al.}, 2017). The converted cDNA was then amplified, for 35 cycles, by PCR using 2 × Rapid Taq Master Mix (Vazyme Biotech, Nanjing, China), after which the PCR products were separated on a 2% agarose gel. The band intensities were quantified using ImageJ (NIH; https://imagej.nih.gov/ij/). The level of expression of luciferase (Luc) mRNA was determined against that of glucose-6-phosphate dehydrogenase (g6pd) mRNA, and the expression levels following treatment with PMR\textsubscript{water} or PMR\textsubscript{EtOH} were measured. The primers used were as follows: g6pd-Fwd: 5’ TGC TTC CAC CAG CTC TGA TG 3’; g6pd-Rev: 5’ CCC TCA ACT CAT CAC TGC GT 3’; Luc-Fwd: 5’ AAA GGC TTC CAC CTA CCA GG 3’; Luc-Rev: 5’ TCC ACG ATC TCC TCG GT 3’.

Computational docking analysis

The chemical structures of phytochemicals were downloaded from Pubchem (https://pubchem.ncbi.nlm.nih.gov/), and the S-protein structure was downloaded from the Protein Data Bank (https://www.rcsb.org). Virtual screening was performed with SEESAR (Version 11.0, https://www.biosolveit.de/) as follows: (i) The binding site was determined according to the residues forming the identified druggable pocket. Ligand binding states including protonation and tautomeric forms were subsequently evaluated using the ProToss method to generate the most accessible hydrogen network. (ii) Docking modulation was performed using the “Compute LeadIT Docking” mode in the FlexX algorithm; ten binding conformations for each ligand were generated. (iii) The binding energy (i.e., ΔG) and estimated HYDE affinity (ΔH\textsubscript{HYDE}) for each ligand pose were calculated using the “Assess Affinity with HYDE in SEESAR” mode in the HYDE rescoring function (Spagnolli \textit{et al.}, 2021).

Results

PMR extracts on S-protein binding and 3CL protease activity

In our HPLC analysis, THSG, emodin, physcion and EGC\textsubscript{G} were utilised as standard markers of the PMR extracts (Fig. 1). PMR\textsubscript{water} was used as a negative control. The inhibition percentage was determined according to the residues forming the identified druggable pocket. Ligand binding states including protonation and tautomeric forms were subsequently evaluated using the ProToss method to generate the most accessible hydrogen network. (ii) Docking modulation was performed using the “Compute LeadIT Docking” mode in the FlexX algorithm; ten binding conformations for each ligand were generated. (iii) The binding energy (i.e., ΔG) and estimated HYDE affinity (ΔH\textsubscript{HYDE}) for each ligand pose were calculated using the “Assess Affinity with HYDE in SEESAR” mode in the HYDE rescoring function (Spagnolli \textit{et al.}, 2021).
consisted of 5.5% THSG, 0.03% emodin, 0.02% physcion and 0.71% EGCG, whereas PMR\textsubscript{EtOH} contained 1.86% THSG, 0.80% emodin, 0.58% physcion and 0.37% EGCG. The extractive efficacy was 41.6% for PMR\textsubscript{water} and 20.5% for PMR\textsubscript{EtOH}. These parameters served as quality controls for subsequent experiments.

An ELISA was utilised to test the binding of S-protein to ACE2. Initially, a standard inhibitor (calibrated to NIBSC code 20/136), provided by the supplier, was tested as a positive control (Fig. 2A). The inhibitor blocked binding to S-protein in a dose-dependent manner. To investigate the binding activity between the PMR extracts and S-protein, PMR\textsubscript{water} or PMR\textsubscript{EtOH} were tested at various concentrations up to 2.5 mg/ml. Both extracts inhibited the binding in a dose-dependent manner (Fig. 2B), with an estimated IC\textsubscript{50} of ~1 to 2 mg/ml.

We also tested the effects of PMR extracts on enzymatic activity of 3CL protease. We used GC-376 as a positive control, which has a broad-spectrum of anti-viral effects (Hu et al., 2021). As shown in Fig. 3, PMR\textsubscript{water} and PMR\textsubscript{EtOH} inhibited 3CL protease activity in a dose-dependent manner, with IC\textsubscript{50} values of ~0.25 mg/ml and ~0.5 mg/ml, respectively. Interestingly, PMR\textsubscript{water} displayed a higher maximal inhibition rate than PMR\textsubscript{EtOH}, i.e., ~100% versus ~70% inhibition.

**PMR extracts on viral entry**

As the S-protein and 3CL protease results indicated that the PMR extracts might prevent viral entry into host cells, we investigated the effect of PMR\textsubscript{water} and PMR\textsubscript{EtOH} on the entry of a Luc-expressing SARS-CoV-2 pseudovirus into ACE2-overexpressing HEK293T cells. The luciferase-generated luminescence was quantified to determine the amount of virus entering the cells. The luminescence decreased as the
pseudovirus was diluted, indicating the efficacy of the assay (Fig. 4A). An anti-SARS-CoV-2 neutralising antibody (positive control) inhibited total pseudoviral entry by ~ 75%. Furthermore, PMR\textsubscript{water} or PMR\textsubscript{EtOH} inhibited pseudoviral entry in a dose-dependent manner (Fig. 4B).

However, maximal inhibition was only achieved by PMR\textsubscript{water}, which displayed ~ 3-fold higher effectiveness than PMR\textsubscript{EtOH}. Neither extract affected the cell viability up to 100 \( \mu \text{g/ml} \) (Supplementary Fig. S1).

The expression of the Luc gene was used as an indicator of successful PEG-pseudovirus entry in zebrafish larvae. The 3-day-old larvae were pre-treated with PMR\textsubscript{water} or PMR\textsubscript{EtOH} for 6 h before being treated with either of these extracts plus PEG-pseudovirus for a further 72 h. Both extracts significantly inhibited the expression of Luc when compared with the respective untreated and DMSO controls (Fig. 5A&B). These results suggest that both extracts can prevent pseudovirus entry into zebrafish larvae.

**Identification of active fractions of PMR extract**

Due to its higher potency, PMR\textsubscript{water} was selected for further fractionation. Thus, it was subjected to EtOH precipitation to obtain PS\textsubscript{enrich} and PS\textsubscript{deplete}. We found that most of our selected marker chemicals from PMR\textsubscript{water} remained in PS\textsubscript{deplete} (Fig. 6A), and that PS\textsubscript{enrich} contained > 86% carbohydrate, of which > 75% were polysaccharides with molecular weights > 10 kDa (Fig. 6B). In the pseudoviral entry assay, PS\textsubscript{deplete} was more potent than PMR\textsubscript{water} in blocking viral entry, with an IC\textsubscript{50} value of ~ 15 \( \mu \text{g/ml} \) (Fig. 7A). Indeed, maximal blocking was revealed by PS\textsubscript{deplete} alone.

The enriched chemicals within PS\textsubscript{deplete} were also tested using the viral entry assays. Several phytochemicals (e.g., THSG, emodin and EGCG) were enriched in PMR\textsubscript{water} (Sun et al., 2018; Yi et al., 2007). Of these, EGCG inhibited pseudovirus entry robustly in a dose-dependent manner, exhibiting complete inhibition at ~ 100 mM and an IC\textsubscript{50} at ~ 30 mM (Fig. 7B). As PMR\textsubscript{water} contained higher amounts of EGCG than PMR\textsubscript{EtOH} (0.71% vs 0.37%), this might account for the different levels of performance of these extracts in the antiviral assays. Compared with EGCG, the other PMR phytochemicals tested, exhibited no significant effects on viral entry (Supplementary Fig. S2). To further validate our hypothesis, the receptor binding domain (RBD) of the S-protein was selected as a binding site for docking analysis. The RBD inhibitor K22 (Xiu et al., 2020), was predicted to bind RBD with a binding energy of -12 kJ/mol. In comparison, EGCG was found to bind the RBD with an energy of -7.5 kJ/mol, whereas no significant binding activity was observed for the other PMR chemicals (Supplementary Fig. S3A). As testing platforms based on the omicron variant of SARS-CoV-2 were not available to our research team, the effect of EGCG on this variant was evaluated by computational docking (Supplementary Fig. S3B). Interestingly, EGCG was predicted to require less energy to bind to this variant than to the wildtype virus (i.e., -15.2 vs -7.5 kJ/mol). This suggests that EGCG, and its parental TCM herb, PMR, might also have potent antiviral effects with regards to the omicron variant of SARS-CoV-2.

**Discussion**

Given that COVID-19 has caused (and is still causing) so many deaths...
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COVID-19. For example, an herbal decoction called PMR has been formally recognised in China as one of the first-line treatments against COVID-19. In vivo experimental approaches, to provide a first step in the identification of TCMs with anti-COVID-19 potential. Using this platform, we established that the water and ethanol extracts of PMR were non-toxic and inhibited the S-protein-ACE2 interaction, the activity of 3CL protease, and entry of the SARS-CoV-2 pseudovirus into HEK293T cells and zebrafish larvae. We also found that EGCG is an active component of PMR, and it is responsible for at least some of the antiviral effects of this herb. Taken together, our new findings might promote future studies on the effect of PMR as a potential preventative measure against infection of the active SARS-CoV-2 virus.

Conclusion

In conclusion, COVID-19 remains a disease of major concern throughout the world and effective treatments are still urgently required to complement vaccination. Here, we established a dual screening platform comprising various in vitro and in vivo experimental approaches, to provide a first step in the identification of TCMs with anti-COVID-19 potential. Using this platform, we established that the water and ethanol extracts of PMR were non-toxic and inhibited the S-protein-ACE2 interaction, the activity of 3CL protease, and entry of the SARS-CoV-2 pseudovirus into HEK293T cells and zebrafish larvae. We also found that EGCG is an active component of PMR, and it is responsible for at least some of the antiviral effects of this herb. Taken together, our new findings might promote future studies on the effect of PMR as a potential preventative measure against infection of the active SARS-CoV-2 virus.

Ethics approval

All the procedures used with zebrafish in this study were conducted in accordance with the guidelines and regulations outlined by the Animal Ethics Committee of HKUST, and the Department of Health, Hong Kong.

CRedit authorship contribution statement

Xiaoyang Wang: Methodology. Shengying Lin: Software, Writing – original draft. Roy Wai-Lun Tang: Investigation. Hung Chun Lee: Investigation. Ho-Hin Chan: Investigation. Sheyne S.A. Choi: Methodology. Ka Wing Leung: Project administration. Sarah E. Webb: Project administration, Writing – review & editing. Andrew L. Miller: Conceptualization, Supervision, Writing – review & editing. Karl Wah-Keung Tsim: Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that there is no conflict of interest associated with this publication.

Acknowledgments

We thank Prof. Jonathan S. Marchant (Medical College of Wisconsin, USA) for providing us with the SARS-related Coronavirus 2 lentiviral kit and ACE2 plasmid.
Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2022.154154.

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