Article

Natural Plant Extracts as Novel Antiviral Candidates: *Citrus* extracts

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Received: 20 November 2024; Revised: 26 December 2024; Accepted: 12 February 2025; Published: 18 February 2025

Abstract: Medicinal plants are the natural and main source of therapeutic drugs today. The biological activity of several plants has been performed, but many remain to be evaluated. Meanwhile, most biological studies on plants used in traditional medicine concern their antiviral and anti-inflammatory activity. In this study, two sets of plant extracts were studied for their biological activities. Various plant extracts, including *Citrus* peels, were evaluated for their antiviral activity. Among them, in the first set, three herbs were tested individually, and the five mixtures in the second set were composed of various traditional Korean herb extracts in several proportions. Through appropriate combinations of traditional Korean herbs, the antiviral activity was improved than single herb extracts.

Keywords: traditional medicine; antiviral activity; plant extract; *Citrus* extract

1. Introduction

Nutritious foods perform essential functions for the growth and development of all living organisms. Plant phytochemicals are known to have beneficial health effects on humans and are used as effective treatments for various diseases. Over 10,000 plant compounds have been isolated and used for diverse health benefits [1–3]. WHO reports that many people worldwide, especially in Asia and Africa, are still finding it difficult to have access to essential medicines. Therefore, they rely heavily on traditional medicine such as using herbs. Additionally, it has been reported that more than 75% of developing countries depend on plant-based medicines for health problems [4–9]. Therefore, many have noted the advantages of the traditional use of plants in medicine, since it is widely known that plants are less dangerous and cheaper than chemicals [1,2].

Phytochemicals are largely divided into primary metabolites such as sugar, fat, and protein, and secondary metabolites such as alkaloids, glycosides, flavonoids, tannins, resins, steroids, saponins, and phenols. In general, secondary metabolites are also called bioactive compounds, and these are attributed to the preservative and antibacterial activities of plants [10–12]. Bioactive compounds such as polyphenols and carotenoids have been noted to help reduce breast cancer risk [13–17]. They have also been shown to be able to prevent the pathogenicity of *Candida* strains by suppressing the formation of biofilm, destructing mature biofilm, inhibiting growth, and altering the virulence of these strains [18]. The antioxidant activity scavenging free radicals is found in various fruits and vegetables [12,19,20].

Korea has had a remarkable experience throughout East Asian history in its healthcare educational system. East Asian medicine is recognized to have its origins in China, dating back over 3000 years. By the 6th century, this medicine system was introduced to Korea and later known as Koryo medicine, also known as *Hanbang* [21–23]. Based on the unique national history of Korea, it built its own traditional medicine like Sasang Constitutional Medicine. It has been reported that nearly 70% of the Korean population has experienced Korean traditional medicine for their health care services [24,25]. It is considered one of the most used systems of traditional medicine along with the Chinese system, Indian (Ayurveda), and Japanese (Kampo) systems [25,26].

Traditional Korean herbs have been used in curing sickness and injuries for thousands of years and have been considered low in adverse effects. Many of these herbs were popular for their cognitive-enhancing, anti-dementia

activities, anti-oxidant activity, pharmacological effect, and other activities like anti-inflammatory, analgesics, anti-spasmodic, and sedatives [23,27–29]. Therefore, several studies have been focused on discovering the exact mechanism of the herbs in these biological and pharmacological activities. Yet, much information on these medicinal plants has not been obtainable.

Citrus peels which are included in traditional Korean herbs are rich in nutrients and contain many bioactive compounds, which have been used as drugs and therapeutic agents [30–32]. Additionally, *Citrus* peels are rich in flavanones, phenolic acids, and poly-methoxylated flavones, which are rarely found in other plants [30,33]. The essential oil from the peel had a higher total phenolic and flavonoid content than other solvent extracts from fruit pulp [34]. *Citrus* peel has been reported to be a potential antioxidant as well as other activities such as antitumor activity, antidiabetic antibacterial, antifungal, antihypertensive, carminative, insect repellent, hypersensitivity, antibacterial, antiviral, anti-yeast, and antihepatotoxic [35–46]. The antibacterial properties of *Citrus* oils found in peels have revealed interesting applications in the food and cosmetics industries [40,45,47–49]. However, in almost all the *Citrus* processing industries like making juice, which is the main industry, a significant portion of *Citrus* peels is wasted worldwide. *Citrus* peels are more than half the weight and 40–50% of the total fruit mass [50,51].

In this study, we characterized the antiviral effects of various traditional Korean herb extracts including *Citrus* on Madin-Darby bovine kidney (MDBK) cells by reducing viral cytopathy of Vesicular stomatitis virus (VSV, Indiana strain). Among them, three herbs were tested alone, and the five mixtures were composed of various herb extracts in several proportions to improve the activity of a single herb extract. These herbs were selected because of their well-documented sterilization properties.

2. Materials and Methods

2.1. Plant Material and Extracts

Peels of *Citrus junos* (Ecoherb, Seoul, Republic of Korea) and *Citrus unshiu* (Ecoherb) were collected and roots of *Coptis japonica* (Donguiherb, Seoul, Republic of Korea) were also harvested, dried, and powdered. These three plant components were prepared as separate solutions for further testing.

The compositional plant extracts of five mixtures (CPE-257, CJP-357, CJE-457, CRE-557, and CJE-157) were derived from *Citrus junos*, *Coptis japonica*, *Eucommia ulmoides* (Donguiherb), *Psidium guajava* (Donguiherb), and *Rubia cordifolia* (Donguiherb). These five mixtures were prepared as solutions ready for evaluation. They could be composed of one plant, part of a plant, or more and might be a crude or purified extract.

2.2. Antiviral Assay

VSV obtained from ATCC (VA, USA) was used as a test virus for MDBK (ATCC) cells to evaluate the antiviral activity of the sample. MDBK were cultured in Dulbecco's Modified Eagle's medium (DMEM), 10% Fetal Bovine Serum (FBS), 100 units penicillin, 100 μg/mL streptomycin, 5% CO_2, water-saturated, and 37 °C (25,000 cells/well in the 96 well cell culture plate). All culture-associated media were obtained from Welgene (Gyeongsan, Republic of Korea). VSV was incubated with 8 samples, which were serially diluted in 2-, 3-, 10-, 20-, or 60-fold steps using the medium, for 10 min. For control, MDBK cells were pretreated with three-fold serial dilutions of porcine interferon α8 (pIFN, 500 ng/mL, 166 ng/mL, 55 ng/mL, 18 ng/mL, 6 ng/mL, 2 ng/mL, 0.6 ng/mL). MDBK cells were infected six hours later by diluting VSV (1.5×10^6 $TCID_{50}/_{mL}$) 1:2000 in the same culture medium. After twenty-four hours from virus infection, the samples' antiviral activity was confirmed by staining live cells using a crystal violet solution. Uninfected and untreated MDBK cells were used as a control.

3. Results

3.1. Antiviral Assay of Citrus junos, Citrus unshiu, and Coptis japonica

The three plant extracts, *Citrus junos*, *Citrus unshiu*, and *Coptis japonica*, were serially diluted 1/2, 1/3, and 1/10 from the same start concentration (1/20) and incubated with VSV for 10 min. The start concentration of three plant extracts was determined by reference to the cytotoxicity assay results where they were treated alone to MDBK cells. After 10 min of incubation, MDBK cells were incubated with these mixtures for 24 h. Crystal violet solution was used to visualize the alive cells after removing all supernatants (Figure 1). The control group consists of cells treated only with VSV at the top, cells treated with VSV after pretreatment with 3-fold serial dilution of pIFNα8 in the left, and cells without any treatment at the bottom. Light purple indicates low, or no alive cell density, and deep purple indicates high alive cell density. Experiments were repeated at least three times.

Even though high concentrations of *Coptis japonica* root extract may cause host cell death due to their cytotoxicity, results showed that three plant extracts have promising antiviral activity against VSV, albeit in a concentration-independent manner. Compared to VSV alone, *Citrus junos* showed the highest antiviral activity among the three plant extracts, and it was hard to determine whether *Coptis japonica* extract exhibited significant antiviral activity because of its cytotoxic effect on cells.

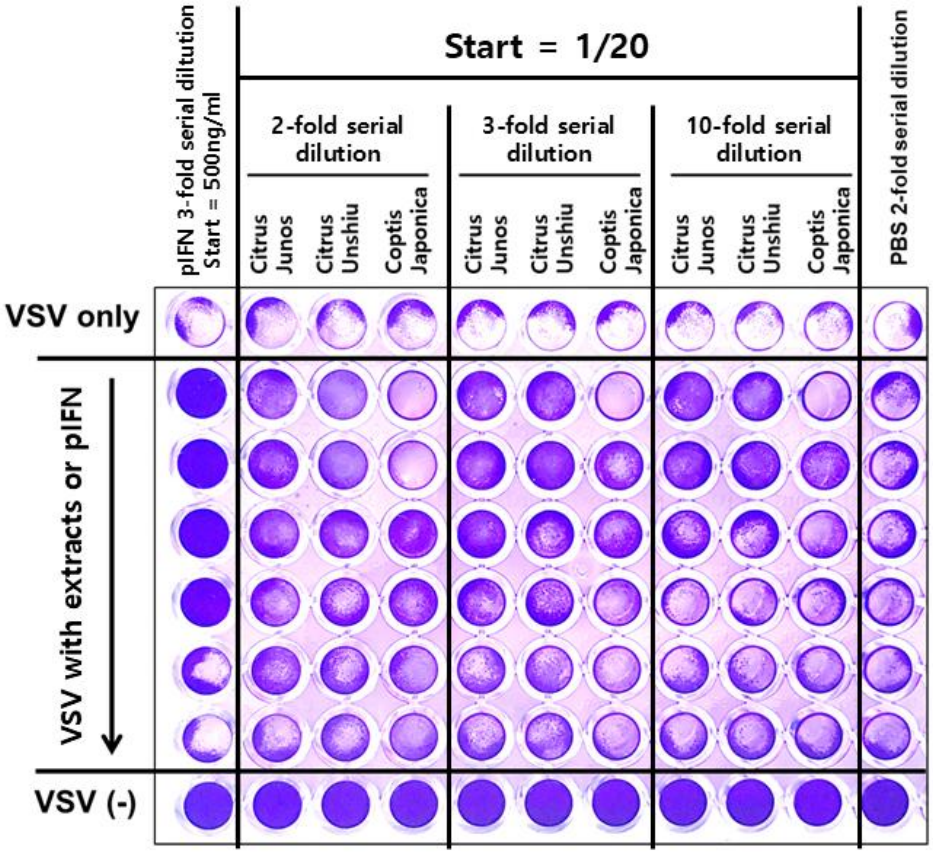

Figure 1. Antiviral activity assay of three plant extracts. *Citrus Junos*, *Citrus Unshiu*, and *Coptis Japonica* extracts were serially diluted to 2-fold, 3-fold, and 10-fold concentrations as indicated at the top, preincubated with VSV, and then treated to MDBK cells. pIFN which is known for its antiviral activity was used for control. The experiments were repeated at least three times.

3.2. Antiviral Assay of the Five Mixtures of Various Plant Extracts (CPE-257, CJP-357, CJE-457, CRE-557, and CJE-157)

The five mixtures of various plant extracts, CPE-257, CJP-357, CJE-457, CRE-557, and CJE-157 were composed of five traditional Korean herbs including *Citrus junos* which showed the highest antiviral activity and *Coptis japonica* which showed different patterns with other two *Citrus* extracts. These mixtures were designed to improve the antiviral activity of extracts. CJP-357, CJE-457, and CJE-157 have *Citrus junos* extract and the other two don't have it. These five mixtures were preincubated in 1/2 serial dilutions with VSV. 10 min later, MDBK cells were incubated with these mixtures for 24 h and visualized using a crystal violet solution (Figure 2). The start concentration of the five mixtures was determined by reference to the results of the cytotoxicity assay where they were treated alone to MDBK cells. The control group consists of cells treated only with VSV at the top, cells treated with VSV after pretreatment with 3-fold serial dilution of pIFNα8 in the left, and cells without any treatment at the bottom. The five mixtures inhibited the cytopathic effect of VSV, except for CRE-557. And CPE-257 showed the highest antiviral activity against VSV among the five and the next was CJE-457.

To determine the limit of antiviral activity of the five mixtures, these results were repeated on 1/20 or 1/60 serial dilution results from start concentration 1/3 or 1/10 respectively. Figure 3 also showed that CPE-257 exhibited the highest protection activity, followed by CJE-457.

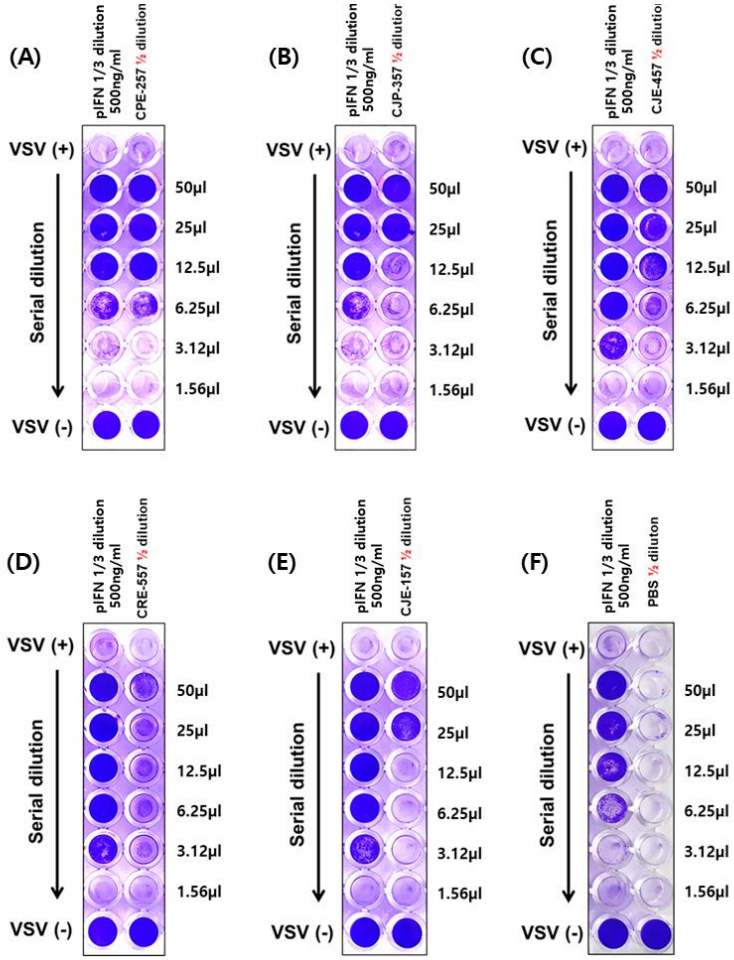

Figure 2. Antiviral activity assay of the five mixtures of various plant extracts**.** CPE-257 (**A**), CJP-357 (**B**), CJE-457 (**C**), CRE-557 (**D**), CJE-157 (**E**), and PBS control (**F**) were pre-diluted in 2-fold serial dilutions. Equal amounts of VSV were then added to each dilution, and the mixtures were incubated for 10 min before being treated to MDBK cells. The experiments were repeated at least three times.

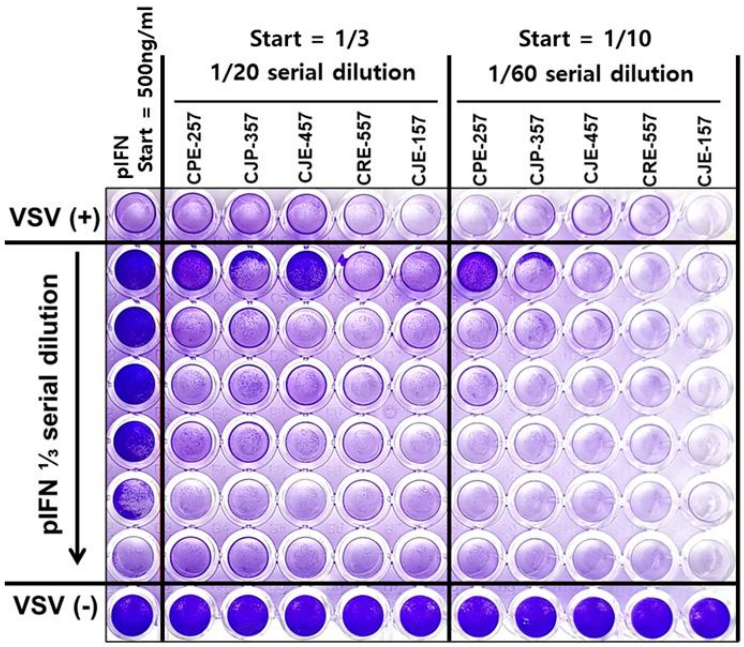

Figure 3. Antiviral activity assay of the five mixtures of various plant extracts in lower concentrations. CPE-257, CJP-357, CJE-457, CRE-557, and CJE-157 were pre-diluted to 20-fold and 60-fold concentrations, preincubated with VSV, and then treated to MDBK cells. The experiments were repeated at least three times.

4. Discussion

In this study, we investigated the antiviral activity of traditional Korean herb extracts. Three single herb extracts, *Citrus junos*, *Citrus unshiu* peel extracts, and *Coptis japonica* root extracts, showed promising antiviral activity (Figure 1). However, the antiviral activities of *Citrus junos* and *Citrus unshiu* weren't concentration-dependent, and *Coptis Japonica* root extracts have a cytotoxic effect on cells. Among five mixtures of herb extracts, four mixtures inhibited the cytopathic effect of VSV depending on their concentration except for CRE-557 (Figure 2). Briefly, the concentrations showing antiviral activity for these extracts were 12.5 μL of CPE-257 (Figure 2A), the 25 μL of CJP-357 (Figure 2B), the 12.5 μL of CJE-457 (Figure 2C), and the 25 μL of CJE-157 (Figure 2E) extract. Each of these extracts was shown to be safe and free from contamination.

Despite the reported studies of the antiviral effect of three traditional Korean herbs [29,38,52,53], in this study, the single-component test extracts did not demonstrate significant antiviral activity (Figure 1). This could be due to inadequate cell uptake of the phytochemicals [54–56]. Among the three extracts, we excluded *Citrus unshiu*, which showed a similar pattern to *Citrus junos*, whose antiviral activity was the highest. We added three other herbs to two herbs and mixed them in various proportions to enhance antiviral activity and reduce cytotoxicity (Figure 2). Among the three mixtures which include *Citrus junos* extract, CJE-457 showed improvement in cytotoxicity and consistency of antiviral activity, showing a concentration-dependent effect (Figure 2C). Even when mixtures included *Coptis japonica*, they didn't show cytotoxicity except for CRE-557 and demonstrated stable antiviral activity. Additionally, contrary to expectations, CPE-257 without *Citrus junos* peel extracts showed the highest activity against VSV (Figure 3). Traditionally, when herbs are used as a medicine, these medical plants are not used in isolation but are usually used in appropriate combinations [21–23]. There is a growing interest and need among scientists for studying herb combinations, however, it seems still insufficient [57,58].

In summary, we confirmed the significant antiviral activity of traditional Korean herb extract mixtures including *Citrus junos* peel against VSV. A certain antiviral efficacy was elicited through combination with other herbs compared to the use of herbs alone, and cytotoxicity could be reduced.

There is no definitive cure for vesicular stomatitis (VS), which occurs periodically and primarily from late spring to early autumn in the U.S. During this period, many livestock shows and events take place, and VS often leads to their cancellation. Consequently, despite the low mortality rate of this virus, VSV has caused significant economic impacts and trade disruptions. So, it is essential to manage this disease using cost-effective methods, which is important in raising livestock [59,60]. More than half of the weight of *Citrus* peel, the main material of this study, is being discarded [50,51]. Therefore, if the antiviral activity and safety are verified in further in vivo experiments, it may be a cost-effective solution to VSV.

In addition, due to the appealing fragrance of most *Cirus* species, *Citrus* peel extracts are adequate for many cosmetic products [61]. If further preclinical research is focused on the skin, a price-effective cosmetic method against many viral skin diseases such as Measles, Rubella, Erythema infectiosum, Roseola infantum et cetera could be developed [62].

Author Contributions: MC, JH: conceptualization, methodology, formal analysis, investigation; KH, HN, BK, TJ: investigation; GC, HL, YK, HR: data curation, formal analysis; SK, HK: conceptualization; SS: conceptualization, supervision, writing—reviewing and editing. MC and JH contributed equally to this work. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Research Foundation of Korea (NRF-2021R1F1A1057397).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Yasmin Hisman contributed to writing the original version of the article. Antiviral testing was performed at the Cytokine Immunology Laboratory at Konkuk University in 2020.

Conflicts of Interest: The authors declare no potential conflict of interest.

Abbreviations

FBS, Fetal Bovine Serum; MDBK, Madin-Darby bovine kidney; PS, Penicillin-Streptomycin; VSV, vesicular stomatitis virus.

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