Vol. 115: 157–164, 2015
doi: 10.3354/dao02891

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published July 23

# *Emilia sonchifolia* extract activity against white spot syndrome virus and yellow head virus in shrimp cell cultures

**Lamai Maikaeo[1], Wilaiwan Chotigeat[1,2,*], Wilawan Mahabusarakam[3]**

[1]Department of Molecular biology and Bioinformatics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

[2]Center for Genomics and Bioinformatics Research Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

[3]Department of Chemistry, Faculty of Science, Prince of Songkla University, HatYai, Songkhla 90112, Thailand

ABSTRACT: *Emilia sonchifolia* (L.) DC is a plant used in traditional medicine to treat several viral and bacterial diseases. The antiviral activities of selected Sephadex LH-20 column fractions and HPLC subfractions of an acetone extract of *E. sonchifolia* leaves were determined in shrimp *Penaeus merguiensis* primary lymphoid cells infected with either white spot syndrome virus (WSSV) or yellow head virus (YHV). WSSV and YHV replication was quantified using quantitative real-time PCR tests targeted to the VP19 and ORF1b gene transcripts, respectively. In lymphoid organ cells exposed to 100 µg $ml^{-1}$ of either the Sephadex fraction F14 or the HPLC F14 subfraction SF4, both fractions caused reduced replication, but YHV replication was reduced only by SF4. In the asthiazolyl blue mitochondrial enzyme activity assays to assess extract cytotoxicity, >60% of primary lymphoid organ cells remained viable following exposure to 100 µg $ml^{-1}$ of either F14 or SF4. GC-MS analysis of the HPLC F14 subfraction SF4 showed that it contained 2,4-di-tert-butylphenol. This study is the first to show that *E. sonchifolia* leaf extracts might be useful as bioactive agents to protect shrimp against viruses such as WSSV and YHV.

KEY WORDS: Antiviral activity · *Emilia sonchifolia* · Lymphoid organ cell culture · WSSV · YHV · *Penaeus merguiensis*



## INTRODUCTION

Diseases caused by white spot syndrome virus (WSSV) and yellow head virus (YHV) can cause serious financial losses for shrimp farmers. Strategies to manage these diseases include the use of specific-pathogen free seed stock, a controlled environment and a bio-secure culture systems as well as various immunostimulants, antimicrobial peptides and RNA interference strategies that have shown experimental promise (Citarasu et al. 2006, Wongprasert et al. 2014). Other strategies include feeding shrimp bioactive compounds extracted from various plants and seaweed species. These bioactive compounds can possess diverse activities such as being antibiotic-like (Direkbusarakom et al. 1995, Direkbusarakom 2004, Citarasu et al. 2006, Balasubramanian et al. 2007), promoting growth (Citarasu et al. 2002, Sankar et al. 2011) and having aphrodisiac properties (Liñán-Cabello et al. 2004, Babu et al. 2008). In addition, plant metabolites of importance to shrimp aquaculture include alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils, all of which have been reported to inhibit pathogenic viruses (Citarasu et al. 2002). For example, an aqueous extract of the short-leaved grass *Cynodon dactylon*, the evergreen tree mangrove *Ceriops tagal*, a methanol extract of the dark-brown seaweed *Sargas-*

*Corresponding author: wilaiwan58@hotmail.com

*sum wightii* and a crude fucoidan extract of dark-brown seaweed *S. polycystum* protect black tiger shrimp *Penaeus monodon* against WSSV (Chotigeat et al. 2004, Balasubramanian et al. 2007, 2008a,b, Huxley & Lipton 2010, Sudheer et al. 2011). Extracts from the small shrub *Phyllanthus amarus* and the tall annual herb *Clinacanthus nutans* have provided *P. monodon* with varying degrees of protection against YHV (Direkbusarakom et al. 1995, 1998). Although these plant extracts have shown promise for controlling viral diseases of shrimp, there remains a need to identify plants that are common and grow easily to allow for the large-scale production of these useful bioactive compounds.

The lilac tassel flower herb *Emilia sonchifolia* (L.) DC occurs commonly throughout Southeast Asia and contains various alkaloids, tannins (Cheng & Röder 1986), flavonoids (Srinivasan & Subramanian 1980) and sterols as well as palmitoic and honey acid (Gao et al. 1993). Extracts from this plant have potent antimicrobial activities (Chen et al. 2009, Yadava & Mamta 2012), and some can act as antioxidants and have anti-inflammatory (Shylesh & Padikkala 1999), anti-tumour (Shylesh & Padikkala 2000, Yadava & Mamta 2012) and anti-cancer activities (Shylesh et al. 2005). However, *E. sonchifolia* extracts have not been examined for their anti-WSSV and anti-YHV activities. Here we show that a Sephadex column fraction and an HPLC-purified subfraction of an *E. sonchifolia* leaf acetone extract invariably inhibit WSSV and/or YHV replication in primary shrimp lymphoid organ cell cultures with low toxicity.

## MATERIALS AND METHODS

### Plant material and acetone extraction

*Emilia sonchifolia* was purchased from the local fresh market in Hatyai, Songkhla, Thailand. Leaves were washed in distilled water and air-dried for 3 d, and then 200 g dried leaves were extracted twice by shaking in 1.7 l acetone for 3 d at room temperature. The combined liquid extracts (3.4 l) were evaporated under vacuum to yield a viscous brown crude extract (11 g).

### Plant extract separation

A portion (1.6 g) of the crude acetone extract was dissolved thoroughly in 3 ml 50% methanol in dichloromethane and applied to a Sephadex LH-20 chromatography column, and material that bound was eluted using 20 ml 10% methanol in dichloromethane. Sequential fractions (n = 14) of eluate were collected and aliquots of each were separated by thin-layer chromatography (TLC) to assess their contents. Each fraction was spotted onto a TLC60 $F_{254}$ silica gel sheet, and compounds were separated using either 50% or 10% methanol in dichloromethane. Sheets were dried and spot patterns were visualized under ultraviolet light at 254 nm. Fractions 1 to 7 (F1–F7) primarily contained chlorophyll, while fractions F8 to F14 possessed an additional small spot that was most pure in fraction F14. The spot from F14 was further purified by HPLC using an Apollo Silica column (150 mm length and 4.6 mm internal diameter) with a 1200 Series HPLC auto-sampler (Agilent Technology). The mobile phase was 1.0% diethyl ether in hexane and compounds in the eluate were detected at $A_{218nm}$. Eight subfractions (SF1–SF8) were collected over an increasing retention time. Each subfraction was concentrated by air-drying and stored at room temperature. The extracts were dissolved at 200 µg $ml^{-1}$ in double-strength Leibovitz-15 (2× L15) culture medium (Gibco®) containing 10% foetal bovine serum, 1% penicillin, 1% streptomycin and 0.1% glucose (adjusted to pH 7.4) to assess their antiviral activity.

### Shrimp primary lymphoid organ cell cultures

Lymphoid organ (LO) tissue was dissected from *Fenneropenaeus merguiensis*, washed 3 times in 2× L15 medium and cut into small pieces (~1 $mm^3$). Tissue pieces (4 or 5) were added to each well of a 24-well culture plate containing 1 ml of 2× L15 medium. Following incubation at 28°C under a 5% $CO_2$ atmosphere for 24 h, tissue explants spread to form a confluent cell monolayer.

### Screening for antiviral activity

WSSV and YHV inoculums were prepared by using gills, heart and LO tissue of either WSSV-infected or YHV-infected shrimp, as described previously (Chotigeat et al. 2004). Tissue homogenates were diluted appropriately in 2× L15 medium to generate inoculums containing either $10^3$ WSSV DNA copies $ml^{-1}$ or $10^3$ YHV RNA copies $ml^{-1}$ as determined by quantitative real-time PCR (qRT-PCR). To infect LO cells, the culture medium was removed and replaced with 1.0 ml WSSV inoculum or YHV inocu-

lum, or with the same inoculums heat inactivated at 100°C for 5 min, and the plates were agitated gently at 28°C for 1 h. Following inoculum removal, cells were washed with 2× L15 medium and covered with 1.0 ml fresh medium alone as a negative control or with 1.0 ml medium containing either 100 µg $ml^{-1}$ or 200 µg $ml^{-1}$ of each extract Sephadex LH-20 chromatography fraction (F1–F14) or HPLC subtraction. Cells were incubated at 28°C for 24 h, at which time they were observed by light microscopy and harvested to extract RNA for qRT-PCR analysis.

Table 1. PCR primer sequences used in white spot syndrome virus (WSSV; VP19) and yellow head virus (YHV; ORF1b) quantitative real-time PCR tests

| Primer name | Sequence (5′–3′) |
|---|---|
| Forward VP19 | CGG GAT CCA TGG CCA CCA CGA CTA A |
| Reverse VP19 | GCC TCG AGC CTG ATG TTG TGT TTC TAT A |
| Forward ORF1b | CCG CTA ATT TCA AAA ACT ACG |
| Reverse ORF1b | AAG GTG TTA TGT CCA GGA AGT |
| Forward GAPDH | CAA GAA GGT CAT CAT CTC CGC T |
| Reverse GAPDH | TCC ACG GTC TTC TGT GTG GC |

### WSSV and YHV qRT-PCR tests

Total RNA was extracted from cells using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol. To prepare cDNA, 4 µg of total RNA were incubated with 200 ng random hexamer primers (Promega) at 70°C for 5 min and cooled on ice for 5 min before addition of a 1 mM dNTP mixture, 1× reaction buffer and 10 U AMV reverse transcriptase (Promega) in a 25 µl reaction that was incubated at 48°C for 2 h. qRT-PCR tests utilized primer sequences targeting the WSSV VP19 gene (Youtong et al. 2011), the YHV ORF1b gene (Cowley et al. 2000) and the host glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as the comparative control (Table 1). Reactions (25 µl) contained 100 ng cDNA, 1× FastStart Universal SYBR Green Master (Roche) and 20 pmol of each primer. Thermal cycling (95°C for 5 min, 40 cycles of 94°C for 30 s, 59°C for 1 min and 72°C for 1 min) and fluorescence detection employed the Mx3000P™ qPCR System (Aligent Technologies). Relative WSSV and YHV RNA amounts detected were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), normalized against GAPDH RNA amounts and expressed as mean ± SD.

### Cell viability assay

The numbers of viable LO cells were quantified by exposure to asthiazolyl blue (MTT) which is converted into a formazan pigment by active mitochondrial enzymes. At 24 h post infection with viruses and post-exposure to the various plant extract fractions, culture medium was replaced with 100 µl of fresh medium to which 10 µl of 5 mg $ml^{-1}$ MTT solution (Invitrogen) were added. Following incubation for 4 h, 100 µl of 10% sodium dodecyl sulphate in 0.01 N HCl were added and the formazan crystals that formed were dissolved by stirring with a pipette. Aliquots of culture medium were transferred to triplicate wells of a 96-well microtitre plate, and the absorbance at $A_{570nm}$ was quantified using a Multiskan FC microplate reader (Thermo Fisher Scientific). The percentage of viable cells was calculated using the formula $[(A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100$.

### Cell cytotoxicity assay

A cytotoxicity concentration 50% end-point ($CC_{50}$) of the active SF4 subfraction was determined using monolayers of primary LO cell cultures grown in wells of 24-well plates. The culture medium was replaced with serial 2-fold dilutions of SF4 in fresh medium ranging from 800 µg $ml^{-1}$ to 3.12 µg $ml^{-1}$. After 24 h, cell viability was quantified using the MTT colorimetric method described above, and the $CC_{50}$ (50% cell viability) for SF4 was calculated from linear regression analysis of the MTT data.

### GC-MS analysis

The composition of the HPLC SF4 subfraction that displayed antiviral activity was examined using gas chromatography (GC, Hewlett Packard 5890) followed by mass spectroscopy (MS, Hewlett Packard 5972). The GC separation conditions used were 260°C inlet temperature in the split-less mode for 0.8 min. The oven temperature was set at 50°C for 5 min before being ramped in consecutive +3°C steps to 260°C, at which temperature it was held for another 5 min. The Innowax GC column (30 m long, 0.25 mm internal diameter, 0.25 µm film thickness) used was connected directly to the mass spectrometer with electron ionization. Identifications were based on a comparison with the standards in the Wiley Library Database (9th edn, Wiley Registry™).

### Statistical analyses

The statistical significance of the MTT assay and viral replication data was determined using 1-way ANOVA with a 95% confidence level ($p < 0.05$) in the SPSS V16.0 software package.

## RESULTS

### Anti-WSSV activity

The anti-WSSV activities of culture media containing either 100 µg ml$^{-1}$ or 200 µg ml$^{-1}$ of the Sephadex LH-20 column fraction F14 or the F14 HPLC subfractions SF1 to SF8 obtained from the *Emilia sonchifolia* leaf acetone extract were examined in primary cultures of *Fenneropenaeus merguiensis* LO cells. Cells were examined for cytopathic effects (CPE) 24 h post infection. While non-infected cells maintained their roundish or fibroblast-like morphologies, most WSSV-infected cells showed evidence of CPE (Fig. 1). CPE was not observed in WSSV-infected cells maintained in media containing either 100 or 200 µg ml$^{-1}$ of extract fraction F14 or its subfraction SF4.

qRT-PCR targeted to mRNA transcribed from the WSSV VP19 gene and the shrimp GAPDH gene were used to quantify WSSV replication levels. As the PCR test detected no significant VP19 mRNA in WSSV-infected primary LO cells maintained in media containing either 100 or 200 µg ml$^{-1}$ of fraction F14 (Fig. 2A), the lower concentration was chosen for use in further studies.

In the WSSV-infected primary LO cells maintained in media containing 100 µg ml$^{-1}$ subfraction SF1 to SF8, VP19 mRNA amounts were reduced compared to WSSV-infected unexposed cells. Among the WSSV-infected LO cell monolayers, viable cells only remained in the cultures exposed to SF4 and SF6 (Fig. 2A).

### Anti-YHV activity

A qRT-PCR test targeted to the YHV ORF1b gene and thus genomic-length RNA transcripts was used to show that YHV replication in primary LO cells maintained in media containing 100 µg ml$^{-1}$ SF4 was decreased significantly with no evidence of CPE compared to untreated YHV-infected cells (Fig. 2B). In contrast, cells maintained in media containing any of the other subfractions resulted in the production of CPE and no significant reduction in YHV RNA levels.

### Plant extract effects on cell viability following virus challenge

The ability of the *E. sonchifolia* extract fractions to protect shrimp LO cells from destruction following challenge with either WSSV or YHV was quantified using a colorimetric MTT mitochondrial enzyme activity assay. MTT data on viable cell numbers were determined at 24 h post challenge with virus for cul-

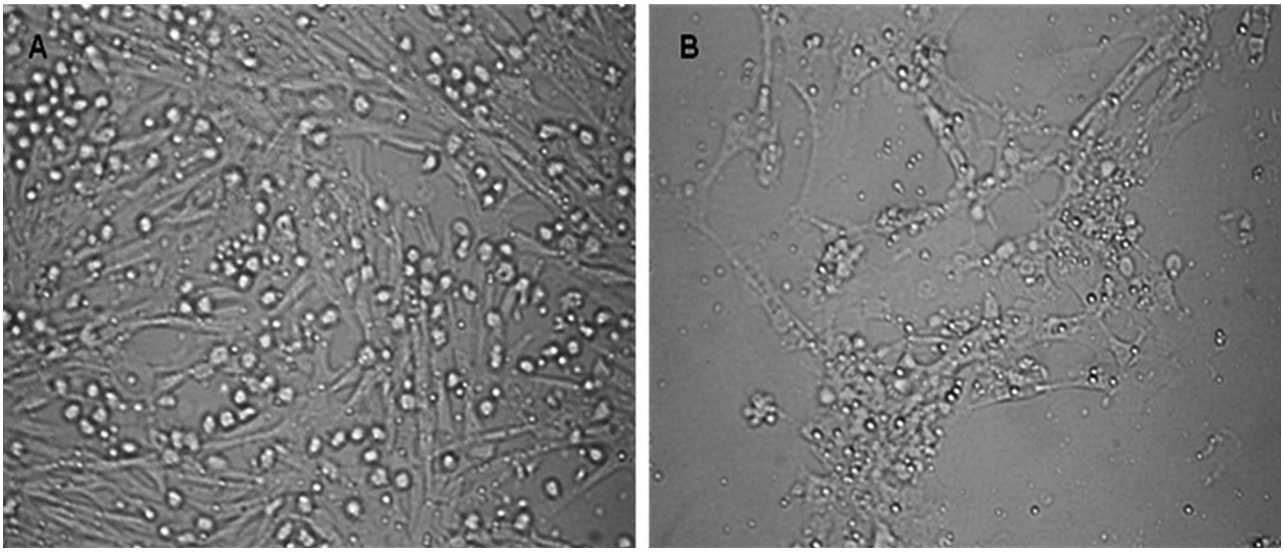


Fig. 1. Micrograph of cytopathic effects in primary cultures of *Fenneropenaeus merguiensis* lymphoid organ cells 24 h post infection with white spot syndrome virus (WSSV). (A) Uninfected monolayer of fibroblast-like cells and (B) cell monolayer partial disintegration caused by WSSV infection

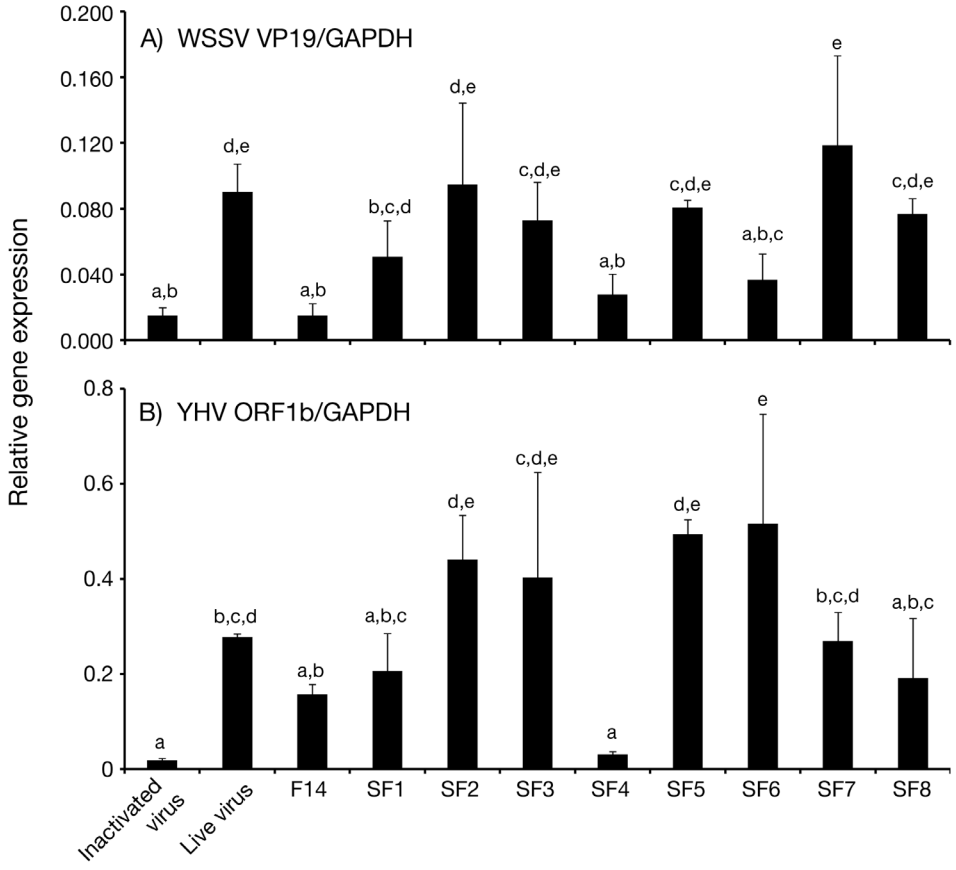


Fig. 2. Relative (A) white spot syndrome virus (WSSV) VP19 gene mRNA amounts and (B) yellow head virus (YHV) genomic RNA (ORF1b gene) amounts in primary lymphoid organ cell cultures of *Fenneropenaeus merguiensis* quantified by quantitative real-time PCR and normalized relative to endogenous GAPDH mRNA levels (mean ± SD, n = 3). Levels differing significantly from each other ($p \leq 0.05$, ANOVA) are identified by different letters above bars

tures using normal media or media containing *E. sonchifolia* extract fraction F14 or F14 subfractions SF2, SF4, SF5, SF6 and SF8. Only those cell monolayers maintained on media containing F14 and SF4 displayed >50% cell viability at 24 h post challenge with either WSSV or YHV (Fig. 3).

### Cell cytotoxicity assay

The MTT cell viability assay was also used to assess the cytotoxicity of the active *E. sonchifolia* extract subfraction SF4 for primary LO cell cultures. MTT data were collected after 24 h exposure of the cell monolayers to media containing 2-fold serial dilutions of SF14 that ranged in final concentrations from 800 to 3.12 µg $ml^{-1}$. While 100 µg $ml^{-1}$ of SF4 had no effect on cell viability, a concentration of 221 µg $ml^{-1}$ was calculated to reduce $CC_{50}$ (50% cell viability) based on the linear regression analysis of MTT data on the cells exposed to various concentrations of SF14.

### Plant extract components

HPLC analysis of the Sephadex LH-20 column fraction F14 from the *E. sonchifolia* extract resolved peaks designated as subfractions SF1 to SF8 (Fig. 4A). Mass spectrum peaks generated from the GC-MS analysis of subfraction SF4 identified the presence of 2,4-di-tert-butylphenol (Fig. 4B) as deduced from comparisons with mass standards in the Wiley Library Database ($9^{th}$ edn, Wiley Registry™).

## DISCUSSION

Pandemics caused by WSSV (DNA virus) and YHV (RNA virus) have seriously impacted shrimp aquaculture. Identification of cheap and effective plant derivatives that inactivate these viruses could thus benefit this industry. Plant compounds including alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils can act to prevent various

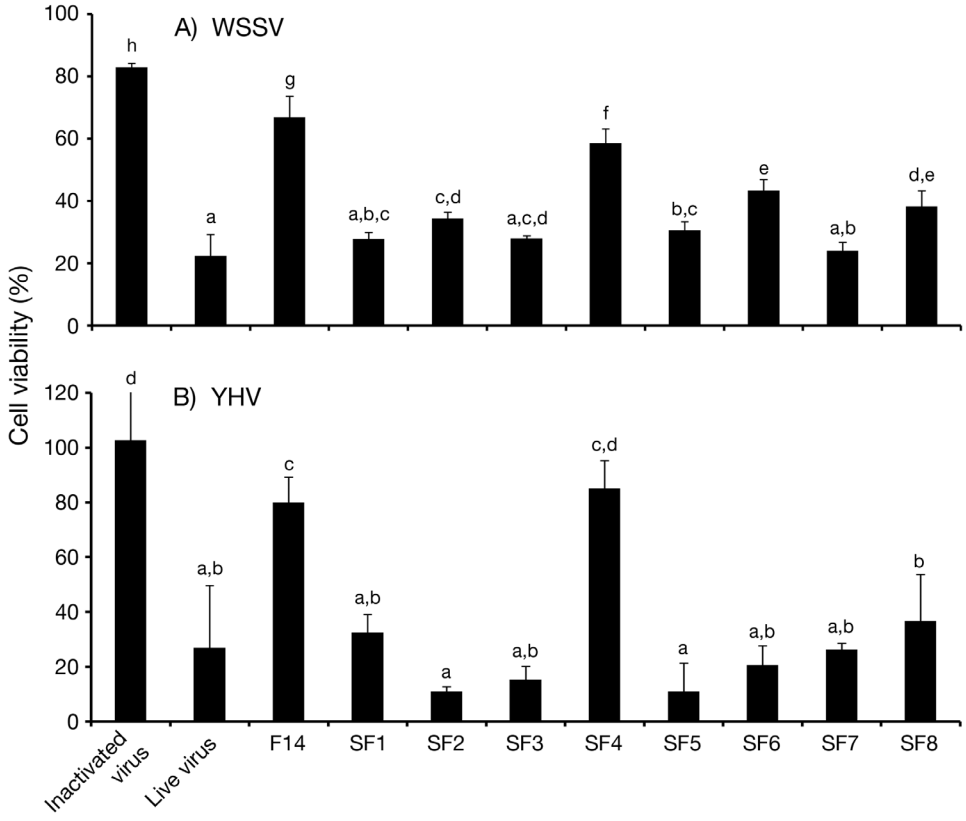


Fig. 3. Viability of lymphoid organ cells of *Fenneropenaeus merguiensis* after infection with (A) white spot syndrome virus (WSSV) or (B) yellow head virus (YHV) and exposed to 100 µg ml$^{-1}$ Sephadex LH-20 column fraction F14 or the F14 HPLC subfractions SF1 to SF8 obtained from *Emilia sonchifolia* leaf acetone extract determined at 24 h post challenge using the asthiazolyl blue (MTT) assay (mean ± SD, n = 3). Levels differing significantly from each other ($p \leq 0.05$, ANOVA) are identified by different letters above bars

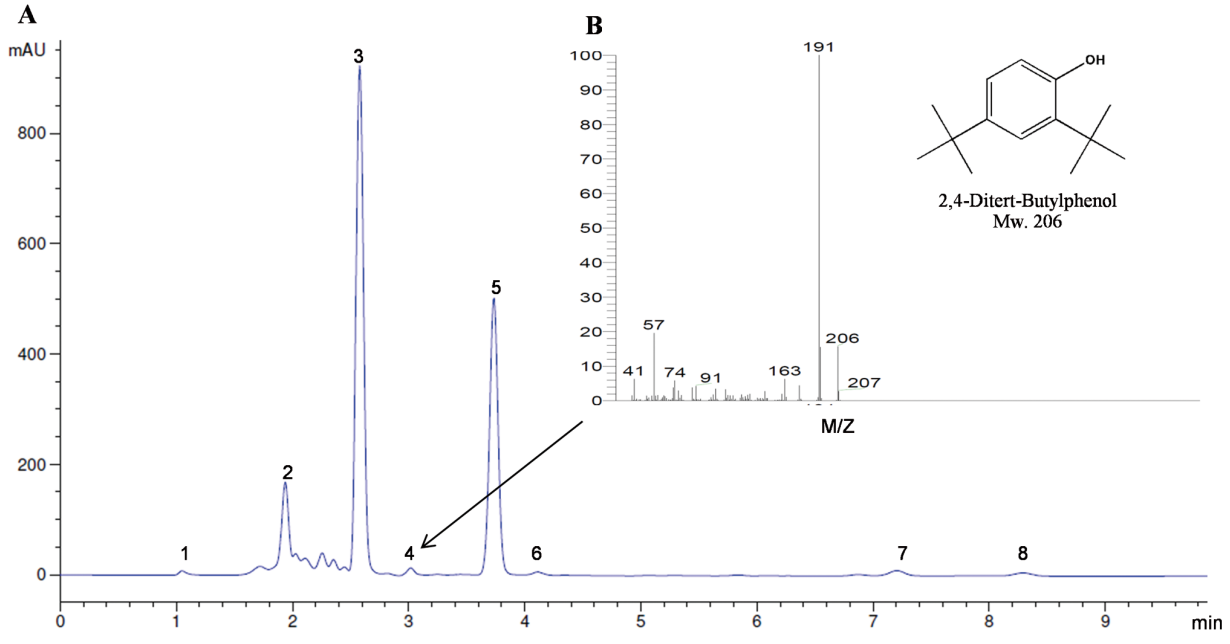


Fig. 4. HPLC fingerprint of the (A) Sephadex LH-20 column fraction F14 showing subfraction SF4 and (B) GC-MS spectrum of subfraction SF4 derived from *Emilia sonchifolia* leaf acetone extract

diseases (Citarasu 2010), and several natural products from plants, shrubs, mangroves and seaweeds are effective inhibitors of virus replication. Here the anti-WSSV and anti-YHV properties of acetone extracts of leaves of the lilac tassel flower herb *Emilia sonchifolia* were examined in primary shrimp LO cell cultures as used to investigate the anti-viral activity of various other compounds (Luedeman & Lightner 1992, Nadala et al. 1993, Lu et al. 1995, Toullec et al. 1996, Tapay et al. 1997).

From an acetone extract of *E. sonchifolia* leaves, a Sephadex LH-20 chromatography column fraction (F14) and an HPLC subfraction (SF4) derived from F14 were assessed for their cell toxicity and antiviral activity against WSSV and YHV. Of these, a 100 µg $ml^{-1}$ concentration of SF4 effectively reduced WSSV and YHV replication as evidenced by reduced VP19 gene and ORF1b gene mRNA levels quantified by qRT-PCR. A 221 µg $ml^{-1}$ concentration of SF4 was determined to reduce LO cell viability by 50% at 24 h post exposure, the antiviral activity delivered by SF4 at a concentration of 100 µg $ml^{-1}$ was accompanied by low cell toxicity. Similarly, a 62.5 µg $ml^{-1}$ concentration of a flavonoid glycoside extracted from *E. sonchifolia* seeds inhibits replication of Japanese encephalitis virus (RNA virus), but its mechanism of action was not reported (Yadava & Mamta 2012).

An ethanol extract from the tall annual herb *Clinacanthus nutans* and methanol extracts from the erect annual herb *Aclypha indica*, the short-leaved grass *Cynodon dactylon*, the small perennial herbs *Picrorhiza kurooa* and *Withania somnifera* and the rhizome perennial herb *Zingiber officinalis* have antiviral activities against YHV and WSSV, respectively (Direkbusarakom et al. 1995, Yogeeswaran et al. 2012). Moreover, 10 µl of an aqueous extract of *C. dactylon* injected intramuscularly into shrimp at 100 mg $kg^{-1}$ shrimp body weight showed strong antiviral activity against WSSV (Balasubramanian et al. 2007). As these ethanol, methanol and aqueous extracts of herbal plants inhibited YHV or WSSV replication, it is possible that *E. sonchifolia* contains other compounds with antiviral activities other than those soluble in acetone. It would be of interest to assess whether *E. sonchifolia*, like other medicinal herbs, might contain immunostimulants that when fed to shrimp can boost resilience against virus-induced disease (Citarasu et al. 2006).

Unlike bacteria, fungi and parasites, viruses need to parasitize host cell machinery to replicate. Thus to avoid generalized cell damage, drugs used to suppress viral replication must have high specificity for viral polymerases, proteases or structural proteins important to virus replication, assembly and cell entry or exit. For the treatment of viral diseases of shrimp, natural compounds therefore need to be examined for their ability to either interfere with these viral processes or to enhance host innate defense mechanisms (Jassim & Naji 2003). GC-MS analysis of subfraction SF4 identified it to primarily comprise the phenolic compound 2,4-di-tert-butylphenol. A polyphenolic extract of a perennial herb, red raspberry *Rubus idaeus*, has been shown to clump virus particles together to form non-infectious aggregates (Cadman 1960). Polyphenols might also bind directly to virus particles and/or host cell membrane proteins to inhibit virus absorption (Van den Berghe et al. 1986, Hudson 1990). Moreover, various polyphenolic compounds directly inhibit the binding of human immunodeficiency virus (RNA virus) and influenza virus (RNA virus) to host cells, thus affording some protection (Sakagami et al. 1995). While it has been concluded that the phenolic OH group present in some plant extracts facilitates binding to either viral nucleic acid or viral capsid proteins, thus inhibiting replication (Naithani et al. 2008), whether 2,4-di-tert-butylphenol identified to have anti-WSSV and anti-YHV properties possesses such activity has yet to be examined.

*Acknowledgements*. This work was supported by a Research professional development project under the Science Achievement Scholarship of Thailand, Prince of Songkla University. Thanks to Dr. Brian Hodgson for suggestions and assistance with the English.

LITERATURE CITED

Babu MM, Sivaram V, Immanuel G, Citarasu T, Punitha SMJ (2008) Effects of herbal enriched *Artemia* supplementation over the reproductive performance and larval quality in spent spawners of the tiger shrimp (*Penaeus monodon*). Turk J Fish Aquat Sci 8:301–307

Balasubramanian G, Sarathi M, Kumar SR, Hameed ASS (2007) Screening the antiviral activity of Indian medicinal plants against white spot syndrome virus in shrimp. Aquaculture 263:15–19

Balasubramanian G, Sarathi M, Venkatesan C, Thomas J, Hameed ASS (2008a) Studies on the immunomodulatory effect of extract of *Cyanodon dactylon* in shrimp, *Penaeus monodon*, and its efficacy to protect the shrimp from white spot syndrome virus (WSSV). Fish Shellfish Immunol 25:820–828

Balasubramanian G, Sarathi M, Venkatesan C, Thomas J, Hameed ASS (2008b) Oral administration of antiviral plant extract of *Cynodon dactylon* on a large scale production against White spot syndrome virus (WSSV) in *Penaeus monodon.* Aquaculture 279:2–5

Cadman CH (1960) Inhibition of plant virus infection by tannins. In: Pridham JB (ed) Phenolics in plants in health and disease. Pergamon Press, Oxford, p 101–105

Chen XW, Wei YY, Zhou WP, Pan MJ, Li JS (2009) Study on separations and the antimicrobial effects of the total flavonoids of *Emilia sonchifolia.* Food Sci Technol 1: 163–165

Cheng D, Röder E (1986) Pyrrolizidine alkaloids from *Emilia sonchifolia.* Planta Med 52:484–486

Chotigeat W, Tongsupa S, Supamataya K, Phongdara A (2004) Effect of fucoidan on disease resistance of black tiger shrimp. Aquaculture 233:23–30

Citarasu T (2010) Herbal biomedicines: a new opportunity for aquaculture industry. Aquacult Int 18:403–414

Citarasu T, Sekar R, Babu M, Marian M (2002) Developing *Artemia* enriched herbal diet for producing quality larvae in *Penaeus monodon.* Asian Fish Sci 15:21–32

Citarasu T, Sivaram V, Immanuel G, Rout N, Murugan V (2006) Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV) infection in black tiger shrimp, *Penaeus monodon* with reference to haematological, biochemical and immunological changes. Fish Shellfish Immunol 21:372–384

Cowley JA, Dimmock CM, Spann KM, Walker PJ (2000) Gill-associated virus of *Penaeus monodon* prawns: an invertebrate virus with ORF1a and ORF1b genes related to arteri- and coronaviruses. J Gen Virol 81:1473–1484

Direkbusarakom S (2004) Application of medicinal herbs to aquaculture in Asia. Walailak J Sci Technol 1:7–14

Direkbusarakom S, Herunsalee A, Boonyaratpalin S, Danayadol Y (1995) Effect of *Phyllanthus* spp. against yellow-head baculovirus infection in black tiger shrimp, *Penaeus monodon.* In: Shariff M, Arthur JR, Subasinghe RP (eds) Disease in Asian aquaculture II. Fish Health Section, Asian Fisheries Society, Manila, p 81–88

Direkbusarakom S, Ruangpan L, Ezura Y, Yoshimizu M (1998) Protective efficacy of *Clinacanthus nutans* on yellow-head disease in black tiger shrimp (*Penaeus monodon*). Fish Pathol 33:401–404

Gao JJ, Cheng DL, Liu XP (1993) Chemical constituents of *Emilia sonchifolia* L. DC. Zhongguo Zhongyao Zazhi 18: 102–103

Hudson JB (1990) Antiviral compounds from plants. CRC Press, Boca Raton, FL, Ann Arbor, MI, and Boston, MA

Huxley VAJ, Lipton AP (2010) Immunomodulatory effect of *Sargassum wightii* on *Penaeus monodon* (Fab.). Asian J Anim Sci 4:192–196

Jassim SAA, Naji MA (2003) Novel antiviral agents: a medicinal plant perspective. J Appl Microbiol 95:412–427

Liñán-Cabello MA, Medina-Zendejas R, Sanchez-Barajas M, Herrera AM (2004) Effects of carotenoids and retinol in oocyte maturation of crayfish *Cherax quadrucarinatus.* Aquacult Res 35:905–911

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Lu Y, Tapay LM, Loh PC, Brock JA, Gose R (1995) Development of a quantal assay in primary shrimp cell culture for yellow head baculovirus (YBV) of penaeid shrimp. J Virol Methods 52:231–236

Luedeman R, Lightner DV (1992) Development of an *in vitro* primary cell culture system from the penaeid shrimp, *Penaeus stylirostris* and *Penaeus vannamei.* Aquaculture 101:205–211

Nadala EC, Lu Y, Loh PC (1993) Primary culture of lymphoid, nerve, and ovary cells from *Penaeus stylirostris* and *Penaeus vannamei.* In Vitro Cell Dev Biol Anim 29: 620–622

Naithani R, Huma LC, Holland LE, Shukla D, McCormick DL, Mehta RG, Moriarty RM (2008) Antiviral activity of phytochemicals: a comprehensive review. Mini Rev Med Chem 8:1106–1133

Sakagami H, Sakagami T, Takeda M (1995) Antiviral properties of polyphenols. Polyphenol Actualites 12:30–32

Sankar G, Elavarasi A, Sakkaravarthi K, Ramamoorthy K (2011) Biochemical changes and growth performance of black tiger shrimp larvae after using *Ricinus communis* extract as feed additive. Int J Pharm Tech Res 3:201–208

Shylesh BS, Padikkala J (1999) Antioxidant and anti-inflammatory activity of *Emilia sonchifolia.* Fitoterapia 70: 275–278

Shylesh BS, Padikkala J (2000) *In vitro* cytotoxic and antitumor property of *Emilia sonchifolia* (L.) DC in mice. J Ethnopharmacol 73:495–500

Shylesh BS, Nair SA, Subramoniam A (2005) Induction of cell-specific apoptosis and protection from Dalton's lymphoma challenge in mice by an active fraction from *Emilia sonchifolia.* Indian J Pharm 37:232–237

Srinivasan KK, Subramanian SS (1980) Chemical investigation of *Emilia sonchifolia.* Fitoterapia 51:241–243

Sudheer NS, Philip R, Singh ISB (2011) *In vivo* screening of mangrove plants for anti WSSV activity in *Penaeus monodon*, and evaluation of *Ceriops tagal* as a potential source of antiviral molecules. Aquaculture 311:36–41

Tapay LM, Lu Y, Gose RB, Nadala ECB Jr, Brock JA, Loh PC (1997) Development of an *in vitro* quantal assay in primary cell cultures for a non-occluded baculo-like virus of penaeid shrimp. J Virol Methods 64:37–41

Toullec JY, Crozat Y, Patrois J, Porcheron P (1996) Development of primary cell cultures from the penaeid shrimps *Penaeus vannamei* and *P. indicus.* J Crustac Biol 16: 643–649

Van den Berghe DA, Vlietinck AJ, Van Hoof L (1986) Plant products as potential antiviral agents. Bull Inst Pasteur 84:101–107

Wongprasert K, Rudtanatip T, Praiboon J (2014) Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp. Fish Shellfish Immunol 36:52–60

Yadava RN, Mamta R (2012) Antiviral activity of a new flavone glycoside from *Emilia sonchifolia* DC. Indian J Chem 15B:635–638

Yogeeswaran A, Velmurugan S, Punitha SMJ, Babu MM, Selvaraj T, Kumaran T, Citarasu T (2012) Protection of *Penaeus monodon* against White Spot Syndrome Virus by inactivated vaccine with herbal immunostimulants. Fish Shellfish Immunol 32:1058–1067

Youtong W, Deachamag P, Phongdara A, Chotigeat W (2011) WSSV: VP26 binding protein and its biological activity. Fish Shellfish Immunol 30:77–83

*Editorial responsibility: Jeff Cowley, Brisbane, Queensland, Australia*

*Submitted: August 14, 2014; Accepted: June 15, 2015*
*Proofs received from author(s): July 20, 2015*