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Antiviral activities of *Artemisia princeps* var. *orientalis* essential oil and its α-thujone against norovirus surrogates

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Abstract *Artemisia princeps* var. *orientalis* is a well-known medicinal food, which has been used for the treatment of several diseases including bacterial infection. We examined the antiviral effects of the essential oil from *A. princeps* var. *orientalis* and its compounds, borneol, α-thujone and camphor, against murine norovirus-1 (MNV-1) and feline calicivirus-F9 (FCV-F9). The time-of-addition plaque assays were used to determine the ability of essential oil to interfere with viral infection. The maximum activities, following the pretreatment of FCV-F9 and MNV-1, reached 48% inhibition on FCV-F9 and 64% inhibition on MNV-1 at 0.1 and 0.01% of the essential oil, respectively. Neither borneol nor camphor exhibited an antiviral activity, whereas α-thujone, a major compound of the essential oil, showed strong inhibition on FCV-F9 and MNV-1.

Keywords Murine norovirus · Feline calicivirus · *Artemisia princeps* var. *orientalis* · Essential oil · α-Thujone

Introduction

Noroviruses frequently cause acute gastroenteritis outbreaks globally, which leads to high morbidity and a heavy economic burden [1]. Norovirus is transmitted through the fecal–oral route or person-to-person contact. It is highly resistant to harsh conditions of a wide range of temperatures (from freezing to 60 °C) and the limit of infectious doses is approximately less than 20 virions [2]. In adults, norovirus-induced gastroenteritis is acute and self-limiting, but in the elderly and in young children, the illness can last longer [2]. Recent studies of immortalized B cells and stem cell-derived organoids allow in-depth analyses in human norovirus replication [3, 4], to provide an opportunity to develop vaccines and antivirals. Nevertheless, murine norovirus-1 (MNV-1) and feline calicivirus-F9 (FCV-F9) are still used as surrogates for studying norovirus biology [5, 6], when no vaccine or effective antivirals to prevent or control norovirus infection are yet available.

Artemisia species are found mainly in temperate climate regions and have frequently been used for the treatment of diseases such as malaria, hepatitis, and bacterial infections [7, 8]. *Artemisia princeps* var. *orientalis* (*ssuk* in Korea), is an aromatic, edible, and medicinal plant belonging to the Compositae family. The major compounds, borneol (12.1%) and α-thujone (8.7%), were identified in the essential oil from *A. princeps* var. *orientalis* [9]. α-Thujone is reported to be a major compound of essential oils from *Artemisia*, which can be as high as 67% depending on its species [10, 11]. Biological activities of α-thujone have been investigated in recent studies; it has an anti-tumor effect and beneficial effect in the treatment of polycystic ovary syndrome [12, 13]. In the plant, borneol is oxidized to camphor which comprises only 2.9% in the essential oil [9]. Camphor derivatives showed an inhibitory effect on hemagglutinin of influenza virus A and B [14]. However, the antiviral activities of *A. princeps* var. *orientalis* essential oil, borneol, α-thujone, and camphor against norovirus surrogates have not been explored. In this study, *A. princeps* var. *orientalis* and its compounds were analyzed the antiviral effects on MNV-1 and FCV-F9.

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Materials and methods

Viruses and cells

RAW 264.7 cells (mouse leukemic macrophage cell line), Crandell Reese feline kidney (CRFK) cells, and FCV-F9 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MNV-1 was obtained from Dr. Herbert Virgin at Washington University School of Medicine, USA. RAW 264.7 and CRFK cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Karlsruhe, Germany) with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin streptomycin (PS) (Invitrogen, Grand Island, NY, USA) in a 5% CO_2 incubator at 37 °C.

Extraction of essential oil

Dried *A. princeps* var. *orientalis* (voucher no. DSNPL-0013) which was purchased at Gyeongdong market in Seoul. The aerial parts were crushed for 10 s using a blender. The essential oil was extracted from *A. princeps* var. *orientalis* by steam distillation using a Clavenger type apparatus (Hanil Labtech Ltd., Incheon, Korea) for 3 h. The essential oil yield was 1.4%. The major compounds of *A. princeps* var. *orientalis* essential oil are in descending order borneol (12.1%), α-thujone (8.7%), T-cadinol (6.7%), and 1, 8-cineole (6.2%) [9]. The essential oil was dried over anhydrous sodium sulfate. Borneol, camphor, and α-thujone were purchased from Sigma–Aldrich.

Cytotoxicity test

RAW 264.7 and CRFK cells were seeded in 96-well tissue culture plates at a density of 1.5×10^5 and 2×10^4 cells per well, respectively. After incubation, 90 μL of DMEM containing 10% FBS-1% PS and 10 μL of the essential oil or its compound was added to the cells in culture and then incubated for 24 h at 37 °C and 5% CO_2. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT: Sigma–Aldrich) solution was added into each well and re-incubated at 37 °C, and followed by the addition of dimethyl sulfoxide (DMSO: Sigma–Aldrich) solution to dissolve the formazan crystals. The absorbance value was determined in a microplate reader (SpectraMax M2, Molecular Devices Corp., CA, USA) at 570 nm. The percentage of cell viability = $(Abs_{treatment}/Abs_{control}) \times 100$.

Plaque assays

Inhibitory effects of the essential oil or its compounds (borneol, α-thujone, and camphor) were determined by using plaque assays. In order to identify the mechanism of action of the essential oil against MNV-1 or FCV-F9, we tested three modes of time-of-addition [15]. For pretreatment of the virus with the essential oil (or its compound), MNV-1 or FCV-F9 (6 $\log_{10}$ plaque-forming unit (PFU)/mL) was mixed in a ratio of 9:1 with the essential oil (or its compound). After 1 h incubation at room temperature, tenfold dilutions of virus mixture were added to each well for 1 h at 37 °C and 5% CO_2 and the virus mixture was removed. And the cells were washed twice with PBS and cells were overlaid with DMEM medium containing 1.5% agarose, 5% FBS, and 0.5% PS. After incubation for 48 h for MNV-1 and 24 h for FCV-F9 at 37 °C in 5% CO_2, the cells were stained with 0.5% crystal violet and plaques were counted on each well. In cotreatment, the confluent monolayers of cultured cells were inoculated with 200 μL of virus (2 $\log_{10}$PFU/mL) simultaneously added with the essential oil. After 1 h incubation at 37 °C in 5% CO_2, the virus and essential oil were removed, and the next procedures were the same as those used in the pretreatment. In posttreatment, the monolayers were inoculated with virus (2 $\log_{10}$PFU/mL) for 1 h and the inocula were removed. After washing the cells, the essential oil was added to the cells for 1 h at 37 °C in 5% CO_2 and then the essential oil was removed. The next procedures were the same as those used in the pretreatment. The untreated control was DMSO which was used as a solvent for the essential oil and its compound. 2-Thiouridine (2TU) was used as a positive control at a concentration of 50 and 200 μM for MNV-1 and FCV-F9, respectively [15]. 2TU showed antiviral activity against MNV-1 and FCV-F9 through binding to the RNA-dependent RNA polymerase of virus [16]. Inhibitory activities were expressed as PFU reduction or relative plaque formation % compared to untreated control.

Statistical analysis

Data were presented as the mean and standard deviation. The significance of differences in the mean was analysed by the Student's *t* test (SPSS software, version 13.0, SPSS Inc., Chicago, IL, USA).

Results and discussion

The cytotoxicity of *A. princeps* var. *orientalis* essential oil or its compound on CRFK and RAW 264.7 cells was determined by MTT assay. The essential oil at 10^{-3} dilution-treated CRFK or RAW 264.7 cells showed ≥85% viability. The cell viability of CRFK or RAW 264.7 was ≥90% at 25 mM of α-thujone, 5 mM of borneol, and 25 mM of camphor. Therefore, inhibitory experiments of

the essential oil and the individual compounds were performed at concentrations below the cytotoxic levels.

In FCV-F9, moderate inhibition of FCV-F9 was achieved upon pretreatment with 0.1% (v/v) the essential oil in a dose-dependent manner, resulting in 48% inhibition (Fig. 1A), whereas 2TU, used as a postive control, showed 25% inhibition at 200 μM. Cotreatment with the essential oil exhibited very low inhibitory effect against FCV-F9, reaching 17% inhibition at the same concentration. With posttreatment, 41% inhibition was achieved by the essential oil at 0.1% (v/v). In the case of MNV-1, a 64% plaque reduction was obtained after pretreatment of MNV-1 with 0.01% (v/v) essential oil (Fig. 1B), where as 2TU at 50 μM resulted in 24% inhibition. Cotreatment with the essential oil at the same concentration showed a 53% inhibition. However, posttreatment revealed no inhibitory effect at 0.01% (v/v) the essential oil. Our results demonstrated that the essential oil was effective in reducing plaque formation by MNV-1 and FCV-F9 and that the inhibitory effects of the essential oil was consistently high with the pretreatment of the virus in the time-of-addition mode.

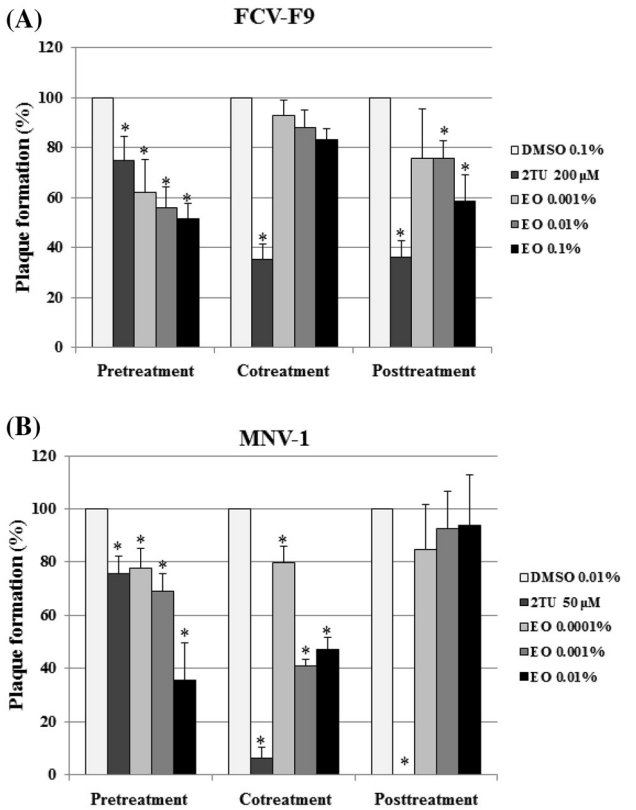

Fig. 1 Inhibitory effects of *Artemisia princeps* var. *orientalis* essential oil on (**A**) FCV-F9 and (**B**) MNV-1. The essential oil was added at different time points during virus infection. The reduction of relative plaque formation (%) was evaluated using plaque assay oil (EO). DMSO and 2TU were used as the untreated control and positive control, respectively. All measurements were analyzed in triplicate. A single *asterisk* denotes significant decrease of plaque formation (%) relative to untreated control ($p < 0.05$)

Next, borneol and α-thujone, major compounds of the essential oil, and camphor, an oxidized form of borneol, were examined in the pretreatment of virus mode in which the essential oil showed the maximal antiviral activity. Neither borneol (5 mM) nor camphor (25 mM), exhibited inhibitory effects on FCV-F9 and MNV-1 (Table 1). However, α-thujone showed an inhibitory effect in a dose-dependent manner: 0.94 $\log_{10}$PFU reduction and 0.50 $\log_{10}$PFU reduction were achieved by α-thujone at 25 mM against FCV-F9 and MNV-1, respectively (Fig. 2), whereas 2TU exhibited 0.06 and 0.16 $\log_{10}$PFU reductions on FCV-F9 and MNV-1, respectively. These results were similar to those obtained with oregano oil and its carvacrol which was effective in inhibiting MNV via directly interfere with the virion capsid [17]. Oregano, clove, and zataria essential oils showed strong inhibitory effects on FCV [18]. However, the essential oils from hyssop, marjoram, clove, zataria, and *Zanthoxylum schnifolium* did not show significant effects on FCV-F9 or MNV-1 infectivity [19–21]. In this study, essential oil was more effective on MNV-1 than FCV-F9, whereas α-thujone caused stronger inhibition against FCV-F9 than MNV-1. Borneol and camphor did not affect these viruses. In this context, only three compounds were tested in this study, suggesting that other compounds in *A. princeps* var. *orientalis* essential oil may play a major role in the inhibitory effect. Nevertheless, to determine the antiviral effect of other compounds, minor compounds of the essential oil need be tested in a future study.

Norovirus is more stable at low temperature (4 °C) in which the antiviral activity of inhibitor against the virus may be lower than the room temperature. Elizaquível et al. reported that the antiviral effects of oregano, clove, and zataria essential oils on FCV were more effective at 37 °C than 4 °C [18]. In the present study, antiviral activities of the essential oil and its compounds were tested at room temperature. More studies are needed to elucidate the effect of temperature on viral inhibition of the essential oil and its compounds. A few studies have been reported on the inactivation of essential oil against FCV-F9 or MNV-1, among them antiviral mechanism of essential oil had not been suggested, except oregano oil and carvacrol which caused disruption of MNV-1 capsid.

Essential oils are mixtures of volatile and odoriferous secondary plant metabolites such as monoterpenes and sesquiterpenes. Essential oils are used extensively as food flavoring and they have potential food biopreservatives [17, 18]. The strength of the study lies in the fact that *A. princeps* var. *orientalis*, one of the most popular edible and medicinal plants in Korea, can be used to control norovirus mediated foodborne diseases. In conclusion, *A. princeps*

Table 1 Inhibitory effects of borneol and camphor from *Artemisia princeps* var. *orientalis* essential oil against norovirus surrogates, FCV-F9 and MNV-1

Norovirus surrogates	Plaque formation (%)			
	DMSO 0.1% (v/v)	2TU	Borneol 5 mM	Camphor 25 mM
FCV-F9	100.00 ± 2.53[a]	80.96 ± 8.38[b]	102.17 ± 13.57[a]	96.32 ± 4.63[a]
MNV-1	100.00 ± 1.15[a]	73.45 ± 5.16[b]	95.12 ± 2.52[a]	105.55 ± 1.73[a]

Data were expressed as the mean ± SD using triplicates. Plaque formation % for the pretreatment of the virus with borneol or camphor was calculated as relative plaque formation using DMSO untreated control. 2TU (200 μM for FCV-F9 and 50 μM for MNV-1) was used as the positive control

[a–b] Means followed by different letters in each row are significantly different according to Tukey's test ($p < 0.05$)

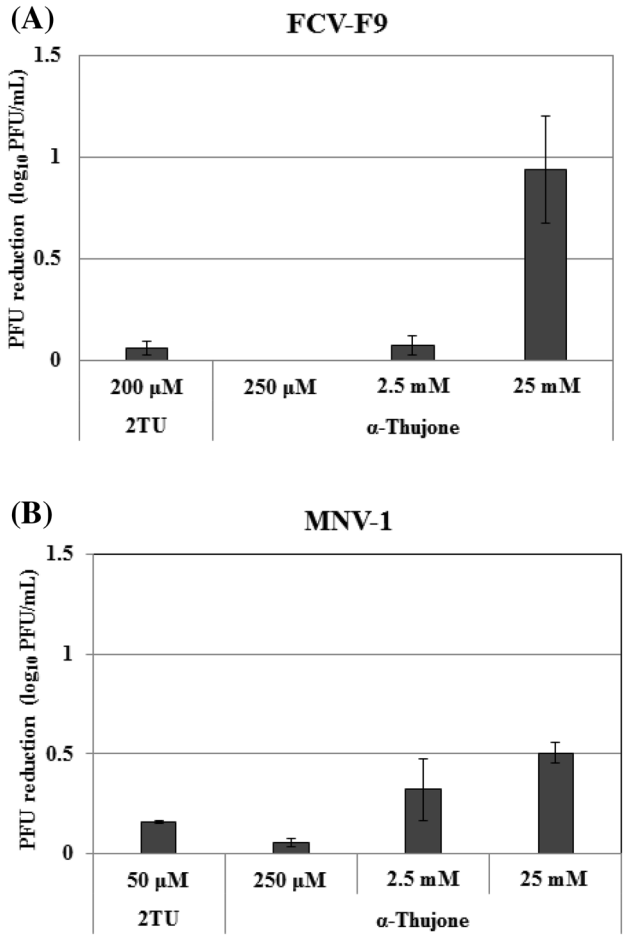

Fig. 2 Antiviral effect of α-thujone from the essential oil against (**A**) FCV-F9 (**B**) MNV-1. The effects of α-thujone against FCV-F9 and MNV-1 were evaluated as PFU reduction ($\log_{10}$ PFU/mL) using plaque assay in the pretreatment of virus with α-thujone at concentration from 250 μM to 25 mM. DMSO and 2TU were used as the untreated and positive control, respectively. All measurements were analyzed in triplicate

var. *orientalis* essential oil or its major compound, α-thujone, in the pretreatment of FCV-F9 and MNV-1 showed strong inhibitory effects. Further studies are required to elucidate antiviral mechanisms of the essential oil and α-thujone on FCV-F9 and MNV-1.

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Compliance with ethical standards

Conflicts of interest The author declares no conflict of interests.

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