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Article

Inhibitory Effect on Replication of Enterovirus 71 of Herb Methanol Extract

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Anti-enterovirus 71 (EV 71) activities of fifteen herb plant species extracts were examined by SRB assay, among which *Origanum vulgare* and *Rosmarinus officinalis* (Anna Rosemary) extracts exhibited the activities with IC_{50} of 8.28 and 8.17 μg/mL, respectively. Their 50% cytotoxicity concentrations (CC_{50}) were 691.89 and 1104.19 μg/mL, and the therapeutic indices were 83.56 and 135.15, respectively. Amantadine (positive control) showed anti-EV 71 activity with 50% inhibitory concentration and CC_{50} of 4.46 and 145.22 μg/mL, respectively. Addition of the methanol extracts of *O. vulgare* and *R. officinalis* (Anna Rosemary) in EV 71-infected Vero cells strongly inhibited the formation of visible cytopathic effects without changing the normal morphology of the cells. These results indicate that methanol extracts of *O. vulgare* and *R. officinalis* (Anna Rosemary) may contain antiviral compound inhibiting the EV 71 replication.

Key words : *antiviral activity, cytopathic effects, enterovirus 71,* Origanum vulgare, Rosmarinus officinalis *(Anna Rosemary)*

Enterovirus 71 (EV 71) is a positive-stranded RNA virus that belongs to the enterovirus genus of the Picornaviridae family [McMinn, 2002]. EV 71 is associated with neurological complications such as aseptic meningitis, brainstem encephalitis, and poliomyelitis-like paralysis, which led to fatalities during the outbreaks in the Asia Pacific region [Ho *et al.*, 1999; Lum *et al.*, 1998]. EV71 infections with neurological complications have been also observed in Malaysia, Singapore, Western Australia, United States, and Europe [Alexander *et al.*, 1994; Gilbert *et al.*, 1988; Ho, 2000]. At present, neither vaccine nor therapeutic treatment is available.

Medicinal plants, because of minor side effects, lower potential to cause resistance, and low costs, are increasingly being projected as suitable alternative sources of antiviral agents [Briskin, 2000; Cowan, 1999; Jassim and Naji, 2003; Vlietinck and Vanden Berghe, 1991; Williams, 2001]. Although several hundreds of plants that have potential as novel antiviral agents have been studied, there still exist innumerable, potentially useful medicinal plants waiting to be evaluated and exploited for therapeutic applications against genetically and functionally diverse virus families; however, no detailed study has been carried out on the efficacy of herb plants against the replication of EV 71 in Vero cells. The aim of this study was to examine the antiviral activities of herbal methanol extracts and antiviral drugs against EV 71. The effects of herbal methanol extracts on EV71-induced CPE were also studied.

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Abbreviations: ATCC, American Type Culture Collection; CC_{50}, cytotoxic concentration; $CCID_{50}$, 50% cell culture infective dose; CPE, cytopathic effect; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HeLa, human epitheloid carcinoma cervix; HRV, human rhinovirus; IC_{50}, 50% inhibitory concentration; ID_{50}, 50% infective dose; MDCK, Madin-Darby canine kidney; MEM, minimal essential medium; SRB, sulforhodamine B; TI, therapeutic index.

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Materials and Methods

Virus, cell line, and reagents. EV 71 was obtained from Chungcheongnam-Do Health and Environment Research Institute in Korea, and was propagated in Vero cells at 37°C. Vero cells were maintained in MEM supplemented with 10% FBS and 0.01% antibiotic-antimycotic solution. Antibiotic-antimycotic solution, trypsin-EDTA, FBS, and MEM were supplied by Gibco BRL (Grand Island, NY). The tissue culture plates were purchased from Falcon (BD Biosciences, Franklin Lakes, NJ). SRB was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of reagent grade.

Plant and sample preparation. Fifteen species (*Melissa officinalis, Origanum vulgare, Rosmarinus officinalis* (Marta Rosemary), *Achillea milleforium, Mentha spp., Salvia officinalis, Citrus berhamia, Aloysia triphylla, Thymus vulgaris, Lavandula angustifolia, Stevia rebaudiana, Rosmarinus officinalis* (Anna Rosemary), *Marrubium vulgare, Ruta graveolens*, and *Alchemilla vularis*) of herb plants were collected from Sangsoo Herb Land (Chungbuk, Korea), air-dried at room temperature, and pulverized. Each 100 g of the test plants was extracted with 600 mL of methanol twice at room temperature for 2 days and filtered (Whatman No. 2). The combined filtrate was concentrated to dryness by rotary evaporation at 40°C. Each extract was solublized in 100 mg/mL DMSO and stored at –20°C.

Cytotoxicity assay. Vero cells grown to confluence in 96-well plates were exposed to different concentrations of the antiviral compounds (three wells per compound concentration) in maintenance medium for 2 days at 37°C, in parallel with the virus-infected cell cultures. For each antiviral compound, three wells were used as controls (non-drug-treated cells). After 2 days of incubation, cytotoxicity was evaluated by the SRB assay as previously described [Lin *et al.*, 1999]. The concentration of antiviral compound that reduced the viability of Vero cells to 50% of the control was estimated as the 50% cytotoxic concentration (CC_{50}). The results were transformed into percentage of the controls, and the CC_{50} values were graphically obtained from the dose–response curves.

Antiviral activity assay. The antiviral activities of herb species-derived materials against EV 71 were determined by the SRB assay. Vero cells in the 96-well tissue culture plates were used when confluent. Culture medium was removed, and the cells were washed with PBS. Subsequently, 0.09 mL of the diluted virus suspension of EV 71 containing $CCID_{50}$ of the virus stock was added to produce the appropriate cytopathic effects within 2 days after infection, followed by the addition of 0.01 mL of medium supplemented with an appropriate concentration of the antiviral compound. The antiviral activity of each test material was determined at four concentrations ranging from 0.1 to 100 μg/mL, a ten-fold dilution scheme for each compound. Three wells each were used as the virus controls (virus-infected non-drug-treated cells) and the cell controls (non-infected non-drug-treated cells). The culture plates were incubated at 37°C in 5% CO_2 for 2 days until appropriate CPE was achieved. Subsequently, the 96-well plates were washed once with 1×PBS, and 100 μL of cold (-20) 70% acetone was added on to the top of each well and left standing for about 30 min at –20°C. After the removal of 70% acetone, the plates were dried in a dry oven for 30 min, followed by the addition of 100 μL of 0.4% (w/v) SRB in 1% acetic acid solution to each well, and left standing at room temperature for 30 min. SRB was then removed, and the plates were washed five times with 1% acetic acid. The plates were then dried in a dry oven for at least 24 h. Bound SRB was solubilized with 100 μL of 10 mM unbuffered Tris-base solution, and the plates were left standing on a table for at least 30 min. The absorbance was read in a 96-well plate reader at 562 nm with subtraction of the background measurement at 620 nm. The results were then transformed into percentage of the controls, and the IC_{50} values were graphically obtained from the dose-response curves (Microsoft Office, 2003). The percent protection achieved by the test compound in the EV 71-infected cells was calculated using the following equation:

$$\frac{(OD_t)_{EV\ 71}-(OD_c)_{EV\ 71}}{(OD_c)_{mock}-(OD_c)_{EV\ 71}} \times 100 \text{ (Expressed in \%)}$$

where $(OD_t)_{EV\ 71}$ is the optical density measured at a given concentration of the test compound in the EV 71-infected cells, $(OD_c)_{EV\ 71}$ is the optical density measured for the untreated EV 71-infected control cells, and $(OD_c)_{mock}$ is the optical density measured for the untreated mock-infected control cells. The concentration achieving 50% protection according to the above equation was defined as the IC_{50}. TI was determined as CC_{50}/IC_{50}.

The effect of herb methanol extracts on EV 71-induced CPE. Vero cells in 96-well tissue culture plates were used when confluent. The culture medium was removed, and the cells were washed with PBS. Subsequently, 0.09 mL of the diluted virus suspension and 0.01 mL of the medium supplemented with 1% FBS containing 10 μg/mL plant extracts were added to the cells. After incubation at 37°C in 5% CO_2 for 2 days, inhibition of virus replication was evaluated by SRB assay. The morphology of the cells was observed under

microscope at 32×40 magnification (Axiovert 10, Zeiss, Germany), and the images were recorded.

Results

Antiviral activity and cytotoxicity of methanol herb extracts against EV 71. The herb extracts were tested for the antiviral activity against EV 71 and examined by SRB assay. *O. vulgare* and *R. officinalis* (Anna Rosemary) extracts exhibited anti-EV 71 activities with IC_{50} values of 8.28 and 8.17 μg/mL, respectively (Table 1). The CC_{50} values of *O. vulgare* and *R. officinalis* (Anna Rosemary) extracts were 691.89 and 1104.19 μg/mL, and the TIs were 83.56 and 135.15, respectively (Table 1). The IC_{50} values of the other extracts including *M. officinalis, R. officinalis* (Marta Rosemary), *A. milleforium, Mentha spp., S. officinalis, C. bergamia, A. triphylla, T. vulgaris, L. angustifolium, S. rebaudiana, M. vulgare, R. graveolens*, and *A. vulgaris* were not determined, because their maximum inhibition rates were under 50% (Table 1).

Antiviral activity of antiviral drugs against EV 71. Amantadine showed strong antiviral activity with IC_{50} of 4.46 μg/mL against EV 71 (Table 1). The CC_{50} of amantadine was 145.22 μg/mL and the TI was 32.56 (Table 1). The IC_{50} values of the other drugs were not calculated, because their maximum inhibition rates were under 50% (Table 1).

The effect of herb methanol extracts on EV 71-induced CPE. During infections of Vero cells with EV 71, mock cells (Fig. 1A) or cells treated with 10 μg/mL herb extract (Fig. 1C, 1E, 1G, 1I, 1M, 1O, 1Q, 1S, 1U, 1W, 1Y, 1AA, 1AC, and 1AE) showed typical spread-out shapes with normal morphology, and cells treated with *R. officinalis* (Anna Rosemary) of 10 μg/mL showed abnormal morphology (Fig. 1K). Infection with EV 71 in the absence of herb extracts resulted in a severe CPE (Fig. 1B). As shown in Fig. 1X and 1AB, the morphology of cells after 48 h of infection with EV 71 in the presence of *O. vulgare* and *R. officinalis* (Anna Rosemary) were virtually distinguishable from that of the EV 71-infected cells. However, addition of the other extracts in the EV

Table 1. Antiviral activity of herb methanol extracts and antiviral drugs against EV 71 in Vero cells

		CC_{50}[a]	IC_{50}[b]	TI[c]
Plant species	*Melissa officinalis*	653.24	-[d]	-
	Origanum vulgare	691.89	8.28±0.18	83.56
	Rosmarinus officinalis (Marta Rosmarinus)	>100	-[d]	-
	Achillea milleforium	492.96	-[d]	-
	Mentha spp.	1708.32	-[d]	-
	Salvia officinalis	>100	-[d]	-
	Citrus berhamia	239.73	-[d]	-
	Aloysia triphylla	>100	-[d]	-
	Thymus vulgaris	759.07	-[d]	-
	Lavandula angustifolia	>100	-[d]	-
	Stevia rebaudiana	>100	-[d]	-
	Rosmarinus officinalis (Anna Rosmarinus)	1104.19	8.17±0.49	135.15
	Marrubium valgare	652.56	-[d]	-
	Ruta graveolens	>100	-[d]	-
	Alchemilla vularis	301.10	-[d]	-
Antiviral drugs	*Ribavirin*	191.64	-[d]	-
	Acyclovir	176.45	-[d]	-
	Amantadine	145.22	4.46±1.48	32.56
	Tamiflu	542.43	-[d]	-
	Relenza	501.87	-[d]	-
	Lamivudine	770	-[d]	-
	Zidovudine	>100	-[d]	-

Values represent the means of three independent experiments.
[a]The 50% cytotoxic concentration for target cells (Vero cells) in μg/mL.
[b]Concentration of compound in μg/mL producing 50% inhibition of virus-induced cytopathic effects.
[c]Therapeutic index (TI)=CC_{50}/IC_{50}.
[d]IC_{50} value within the concentration of compound to test not determined due to maximum inhibition rate under 50%.

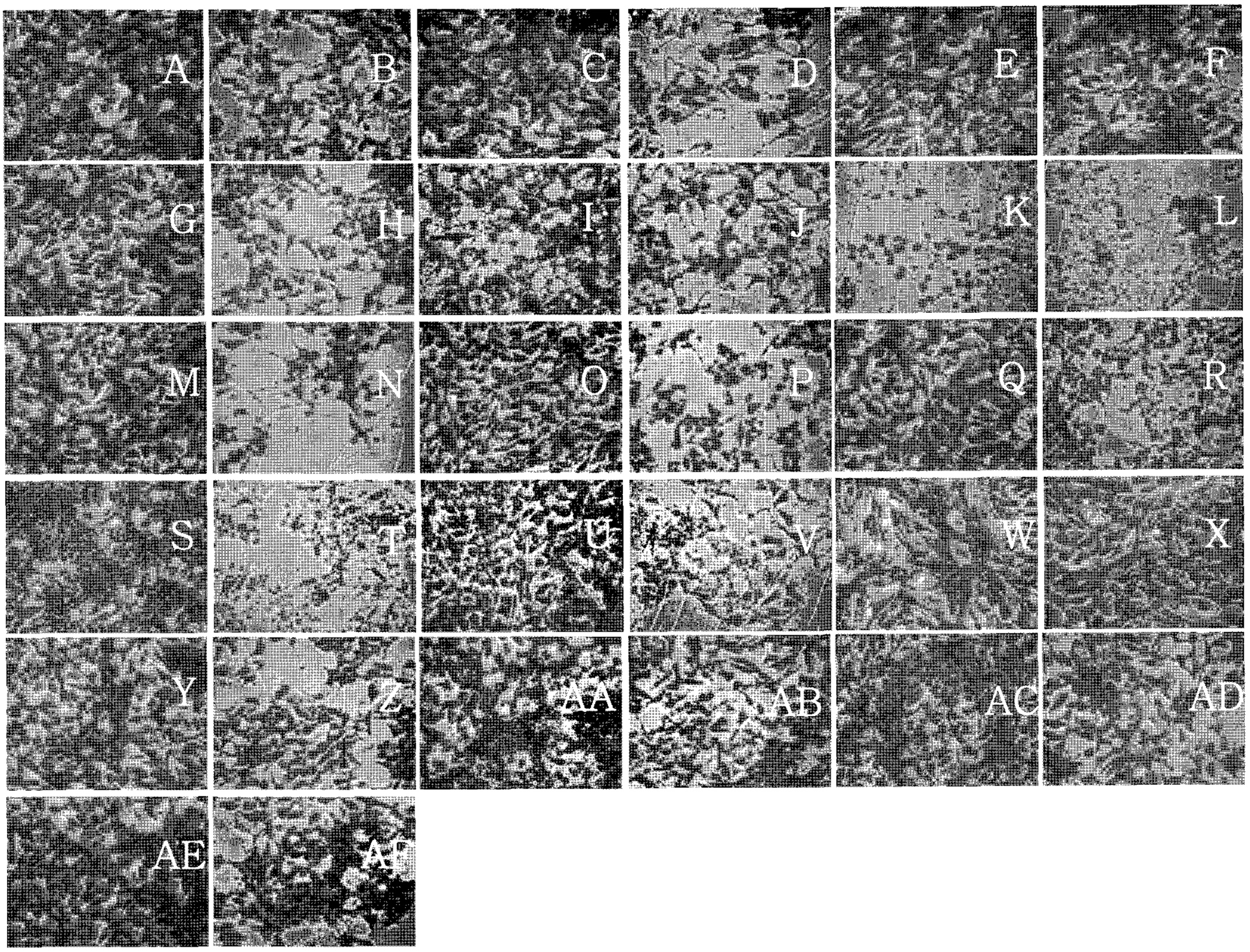

Fig. 1. The effect of herb methanol extracts on EV 71-induced CPE. Herbal methanol extracts (10 μg/mL each) were treated, and after incubation at 37°C in 5% CO_2 for 2 days, inhibition of the virus replication was evaluated by the SRB assay. Microscopic images of the cell morphology were taken. (A) non-infected cells; (B) virus-infected cells; (C) non-infected cells with *A. triphylla*; (D) virus-infected cells with *A. triphylla*; (E) non-infected cells with *M. officinalis*; (F) virus-infected cells with *M. officinalis*; (G) non-infected cells with *A. vulgaris*; (H) virus-infected cells with *A. vulgaris*; (I) non-infected cells with *R. graveolens*; (J) virus-infected cells with *R. graveolens*; (K) non-infected cells with *R. officinalis* (Marta Rosemary); (L) virus-infected cells with *R. officinalis* (Marta Rosemary); (M) non-infected cells with *L. angustifolium*; (N) virus-infected cells with *L. angustifolium*; (O) non-infected cells with *C. bergamia*; (P) virus-infected cells with *C. bergamia*; (Q) non-infected cells with *S. officinalis*; (R) virus-infected cells with *S. officinalis*; (S) non-infected cells with *Mentha spp.*; (T) virus-infected cells with *Mentha spp.*; (U) non-infected cells with *S. rebaudiana*; (V) virus-infected cells with *S. rebaudiana*; (W) non-infected cells with *R. officinalis* (Anna Rosemary); (X) virus-infected cells with *R. officinalis* (Anna Rosemary); (Y) non-infected cells with *A. milleforium*; (Z) virus-infected cells with *A. milleforium*; (AA) non-infected cells with *O. vulgare*; (AB) virus-infected cells with *O. vulgare*; (AC) non-infected cells with *T. vulgaris*; (AD) virus-infected cells with *T. vulgaris*; (AE) non-infected cells with *M. vulgare*; (AF) virus-infected cells with *M. vulgare*.

71-infected Vero cells did not prevent CPE.

Discussion

The current armamentarium for the chemotherapy of viral infections consists of 37 licensed antiviral drugs and amantadine used in the treatment of influenza A virus [Erik, 2004]. In the present study, the antiviral activities of the herb plant extracts were comparable to those of the antiviral drugs, in particular, *O. vulgare* and *R. officinalis* (Anna Rosemary) were more effective than amantadine. Many viruses are capable of inducing cell death, which leads to the lysis of infected cells [Agol, 1998; Connolly, 2000; Levine, 1993], and the addition of *O. vulgare* and *R. officinalis* (Anna Rosemary) extracts to the EV 71-infected Vero cell were proved to be impossible in preventing CPE. Furthermore, *O. vulgare* and *R. officinalis* have been reported to possess anti-HIV activity

[Aruoma *et al.*, 1996] as well as anti-inflammatory effect [Umezu, 2003], but no relevant pure constituent has yet been reported. Therefore, *O. vulgare* and *R. officinalis* (Anna Rosemary) may possess strong antiviral materials against EV 71. Therefore, further studies on the isolation of antiviral compounds from *O. vulgare* and *R. officinalis* (Anna Rosemary) are necessary.

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