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Research Article

Antiviral Activity and Synergy of Herba *Andrographidis* and Radix *Eleutherococci* Preparations against SARS-CoV-2 Infected Vero E6 Human Primary Embryonic Kidney Epithelial Cells

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Abstract

Background: Preparations from Herba *Andrographidis* (AP) and Radix *Eleutherococci* (ES) are known to exhibit antiviral, immunomodulatory and anti-inflammatory effects and are commonly used for prevention and treatment of viral respiratory diseases.

The aim of the study: The aim of our study was to assess antiviral activity of AP (SHA-10), ES (SHE-3) extracts and their fixed combination Kan Jang against coronavirus SARS-CoV-2 infected epithelial cells.

Methods: The antiviral activity of herbal extracts against coronavirus SARS-CoV-2 (MOI=0.05) was assessed in Vero E6 human primary embryonic kidney epithelial cells using the cytopathic effect (CPE) inhibition assay in which infection preventive, mitigating and curative models were applied.

Results: Significant antiviral activity was observed in the infection preventive in vitro model. Kan Jang combination at the concentration corresponding to the therapeutic daily dose used for the treatment of the common cold, effectively inhibited SARS-CoV-2 viral growth by 33.06±4% vs control ($p<0.05$). Kan Jang combination that contains higher content of ES (1 μM ES and 2 μM AP) resulted in a 42.87±4.5% inhibition in antiviral activity ($p<0.05$). The most potent antiviral effect (63.43±4.5% viral inhibition vs control) resulted from a 5-fold higher dose of the Kan Jang combination that corresponds to 5 μM ES and 10 μM AP, respectively.

Conclusion: Our study for the first time, demonstrates that ES, AP, and their combinations significantly inhibits SARS-CoV-2 viral growth in Vero E6 cells in a dose-dependent manner. The antiviral activity of the combinations of ES and AP are greater than expected.

Keywords: Andrographis; Coronavirus SARS-COV-2; Eleutherococcus; Epithelial Cells; Kan Jang; Synergy

Abbreviations

AP - Herba *Andrographidis*
ES - Radix *Eleutherococci*

Introduction

The COVID-19 pandemic has raised new challenges in the field of biomedical science, particularly in the development of effective therapeutics for the prevention and treatment of acute viral and stress-induced diseases, which were most severe in elderly people [1,2]. Conventional drugs including anti-viral (remdesivir), anti-inflammatory (dexamethasone) and anti-malarial (hydroxychloroquine) drugs, show only moderate benefit along with many adverse effects [3,4]. Several complex Traditional Chinese medicine (TCM) formulations were successfully used in seven original studies in which a total of 732 adults with COVID-19 in China participated. The meta-analysis shows that TCM adjunct treatment with standard care helps to improve treatment outcomes in patients with COVID-19 [5]. Using in silico modeling, several medicinal plants and natural compounds have been predicted to exert antiviral activity against SARS-Cov-2 [6-10].

Importantly, some herbal preparations used for the treatment of viral respiratory diseases have also been recommended for prevention [11,12], mitigation [11,13], as adjuvant therapy [11,14] and for the recovery of patients with COVID-19 [11]. Among these preparations, two adaptogenic plants, *Andrographis paniculata* (*Burm. F.*) Wall. ex. Nees, Acanthaceae (AP), *Eleutherococcus senticosus* (*Rupr. & Maxim.*) *Maxim, Araliaceae* (ES) and their fixed combination Kan Jang, are known to exhibit antiviral, immunomodulatory and anti-inflammatory effects [11] and clinical efficacy in the respiratory tract of patients with infectious diseases [15-26]. The aim of our study was to assess the antiviral activity of AP (SHA-10), ES (SHE-3) extracts and their fixed combination Kan Jang against SARS-CoV-2 infection of Vero E6 human primary embryonic kidney epithelial cells.

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Materials and Methods

Herbal Extracts and Positive Control

Pharmaceutical grade standardized extracts of *A. paniculata* and *E. senticosus* genuine extracts and their fixed combination, Kan Jang, were manufactured in accordance to ICHQ7A and EMEA guidelines for Good Agricultural and Collecting Practice (GACP) and Good Manufacturing Practice (GMP) of active pharmaceutical ingredients [27].

The stock solution (SS-A) of herba *Andrographidis* extract in the concentration of 30 mg/ml was obtained by dilution of 1,000 mg of 30% Herba *Andrographidis spissum* (soft genuine extract SHA-10) in 10 ml of distilled water. It was used for further dilutions to obtain final concentrations shown in table 1. Similarly, the stock solution (SS-E) of Radix *Eleutherococcici* extract in the concentration of 30 mg/ml was obtained by dilution of 577 mg of Radix *Eleutherococcici spissum* (soft genuine extract SHE-3) in 10 ml of distilled water. It was used for further dilutions to obtain final concentrations shown in table 1. The concentration of Kan Jang in incubation media is based on the results of pharmacokinetic study of Kang Jang-derived and rographolide in human blood plasma, where it was detected in concentrations of ~ 0.7 μg/ml = 2μM [28]. The concentrations of the total extracts of both herbal ingredients and their active constituents were compatible in all test samples. The concentrations of genuine extracts have been calculated using specifications of Kan Jang to ensure that they correspond to therapeutically effective doses.

The antiviral agent, remdesivir (GS-5734; Gilead Science Inc., Foster City, CA), an inhibitor of the viral RNA-dependent, RNA polymerase with in vitro inhibitory activity against SARS-CoV-1 and the Middle East respiratory syndrome (MERS-CoV) [29], was used as a positive control and PBS used as a negative control.

Cell line

Vero E6 human primary embryonic kidney epithelial cell line (CRL-1586TM, American Type Cell Culture; Rockville, MD) was obtained from the ATCC (Rockville, MD), which routinely performs cell line characterization. Cells were passaged in our lab for not more than 3 months after receiving them from ATCC and maintained and grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal calf serum (FCS) (Gibco, Gaithersburg, MD). Cells were maintained in an incubator adjusted to 37°C with humidified atmosphere and 5% CO2.

Cytopathic Effect (CPE) inhibition assay and herbal antiviral model systems

***In vitro* preventative model**: Vero E6 human primary embryonic kidney epithelial cells (CRL-1586™) were grown as monolayers in 96-well plates. Herbal extracts were added to Vero cells and incubated for 24 h at 37°C with 5% CO_2. Remdesivir was added as a positive control. The plates were then washed three times with PBS. SARS-CoV-2 (MOI=0.05) was then added to the plates and incubated for a further 2 h at 37°C with 5% CO_2. After incubation, the inoculum was removed by washing three times with PBS. Cells were subsequently incubated for an additional 72 h at 37°C with 5% CO_2, as which time the infected cells shown 100% CPE under the microscope. The percentage (%) inhibition of CPE in herbal extract-treated cells was calculated by the GraphPad Prism 7.0 software.

***In vitro* mitigating model**: Vero E6 human primary embryonic kidney epithelial cells (CRL-1586™) were grown as monolayers in 96-well plates. SARS-CoV-2 (MOI=0.05) was then added to the plates and incubated for 2 h at 37 °C with 5% CO_2. After incubation, the inoculum was removed by washing three times with PBS. Herbal extracts were then added to Vero cells for 0 h, 1 h and 3 h at 37°C with 5% CO_2. The cell monolayers were then washed three times with PBS and incubated for an additional 72 h at 37°C with 5% CO_2. The percentage (%) inhibition of CPE in herbal extract-treated cells was calculated by the GraphPad Prism 7.0 software.

***In vitro* curative model**: Vero E6 human primary embryonic kidney epithelial cells (CRL-1586™) were grown as monolayers in 6-well plates. SARS-CoV-2 (MOI=0.05) was then added to the plates and incubated for 2 h at 37°C with 5% CO_2. After incubation, the inoculum was removed by washing three times with PBS. Herbal extracts were then added to Vero cells for 24 h, 48 h and 72 h at 37°C with 5% CO_2. The cell monolayers were then washed three times with PBS and incubated for an additional 72 h at 37°C with 5% CO_2. The percentage (%) inhibition of CPE in herbal extract-treated cells was calculated by the GraphPad Prism 7.0 software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software on the comparison between two groups, the SARS-CoV-2-infected or SARS-CoV-2-non-infected groups, under equivalent conditions. Therefore, significant differences in viral titer were determined by an unpaired two-tailed Student's t-test and two-way analysis of variance (ANOVA) throughout this study (p values <0.05 were considered significant).

Results

Dose dependence of ginseng induced excitatory neurotransmission in rat hippocampal slice preparations *ex vivo*

To determine the antiviral activity of herbal extracts on SARS-CoV-2, we initially applied the *in vitro* preventative model system in which various concentrations of herbal extracts were added to Vero cells 24 h prior to infestation by SARS-CoV-2 (MOI=0.05), table 1.

We demonstrate that the Kan Jang combination at a concentration of 0.1 μM ES and 2 μM AP, which corresponds to the therapeutic daily dose used for the treatment of the common cold, effectively inhibited the viral load of SARS-CoV-2 in Vero E6 human primary embryonic kidney epithelial cells by 33.06±4% vs control ($p<0.05$), as determined by the CPE inhibition assay in which remdesivir was used as a positive control (Figure 1).

We further observed that the Kan Jang combination that contains a higher content of ES (1 μM ES and 2 μM AP) resulted in a 42.87±4.5% inhibition in antiviral activity against SARS-CoV-2, as compared to control ($p<0.05$) (Figure 1 and Table 2). Interestingly, we demonstrated that the most potent antiviral activity (63.43±4.5% viral inhibition vs control) resulted from a 5-fold higher dose of the Kan Jang combination that corresponds to 5 μM ES and 10 μM AP, respectively (Figure 1 and Table 2). However, similar concentrations of herbal extracts did not significantly inhibit SARS-CoV-2 using the *in vitro* mitigation and in vitro curative models (Table 3).

Eleutherococcu ES (SHE-3)	Eleutherosides	Andrographis AP (SHA-10)	Andrographolides	Kan Jang combination	Active markers
µg/ml	µM (ES)	µg/ml	µM (AP)	µg/ml (AP+ES)	µM (AP+ES)
3	0.1	6	0.4	9 (6+3)	0.4 (AP) + 0.1 (ES)
30	1	30	2	33 (30+3)	2.0 (AP) + 0.1 (ES)
150	5	150	10	60 (30+30)	2.0 (AP) + 1.0 (ES)
				180 (150+30)	10 (AP) + 1 (ES)
				300 (150 + 150)	10 (AP) + 5 (ES)

Table 1: Final concentration of the AP (SHA-10), ES (SHE-3) extracts (µg/ml) and active markers (µM) in incubation media.

Remdesivir		Eleutherococcus		Andrographis		Kan Jang combination	
		Eleutherosides (ES)		Andrographolides (AP)		AP + ES	
Conc. µM	Inhibition, %	Conc. µM	Inhibition, %	Conc. µM	Inhibition, %	Conc. µM	Inhibition, %
0	4.37±2.25	0	4.37±2.25	0	4.37±2.25	0	4.37±2.25
0.01	5.85±2	0.1	3.6±2	0.4	7.05±2.5	0.4 (AP) + 0.1 (ES)	7.76±2.5
0.1	21.08±3.5	1	11.39±4.5	2	21.57±4.5	2.0 (AP) + 0.1 (ES)	33.06 ±4
0.5	43.31±3.5	5	23.9±5	150	63.43±4.5	2.0 (AP) + 1.0 (ES)	42.87±4.5
1	86.59±5					10 (AP) + 1 (ES)	49.7±4.5
5	97.26±6					10 (AP) + 5 (ES)	62.93±4.5
10	97.78±4.5						

Table 2: Antiviral Activity of Herbal Extracts Against SARS-CoV-2 as Determined in an In Vitro Preventative Model[1] using the CPE inhibition assay.

[1]Agents were added to Vero cells and incubated for 24 h at 37°C. SARS-CoV-2 (MOI=0.05) was then added to the cells and incubated for 2 h at 37°C. Remdesivir was used as a positive control. The CPE inhibition assay was performed 48 h post infection as described in detail in the Materials and Methods section.

	Eleutherococcus	Andrographis	Kan Jang combination
Time, h	Eleutherosides (ES) 5 µM	Andrographolides (AP) 150 µM	AP (10 µM) + ES (5 µM)
0	17.59±3.0	41.62±4.5	83.32±6.5
1	11.26±5.5	26.08±5.0	64.08±6.0
3	6.34±1.75	11.09±3.5	16.75±3.5
24	3.32±1.5	6.98±2.25	20.44±4.0
48	3.0±1.5	2.37±2.0	9.12±1.75
72	4.89±1.5	1.99±1.35	2.34±1.75

Table 3: Antiviral Activity of Herbal Extracts against SARS-CoV-2 as Determined in an In Vitro Mitigating and Curative Models1 using the CPE Inhibition Assay.

[1]SARS-CoV-2 (MOI=0.05) was added to Vero cells and incubated for 48 h at 37°C with 5% CO2. Agents were then added to the cells for 0 h, 1 h, 3 h (mitigating model), and 24 h, 48 h and 72 h (curative model) at 37°C with 5% CO2. The CPE inhibition assay was performed 48 h after the end of each incubation cycle as described in detail in the Materials and Methods section.

Discussion

The earliest evidence for the clinical efficacy of ES against respiratory infections was reported in the 1970-80s during the influenza virus epidemic in the Soviet Union [30-33]. It was demonstrated that the prophylactic treatment with the ES extract reduced a number of complications associated with the influenza infection, including pneumonia, bronchitis, otitis, as well as morbidity and mortality rates [34-36].

Antiviral effect of Eleutherococcus extracts were further demonstrated in experimental model systems in which rodents were infected with H1N1 influenza A virus [37-41], human rhinovirus and respiratory syncytial virus [38]. Recently, in silico studies have predicted the antiviral actions of ES against SARS-COV-2 virus docking and replication by targeting Nsp5 (3-chymotrypsin-like protease 3Clpro) and Nsp3 (papain like protease Plpro) structural proteins [41,42].

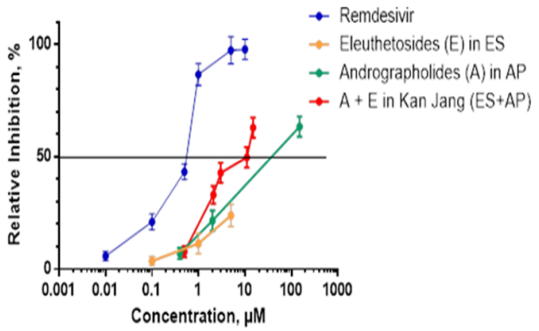

Figure 1: Antiviral Activity of Herbal Extracts against SARS-CoV-2 Measured in an *In vitro* Preventative Model using the Cytopathic Effect (CPE) Inhibition Assay. Vero cell monolayers were incubated without or in the presence of different dilutions (expressed in µM concentrations of Eleutherosides (E) and Adrofrapholides (A), ordinate), of ES (SHE-3), AP (SHA-10) extracts, their combination Kan jang or Remdesivir (positive control) for 24 h and infected with coronavirus SARS-CoV-2 (MOI of 0.05). The antiviral activity of test samples against the virus was determined using the CPE inhibition assays. Infected cells showed 100% CPE under the microscope. The percentage of CPE in herbal extract-treated cells was recorded. Data are mean ± SD of four independently performed experiments. Data represent six replicates derived from four independently performed experiments.

The antiviral activity of AP was demonstrated against H1N1 influenza A [43-45], H5N1 avian influenza [46], Chikungunya [47] and Dengue [48,49] viruses. The predicted antiviral effect of AP against SARS-COV-2 virus docking and replication by targeting Nsp5 (3-chymotrypsin-like protease 3Clpro), Nsp3 (papain like protease Plpro), Nsp12 (RNA-dependent RNA polymerase RdRp), Nsp1 (the most N-terminal gene 1 protein) structural proteins and S2 Spike glycoprotein receptor to type-II Transmembrane Serine Protease Enzymes (TMPRSS2) of host cells was also demonstrated by in silico modeling [50,51].

In this study, our group was the first to demonstrate potent antiviral effects of ES and AP extracts as well as their fixed combination Kan Jang against SARS-CoV-2 infection of Vero E6 human primary embryonic kidney epithelial cells. The highest antiviral activity was observed in the *in vitro* infection preventive model. These results, however, do not exclude the possibility that preparations are effective *in vivo* models, e.g., when orally applied after viral exposure of subjects. We hypothesize that this could be due to activation of innate immunity and other immune defense mechanisms described in numerous publications, for review see [11].

In this study, we demonstrated that Kan Jan combination is significantly more effective than its ingredients (ES or AP) in the prevention of growth of SARS-COV-2 in isolated Vero E6 human primary embryonic kidney epithelial cells (Figure 1). These results are intriguing. Although the exact reason is currently unknown. We hypothesize that ES and AP's mutual potentiation and synergistic interactions within target cells could be a cause. A similar effect was recently observed in isolated neuroglia cells [27,52-55]. This suggestion is in line with recent publications where synergistic interaction of ES and AP were demonstrated in isolated neuroglia cells [27,52-55]. The synergistic effect is hypothesized to be associated with their effects on tumor cell proliferation [53], expression on genes involved in inflammatory and immune responses [27], neuroprotection [52], regulation of Nrf2-mediated signaling proteins [55] and on enzymes associated with antioxidants, metabolization and detoxification [54,56].

Table 2 demonstrates that the Kan Jang combination at concentrations of ES (0.1 μM) and AP (2 μM), that corresponds to the therapeutic daily dose used for the treatment of common cold, significantly inhibits the viral load by 33.06±4% vs control ($p<0.05$). On the other hand, the Kan Jang combination with a higher content of ES (1 μM ES and 2 μM AP) significantly inhibits antiviral activity by 42.87±4.5% vs control ($p<0.05$). Importantly, the Kan Jang combination with 5-fold higher concentrations of ES and AP (5 μM ES and 10 μM AP) exhibited the highest level of SARS-CoV-2 antiviral inhibition, 63.43±4.5% vs control ($p<0.05$).

Interestingly, the effective concentrations of Kan Jang are higher than effective concentrations of positive control Remdesivir in our experiments, Table 2 and Figure 1. Importantly, it should be noted that Kan Jang has at least two important advantages compared to Remdesivir: (i) beneficial effects on immune, antioxidant and detoxifying systems [11], which are of importance on all phases of infection and recovery of COVID-19 patients, and (ii) lack of serious adverse events [11].

Further experimental studies on animals and clinical studies in COVID-19 are required to confirm efficacy of Kan Jang for prevention and amelioration of symptoms of COVID-19.

Conclusion

ES, AP, and their combinations inhibit SARS-CoV-2 replication *in vitro* infected Vero E6 human primary embryonic kidney epithelial cells in a dose-dependent manner. The antiviral activity of the combinations of ES and AP are greater than expected.

Declarations

Ethics approval

No ethic approvals are required for in vitro studies of commercially available Vero E6 human primary embryonic kidney epithelial cell line (CRL-1586TM).

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author A.A. upon reasonable request.

Conflict of interest statement

The authors AA and PK declare no conflicts of interest. KGW is the founder of Swedish Herbal Institute AB and Swedish Herbal Institute Research and Development AB. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Authors' contributions

Conceptualization AA and GW; methodology AA, PK; software AA and PK; validation AA and PK; formal analysis AA and PK; investigation AA and PK; resources AA, GW; data curation AA and PK; writing original draft preparation AA and PK; writing review and editing AA and PK; visualization AA GW and PK; supervision AA and PK; project administration AA; funding acquisition GW. All authors have read and agreed to the submitted version of the manuscript.

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