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Effectiveness of neutral electrolyzed water in inactivating HCoV-OC43 and SARS-CoV-2 on the surfaces of plastic and the medicinal plant *Centella asiatica* (L.) urban

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ABSTRACT

Concerns have been raised about viral contamination, including in crops due to the recent coronavirus disease 2019 pandemic. Limited evidence is available to support the use of sanitizing agents for human coronavirus-contaminated medicinal plants. Thus, we aimed to investigate the persistence of infectious human coronavirus OC43 (HCoV-OC43) as a SARS-CoV-2 surrogate in storage conditions and the capability of neutral electrolyzed water (NEW) to inactivate coronavirus, including in fresh plants such as *C. asiatica*. The levels of infectious HCoV-OC43 and the triterpenoid content of *C. asiatica* were quantified using a plaque assay and high-performance liquid chromatography, respectively. The results showed that the persistence of HCoV-OC43 on *C. asiatica* leaves is identical to that on inert polystyrene. When covered and kept at room temperature with high humidity (>90% RH), HCoV-OC43 can be stable on *C. asiatica* leaves for at least 24 h. NEW with 197 ppm of available chlorine concentration (ACC) was effective in inactivating both infectious HCoV-OC43 and SARS-CoV-2 in suspension ($\geq$3.68 and $\geq$4.34 log reduction, respectively), and inactivated dried HCoV-OC43 on the surfaces of *C. asiatica* leaves ($\geq$2.31 log reduction). Soaking *C. asiatica* leaves for 5 min in NEW with 205 ppm of ACC or water resulted in significantly higher asiaticoside levels (37.82 $\pm$ 0.29 and 35.32 $\pm$ 0.74 mg/g dry weight, respectively), compared to the unsoaked group (29.96 $\pm$ 0.78 mg/g dry weight). These findings suggest that although coronavirus-contaminated *C. asiatica* leaves can pose a risk of transmission, NEW could be an option for inactivation.

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a human coronavirus belonging to *Coronaviridae* family. The coronavirus disease 2019 (COVID-19 pandemic), caused by SARS-CoV-2, has largely increased public awareness of pathogenic viruses and generated concerns regarding the viral contamination of fresh vegetables and foods (Aboubakr et al., 2021; Ming et al., 2021). Additionally, patients with COVID-19 have had problems with the gastrointestinal tract, including loss of taste and appetite, diarrhea, nausea, and vomiting (Guo et al., 2021). The public has changed their working and eating behaviors and expressed concern about the possibility of coronaviruses contaminating public places and fresh vegetables (Carroll et al., 2020; Ming et al., 2021). Thus, the viral contamination has a significant effect on economic crops, including the supply of medicinal plants.

While coronaviruses are primarily transmitted between people through respiratory droplets, some evidence suggests that everyday materials can act as carriers (Castaño et al., 2021; Yekta et al., 2021). The infectivity of coronavirus can last for several hours up to days outside its host (Aboubakr et al., 2021; Blondin-Brosseau et al., 2021; Ming et al., 2021). SARS-CoV-2 genomes were found on the surfaces of everyday materials and food products during the COVID-19 pandemic; however, their infectivity levels remain unknown (Qu et al., 2020; Singh et al., 2021). Lee et al. (2020) reported that SARS-CoV-2 at 10^5 plaque-forming

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units (pfu) can infect Syrian hamsters via oral inoculation, leading to viral replication and propagation to the oral mucosa, tongue, esophagus, and stomach. People may come into contact with coronaviruses or ingest food contaminated with the virus during a large outbreak (Yekta et al., 2021), as the secretions of patients infected with SARS-CoV-2, such as saliva, can contain up to 1.2×10^8 copies/mL of culturable SARS-CoV-2 (Kai-Wang et al., 2020).

Heating is a straightforward and effective method to inactivate pathogens (Hemati et al., 2021). However, high temperatures can destroy thermally labile compounds, physical appearance, and pharmaceutical value of medicinal plants. To improve the safety of thermally labile compounds, non-thermal sanitization could be advantageous for overcoming the risk of pathogen transmission associated with fresh foods and herbs, in addition to increasing their shelf-life during transportation and distribution (Huang et al., 2008; Nowsad et al., 2020). Electrolyzed water (EW) produced by the electrolysis of sodium chloride (NaCl) is a non-thermal sanitizing agent capable of inactivating pathogens. EW has emerged as an interesting non-thermal sanitizing agent because of its low cost, ease of preparation, and safety for use in food products (Huang et al., 2008). However, the mechanisms driving pathogen inactivation by EW remain unclear. Chlorine, hypochlorite, and hypochlorous acid play crucial roles in the anti-pathogenic activity of EW. Hypochlorous acid is believed to be the most effective among them because of its strong oxidizing power. However, it is less corrosive than the hypochlorite. In addition, the concentration of hypochlorous acid in the solution increased proportionally with the acidity of the solution. EW denatures and aggregates the proteins of pathogens (Moorman et al., 2017; Slaughter et al., 2019; Yan et al., 2021). Due to the lower pH, EW may have the same, or better, broad spectrum anti-pathogenic activities than sodium hypochlorite solution, which is commercially available at high concentrations and alkalinity. Dilution of the corrosive stock is required to prepare the working concentration of a sodium hypochlorite solution, indicating that chlorination and corrosion are less likely to occur when EW is used instead of it (Abadias et al., 2008; Yan et al., 2021). However, neutral electrolyzed water (NEW) is more stable than acidic EW (Cui et al., 2009).

According to Rosli et al. (2021), washing fresh *Centella asiatica* with an undefined concentration of acidic EW is efficient in reducing unspecific microbial contamination and does not change the quality of *C. asiatica* in terms of taste, texture, antioxidant capacity, and phenolic content. Concurrently, acidic EW with 100 ppm of available chlorine concentration (ACC) efficiently reduced *Salmonella typhimurium* contamination in fresh red cabbage without reducing its color, pH, and total phenolic content (Chen et al., 2018). However, EW treatment may alter the levels of plant bioactives. For example, acidic EW with 40 ppm of ACC increased sulforaphane levels in broccoli sprouts by stimulating glucosinolate levels and myrosinase activity (Li et al., 2018). SARS-CoV-2 was reported to be inactivated with acidic EW (up to $\geq$4.25 log reduction) in suspension, depending on the free chlorine concentration (Takeda et al., 2020). A clinical study by Gutié rrez-Garc í a et al., 2022 observed that rinsing the nose and mouth with NEW containing 0.0015% of reactive species of chlorine and oxygen significantly reduced the incidence of SARS-CoV-2 infection. Moreover, NEWs with 50–120 ppm of ACC can also inactivate pathogens that contaminate vegetables, such as *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* (Abadias et al., 2008), while a NEW with 250 ppm of ACC can completely inactivate norovirus on a stainless-steel surface (Moorman et al., 2017). However, whether NEW can be used to inactivate human coronaviruses in medicinal plants remain unclear.

Human coronavirus OC43 (HCoV-OC43) is a member of the *Coronaviridae* family and usually causes common cold with non-severe symptoms (Vabret et al., 2003). However, in immunocompromised patients such as children with pre-B acute lymphoblastic leukemia, HCoV-OC43 can be fatal (Nilsson et al., 2020). Although infective SARS-CoV-2 testing requires a biosafety level 3 laboratory (BSL-3), infective HCoV-OC43 testing requires BSL-2. Thus, HCoV-OC43 might be an interesting surrogate for SARS-CoV-2 to examine viral persistence and decontamination (Schirtzinger et al., 2022). To elucidate the risk of a medicinal plant acting as a coronavirus fomite and the efficacy of NEW on virus inactivation on its surface, this study aimed to investigate 1) the persistence of HCoV-OC43 as a surrogate for SARS-CoV-2, including on fresh *C. asiatica* leaves, and 2) the effects of NEW on HCoV-OC43 inactivation and the levels of the pharmaceutically important triterpenoids of *C. asiatica*.

2. Materials and method

2.1. Cell and virus cultures

HCoV-OC43 (ATCC VR-1558) was propagated in HCT-8 cells (ATCC CCL-244) at the BSL-2 of the Center for Vaccine Development, Institute of Molecular Biosciences, Mahidol University, Thailand. SARS-CoV-2 (MUMT64019039) was propagated in Vero cells (ATCC No. CCL-81) in the BSL-3 of the Faculty of Veterinary Science, Mahidol University. The viral suspensions were obtained from the cultured supernatants and stored at −80 °C before use.

2.2. Plant samples

Fresh *C. asiatica* was purchased from a local market in Nakhon Pathom, Thailand, and then covered with absorbent paper. *C. asiatica* leaves were cut to separate the petioles.

2.3. Pre-cleaning of C. asiatica leaves

For pre-cleaning, *C. asiatica* leaves were soaked in pH-adjusted bleach (pH 4.5) with 200 ppm of ACC for 10 min before being rinsed with sterile 1% sodium thiosulfate and sterile water. They were then placed on sterile absorbent paper to remove excess liquid. Pre-cleaned *C. asiatica* leaves were used to test the persistence of HCoV-OC43 on leaves and the efficacy of NEW.

2.4. Plaque assays

Plaque assays were used to measure levels of HCoV-OC43 and SARS-CoV-2. For HCoV-OC43, 100 μL of a 10-fold diluted sample was adsorbed onto monolayer Vero cells in 6-well plates (Corning, USA) and incubated for 60 min at 33 °C. Then, the cells were overlaid with MEM/EBSS (Hyclone™) containing 1.2% Avicel® and incubated for 6–7 days under 5% CO_2 at 33 °C. After incubation, the cells were fixed with 4% formaldehyde and stained with crystal violet. Virus titers were calculated in pfu/mL. For SARS-CoV-2, the same procedures were performed, except that the overlay medium used was MEM/EBSS containing 1.5% carboxymethylcellulose, and the incubation temperature was set at 37 °C.

2.5. Evaluation of HCoV-OC43 persistence

To evaluate the effect of temperature on the persistence of HCoV-OC43 in inert containers, 40 μL of virus suspension (1×10^6 pfu/mL) was inoculated in a closed 1.5 mL polypropylene microtube or an open polystyrene cell culture plate. The samples were stored in an airtight container at 4 °C with 80% relative humidity (RH), 22 °C with 64% RH, or 37 °C with 58% RH, for 0–7 days. Then, the virus residue was recovered and serially diluted with 30% fetal bovine serum (FBS) in phosphate-buffered saline (PBS) for virus quantification.

The 2-h dried virus samples from the *C. asiatica* leaves and polystyrene surfaces were performed separately and used as controls to test viral inactivation on surfaces by NEW. Briefly, these samples were treated with 100 μL of virus at 5×10^5 pfu/mL and recovered with water before virus quantification.

2.6. Antiviral activity on surfaces

Measuring anti-HCoV-OC43 activity on surfaces was evaluated using the method of ISO 21702-2019 2019, with some modifications. Briefly,

100 μL of virus suspension (5×10^5 pfu/mL) was inoculated onto the surface of a pre-cleaned *C. asiatica* leaf or polystyrene cell culture plate. The virus suspension was covered with a 2 cm × 2 cm, 0.07 mm thick sterile polypropylene. Subsequently, the samples were stored at 22 °C with a relative humidity of >90% in an airtight container for 24 h before recovery with 30% FBS in PBS for virus quantification.

2.7. Preparation of NEW

EW with approximately 100 or 200 ppm of ACC was freshly prepared by adding 1 L or 500 mL of 0.2% (w/v) NaCl solution, respectively, to a portable EW generator (HOME GUARD, Ecowell Co., Ltd., Korea) for 5 min. To obtain NEW, 5% (v/v) acetic acid was added to EW to adjust the pH to 7. The ACC of NEW was measured using the HI771 Checker® HC and HI771 reagents (HANNA Instruments, USA). The NEW was filtered through a 0.2 μm syringe filter and used as the test substance for the virus inactivation test.

2.8. Virus inactivation test in suspensions

NEW with 76 and 197 ppm of ACC were used for the virus inactivation tests performed according to the ASTM E1052-20 method, with some modifications. Briefly, 5×10^5 pfu/mL of HCoV-OC43 or 2×10^6 pfu/mL of SARS-CoV-2 suspension was used. One part of the viral suspension was mixed with nine parts of the test substance for 5 min. Subsequently, 1% sodium thiosulfate was immediately added as a neutralizer before performing 10-fold serial dilutions with 30% FBS in PBS. Then, 200 μL aliquot from the serial dilutions were quantified using a plaque assay. For the untreated group, ultrapure water or 30% FBS in PBS was used as the test substance.

2.9. Viral inactivation activity of NEW on surfaces

One hundred microliters of HCoV-OC43 suspension (5×10^5 pfu/mL) were inoculated onto a pre-cleaned *C. asiatica* leaf or polystyrene cell culture plate. The virus was air dried at room temperature in a biosafety cabinet. The test substance (500 μL) was pipetted onto the virus-dried spot and left for 5 min, followed by neutralization with 50 μL of 1% sodium thiosulfate. The samples were recovered, and 10-fold serial dilution with 30% FBS in PBS was performed for quantification using a plaque assay. Sterile ultrapure water was used as the test substance for virus control.

2.10. Effect of NEW on triterpenoid levels

To study the effect of NEW at the effective concentration on triterpenoid levels in *C. asiatica,* NEW was prepared from 500 mL of 0.2% NaCl solution using the same method as that used for virus inactivation. Five grams of fresh *C. asiatica* petioles or leaves were either unsoaked, soaked in 250 mL of ultrapure water, or soaked in 250 mL of NEW, for 5, 30, or 60 min. The samples were washed three times with ultrapure water and placed on absorbent paper to remove the excess liquid. Subsequently, the samples were prepared, extracted, and analyzed based on the methods of Buraphaka and Putalun (2020), with some modifications. Briefly, *C. asiatica* samples were dried overnight at 50 °C and ground into a fine powder for the extraction of triterpenoids. A total of 25 mg of *C. asiatica* powder was extracted with 2 mL of 90% methanol and sonicated for 20 min, followed by centrifugation at 6,000 rpm for 10 min. Extraction was performed twice. The supernatant was collected and filtered before determination of the triterpenoid content.

2.11. Determination of triterpenoid content using high-performance liquid chromatography (HPLC)

Triterpenoids were analyzed using an HPLC system equipped with a UV detector, binary pump, autoinjector, and column thermostat (Shimadzu LC-10AD, Japan). Triterpenoids were separated using the COSMOSIL C18-MS-II reversed-phase column (250 × 4.6 mm, 5 μm) and detected at 206 nm. The mobile phases were water (eluent A) and acetonitrile (eluent B), both acidified using 0.01% formic acid. For eluent B, the following gradient elution program was used: 24% (0–10 min), 24–45% (10–15 min), 45–65% (15–30 min), and 65% (30–45 min). The flow rate of the system was set at 1 mL/min. The levels of triterpenoids in extracts were determined using the retention time and peak area of the standards asiaticoside and madecassoside (98% purity, Chengdu Biopurify Phytochemicals Ltd., China) at five different concentrations ranging from 7.81 to 250 μg/mL. Standard curves of asiaticoside and madecassoside were established as $y = 182.93x - 459.81$ ($R^2 = 0.9997$) and $y = 521.41x - 1463.2$ ($R^2 = 0.9998$), respectively. The triterpene levels in the samples (μg/mL) were calculated using standard curve equations and then converted to mg/g dry weight of *C. asiatica* powder (Buraphaka and Putalun, 2020).

2.12. Statistical analysis

Data analysis was performed using PASW Statistics for Windows, version 18.0 software (SPSS Inc., Chicago, USA). A paired t-test was used to evaluate the statistical significance of the differences in virus titers between the groups. One-way ANOVA followed by Tukey's post-hoc test was used to compare the different triterpenoid levels among the groups. Statistical significance was set at $p < 0.05$. Each test was performed in triplicate.

3. Results and discussion

3.1. Persistence of infectious HCoV-OC43

Limited data is available on coronavirus persistence on fresh food and medicinal plant surfaces, as well as their risk as coronavirus fomites. Conventional polymerase chain reaction methods can detect the presence of viruses in food; however, they cannot distinguish between infectious and non-infectious viruses (Ming et al., 2021). Cell-based techniques, such as plaque assays and the median tissue culture infectious dose ($TCID_{50}$) method, are ideal for quantifying the level of virus infectivity. However, accurately quantifying the level of virus infectivity using a cell-based method from uncleaned samples contaminated with various microbes would be difficult (ISO, 2019). Blondin-Brosseau et al. (2021) previously showed that the human coronavirus 229E left on the pre-cleaned surfaces of fresh fruits can remain culturable for 16–72 h.

In this study, to examine the persistence of HCoV-OC43 on a medicinal plant surface, the levels of infectious HCoV-OC43 maintained under common storage conditions and a pre-cleaned *C. asiatica* leaf surface were quantitatively determined. Infectious HCoV-OC43 suspensions were stored for up to 7 days under common conditions for fresh vegetable storage. This study showed the HCoV-OC43 persistence on an inert surface increased at low temperatures (Table 1). Following storage, the levels of infectious HCoV-OC43 decreased in a time- and temperature-dependent manner. When infectious HCoV-OC43 was kept in a closed container at a low temperature, its persistence was greater than that at a high temperature or dried on a polystyrene surface. In the closed polypropylene tubes, a significant reduction in virus levels occurred after 7 days of storage at 4 °C and within a day at 22 °C. However, infectious HCoV-OC43 was not detected after storage at 37 °C for 24 h.

Surface and environmental factors influence the persistence of infectious coronaviruses. The infectivity of HCoV-OC43 was reported to last less than 1–3 h on aluminum, sterile latex surgical gloves, and sterile sponge surfaces after drying (Sizun et al., 2000). It can remain for 6–48 h on leathers (Shivkumar et al., 2021), ≥72 h on polyester, and ≥24 h on cotton textiles (Owen et al., 2021). As shown in Table 2, this study indicated that the *C. asiatica* surface had no virus inactivation effect against HCoV-OC43, similar to inert polystyrene. It should be noted that under the environmental conditions of the ISO21702-2019 method, in which the virus suspension was covered and maintained at high humidity (>90% RH), the levels of infectious HCoV-OC43 were stable in the

Table 1. The effect of temperature on the persistence of infectious HCoV-OC43 on inert containers.

Container	Time (day)	Temperature (°C)	Virus level (pfu/mL)
Closed polypropylene tube	0 (Immediately)[a]	22 °C	$1.00 \pm 0.30 \times 10^6$
	1	4 °C	$3.17 \pm 0.42 \times 10^5$
	3	4 °C	$2.63 \pm 0.06 \times 10^5$
	7	4 °C	$4.63 \pm 0.46 \times 10^4$*
	1	22 °C	$9.00 \pm 4.00 \times 10^4$*
	3	22 °C	$1.70 \pm 0.56 \times 10^4$*
	7	22 °C	<100*
	1	37 °C	<100*
Opened polystyrene cell culture plate	30 min	22 °C	$7.67 \pm 2.08 \times 10^5$
	1	22 °C	$5.33 \pm 1.53 \times 10^2$*
	3	22 °C	<100*

* Significantly reduced in comparison with the virus level of immediate contact (control), paired t-test ($P < 0.05$).
[a] the infectivity titer of virus immediately after inoculation.

Table 2. Persistence of infectious HCoV-OC43 on surfaces.

Surface	Storage condition	Surface contact time	Virus level (pfu/mL)
C. asiatica leaf	Covered[a], >90% RH	Immediately	$1.43 \times 10^5 \pm 0.06 \times 10^5$
		2 h	$1.97 \times 10^5 \pm 0.25 \times 10^5$
		6 h	$1.77 \times 10^5 \pm 0.57 \times 10^5$
		24 h	$2.47 \times 10^5 \pm 0.21 \times 10^5$
	Air-dried in a biosafety cabinet[b]	2 h	$2.07 \times 10^4 \pm 0.75 \times 10^4$*
Polystyrene cell culture plate	Air-dried in a biosafety cabinet[b]	2 h	$5.85 \times 10^4 \pm 0.49 \times 10^4$*

* Significantly reduced in comparison with the virus level of immediate contact (control), paired t-test ($P < 0.05$).
[a] Virus suspension was covered with a polypropylene film.
[b] Virus suspension was dried on the surface.

C. asiatica leaves. This was in contrast to the increasing trend in SARS-CoV-2 inactivation when dried and left under high humidity (within the range of 20–60% RH) without covering (Biryukov et al., 2020). The levels of infectious HCoV-OC43 were reduced by approximately 10-fold when air-dried on the surfaces for 2 h. No significant difference was observed between the levels of infectious HCoV-OC43 dried on *C. asiatica* leaves and on polystyrene surfaces (Table 2).

Low temperature and closed conditions under high humidity are common conditions for fresh crop storage. Based on these findings, infectious HCoV-OC43 can persist on *C. asiatica* leaves for ≥24 h at room temperature or for at least a week in a refrigerator (Table 1 and Table 2). Consequently, when contaminated with the virus, the surface of fresh *C. asiatica* can act as a coronavirus fomite.

3.2. Efficacy of NEW on HCoV-OC43 and SARS-CoV-2 inactivation

Although dried HCoV-OC43 has not been transmitted through gloved hands, fruits, and inorganic surfaces in the absence of organic media as the transfer matrix (Dallner et al., 2021), contamination of fresh crops with coronaviruses may increase the risk of transmission via touching with bare hands or eating the crops directly. Water is commonly present on the surface of raw plant materials. Furthermore, HCoV-OC43 can remain infectious in simulated gastrointestinal fluids during feeding, and food may increase its stability (Harlow et al., 2022). In this study, water showed no or low antiviral activity against dried HCoV-OC43 compared with NEW. Thus, water may act as a virus-extracting agent and a virus-transfer medium that leads to fomite transmission or infection via oral intake.

For safety, it is critical to have a reliable virus sanitizing method. According to Takeda et al. (2020), the efficacy of acidic EW for SARS-CoV-2 inactivation is dependent on the amount of the available active components in the free chlorine. NEW has been reported to cause virus inactivation as well as denaturation, and aggregation of human norovirus proteins in a dose-dependent manner (Moorman et al., 2017). However, evidence proving the efficacy of NEW for SARS-CoV-2 inactivation is lacking. In this study, HCoV-OC43 and SARS-CoV-2 were used to test the inactivation activity of NEW. As shown in Table 3 and Figure 1, NEW with 197 ppm of ACC completely inactivated HCoV-OC43 and SARS-CoV-2 in suspension within 5 min. In contrast, EW with 76 ppm of ACC showed lower efficacy (Table 3), and at 5-fold dilution, NEW did not exhibit inactivation activity against HCoV-OC43 (data not shown).

Contact NEW with 197 ppm of ACC for 5 min also significantly reduced the infectious virus titer of dried HCoV-OC43 below the limit of detection (<100 pfu/mL) on both *C. asiatica* leaves (≥2.31 log reduction) and polystyrene surfaces (≥2.77 log reduction) (Figure 1). The concentration of NEW (197 ppm of ACC) for SARS-CoV-2 inactivation might be higher than the concentration of acidic EW (74 ppm of free available chlorine) reported in a previous study (Takeda et al., 2020). This could be due to the solution containing a lower ratio of hypochlorous acid to hypochlorite or the differences in testing conditions. Nevertheless, NEW has greater advantages than acidic EW in terms of stability and neutral pH (Cui et al., 2009).

3.3. Effects of NEW on the levels of C. asiatica triterpenoids

Pennywort (*Centella asiatica* (L.) Urban) is commonly consumed raw and is widely used in folk medicine. It contains pharmaceutically

Table 3. Virus inactivation activity of NEW in suspensions.

Available chlorine concentration (ppm)	Virus	Virus inactivation activity[1]
76	HCoV-OC43	3.26
197	HCoV-OC43	≥3.68*
197	SARS-CoV-2	≥4.34*

[1] Virus inactivation activity is defined as the difference in the logarithm of the infectivity titer of the virus found in the treatment and control groups. *Viruses were not detected ($<10^2$ pfu/mL).

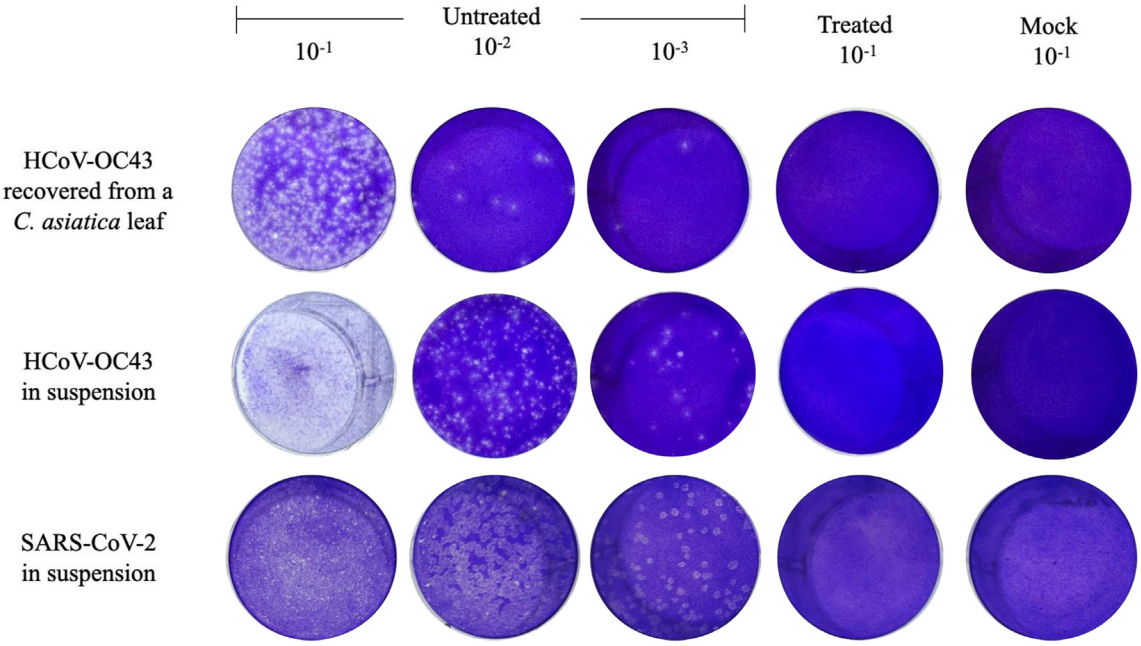
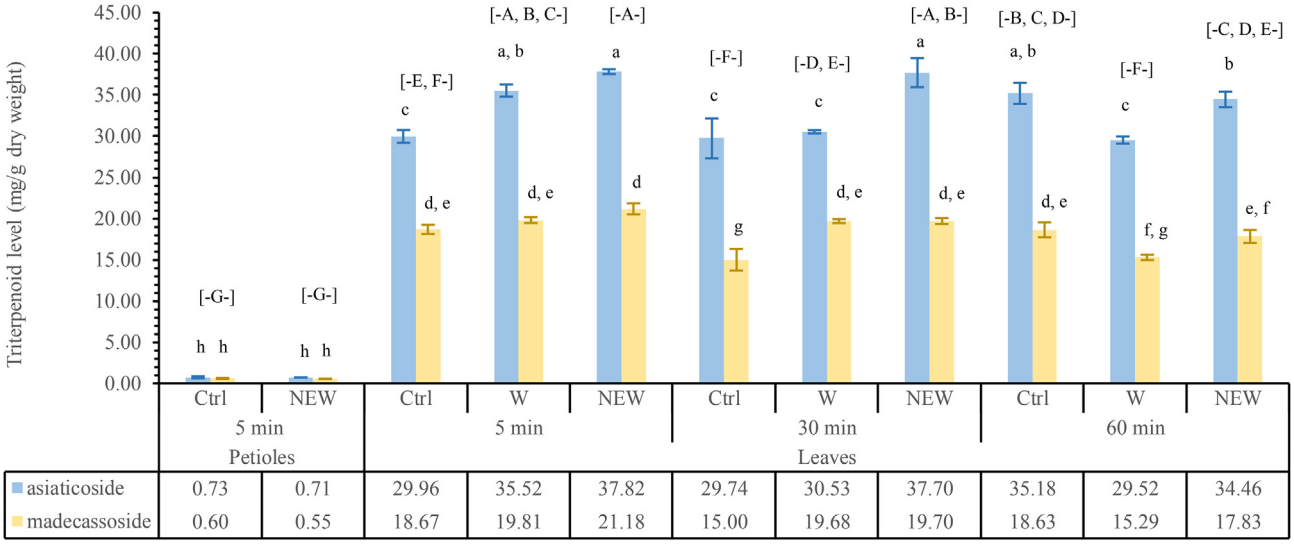


Figure 1. Plaque assays of HCoV-OC43- and SARS-CoV-2-infected Vero cells in 6-well plates. The cells were inoculated with 10-fold dilutions of the untreated or NEW-treated samples.



	Ctrl (5 min, Petioles)	NEW (5 min, Petioles)	Ctrl (5 min, Leaves)	W (5 min, Leaves)	NEW (5 min, Leaves)	Ctrl (30 min, Leaves)	W (30 min, Leaves)	NEW (30 min, Leaves)	Ctrl (60 min, Leaves)	W (60 min, Leaves)	NEW (60 min, Leaves)
asiaticoside	0.73	0.71	29.96	35.52	37.82	29.74	30.53	37.70	35.18	29.52	34.46
madecassoside	0.60	0.55	18.67	19.81	21.18	15.00	19.68	19.70	18.63	15.29	17.83

Figure 2. Triterpenoid levels in *C. asiatica* samples after being unsoaked (control), soaked with ultrapure water (W), or soaked with neutral electrolyzed water (NEW) with 205 ppm of ACC. The alphabets represent significant differences (lowercase for individual asiaticoside and madecassoside levels, and uppercase for total triterpenoid levels) at $P \leq 0.05$ (one-way ANOVA, Tukey's post hoc test).

important triterpenoids, such as madecassoside and asiaticoside, which exert strong anti-inflammatory and wound healing effects (Chandrika and Kumara, 2015; Tan et al., 2021). The levels of madecassoside and asiaticoside are key biomarkers of *C. asiatica* products, which should be standardized for pharmaceutical use (Tan et al., 2021).

To examine whether virus inactivation with NEW changes the levels of *C. asiatica* triterpenoids, a slightly higher concentration of NEW (205 ppm of ACC) was used to soak the *C. asiatica* samples. No physical changes in appearance, including color or damage, were observed among *C. asiatica* samples after being soaked or unsoaked in NEW. Figure 2 shows that the levels of total triterpenoids in *C. asiatica* leaves were 36.6-fold higher than those in its petioles (1.33 ± 0.13 and 48.63 ± 0.96 mg/g dry weight, respectively). Therefore, this study focused on the effects of NEW on its leaves. After 5 min of soaking, the level of total triterpenoids in the NEW-soaked group was significantly higher than those in the unsoaked group (59.00 ± 0.72 and 48.63 ± 0.96 mg/g dry weight, respectively). However, the increase in total triterpenoid level was comparable to that of the water-soaked group (55.33 ± 0.82 mg/g dry weight). The increase in both groups was primarily due to a significant increase in asiaticoside levels compared to the control. Compared with the triterpenoid levels of the 5-min-soaked groups, a significant decrease was found only in the asiaticoside levels of the 30-min water-soaked group, but not in the 30-min NEW-soaked group. Furthermore, a significant decrease in total triterpenoid levels was observed in the NEW-soaked groups at 60 min (52.29 ± 1.23 mg/g dry weight). The increase in triterpenoid levels after soaking may be due to the soaking process, which stimulates the intrinsic enzymatic activity of plants (Guajardo-Flores et al., 2012). On the other hand, those with a long

soaking time may decrease due to the dissolution of triterpenoids and/or loss due to enzymatic degradation (Singh et al., 2017).

The asiaticoside level in the 60-min unsoaked group was significantly higher than that in the 5- and 30-min unsoaked groups. This might be caused by light or environmental elicitation during the longer experiment time (Yendo et al., 2010); however, the increase at 60 min did not occur in the soaked groups since the levels were already increased at 5 min. The triterpenoid levels of the 60-min NEW soaked group did not significantly differ from those of the 60-min unsoaked group (52.29 ± 1.23 and 53.82 ± 1.56 mg/g dry weight, respectively).

NEW has the potential to reduce foodborne pathogens on vegetable surfaces (Abadias et al., 2008). However, information regarding its effects on bioactive compounds in medicinal plants is lacking. A significant change in total triterpenoid levels led to a significant change in the quality and pharmaceutical value of *C. asiatica*. These findings suggest that NEW can also be used to effectively inactivate infectious HCoV-OC43 in suspension and on a *C. asiatica* leaf, while having no negative effects on the accumulation of its major triterpenoids. Moreover, the soaking process can simply increase asiaticoside levels compared to the unsoaking process. Collectively, these results show that NEW has the potential to improve worker hygiene and consumer confidence while reducing the risk of virus transmission.

The effects of NEW may differ depending on the type of vegetable used and its constituents. Therefore, the applicability and appropriateness of using NEW or other virus inactivation methods for cleaning medicinal plants should be proven individually. It would be interesting to determine how well NEW works against other viruses that are a concern for valuable vegetables or other food products.

4. Conclusion

HCoV-OC43, a betacoronavirus belonging to the same family as SARS-CoV-2, can remain infectious in medicinal plants, such as *C. asiatica* leaves, for a sufficient period, thereby posing a risk of transmission. In suspension tests, NEW with 197 ppm of ACC efficiently inactivated SARS-CoV-2 and HCoV-OC43. At this concentration, NEW can also efficiently inactivate dried HCoV-OC43 on contaminated *C. asiatica* leaves. Soaking *C. asiatica* leaves with this effective concentration of NEW had no negative effects on triterpenoid levels. Therefore, using NEW for 5–30 min could be an option for cleaning contaminated *C. asiatica* leaves.

Declarations

Author contribution statement

Jukrapun Komaikul; Tharita Kitisripanya: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sasiporn Ruangdachsuwan; Sanjira Juntarapornchai; Kunjimas Ketsuwan: Performed the experiments; Analyzed and interpreted the data.

Duangnapa Wanlayaporn: Performed the experiments.

Promsin Masrinoul; Suthee Yoksan; Pilaipan Puthavathana: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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