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Antiviral Activity of Several Thai Traditional Herb Extracts against Fish Pathogenic Viruses

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Extracts from 18 Thai traditional herbs were prepared by boiling with ethanol under a soxhlet apparatus and virucidal activity against fish pathogenic viruses (IHNV, OMV and IPNV) was tested by plaque reduction method using CHSE-214 cells.

When the viruses were exposed to herb extract at 500 μg/ml before inoculation to CHSE-214 cells, all herbs showed antiviral activity against IHNV and OMV, reducing plaques by 65-100% and 20-100%, respectively. However, in the case of IPNV, no plaque reduction was observed by any herbs tested.

Some kinds of the herb extracts prevented viral infection, when CHSE-214 cells were treated with 100 μg/ml of herbs for 3 h before viral infection. The percent of plaque reduction above 50% was observed in 6, 8 and 6 kinds of herb extracts for IHNV, OMV and IPNV, respectively. These extracts may inhibit the viral adsorption to the cells.

When 100 μg/ml of the herb extracts was applied for infected cells, the percent of plaque reduction above 50% was observed in 0, 8 and 5 kinds of herb extracts for IHNV, OMV and IPNV, respectively. This means that some of the herb extracts may inhibit the replication of OMV and IPNV in CHSE-214, but no herb has any effect for IHNV infection.

Moreover, all of the tested herb extracts showed low toxicity to CHSE-214 cell line, the cytotoxic 50% value being 1,200-41,500 μg/ml.

Key words: antiviral activity, herb extract, fish virus, IHNV, IPNV, OMV

In recent years, aquaculture in the world has been rapidly developed. At the same time, the most serious problem that it is now facing is disease especially viral disease. That causes a lot of economic losses for the farmer.

Thailand has realized for a long time the potential value of traditional remedies including medicinal plants (crude drugs) which constitute the great part of traditional medicine. In dealing with medicinal plants, people have known to make use of them in treating illness. There are a lot of plants or herbal medicines that are claimed by traditional medical practitioners to be effective for the treatment of bacterial and viral infections.

For example, plants of the genus *Phyllanthus* have been used wildly by traditional medical practitioners for the treatment of jaundice and other disease (Thyagarajan *et al.*, 1988). Moreover, Venkateswaran *et al.* (1987) found that *P. niruri* have the antiviral activities against hepatitis B virus and woodchuk hepatitis virus. And the antiviral activities against herpes simplex virus (HSV) have been reported by Jayavasu *et al.* (1992). However, scientific evidence to support the efficacy of these herbal medicines in mankind. Recently, effect of guava (*Psidium guajava* L.) on prawn pathogenic *Vibrio* spp. was reported (Direkbusarakom and Aekpanithanpong, 1992). The ways in which such herbal medicines are applied in animals are obscure and more researches are needed.

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Even if the antiviral activity of some herbs against yellow head baculovirus have been reported by Direkbusarakom *et al.* (1993, 1995). But there are many problem for study of antiviral activity in shrimp and marine fish pathogenic viruses such as fish nodavirus, yellow head baculovirus (YHV) and due to quality control of fish and shrimp that use in the study and the lack of the cell line for virus replication. While the cell line for salmon pathogenic viruses are available and the standard method have been established (Kamei *et al.*, 1987).

This experiment attempts to determine the *in vitro* activities of the extracts from the herbs against three salmon pathogenic viruses and one fish cell line.

Materials and Methods

Viruses and cell culture

Three kinds of salmonid fish pathogenic viruses, infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and *Oncorhynchus masou* virus (OMV) (Kimura and Yoshimizu, 1991), were used in this study. All viruses were inoculated in CHSE-214 cell line (Fryer *et al.*, 1965) grown in 75 cm^2 plastic flasks containing 25 m*l* of MEM10-Tris medium, composed of Eagle's minimum essential medium (MEM, Gibco), 10% fetal bovine serum (M.A. Bioproduct), 0.075% $NaHCO_3$, 100 IU/m*l* penicillin (Sigma), 100 mg/m*l* streptomycin (Sigma), and 1.6% Tris buffer (Tris hydroxymethyl aminomethane (Tris-hydrochloride) (Sigma) adjusted to pH 7.8. When the cytopathic effect was the maximum, the culture fluid was removed from the flask and filtrated by through a 0.45 μm pore sized membrane filter (Millipore) and stored at $-80°C$ until used.

Preparation of the herb extract

Eighteen of Thai traditional herbs were selected for this study. They were *Cassia alata*, *Calophyllum inophyllum*, *Clinacanthus* sp., *Clinacanthus nutans*, *Glinus oppositifolis*, *Hura crepitan*, *Momordica charantina*, *Ocimum sanctum* (red), *Ocimum sanctum* (white), *Orchocarpus siamensis*, *Phyllanthus acidus*, *Phyllanthus amarus*, *Phyllanthus debelis*, *Phyllanthus reticulatus*, *Phyllanthus urinaria*, *Psidium guajava*, *Tinospora cordifolia* and *Tinospora crispa*. Each dried plant was extracted by ethanol using a soxhlet apparatus. The crude extract was further prepared as complex granule with polyvinylpyrolidone (PVP) and used for antiviral study.

Direct virucidal of the herb extracts

Eighteen Thai traditional herb extracts were used for screening of the antiviral activity by plaque assay according to Kamei *et al.* (1987). Briefly, 1 mg of each extract was dissolved in 1 m*l* of Hanks' balanced salt solution (Hanks' BSS). A mixture of 0.2 m*l* of the solution of the herb extract and equal volume of virus suspension (approximately 200 PFU/0.1 m*l*) was reacted at 15°C for 3 h. A 0.2 m*l* aliquot of the mixture was inoculated into each of 2 wells of 24-well microplate (Falcon) containing confluent monolayer of CHSE-214 cells and kept for 1 h at 15°C. The inocula were removed and washed 3 times with Hanks' BSS. Then 1 m*l* of 0.8% methyl cellulose overlay medium was added to each cell culture. After 10 day incubation, the cells were fixed with 10% formalin, stained with 0.1% crystal violet, and plaques were counted. The plaque reduction rate was calculated by comparison with positive control which was inoculated with the virus suspension only.

Effect of the herb extracts on virus adsorption

Each well of 24-well plate containing CHSE-214 cells grown to confluence for 24 h was added with one hundred microgram of each extracts and incubated for 3 h at 15°C. The cells were washed with Hanks' BSS, inoculated with 100 PFU of virus to the well and kept for 1 h at 15°C. The inocula were removed and washed 3 times with Hanks' BSS. The 1 m*l* of 0.8% methyl cellulose overlay medium was added to the cell culture. After incubation for 10 days, the cells were fixed and stained as above, and the plaque reduction rate was calculated.

Effect of the herb extracts on viral replication

CHSE-214 cells grown to confluence in 24-well plate for 24 h were washed with Hanks' BSS, inoculated with 100 PFU of virus and kept for 1 h at 15°C. The inocula were removed and washed 3 times with Hanks' BSS. Then 1 m*l* of 0.8% methyl cellulose overlay medium which contained 100 μg of herb extract was add to the cell culture. After incubation for 10 days, the cells were fixed and stained as above, and the plaque reduction rate was calculated.

Cytotoxic assay

Cytotoxicity of each herb extract was estimated by using CHSE-214 cells according to Fernandez *et al.* (1993). Briefly, each well of 96-well plate was seeded with 0.1 ml of CHSE-214 cells (1×10^5 cell/ml) and cultured in the medium containing the extract at the final concentration of 0, 10, 100, 1000, 10000 and 50000 μg/ml. After 5 days of incubation at 15°C, cells in the microplate were fixed with 10% formalin for 30 min and washed with tap water. Cells were then stained with 0.1% crystal violet for 1 h and rinsed again with several washing. Rinsed microplates were then thoroughly airdried. Absorbance of stained microplates was measured by using a microplate spectrophotometer (Corona MTP-22) at 600 nm. The 50% cytotoxic dose of each extract was analysed by using probit analysis.

Results

Direct virucidal activity

The results of direct antiviral activity of each herb extract against IHNV, OMV and IPNV are shown in Table 1. All of the herbs showed antiviral activity against INHV and OMV reducing plaques by 65–100% and 21–100%, respectively. But in the case of IPNV, no plaque reduction was observed among the herbs.

Effect on virus adsorption

The effect of herb extracts on virus adsorption was determined by using CHSE-214 cells pre-treated with the extracts. The result are shown in Table 2. Percent of plaque reduction above 50% was observed in 6, 8 and 6 kinds of herb extracts for IHNV, OMV and IPNV, respectively. Especially extracts of *C. alata*, *P. acidus*, *P. amarus* and *P. guajava* showed 100% plaque reduction for IHNV, and *C. alata* and *P. acidus* showed 92–100% plaque reduction for IPNV.

Effect on viral replication

Effect of the herb extracts on viral replication in the infected cell line was estimated (Table 3). The percent of plaque reduction above 50% was observed in 0, 8 and 5 kinds of herb extracts for IHNV, OMV and IPNV, respectively. Extracts from *O. siamensis* and *P. acidus* showed 100% plaque reduction for OMV and IPNV.

Cytotoxicity

These extracts were found to be low toxic to

Table 1. Direct virucidal activity of 18 Thai traditional herbs against IHNV, IPNV and OMV by plaque method

Herb	Plaque reduction rate (%)		
	IHNV	IPNV	OMV
Cassia alata	99	≤0	100
Calophyllum inophyllum	97	≤0	92
Clinacanthus nutans	100	≤0	100
Clinacanthus sp.	100	≤0	100
Glinus oppositifolis	97	≤0	76
Hura crepitan	65	≤0	21
Momordica charantina	98	≤0	47
Ocimum sanctum (red)	100	≤0	100
Ocimum sanctum (white)	99	≤0	100
Orchocarpus siamensis	97	≤0	91
Phyllanthus acidus	100	≤0	100
Phyllanthus amarus	100	≤0	100
Phyllanthus debelis	97	≤0	93
Phyllanthus reticulatus	100	≤0	99
Phyllanthus urinaria	100	≤0	100
Psidium guajava	100	≤0	100
Tinospora crispa	100	≤0	90
Tinospora cordifolia	97	≤0	91

Table 2. Effect of 18 Thai traditional herbs on virus adsorption against IHNV, IPNV and OMV by plaque method

Herb	Plaque reduction rate (%)		
	IHNV	IPNV	OMV
Cassia alata	100	92	61
Calophyllum inophyllum	≤0	37	25
Clinacanthus nutans	31	74	54
Clinacanthus sp.	34	≤0	75
Glinus oppositifolis	15	7	32
Hura crepitan	11	≤0	25
Momordica charantina	9	23	15
Ocimum sanctum (red)	27	≤0	≤0
Ocimum sanctum (white)	0	7	48
Orchocarpus siamensis	9	30	65
Phyllanthus acidus	100	100	32
Phyllanthus amarus	100	74	64
Phyllanthus debelis	42	42	67
Phyllanthus reticulatus	51	58	72
Phyllanthus urinaria	56	37	15
Psidium guajava	100	57	10
Tinospora crispa	39	39	67
Tinospora cordifolia	16	≤0	38

Table 3. Effect of 18 Thai traditional herbs on viral replication against IHNV, IPNV and OMV by plaque method

Herb	Plaque reduction rate (%)		
	IHNV	IPNV	OMV
Cassia alata	8	25	59
Calophyllum inophyllum	20	≦0	59
Clinacanthus nutans	25	3	48
Clinacanthus sp.	≦0	≦0	56
Glinus oppositifolis	2	12	44
Hura crepitan	17	≦0	35
Momordica charantina	≦0	10	60
Ocimum sanctum (red)	≦0	56	48
Ocimum sanctum (white)	≦0	38	42
Orchocarpus siamensis	≦0	100	100
Phyllanthus acidus	29	100	100
Phyllanthus amarus	5	8	30
Phyllanthus debelis	8	34	17
Phyllanthus reticulatus	≦0	59	61
Phyllanthus urinaria	36	14	10
Psidium guajava	3	61	26
Tinospora crispa	≦0	≦0	26
Tinospora cordifolia	≦0	≦0	78

Table 4. Cytotoxicity of 18 Thai traditional herbs to CHSE-214 cell line

Herb	Concentration of 50% cytotoxic (μg/ml)
Cassia alata	4,895
Calophyllum inophyllum	6,439
Clinacanthus nutans	2,124
Clinacanthus sp.	2,124
Glinus oppositifolis	10,628
Hura crepitan	41,465
Momordica charantina	3,759
Ocimum sanctum (red)	3,024
Ocimum sanctum (white)	7,167
Orchocarpus siamensis	10,979
Phyllanthus acidus	1,475
Phyllanthus amarus	1,237
Phyllanthus debelis	10,718
Phyllanthus reticulatus	2,582
Phyllanthus urinaria	2,336
Psidium guajava	1,923
Tinospora crispa	8,923
Tinospora cordifolia	8,149

CHSE-214 because the cytotoxic 50% value of 18 herb extracts were about 1,237–41,465 μg/ml (Table 4).

Discussion

The result of this study showed that OMV and IHNV could be inactivated by direct reaction with 500 μg/ml of herb extract. While these herbs could not inactivated IPNV. Both OMV and IHNV are enveloped viruses but IPNV is non enveloped virus. Yellow head baculovirus which is an enveloped virus of black tiger prawn (*Penaeus monodon*) (Boonyaratpalin *et al.*, 1993) were also inactivated by some kinds of the herbs such as *P. amarus*, *P. urinaria*, *P. reticulatus* and *C. nutans* (Direkbusarakom *et al.*, 1993, 1995). These suggests that virus inactivation of the herbs might be due to the reaction to the envelope of virus.

Extracts of *C. alata*, *P. acidus*, *P. amarus* and *P. guajava* showed 70–100% plaque reduction rate for IHNV and IPN, when CHSE-214 cells were treated with 100 μg/ml of them before viral infection. It is considered that the extracts inhibit the viral adsorption to CHSE-214 and may block the viral binding sites on the cells.

In addition, extracts of *O. siamensis* and *P. acidus* showed 100% plaque reduction of OMV and IPNV in CHSE-214 cells which were treated with 100 μg/ml of the extracts after the viral infection. This result suggests that the extract inhibits the replication of OMV and IPNV in the cells.

All of the herb extracts showed low toxicity to CHSE-214, but the toxicity of some herbs such as *P. amarus*, *P. acidus* and *P. guajava* to CHSE-214 were higher than those to *Penaeus monodon*. The cytotoxic 50% values to CHSE-214 of *P. amarus*, *P. acidus* and *P. guajava* are 1,237, 1,475 and 1,923 μg/ml respectively, while LD_{50} of these herbs for *P. monodon* are 2,471, 2,564 and 2,468 μg/ml. (Direkbusarakom *et al.*, 1993, 1995). However, this study could not establish the antiviral mechanism of herb extracts. So further studies are required to determine the mechanism of the herbs tested in this study, to estimate therapeutic value in aquatic animals and to determine the possibility of therapeutic use in aquaculture.

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