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In vitro Study of Antiviral Activity of Plant Crude-extracts against the Foot and Mouth Disease Virus

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ABSTRACT

The forty-seven ethanol crude-extracts of 42 plants were studied the antiviral activity against the Foot and Mouth Disease virus (FMDV) type O, local strain KPS/005/2545. The concentration of crude-extracts were 0.2 g/ml in mixed solvents (10% Ethanol in 1% Tween 80®). Each of dissolved crude was two fold diluted with DMEM (Dulbecco's modification of Eagle's medium, Gibco) adding 5% fetal bovine serum for cytotoxicity testing on BHK-21 (Baby Hamster kidney cell line). The preliminary antiviral activity test, each of dissolved crude-extracts was mixed with DMEM containing FMDV at concentration $1\times10^{6.37}$ $TCID_{50}$ (50% Tissue culture infective dose) for 1 hr in CO_2 incubator at 37 °C. The preliminary antiviral crude-extracts were tested the antiviral activity at 20-120 second. The crude-extract of the immature fruits of *Morinda elliptica* L. was the highest anti-FMDV ($1\times10^{3.65}$ $TCID_{50}$) at 0.39 μg/μl concentration. The *Morinda citrifolia* L. crude-extract had the anti-FMDV ($1\times10^{3.35}$ $TCID_{50}$) at 0.19 μg/μl concentration. This indicated that the crude-extracts of the *Morinda elliptica* L. and the *M. citrifolia* L. might be the further study for practical use.

Key words: plant crude-extracts, antiviral, FMDV

INTRODUCTION

The inactivated vaccines from Foot and Mouth Disease virus (FMDV) are not complete success to control the disease (Barteling and Vreeswijk, 1991) since the FMDV is RNA virus which it has continuous antigenic drift (Belsham, 1993). The virucidal drugs have been studied for decreasing the outbreaks, but the slaughter policy should be mandatory. Thus the antiviral substances from plants are in current studies. The cyclic peptide from the leaves of *Melia azedarach* Linn. had anti-replication activity against FMDV (Andrei *et al.,* 1994) and anti-viral reproducing cycle within 1 h against several serotypes of FMDV (Wachsman and Coto, 1995; Wachsman *et al.,* 1998). The antiviral activity of medicinal plants on the other virus had many previous reports such as the crude-extracts from *Acacia catechu* Willd., *Cassia fistula* Linn., *Tamarindus indicus* Linn. and *Imperata cylindrica* Linn. showed the antiviral activity on Ranikhet and or vaccinia virus

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(Dhar *et al.,* 1968; Bhakuni *et al.,* 1971), the water crude-extract of *Punica granatum* Linn. was against the Red virus type I and Cosackie B9 virus (Konowalchuk and Speirs, 1976), and the hot water crude-extract of *Carthamus tinctorius* Linn. was antiviral and anti-tumor activities (Kojima *et al.,* 1980, 1984). Therefore, this study try to search the effective common local plants for anti-FMDV type O which is the most outbreak serotype in Thailand.

MATERIALS AND METHODS

The forty seven ethanol crude-extracts of 42 plant species were tested the cytotoxicity on BHK-21 and bioassayed the antiviral activity against the Foot and Mouth Disease type O (FMDV), local strain KPS/005/2545. The concentration of crude-extracts were 0.2 g/ml of solvent containing 10% EtOH in 1% Tween 80®. Each of dissolved crude-extract was two fold diluted by DMEM (Dulbecco's modification of Eagle's medium, Gibco) adding 5% fetal bovine serum till 4096 dilution for cytotoxicity test. The preliminary antiviral activity screening test (Neutralization test), each of dissolved crude-extract was mixed by DMEM containing FMDV concentration $1\times10^{6.37}$ $TCID_{50}$ for 1 hr in CO_2 incubator at 37 °C. Then each of them was two fold diluted by DMEM and poured each non-cytotoxic dilution into monolayer BHK-21 cells plates. The cytopathic effect (CPE) on BHK-21 cells of each crude-extracts was observed under light microscope after incubation for 24 hr in CO_2 incubator at 37 °C.

The crude-extracts having antiviral activity in preliminary test were immediately two fold diluted after mixing with DMEM containing FMDV concentration $1\times10^{6.37}$ $TCID_{50}$ and poured each non-cytotoxic dilution on monolayer of BHK-21 cells which it took 20-120 second. The control groups were the mixed with solvents (10% EtOH in 1% Tween 80®) adding virus as positive control and non-adding virus as negative control. The antiviral activity tests were two replicated bioassays.

RESULTS

The cytotoxic dilution of mixed solvents (10% ethanol in 1% Tween 80®) was the 16 dilution. The cytotoxic dilution of crude-extracts on BHK-21 cells were the 16 to 2048 dilutions which their concentrations were 12.5 - 0.097 μg/μl. (Table 1). The non-cytotoxic dilution of these crude-extracts were the succeeded two fold dilution; 64 to 4096, respectively.

The preliminary antiviral activity screening of 42 plant species, the activity after mixing the virus with crude-extract for 1 hr in CO_2 incubator at 37 °C, the crude-extracts of 24 plants showed the antiviral activity (Table 1). The high to low antiviral activity were the following crude-extracts; the *Phaseolus atropurpureus* Moc et Ses. ex De (leaves and stems), the *Morinda citrifolia* L. (immature fruits), the *Citrus reticulata* Blanco. (fruits), the *Carthamus tinctorius* L. (flowers), the *Lindenbergia philippensis* Benth. (leaves and stems), the *Boerhavia erecta* L. (leaves and stem), the *Veronia cinerea* L. Less (whole plant), the *Morinda elliptica* L. (immature fruits), the *Azadirachta indica J.v.s.v.* (leaves), the *Cayratia trifolia* L. (leaves and stems), the *Acanthus ebracteatus* V. (leaves), the *Tagetes erecta* L. (flowers), the *Nicotiana tabacum* L. (dry leaves), the *Eclipta prostata* L. (whole plant), the *Cassia fistura* L. (fruits), the *Boesenbergia rotunda* L. (rhizomes), the *Dioscorea hispida* D. (tuber), the *Elaeocarpus hygrophilus* Kurz (fruits), the *Tribulus terrestris* L. (leaves and stems), the *Curcuma zedoaria* R. (rhizomes), the *Eugenia cumini* Druce (leaves), the *Coccinia grandis* L. (leaves and stems), the *Amaranthus viridis* L. (leaves and stems) and *Malachra capitata* L. (leaves and stems).

Table 1 The cytotoxic dilution on BHK-21 cells (X) and anti-virus (-) or non anti-virus FMD (+) dilution after mixing the plant crude-extracts with FMDV for 1 hr.

Serial No.	Scientific name of plants (Local name)	Plant part	Cytotoxic (X) and CPE positive (+) or CPE negative (-) dilution on BHK-21 cells of plant crude-extracts mixing with FMDV								
		Dilution (Conc., µg/µl)	32 (6.25)	64 (3.12)	128 (1.56)	256 (0.78)	512 (0.39)	1024 (0.195)	2048 (0.097)	4096 (0.048)	8192 (0.024)
		FMDV conc. (particles/100 µl)	$10^{4.85}$ 71,759	$10^{4.55}$ 35,879	$10^{4.25}$ 17,939	$10^{3.95}$ 8,969	$10^{3.65}$ 4,484	$10^{3.35}$ 2,242	$10^{3.05}$ 1,121	$10^{2.74}$ 560	$10^{2.44}$ 280
	Positive control (solvent adding virus)		+	+	+	+	+	+	+	+	+
	Negative control (solvent non-adding virus)		-	-	-	-	-	-	-	-	-
19	*Acanthus ebracteatus* V. (Ngueak-pla-moa-dok-khaao)	L	+	+	+	+	+	+	-	-	-
46	*Allium cepa* L. (Hom-hua-yai)	T	+	+	+	+	+	+	+	+	+
75	*Amaranthus viridis* L (Phak-khom-had)	L St	X	X	X	+	+	+	+	+	-
49	*Andrographis paniculata* Wall ex Nees (Faa-tha-lai-Chorn)	L St	X	X	X	X	X	+	+	+	+
16	*Azadirachta indica J.v.s.v.* (Sa-dao)	L	X	X	X	+	+	-	-	-	-
15	*Barleria lupulina* Lindl. (Sa-led-phang-phon-mia , Pha-yaa-yo)	L Br St	X	X	X	+	+	+	+	+	+
29	*Barleria lupulina* Lindl. (Sa-led-phang-phon-puu)	L Br St	X	X	X	X	+	+	+	+	+
65	*Bidens pilosa* L. (Puen-nok-sai)	L St	X	X	X	X	X	+	+	+	+
60	*Boerha erecta* L. (Phak-khom-hin)	L St	X	X	X	X	-	-	-	-	-
33	*Boesenbergia rotunda* L. (Kra-chaai)	Rh	X	X	X	+	+	+	+	-	-
30	*Carthamus tinctorius L.* (Kham-phoi)	Fl	X	X	X	+	-	-	-	-	-
32	*Cassia fistura* L. (Khuun)	Fr	X	X	X	X	X	X	X	-	-
71	*Cayratia trifolia L.* (Thao-khan)	L St	X	X	X	X	+	-	-	-	-
44.1	*Citrus reticulata* Blanco. (Som-khiea-waan)	P Fr	X	X	X	+	+	+	+	+	+
44.2	*Citrus reticulata* Blanco. (Som-khiea-waan)	P Fr	X	+	+	-	-	-	-	-	-
50	*Coccinia grandis* L. (Tam-lueng)	L St	X	X	X	+	+	+	+	+	-
36	*Curcuma zedoaria* R. (Kha-min-oi)	Rh	X	X	X	X	+	+	+	+	-
38	*Dioscorea hispida* D. (Kloi)	Tu	X	X	X	+	+	+	+	-	-
11	*Eclipta prostata* L. (Ka-meng-tua-mia)	R L St	X	X	X	X	X	X	+	-	-
43	*Elaeocarpus hygrophilus* Kurz (Ma-kok-nam)	Fr	X	X	X	X	X	X	X	-	-
37	*Eugenia cumini* Druce (Waa)	L	X	X	X	X	X	X	+	+	-
82	*Croton bonplandianus* Baill. (Pao-thung)	L St Fl	X	X	X	X	X	X	+	+	+
21	*Imperata cylindrica* L. (Yaa-kha)	L	X	X	X	X	X	+	+	+	+
42	*Lindenbergia philippensis* Benth. (Yaa-nam-dap-phai.)	L & St	X	X	X	+	-	-	-	-	-
26	*Lonicera japonica* Thonb. (Saai-nam-phueng)	L	X	X	X	X	X	+	+	+	+
7.1	*Luffa cylindrica* Roem. (Buab-klom)	Mat. Fr.	+	+	+	+	+	+	+	+	+
7.2	*Luffa cylindrica* Roem. (Buab-klom)	Imm Fr	X	+	+	+	+	+	+	+	+
83	*Malachra capitata* L.	St L	X	X	X	+	+	+	+	+	-
9	*Melia azedarach* L. (Lian)	L	X	X	X	X	+	+	+	+	+
10.1	*Momordica charantia* L. (Ma-ra-khee-nok)	Mat. Fr	X	X	X	X	X	+	+	+	+
10.2	*Momordica charantia* L. (Ma-ra-khee-nok)	Imm. Fr	X	X	X	X	X	+	+	+	+
5.1	*Morinda citrifolia* L. (Yo-baan)	L	X	X	X	X	+	+	+	+	+
5.2	*Morinda citrifolia* L. (Yo-baan)	Imm. Fr	X	X	X	X	X	-	-	-	-
12.1	*Morinda Elliptica* L. (Yo-paa)	Imm. Fr	X	X	X	-	-	-	-	-	-
12.2	*Morinda Elliptica* L. (Yo-paa)	L	X	X	X	X	X	X	+	+	+
47	*Nicotiana tabacum* L. (Yaa-suup)	D L	X	X	X	X	+	+	-	-	-
61	*Phaseolus atropurpureus* Moc et Ses. ex De (Thua-ser-ra-too)	L St	X	-	-	-	-	-	-	-	-

78	*Phaseolus lothyroides* L.f. (Thua-phee)	L St	X	X	X	X	X	+	+	+	+
41	*Phyllanthus pulcher* W.E.M.A. (Waan-thoranee-saan)	L	X	X	X	X	+	+	+	+	+
28	*Rhoeo discolor* Hance (Waan-kap-hoi-yai)	L	X	X	X	X	X	+	+	+	+
76	*Richardia brasiliensis* G. (Kra-dum-bai)	L St	X	X	X	X	+	+	+	+	+
69	*Ruellia tuberosa* L. (Toi-ting)	L St	X	X	X	X	+	+	+	+	+
31	*Sesbania grandiflora* D. (Khae-khaao)	Fl	X	X	+	+	+	+	+	+	+
72	*Sida subcordata* Span (Yaa-khat-Luang)	L St	X	X	X	X	+	+	+	+	+
34	*Tagetes erecta* L. (Daao-rueang)	Fl	X	X	X	X	+	+	-	-	-
55	*Tribulus terrestris* L. (Kok-kra-suun)	L St	X	X	X	X	X	+	+	-	-
73	*Veronia cinerea* L. Less (Mo-noi)	L St Fl	X	X	X	+	-	-	-	-	-

X, Cytotoxic dilution of two replications on BHK-21 cells

+, CPE positive of two replications; Non anti-FMDV type O

-, CPE negative of two replications; Anti-FMDV type O

Br, Branches; DL, Dry leaves; Fl, Flower; Fr, Fruits; L, Leaves; Imm, Immature; Mat, Mature; P, Peel; R, Roots; Rh, Rhizomes; St, Stem; T, Tuber.

The thirteen plant crude-extracts showed the antiviral activity at 20-120 second after mixing with FMDV (Table 2). The crude-extracts of the immature fruits of *Morinda elliptica* L. was inhibited the highest FMDV concentratioin ($1\times10^{3.65}$ $TCID_{50}$) at 0.39 μg/μl of crude-extracts (512 dilution). The crude-extract of *Morinda citrifolia* L. inhibited the FMDV concentration $1\times10^{3.35}$ $TCID_{50}$ at 0.19 μg/μl of crude-extracts (1024 dilution). The concentration 0.024 μg/μl (8192 dilution) of *Amaranthus viridis* L. leaves and stems inhibited the FMDV concentration $1X10^{2.44}$ $TCID_{50}$. The crude-extract concentration 0.012 μg/μl (16384 dilution) inhibited the FMDV concentration $1\times10^{2.14}$ $TCID_{50}$ were as follows; the fruits of the *Boesenbergia rotunda* L. rhizomes, the *Carthamus tinctorius* L. flowers, the *Citrus reticulata* Blanco., and the *Elaeocarpus hygrophilus* Kurz. The *Nicotiana tabacum* L. dry leaves crude-extract inhibited the FMDV concentration $1\times10^{1.84}$ $TCID_{50}$ at 0.006 μg/μl of crude-extracts (32768 dilution). The lowest antiviral activity of crude-extracts were as follows; the *Lindenbergia philippensis* Benth. leaves and stems, the leaves and stems of the *Phaseolus atropurpureus* Moc et Ses. ex De, the *Tagetes erecta* L. flowers, the *Tribulus terrestris* L. leaves and stems, and the whole plant of *Veronia cinerea* L. Less. They inhibited the FMDV concentration $1\times10^{1.54}$ $TCID_{50}$ at 0.003 μg/μl of crude-extracts (65536 dilution).

DISCUSSION

The preliminary antiviral activity screening of crude-extracts after mixing with FMD virus for 1 hr exhibited the crude-extracts having the antiviral activity for further selection the high effective antiviral plants. The high effective antiviral activity should be the most rapid action and inhibition the large amount of virus. Therefore, this experiment was immediately diluted and poured the non-cytotoxic dilution of each crude-extract on the BHK-21 cells after mixing the crude-extract and virus. They took 20-120 second for making 16 dilution. In this short exposure period, the crude-extracts of the immature fruits of *Morinda elliptica* L. showed the highest FMDV concentration inhibition ($1\times10^{3.65}$ $TCID_{50}$) with 0.39 μg/μl of crude-extracts (Table 2). The root extract of *Morinda elliptica* L. has been found the methylanthraquinone having the antivirus HIV (Ali *et al.,* 2000). It was not done in this experiment because it might be not practical to use the crude-extract from the roots of tree. The more common plant of the genus *Morinda* in Thailand*;* the *Morinda citrifolia* L. also inhibited the FMDV concentration $1\times10^{3.35}$ $TCID_{50}$ at 0.19 μg/μl of crude-extracts (1024 dilution) (Table 2). This plant has also had the anti- HIV-1 reverse transcriptase enzyme (Tan *et al.,* 1991).

The lower inhibition the FMDV concentration ($1\times10^{2.44}$ $TCID_{50}$) was the crude-

extract from leaves and stems of *Amaranthus viridis* L. with 0.024 µg/µl concentration (8192 dilution). It has antiviral proteins against tobacco mosaic virus (Kwon *et al*., 1997). The close related species, *Amaranthus spinosus* L. has antiviral activity against the polio and parvo virus (Kudi and Myint, 1999). It is being study for practical use of these weeds.

The crude-extract of the *Boesenbergia rotunda* L. rhizomes, the *Carthamus tinctorius* L. flowers, the *Citrus reticulata* Blanco. fruits and the *Elaeocarpus hygrophilus* Kurz fruits, inhibited the FMDV concentration $1\times10^{2.14}$ $TCID_{50}$ with 0.012 µg/µl of crude-extract (16384 dilution). These plants were also the common plants in Thailand and having the antiviral activity. The crude-extract of the *Boesenbergia rotunda* L. rhizomes has inhibited dengue 2 virus NS3 protease (Kiat *et al*., 2006). The flavonontriglycoside substances from the *Citrus*

Table 2 The cytotoxic dilution on BHK-21 cells (X) and anti-virus (-) or non anti-virus FMD (+) dilutions after mixing the plant crude-extracts with FMDV for 20-120 sec.

Serial No.	Scientific name (Local plant name)	Plant part	Cytotoxic (X) and CPE positive (+) or CPE negative (-) dilutions on BHK-21 cells of plant crude-extracts mixing with FMDV							
		Crude dilution (Conc., µg/µl)	512 (0.39)	1024 (0.195)	2048 (0.097)	4096 (0.048)	8192 (0.024)	16384 (0.012)	32768 (0.006)	65536 (0.003)
		FMDV conc. (particles/100 µl)	$10^{3.65}$ (4,484)	$10^{3.35}$ (2,242)	$10^{3.05}$ (1,121)	$10^{2.74}$ (560)	$10^{2.44}$ (280)	$10^{2.14}$ (140)	$10^{1.84}$ (70)	$10^{1.54}$ (35)
	Positive control (solvent adding virus)		+	+	+	+	+	+	+	+
	Negative control (solvent non-adding virus)		-	-	-	-	-	-	-	-
19	*Acanthus ebracteatus* V. (Ngueak-pla-moa-dok-khaao)	L	+	+	+	+	+	+	+	+
75	*Amaranthus viridis* L. (Phak-khom-had)	L St	+	+	+	+	-	-	-	-
16	*Azadirachta indica* J.v.s.v. (Sa-dao)	L	+	+	+	+	+	+	+	+
60	*Boerhavia erecta* L. (Phak-khom-hin)	L St	+	+	+	+	+	+	+	+
33	*Boesenbergia rotunda* L. (Kra-chaai)	Rh	+	+	+	+	+	-	-	-
30	*Carthamus tinctorius* L. (Kham-phoi)	Fl	+	+	+	+	+	-	-	-
32	*Cassia fistura* L. (Khuun)	Fr	X	X	X	+	+	+	+	+
71	*Cayratia trifolia L.* (Thao-khan)	L St	+	+	+	+	+	+	+	+
44.2	*Citrus reticulata* Blanco. (Som-khiea-waan)	Fr	+	+	+	+	+	-	-	-
50	*Coccinia grandis* L. (Tam-lueng)	L St	+	+	+	+	+	+	+	+
36	*Curcuma zedoaria* R. (Kha-min-oi)	Rh	+	+	+	+	+	+	+	+
38	*Dioscorea hispida* D. (Koi)	T	+	+	+	+	+	+	+	+
11	*Eclipta prostata* L. (Ka-meng-tua-mia)	R L St	X	X	+	+	+	+	+	+
43	*Elaeocarpus hygrophilus* Kurz (Ma-kok-nam)	Fr	X	X	X	+	+	-	-	-
37	*Eugenia cumini* Druce (Waa)	L	X	X	+	+	+	+	+	+
42	*Lindenbergia philippensis* Benth. (Yaa-nam-dap-phai)	L & St	+	+	+	+	+	+	+	-
83	*Malachra capitata* L.	St L Fl	+	+	+	+	+	+	+	+
5.2	*Morinda citrifolia* L. (Yo-baan)	Imm Fr	X	-	-	-	-	-	-	-
12.1	*Morinda elliptica* L. (Yo-paa)	Imm. Fr	-	-	-	-	-	-	-	-
47	*Nicotiana tabacum* L. (Yaa-suup)	D L	+	+	+	+	+	+	-	-
61	*Phaseolus atropurpureus* Moc et Sesse ex De (Thua-ser-ra-too)	L St	+	+	+	+	+	+	+	-
34	*Tagetes erecta* L. (Daao-rueang)	Fl	+	+	+	+	+	+	+	-
55	*Tribulus terrestris* L. (Khok-kra-suun)	L St	X	+	+	+	+	+	+	-
73	*Veronia cinerea* L. Less (Mo-noi)	L St Fl	+	+	+	+	+	+	+	-

+, CPE positive of two replications, Non anti-FMDV type O

-, CPE negative of two replications, Anti-FMDV type O

X, Cytotoxic dilution of two replications on BHK-21 cells

Br, Branches; DL, Dry leaves; Fl, Flower; Fr, Fruits; L, Leaves; Imm, Immature; Mat, Mature; P, Peel; R, Roots; Rh, Rhizomes; St, Stem; T, Tuber

reticulata Blanco. fruits has inhibited the influenza A virus (Kim *et al.,* 2001).

The crude-extract of the *Lindenbergia philippensis* Benth. (leaves and stems), the *Nicotiana tabacum* L. (dry leaves), the *Phaseolus atropurpureus* Moc et Ses. ex De (leaves and stems), the *Tagetes erecta* L. (flowers), the *Veronia cinerea* L. Less (whole plant), and the *Tribulus terrestris* L. (leaves and stems) were the lowest inhibition FMDV concentrations after mixing with virus for 2-20 second ($1\times10^{1.84}$ $TCID_{50}$ and $1\times10^{1.54}$ $TCID_{50}$) but they were higher antiviral activity after mixing with the virus for 1 hr (1×10 (Table 1). They might have slow antiviral action. Some of them, the *Lindenbergia philippensis* Benth., the *Tribulus terrestris* L., the *Phaseolus atropurpureus* Moc et Sesse ex De and the *Veronia cinerea* L. Less are the weeds and no previous antiviral reports. They will be further study for the practical use.

CONCLUSION

The high effective antiviral activity should be the most rapid action against the large amount of virus. Thus this rapid action screening study, 20-120 second after mixing the crude-extract and virus, the crude-extracts of the immature fruits of *Morinda elliptica* L. (0.39 μg/μl concentration) showed the highest inhibition of FMDV concentration $1\times10^{3.65}$ $TCID_{50}$. While the 0.19 μg/μl concentration of crude-extracts of *Morinda citrifolia* L. inhibited the FMDV concentration $1\times10^{3.35}$ $TCID_{50}$. These two plants and the lower antiviral activity weeds are being study for practical use.

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