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Full Length Research Paper

Anti-enterovirus 71 activity screening of Taiwanese folk medicinal plants and immune modulation of *Ampelopsis brevipedunculata* (Maxim.) Trautv against the virus

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Enterovirus 71 (EV71) infection caused host immune responses and resulted in severe pathogenicity. In this study, a preliminary anti-EV71 activity of 58 Taiwanese folk medicinal plants and further investigations of the anti-EV71 activity and immunomodulatory effect of *Ampelopsis brevipedunculata* (Maxim.) Trautv (ABT) were performed. Of the 58 plant extracts, 37 extracts exhibited anti-EV71 activity and strong activity was demonstrated by six extracts with average 50% inhibitory concentrations (IC_{50}) below 80 µg/ml. The ABT (one of the most effective plants) acetone extract exhibited relatively high anti-EV71 activity with 26.11 µg/ml of average IC_{50}, 3.6% of that of the positive control (ribavirin, 734.52 µg/ml). The anti-EV71 modes of the ABT extract included viral infection prevention, viral inactivation and anti-viral replication. Acidic pH treatments slightly decreased anti-EV71 activity of the extract. The ABT extract significantly increased the interlukin-6 (IL-6) and IL-1β and decreased the IL-8 levels in the EV71-infected cells. In summary, a high rate of the screened plants exhibited anti-EV71 activity and the greatest activity was demonstrated by ABT. High amounts of IL-6 induction in the infected-cells had high correlation with the anti-viral replication of the ABT extract.

Key words: Antienterovirus 71 activity, *Ampelopsis brevipedunculata* (Maxim.) Trautv, immune modulation, medicinal plant.

INTRODUCTION

Enterovirus 71 (EV71) is an important pathogenic virus with single-stranded RNA belonging to the picornavirus family. EV71 infection occurs worldwide and has caused large epidemics in the Asia-Pacific region (Lum et al., 1998; Ho et al., 1999). A great outbreak of EV71 infection occurred in Taiwan in 1998 resulting in 130,000 infection cases (Wu et al., 1999) and 78 child deaths (Liu et al., 2000). Until now, enterovirus infections recur annually and EV71 has been the most common isolate (Kuo, 2006). Manifestations of EV71 infection vary from mild-hand-food-and-mouth disease and herpangina to severe meningitis and cardiopulmonary diseases. EV71 is transmitted from person to person mainly by the faecal-oral route. The virus initially colonizes in the intestines and then spreads to the spinal cord and ascends to the brain (Chen et al., 2007). EV71 in the brainstem induces high amounts of cytokines (IL-1β, IL-6, IL-8 and TNF) for the elimination of the virus. On the other hand, these cytokines pass through the blood-brain barrier to enter

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Abbreviations: ABT, *Ampelopsis brevipedunculata* (Maxim.) Trautv; **IC_{50}, CC_{50},** 50% cytotoxic concentrations; **EV71,** enterovirus

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **RD cell,** human embryonal rhabdomyosarcoma cell; **SI,** selective index; **$TCID_{50}$,** 50% tissue culture infection dose.

the systemic circulation resulting in cardiopulmonary failure and pulmonary edema (Chung et al., 2006; Lin et al., 2002a, 2003; Wang et al., 2006). There is no available anti-EV71 drug for clinical use today. Medicinal plants have been widely used to treat various infections for a long time.

In literatures, *Arctium lappa* L and *Lonicera japonica* were studied for their anti-inflammatory activity (Zhao et al., 2009; Tae et al., 2003) as well as *Allium sativum* L, *Morus alba* and *Polygonum multiflorum* for their anti-bacterial activity (Curtis et al., 2004; Sohn et al., 2004; Zuo et al., 2008). Furthermore, *Isatis indigotica*, *Forsythia suspense, Glycyrriza uralensis*, *Platycodon grandiflorus*, and *Polygonum multiflorum* were found to have anti-SARS-coronavirus and respiratory syncytial virus activity (Lin et al., 2005; Ma et al., 2002). A great anti-EV71 potential is possibly presented in the traditional medicinal plants. *Ampelopsis brevipedunculata* (Maxim.) Trautv (Vitaceae) (ABT) is traditionally distributed in Asia for the treatment of liver disease and inflammation. Furthermore, the ABT root is a food ingredient commonly used in the preparation of meat soup and blended wine in Taiwan. In pharmacological studies, ABT exhibits anti-viral, anti-oxidant and anti-genetic mutation activities as well as liver protection (Sun et al., 1986; Lee and Lin, 1988; Yabe et al., 1998; Wu et al., 2004). The aim of this study was to examine primary anti-EV71 activity of 58 Taiwanese folk medicinal plants and proceed to further investigate anti-EV71 activity and immunomodulatory effect of ABT. Different polarity solvents were used for ABT extraction.

50% cytotoxic concentrations (CC_{50}), 50% inhibitory concentrations (IC_{50}), selective index (SI), pH stability, time-of-addition assay and cytokine levels in the EV71-infected cells were determined.

MATERIALS AND METHODS

Plant extract preparation

On the basis of the traditional uses of the Taiwanese folk medicinal plants, 58 plants were collected from local herbal markets (Taichung, Taiwan). Their abbreviations, parts used in pharmaceuticals and traditional uses are listed in Table 1. The plant extracts were prepared according to Wang and Huang (2005). In brief, 200 ml of extraction solvent (water, 95% ethanol, acetone and ethyl acetate) were added to 30 g of ground specimen followed by stirring for 1 h at room temperature. Subsequently, the mixture was centrifuged at 12,300 g for 15 min at 4°C and the residue was extracted twice more with 200 ml of extraction solvent each time. The supernatants were mixed and concentrated to dryness in a rotary vacuum evaporator at less than 40°C. The plant extracts were dissolved in DMSO (Echo, Miaoli, Taiwan) and diluted with DMEM (Grand Island, Gibco, NY, USA) supplemented with 2% FBS (Gibco, Grand Island, NY, USA).

Cell and viruses

Human embryonal rhabdomyosarcoma (RD) cells were obtained from American Type Culture Collection (ATCC; accession no. CCL-136, Manassas, VA, USA). The cells were cultivated in DMEM supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2. The EV71 standard strain (ATCC-VR784) was purchased from ATCC and the clinical strains (CMUH-527-1, CMUH-V4079 and CMUH-627-14) were obtained from China Medical University Hospital (Taichung, Taiwan). EV71 was propagated in RD cells. Virus titer was determined by cytopathic effect and expressed as 50% tissue culture infection dose ($TCID_{50}$). Virus stocks (10^{5-7} $TCID_{50}$/ml) were stored at -80°C until use.

Cytotoxicity assay

CC_{50} was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Ameresco, Solon, Ohio, USA) method (Garozzo et al., 2000). Briefly, RD cells were seeded onto a 96-well plate (3×10^4 cells/well) and cultivated at 37°C in an atmosphere of 5% CO_2 for 24 h (90% confluence). After washing with PBS, 0.2 ml of 2-fold dilutions of test sample were added and subsequently incubated at 37°C in an atmosphere of 5% CO_2 for 72 h. No test sample contained and ribavirin (Sigma, St Louis, MO, USA) were used as the negative and positive controls, respectively. After washing with PBS, MTT reagent (20 µl, 0.5 mg/ml) was added and kept in the dark at room temperature for 2 h. MTT was removed and 150 µl DMSO were added to dissolve the crystal formazan. The optical density (OD) was measured with a FLUOstar spectrophotometer (BMG Labtech, Offenburg, Germany) at 550 nm.

The CC_{50} for the test sample was defined as the concentration of the sample to reduce the viable cell by 50% relative to the negative control. Three experiments were performed each in triplicates.

Antiviral assay

A neutralization method (Shih et al., 2003) was used for the determination of IC_{50} for 58 plant extracts against EV71. In brief, 90% confluent cell monolayers on a 96-well plate were washed with PBS and 0.2 ml of virus suspensions ($100TCID_{50}$ in DMEM supplemented with 2% FBS) containing 2-fold dilutions of the test sample were added to each well. No test sample contained in the virus-infected and virus-uninfected cells were used as the virus-infected and mock-infected controls, respectively; and ribavirin was used as the positive control. The plate was incubated in an atmosphere of 5% CO_2 for 72 h. After washing with PBS, MTT reagent was added as aforementioned and OD_{550} was measured with a FLUOstar spectrophotometer.

The inhibition rate was calculated by the following formula:

$$\text{Inhibition (\%)} = \frac{[(OD_S)_{vir} - (OD_C)_{vir}]}{[(OD_C)_{mock} - (OD_C)_{vir}]} \times 100\ (\%)$$

Where $(OD_S)_{vir}$, $(OD_C)_{vir}$, and $(OD_C)_{mock}$ are the OD_{550} of the test sample, the virus-infected control and the mock-infected control, respectively. The IC_{50} was calculated by regression analysis of the dose-response curve of inhibition. Three experiments were performed each in triplicates. A plaque reduction assay according to Shih et al. (2003) was used for the determination of IC_{50} for four solvent ABT extracts against EV71. In brief, 90% confluent cell monolayers on a 6-well plate (6×10^5 cells/well) were washed with PBS and virus suspensions (100 PFU/well) containing 2-fold dilutions of the extract were added. No extract (0%) and ribavirin were used as the negative and positive controls, respectively. After 1 h adsorption at 4°C, the culture supernatants were removed and an overlay medium containing the same concentrations of the extract were overlaid. The plate was incubated at 37°C for 72 h.

Table 1. The reference of scientific names, abbreviations, parts used in pharmaceuticals and traditional uses of the 58 Taiwanese folk medicinal plants.

Scientific name	Abbreviation	Part used in pharmaceuticals	Traditional use
Abutilon indicum (L.) Sweet	AIS	Whole plant [a]	Cold
Achyranthes aspera	AA	Whole plant	Cold
Allium sativum L.	ASL	Tuber	Bacterium
Amomum villosum Lour	AVL	Fruit	Vomit
Ampelopsis brevipedunculata (Maxim.) Trautv	ABT	Stem, root	Inflammation
Arctium lappa L.	ALL	Seed	Inflammation
Armeniaca mume Sieb.	AMS	Fruit	Cough
Astragalus membranaceus Bunge	AMB	Stem, root	Cold
Catharanthus roseus (L.)G. Don	CRGD	Whole plant	Inflammation
Cayratia japonica (Thunb.) Gagnep	CJG	Whole plant	Inflammation
Cibotium barometz (L.) J. Sm	CB	Stem, root	Muscle ache
Citrus reticulate Blanco	CRB	Fruit peel	Cough
Coptis chinensis Franch	CCF	Stem, root	Inflammation
Dysosma pleiantha (Hance) Woodson	DPW	Stem, root	Cold
Echinops grijsii Hance	EGH	Stem, root	Inflammation
Elaeagnus oldhamii Maxim	EOM	Root	Cold
Euchresta formosana (Hayata) Ohwi	EFO	Stem, root	Inflammation
Fagopyrum esculentum Moench	FEM	Whole plant with flower	Fever
Ficus pumila L.	FPL	Stem, root	Muscle ache
Forsythia suspensa (Thunb.) Vahl	FSV	Fruit	Virus
Glycine tomentella Hayata	GTH	Root	Muscle ache
Glycyrrhiza Uralensis Fisch.G. Glabra L.	GUFGL	Stem, root	Cough
Hemerocallis fulva Linn	HFL	Stem, root	Inflammation
Hibiscus rosa-sinensis L.	HRSL	Flower	Cold
Hibiscus Sabdariffa Linn.	HSL	Flower	Fever
Impatiens balsamina L	IBL	Whole plant	Inflammation
Imperata cylindrica (Linn.) Beauv	ICB	Root	Inflammation
Isatis indigotica Fortune	IIFL	Leaf	Fever
Isatis indigotica Fortune	IIFS	Stem	Virus
Laminaria japonica Aresch	LJA	Leaf	Cough
Ledebouriella divaricata (Turcz.) Hiroe	LDH	Root	Cold
Lemmaphyllum microphyllum Presl	LMP	Whole plant	Virus
Lilium formosanum Wall	LFW	Stem, root	Cough
Lonicera japonica Thunb	LJT	Flower	Inflammation
Melastoma candidum D. Don	MCDD	Stem, root	Inflammation
Mentha arvensis var. *piperascens*	MAP	Leaf	Cold
Moghania macrophylla (Willd.) O. Kuntze	MMK	Root	Muscle ache
Morus alba L	MAL	Leaf	Cold
Oxalis corniculata Linn.	OCL	Stem, leaf	Virus
Phragmites communis (L.) Trin	PCT	Root	Fever
Platycodon grandiflorus A.DC	PGAD	Stem, root	Cold
Polygonum chinense Linn	PCL	Root	Cold
Polygonum multiflorum Thunb.	PMT	Root	Cold
Prunus amygdalus	PA	Seed	Cough
Psidium Guajava Linn.	PGL	Leaf	Bacterium
Rheum officinale Baill	ROB	Stem, root	Virus
Rhinacanthus nasutus (L.) Kurz.	RNK	Leaf	Bacterium
Schisandra chinensis (Turcz.) Baill.	SCB	Fruit	Cold
Schizonepeta tenuifolia (Benth.) Briquet	STB	Stem	Cold

Table 1. Contd.

Scutellaria baicalensis Georgi	SBG	Root	Cold
Spatholobi Caulis	SC	Whole plant	Cold
Toona sinensis (A.Juss.) Roem.	TSR	Stem, leaf	Inflammation
Vaccaria segetalis Garcke	VSG	Seed	Inflammation
Vernonia cinerea (L.) Less	VCL	Whole plant	Cold
Vicia radiatus L.	VRL	Seed	Inflammation
Vitis thunbergii S. et Z. var. adstricta (Hance) Gagnep	VTAG	Root	Muscle ache
Vitex rotundifolia Linn. F.	VRLF	Stem, root	Cold
Wikstroemia indica (L.) C.A.Mey	WICAM	Stem, root	Inflammation

a) Roots, stems and leaves were involved.

The overlay medium was removed, fixed with 10% formaldehyde for 30 min and stained with 1% crystal violet. Virus plaques that had formed on the plate were enumerated.

Inhibition rate was calculated by the ratio of the reduction of the plaques of the extract to the plaques of the control. The IC_{50} was calculated by regression analysis of the dose-response curve of inhibition. Three experiments were performed each in duplicate.

pH stability

pH stability for the ABT acetone extract on the anti-EV71 ATCC-VR784 activity was determined. The extract (500 mg/ml) mixed with different pH of buffers (Clark and Lubs buffer: pH 2 and 8, McIlvaine buffer: pH 3 to 7) at a ratio of 1:10 (v/v) and stood at room temperature for 2 h (Dawson et al., 1978). Subsequently, the pH treated extracts were subjected to determine inhibitory effect against EV71 using the plaque reduction assay. Three experiments were performed each in duplicate.

Time-of-addition assay

The plaque reduction assay was used for the time-of-addition testing for the extract against EV71. EV71 ATCC-VR784 suspensions (100 PFU/well) were absorbed onto 90% confluent cell monolayers at 4°C for 1 h. Different concentrations (0, 20, 40, 62.5 and 100 µg/ml) of the extracts were added to culture cells at various times of pre-infection (-2, -1 h), co-infection (0 h), or post-infection (1 to 8 h) and were then removed before viral infection and after viral absorption for the pre-infection and co-infection treatments, respectively; whereas the extract was persisted in the culture for the post-infection treatment. No extract contained (0%) was used as the control. Inhibition rates of the treatments were determined as described above. Three experiments were performed each in duplicate.

Cytokine measurements

Cytokine (TNF-α, IL-1β, IL-2, IL-6 and IL-8) levels in the EV71 ATCC-VR784-infected cell culture supernatants were determined. The EV71 suspensions (100 PFU/well) containing the ABT acetone extract (0, 5, 10, 20, 40 and 80 µg/ml) were added to RD cell monolayers on a 24-well plate (1.5×10^5 cells/well). No virus contained with the same concentrations of the extract were used as the mock-infected controls. The plate was incubated in an atmosphere of 5% CO_2 at 37°C for 48 h. The culture supernatants were collected and centrifuged at 7,425 g for 10 min at 4°C. The cytokine levels in the supernatants were measured by a commercial ELISA kit (R&D system, Minneapolis, MN, USA) according to the manufacturer's instructions. Three experiments were performed.

Statistical analysis

Data for the cytokine levels were subjected to analysis of variance with Dunnett's test to identify significant differences from the controls ($P < 0.01$).

RESULTS

Anti-EV71 activity of 58 Taiwanese folk medicinal plants

As shown in Table 2 of the 58 extracts, 37 extracts exhibited anti-EV71 activity with six extracts to inhibit four EV71 strains (Group 1), three extracts to inhibit three EV71 strains (Group 2), eight extracts to inhibit two EV71 strains (Group 3), and the remaining 20 extracts to inhibit one strain (Group 4). The strongest anti-EV71 activity was demonstrated by the Group 1 (ABT, EFO, FPL, LMP, MCDD and SC) extracts, for which all four test EV71 strains were inhibited with average IC_{50} below 80 µg/ml. Of these values from lowest to highest were the ABT, FPL, EFO, MCDD, SC and LMP extracts. The average IC_{50} for the Group 2 (LJA, PCL and LJT) extracts ranged from 51.48 to 104.07 µg/ml. The values for six (TSR, AVL, LDH, ROB, PGL and GTH) extracts of Group 3 were lower than 100 µg/ml. 55% (11/20) average IC_{50} of the Group 4 extracts were lower than 50 µg/ml. Notably, all the average IC_{50} values for these 37 active extracts were lower than that of the positive control (ribavirin, 717.98 µg/ml). Selective index (SI) is an index for the evaluation of anti-viral agent's activity and cytotoxicity, higher SI indicating higher activity with lower cytotoxicity. As shown in Table 2, 76% (28 of 37) SI of the active extracts were higher than or equal to the positive control (ribavirin, 4.47). Of the average SI for the Group 1 extracts, the FPL and SC extracts had higher SI (13.35

Table 2. The IC_{50} and SI for the 58 plant 59% ethanol extracts against EV71 in which the neutralization method was used.

Plant extract	CC_{50} (μg/ml)	IC_{50} (μg/ml)				Average IC_{50} (μg/ml)	Average SI[b]
		CMUH-527-1	CMUH-V4079	CMUH-627-14	ATCC-VR784		
Ribavirin	2593.37 ± 183.22	1000.00 ± 0.00	509.68± 11.86	1024.08 ± 41.71	338.15 ± 3.68	717.98	4.47
Group 1							
ABT	193.82 ± 8.9	43.16 ± 0.52	31.73± 1.48	22.26 ± 4.51	31.63 ± 2.12	32.20	6.36
EFO	157.59 ± 1.27	61.41 ± 0.61	53.68± 0.94	45.30 ± 2.47	49.47 ± 2.08	52.47	3.19
FPL	639.80 ± 19.8	56.94 ± 0.04	43.63± 0.71	40.30 ± 0.13	55.12 ± 1.74	49.00	13.35
LMP	410.49 ± 7.96	96.05 ± 0.55	91.97± 3.13	53.99 ± 0.58	62.87 ± 0.09	76.22	5.72
MCDD	278.84 ± 4.65	49.82 ± 1.55	48.46± 1.85	60.83 ± 0.95	53.64 ± 0.81	53.19	5.28
SC	576.36 ± 9.36	107.80 ± 3.88	75.75± 2.09	40.87 ± 1.32	61.85 ± 0.98	71.57	9.10
Group 2							
LJA	256.75 ± 2.86	79.85 ± 1.98	59.72± 3.40	14.88 ± 0.13	_	51.48	8.26
LJT	399.02 ± 21.8	190.76 ± 3.91	73.25± 3.78	48.19 ± 3.05	_	104.07	5.27
PCL	1484.77 ± 14.1	79.32 ± 0.83	119.32 ± 0.64	_	55.60 ± 0.18	84.75	19.29
Group 3							
AVL	142.28 ± 0.53	9.97 ± 0.31	_	38.44 ± 1.76	_	24.21	8.99
EOM	306.61 ± 16.4	_[a]	105.21 ± 2.91	227.51 ± 2.34	_	166.36	2.13
GTH	403.76 ± 14.9	_	72.89± 0.78	118.07 ± 4.86	_	95.48	4.48
LDH	127.74 ± 1.68	37.86 ± 1.18	_	30.28 ± 0.17	_	34.07	3.79
PCT	2134.50 ± 47.1	250.00 ± 15.6	_	338.42 ± 8.49	_	294.21	7.42
PGL	158.95 ± 6.10	49.91 ± 2.24	_	68.54 ± 1.84	_	59.23	2.75
ROB	356.90 ± 19.0	_	32.29± 0.93	52.78 ± 0.27	_	42.54	8.91
TSR	103.80 ± 6.55	9.17 ± 0.28	_	_	24.13 ± 0.77	16.65	7.81
Group 4							
AIS	400.00 ± 5.42	_	105.83 ± 3.71	_	_	105.83	3.78
AA	1004.45 ± 50.2	_	401.71 ± 21.6	_	_	401.71	2.50
ASL	449.00 ± 12.6	33.47 ± 1.91	_	_	_	33.47	13.41
CJG	139.18 ± 1.68	31.25 ± 1.25	_	_	_	31.25	4.45
CRB	817.34 ± 26.9	190.16 ± 5.41	_	_	_	190.16	4.30
FEM	844.15 ± 1.90	31.25 ± 3.89	_	_	_	31.25	27.01
FSV	125.00 ± 8.05	12.90 ± 0.25	_	_	_	12.90	9.69
HRSL	127.69 ± 2.49	52.56 ± 1.09	_	_	_	52.56	2.43
HSL	1420.57 ± 16.6	250.00 ± 16.9	_	_	_	250.00	5.68

Table 2. Contd.

LFW	303.67 ± 19.4	25.54 ± 0.98	_	_	_	25.54	11.89
MMK	286.00 ± 3.77	_	31.25 ± 0.16	_	_	31.25	9.15
OCL	196.28 ± 8.89	52.51 ± 0.53	_	_	_	52.51	3.74
PMT	1415.29 ± 16.5	163.27 ± 4.04	_	_	_	163.27	8.67
PA	1697.50 ± 64.4	69.96 ± 7.57	_	_	_	69.96	24.26
RNK	251.58 ± 11.0	_	49.21 ± 0.07	_	_	49.21	5.11
SCB	261.67 ± 4.99	15.63 ± 0.13	_	_	_	15.63	16.74
STB	200.66 ± 7.05	_	19.85 ± 1.03	_	_	19.85	10.11
VSG	103.61 ± 8.23	12.58 ± 1.37	_	_	_	12.58	8.24
VRL	>2000	83.75 ± 3.46	_	_	_	83.75	>23.88
WICAM	91.26 ± 2.31	43.73 ± 1.37	_	_	_	43.73	2.09
Inactive group							
ALL	_	_	_	_	_		
AMS	_	_	_	_	_		
AMB	_	_	_	_	_		
CRGD	_	_	_	_	_		
CB	_	_	_	_	_		
CCF	_	_	_	_	_		
DPW	_	_	_	_	_		
EGH	_	_	_	_	_		
GUFGL	_	_	_	_	_		
HFL	_	_	_	_	_		
IBL	_	_	_	_	_		
ICB	_	_	_	_	_		
IIFL	_	_	_	_	_		
IIFS	_	_	_	_	_		
MAP	_	_	_	_	_		
MAL	_	_	_	_	_		
PGAD	_	_	_	_	_		
SBG	_	_	_	_	_		
VCL	_	_	_	_	_		
VTAG	_	_	_	_	_		
VRLF	_	_	_	_	_		

a: not detectable below the CC_{50} of the extract. Data presented are the mean ± standard derivation (SD) (n = 3); b. SI = CC_{50}/IC_{50}.

Table 3. IC_{50} and SI for the *A. brevipedunculata* different solvent extract against EV71 in which the plaque reduction assay was used.

Solvent extract	CC_{50} (µg/ml)	IC_{50} (µg/ml)[a]			Average IC_{50} (µg/ml)	Average SI
		CMUH -527-1	CMUH -V4079	ATCC -VR784		
Ribavirin	2593.37 ± 183.22	776.83 ± 4.03	954.82 ± 42.17	471.92± 20.49	734.52	3.70
Water	304.64 ± 16.35	75.04 ± 3.33	54.63 ± 2.93	159.89± 26.35	96.52	3.83
Acetone	128.89 ± 5.50	18.52 ± 0.42	39.98 ± 0.45	19.84± 1.57	26.11	5.56
95% ethanol	193.82 ± 8.9	33.77 ± 0.31	46.91 ± 3.37	66.40± 2.65	49.03	4.26
Ethyl acetate	143.42 ± 3.97	41.71 ± 0.26	45.11 ± 2.51	39.85± 2.57	42.22	3.41

a: values presented are the mean ± SD (n = 3), each value was obtained from three experiments each in triplicates.

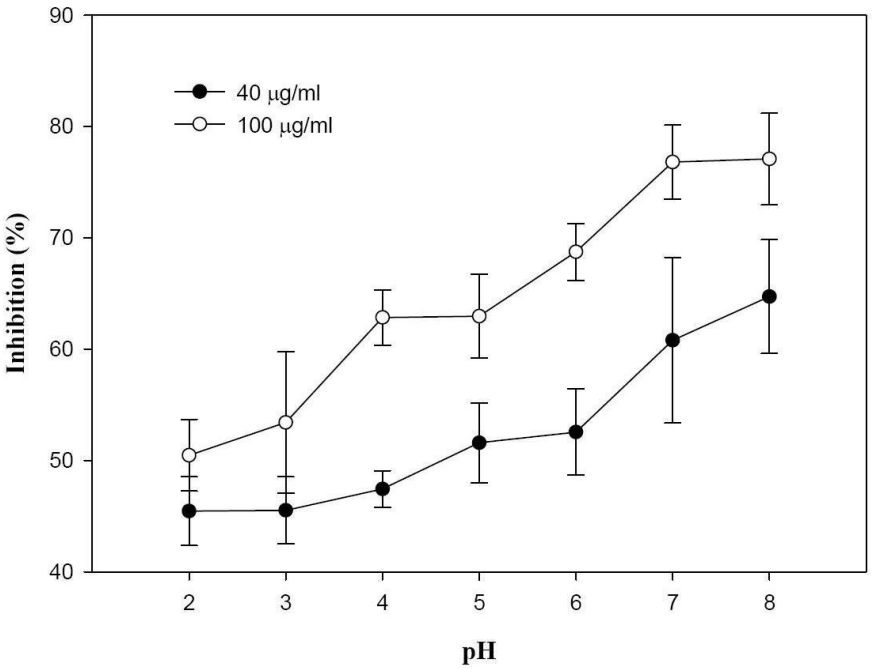

Figure 1. pH stability for the *A. brevipedunculata* acetone extract on the anti-EV71 ATCC-VR784 activity. The extract was treated with different pH buffers and then subjected to determine anti-EV71 activity at 40 and 100 µg/ml of treated doses. Data points presented are the mean ± SD (n = 3).

and 9.10, respectively) and the other 4 extracts ranged from 3.19 to 6.36.

The average SI values for the Group 2, 3, and 4 extracts were in the ranges of 5.27 to 19.26, 2.13 to 8.99 and 2.09 to 27.01, respectively.

Anti-EV71 activity of the ABT extracts

From Table 2, the ABT extract exhibited the strongest anti-EV71 activity among 37 active extracts, therefore, we proceeded to further examine anti-EV71 activity of ABT. The IC_{50} and SI for the different solvent ABT extracts were presented in Table 3. The lowest average IC_{50} (26.11 µg/ml) was demonstrated in the acetone extract, followed by (from lowest to highest) the ethyl acetate (42.22 µg/ml), the 95% ethanol (49.03 µg/ml), and the water (96.52 µg/ml) extracts, for which 3.6, 5.8, 6.7 and 13.1% of the positive control IC_{50}. The average SI for the acetone extract (5.56) was higher than the other extracts (3.41 to 4.26) including ribavirin (3.70). Of these four solvent extracts, the acetone extract had the lowest IC_{50} with the highest SI, therefore, the ABT acetone extract was selected for the following tests.

pH stability for the ABT acetone extract

As shown in Figure 1, anti-EV71 activity of the ABT extract for both 40 and 100 µg/ml of treated doses slightly decreased after acidic pH treatments. Inhibition rates decreased from 60.80 ± 7.43% for the pH 7 treatment at

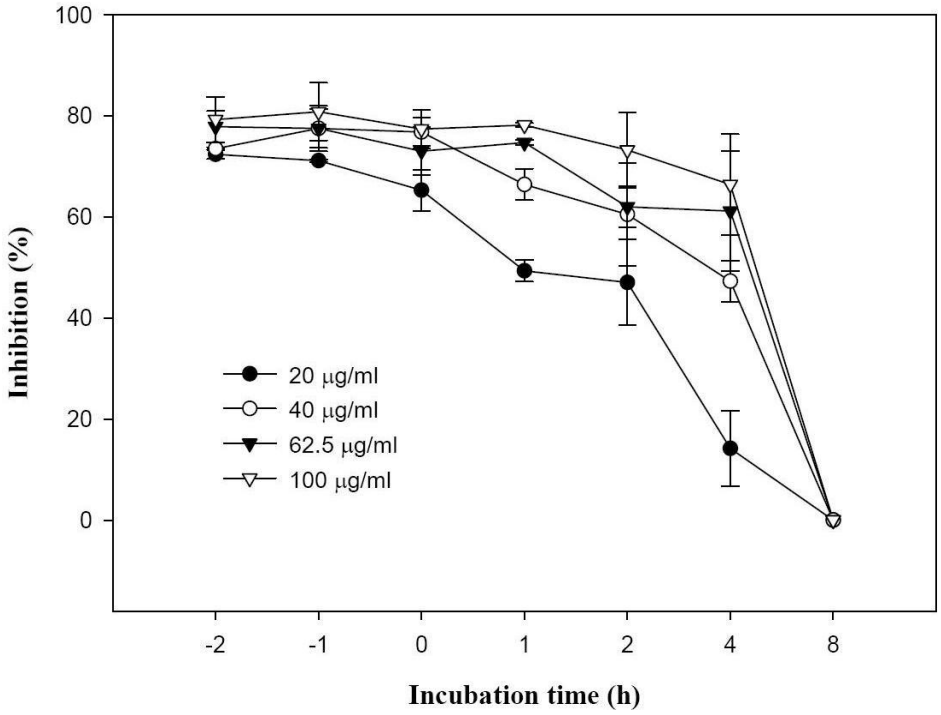

Figure 2. Time-of-addition assay for the *A. brevipedunculata* acetone extract against EV71 ATCC-VR784. Different concentrations (20, 40, 62.5, 100 µg/ml) of the extract were added to the culture cells at various times of pre-infection (-1, -2 h), co-infection (0 h) or post infection (1 to 8 h). Data points presented are the mean ± SD (n = 3).

40 µg/ml of treated dose to 45.43 ± 3.09% for that of the pH 2 treatment.

Time-of-addition assay for the ABT acetone extract

As shown in Figure 2, the inhibition rates for the ABT acetone extract against EV71 ATCC-VR784 increased with increasing treatment doses (20 to 62.5 µg/ml), and those rates maintained at a plateau when the treated dose raised to 100 µg/ml, indicating that 62.5 µg/ml of treated dose was sufficient. The relatively high rates (73.54 ± 0.28 to 80.84 ± 5.71% at 40 to 100 µg/ml of treated doses) were observed for the pretreatments (-2, -1 h) as well as high rates of 76.83 ± 2.79 to 77.45 ± 3.80% and 47.06 ± 8.45 to 78.21 ± 0.40% for those of co-infection (0 h) and post infection (1 to 4 h) treatments, respectively. After 4 h of post-infection treatment, the inhibition rates dramatically decreased and dropped to zero at 8 h post-infection treatment.

Cytokine levels in EV71-infected cells

As shown in Figure 3, the ABT acetone extract increased the IL-6 and IL-1β and decreased the IL-8 levels in the EV71 ATCC-VR784-infected culture supernatants, but the other cytokine (IL-2 and TNF-α) levels were not affected.

The IL-6 level in the culture supernatants significantly increased after the EV71 infection (82.67 ± 24.68 pg/ml) (Figure 3A), and those levels significantly increased with increasing treatment doses (10 to 20 µg/ml) ($P < 0.01$), 218 to 237% of that of the control. When the treated doses (40 to 80 µg/ml) were higher than the IC_{50} (19.84 ± 1.57 µg/ml), the levels decreased due to the diminished viral titer by the extract. In Figure 3B, RD cells could secrete high amounts of IL-8 (1305 ± 111 pg/ml), which increased (1817 ± 57 pg/ml) after the EV71 ATCC-VR784 infection. IL-8 levels in both mock- and EV71-infected culture supernatants significantly decreased with increasing treatment doses ($P < 0.01$,), 27 to 85 and 15 to 62%, respectively, of that of the controls at 5 to 80 µg/ml treatments.

The IL-1β levels in the EV71-infected culture supernatants elevated with increasing treatment doses of 5 to 10 µg/ml (250 to 291%, $P < 0.01$) but declined at 20 to 80 µg/ml of treated doses (Figure 3C).

DISCUSSION

In this study, 64% (37 of 58) Taiwanese folk medicinal plants exhibited anti-EV71 activity in which strong activity was demonstrated by the Group 1 extracts (32.20 to 76.22 µg/ml of IC_{50}) with all four test EV71 strains being inhibited (Table 1). A few related anti-EV71 studies have

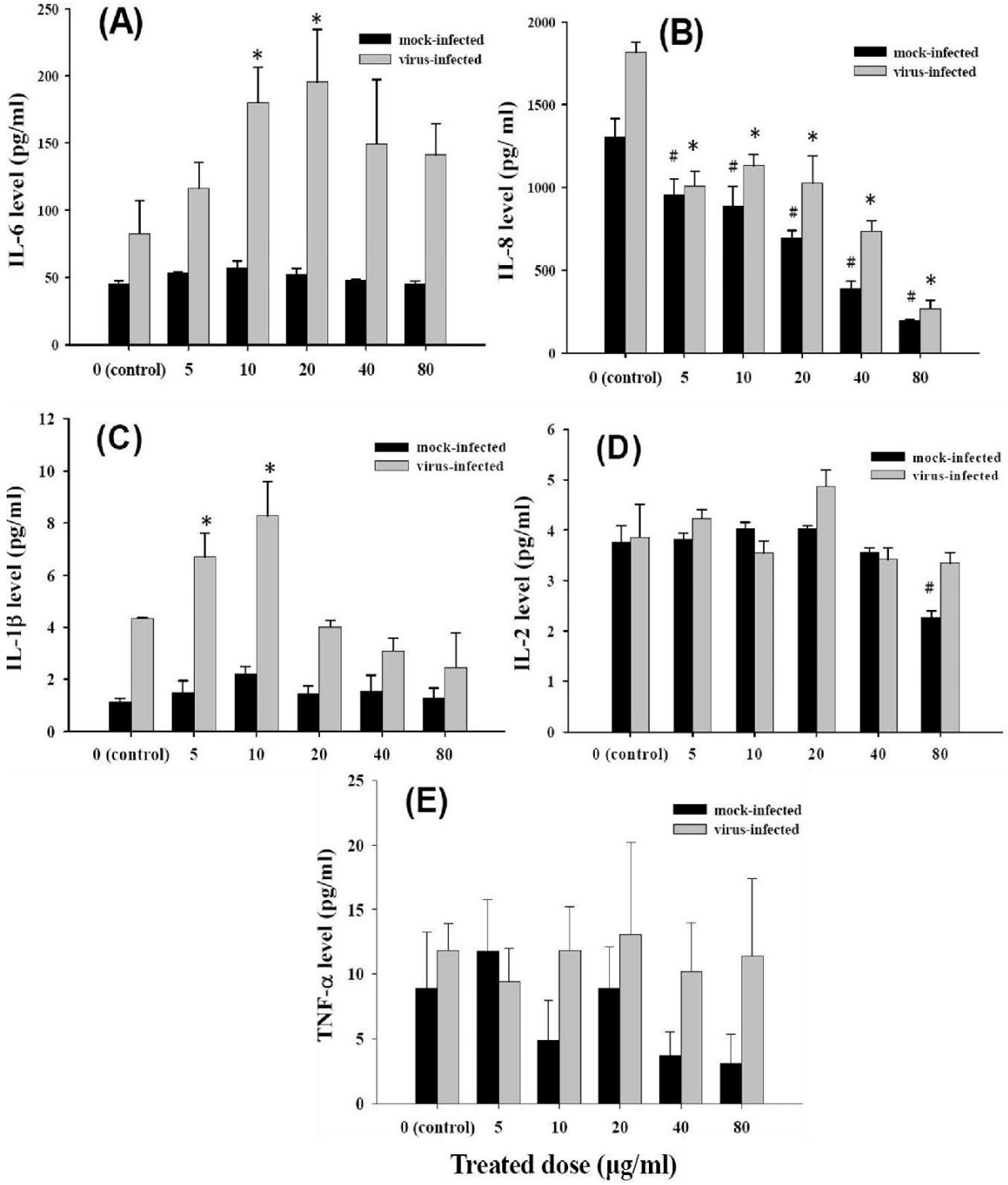

Figure 3. Effect of the *A. brevipedunculata* acetone extract on the cytokine levels of (A) IL-6, (B) IL-8, (C) IL-1β, (D) IL-2, and (E) TNF-α in the EV71 ATCC-VR784-infected and mock-infected culture supernatants. Bars presented are the mean ± SD (n = 3) with the symbols showing the significant difference from the controls at $P < 0.01$.

been reported. The IC_{50} for the *Houttuynia cordata* extract, the *Salvia miltiorrhiza* fractions, and the Sheng-Ma-Ge-Gen-Tang prescription extract against EV71 are 125.92 ± 27.84, 364 ± 205 to 847 ± 457 and 0.21 µg/ml, respectively (Lin et al., 2009a; Wu et al., 2007; Chang et al., 2008). The *Ocimun basillicum* extracts and its constituents (linalool, apigenin and ursolic acid) are reported by their anti-EV71 activity with 198.9 ± 1.8 to 200.2 ± 3.2 and 0.5 ± 0.2 to 42.2 ± 1.8 µg/ml of IC_{50}, respectively (Chiang et al., 2005). The bovine and human

lactoferrins have the IC_{50} of 10.5 ± 2.1 to 24.5 ± 6.2 and 103.3 ± 22.5 to 185.0 ± 37.7 µg/ml, respectively (Lin et al., 2002b). Allophycocyanin from blue-green algae, aloe-emodin from *Rheum palmatum*, and BPROZ-194 (a synthetic compound) are found to have anti-EV71 activity with IC_{50} of 4.68 ± 1.24 µg/ml, 0.14 ± 0.04 to 0.52 ± 0.03 µg/ml, and 0.027 to 1.21 ± 0.11 µM, respectively (Shih et al., 2003, 2004; Lin et al., 2008).

Comparing our results to those references, the IC_{50} for the ABT, FPL, EFO, and MCDD extracts were much lower than the *S. miltiorrhiza* fractions, the *O. basillicum* and *H. cordata* extracts, and human lactoferrin (Lin et al., 2002b, 2009a; Wu et al., 2007; Chiang et al., 2005); similar to bovine lactoferrin (Lin et al., 2002b) and higher than the Sheng-Ma-Ge-Gen-Tang prescription extract and the other pure compounds (Shih et al., 2003, 2004; Chang et al., 2008; Chiang et al., 2005; Lin et al., 2008). From Table 3, the anti-EV71 activity of the moderately polar solvent (ethyl acetate, acetone and 95% ethanol) extracts of ABT was better than that of the polar (water) extract. Moreover, the non-polar (n-hexane) extract of ABT did not show any inhibitory effect against EV71. These consequences indicated that the moderately polar compound may be the main contributor of the anti-EV71 activity of ABT. As reported, the chemical compounds isolated from ABT included resveratrol, benzoic acid derivatives (gallic acid, ethyl gallate, vanillic acid), terpenoids (β-sitosterol, oleanolic acid, lupeol, β-amyrin, betulin) and flavonoids (myricetin, luteolin, catechin, kaempferol) (Tang and Wu, 2003; Hsieh et al., 1998). With exception to terpenoids, most of their polarities were moderate. Notably, kaempferol and luteolin have been reported to possess anti-EV71 activity (Tsai et al., 2011). Thus, we suppose that these moderately polar compounds possibly including kaempferol and luteolin may contribute to the anti-EV71 activity of ABT.

The anti-EV71 activity of the ABT acetone extract decreased with decreasing treatment pH (Figure 1). These results indicated that the active compounds of ABT were unstable in the acidic conditions which might be degraded into inactive forms and lose their anit-EV71 activity. In Figure 2, high inhibition rates were observed in the pretreatments (-2 and -1 h), indicating that the ABT extract has the ability to pre-penetrate the host cells against the following viral infection. Luteolin, kaempferol, ursolic acid, and the Sheng-Ma-Ge-Gen Tang and *H. cordat* extracts also have the similar viral infection prevention effects (Tsai et al., 2011; Chang et al., 2008; Lin et al., 2009a; Chiang et al., 2005). Relatively high inhibition rates of ABT were found in the co-infection treatment (0 h), the extract was possibly able to inactivate the viral titers by direct action. In our supplementary results, the EV71 titers were reduced by 80% when the virus was co-incubated with the extract in an atmosphere of 5% CO_2 at 37°C for 1 h. The similar action mode was also demonstrated in the *S. miltiorrhiza* extract and ursolic acid (Wu et al., 2007; Chiang et al., 2005). When the EV71 enters the host cells (post-infection treatment), the effectiveness of the extract gradually decreased and a dramatic decrease occurred at the 4 to 8 h post-infection treatments. The ABT extract had anti-EV71 replication activity, however, such activity only persisted in the early replication stage.

The anti-EV71 replication effect was also observed in ursolic acid (Chiang et al., 2005). The anti-EV71 modes of the ABT extract included viral infection prevention, viral inactivation and anti-viral replication. The severe pathogenicity of EV71 is mainly caused by the high viral replication rate in the host cells along with the host's immune response cascade. High IL-1β, IL-6 and IL-8 levels are detected in the EV71-infected patients (Lin et al., 2002a, 2003; Wang et al., 2006). IL-1β is able to elicit fever and induce the IL-6 expression to protect host cells (Sehgal, 1990). IL-6 has an ability to destroy human immunodeficiency virus RNA and remarkably suppress hepatitis B virus genome-containing nucleocapsid level (Rogez-Kreuz et al., 2005; Kuo et al., 2009). IL-6 also plays an important role to protect mice from the herpes simplex virus and EV71 infections (Carr and Campbell, 1999; Leblanc et al., 1999; Lin et al., 2009b) as well as increases the severity of chronic autoimmune myocarditis in the coxsackievirus-infected IL-6 deficient mice (Poffenberger et al., 2009). In our results, the ABT extract had strong anti-EV71 replication effect in the early stage and significantly increased the IL-6 levels in the EV71-infected cells (Figure 3A). A possibly high correlation was demonstrated in the high amounts of IL-6 induction in the EV71-infected cells and the anti-viral replication of the extract.

IL-8, a chemokine leads neutrophil infiltration into the infected sites to produce high reactive oxygen species (ROS) levels, causing inflammation and cell damage (Srivastava et al., 1999). The IL-8 expression was enhanced by oxidative stress and inhibited by antioxidants (Deiana et al., 2002). As reported, ABT has anti-oxidant activity against linoleic acid peroxidation and prevention cells from H_2O_2-induced oxidative stress (Wu et al., 2004). In Figure 3B, IL-8 levels in both mock- and EV71-infected RD cells were significantly decreased by the ABT treatments which were mostly contributed by the ABT acetone extract antioxidant activity. Such results indicated that the ABT acetone extract has the ability to alleviate IL-8-induced inflammation. The ABT extract exhibited the best anti-EV71 activity among 58 screening plants with the viral infection prevention, viral inactivation, and anti-viral replication of action modes. Furthermore, the anti-EV71 replication effect of the ABT extract may result from the increasing IL-6 levels in the EV71-infected cells.

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