Songklanakarin J. Sci. Technol.
45 (2), 189–196, Mar. – Apr. 2023

http://www.sjst.psu.ac.th

Original Article

In vitro and *in vivo* characterisations of *Centella asiatica* extract against *Vibrio alginolyticus* infection in whiteleg shrimp, *Penaeus vannamei*

Wan Nurhafizah Wan Ibrahim[1], Hasnazifa Huzmi[1], Kok Leong Lee[1], Chen Fei Low[2], Alia Syafiqah Aznan[1], Nurul Aqilah Iberahim[1], Arief Izzairy Zamani[2], A. Abdul Razzak Laith[1], Abu Hena Mustafa Kamal[1], Hassan Ibrahim Sheikh[1], Musa Nadirah[1], Wan Sembok Wan-Zaliha[1], and Musa Najiah[1*]

[1] *Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Kuala Nerus, Terengganu, 21030 Malaysia*

[2] *Institute of Systems Biology* (*INBIOSIS*), *Universiti Kebangsaan Malaysia, Bangi, Selangor, 43600 Malaysia*

Received: 5 October 2021; Revised: 15 December 2022; Accepted: 17 February 2023

Abstract

Methanolic leaf extracts of the medicinal herb *Centella asiatica* (Asiatic pennywort) were screened for *in vitro* and *in vivo* antimicrobial activities against *Vibrio alginolyticus* isolated from whiteleg shrimp, *Penaeus vannamei*. Phytochemical analyses were positive for tannins, saponins, steroids, and cardiac glycosides. Antioxidant test revealed antioxidant activity nearly as potent as that of 0.8 mg/mL ascorbic acid. Minimum inhibitory and bactericidal concentrations against *V. alginolyticus* were determined to be 0.79 and 12.50 mg/mL respectively. Scanning electron microscopy demonstrated disruptions of treated bacterial cells. 30 mg/kg extract supplementation achieved 85% survival of juvenile shrimp in a feeding trial. Histopathology showed increasingly fewer alterations in hepatopancreas from 10, 20 to 30 mg/kg supplementation, where 30 mg/kg preserved the tissues most with relatively complete structure including the star-shaped tubule lumen and various cell types. The present findings suggest the potential of *C. asiatica* as an alternative antimicrobial agent against *V. alginolyticus* as well as other *Vibrio* spp. and gram-negative bacteria in aquaculture.

Keywords: medicinal herb, Asiatic pennywort, methanolic leaf extract, alternative antimicrobial, antioxidant, aquaculture use

1. Introduction

Vibrios are halophilic gram-negative bacteria that can cause zoonosis and substantial mortality in domestic marine fish and invertebrates (Nurhafizah *et al*., 2021; Zhang & Austin, 2000). The widespread use of antibiotics in aquaculture has caused emergence of antibiotic resistance, and prompted a shift to alternatives like phage therapy (Nurhafizah *et al*., 2017), whose benefits however are disputable due to the obscure health effects on consumer, and the lack of a regulatory framework (Plaza *et al*., 2018). The use of probiotics to improve animal health has grown worldwide. Probiotics colonise the gut environment, and create biocidal effects against pathogens, for example in shrimp (D'Arienzo *et al*., 2006), for which the probiotics are either applied to feed or directly into pond water to improve water quality, and to reduce environmental stress (Rico *et al*., 2013). Probiotic bacteria recovered from shrimp aquaculture were nevertheless found to harbour an antibiotic resistance gene in a recent study (Noor Uddin *et al*., 2015).

These findings encourage the search for plant-based natural remedies for shrimp aquaculture use. Biomedicines of plant origin could provide an alternative approach against infectious diseases in aquaculture, supporting aspects of treatment and health improvement (Citarasu, 2010). Plant bioactive compounds with antimicrobial, anti-inflammatory and antioxidant activities can be added to pelleted feed as preventive and therapeutic medications to improve the health of aquatic animals. Commonly known as Asiatic pennywort, Indian pennywort, or Gotu kola, *Centella asiatica* is a low-

*Corresponding author
Email address: najiah@umt.edu.my

growing perennial plant of pan-tropical distribution. Also native to Malaysia, *C. asiatica* is commonly cultivated and consumed crude as salad, and used in folk medicine.

Methanolic extracts of *C. asiatica* has been found to be more inhibitory to bacteria than extracts done using acetone, chloroform, or water (i.e., aqueous extracts), because the methanolic one contains terpenoids, saponins, phenols, flavonoids, and tannins (Idris & Nadzir, 2021). Aqueous extract bath treatment at 100 mg/L has been demonstrated to reduce the mortality of columnaris-infected Nile tilapia without negative effects (Rattanachaikunsopon & Phumkhachorn, 2010). On the other hand, Nuwansi, Verma, Chandrakant, Prabhath, and Peter (2021) optimised koi carp stocking density in aquaponics with *C. asiatica* phytoremediation. *Centella asiatica* supplementation in the diet has also been shown to improve serum and mucosal immunity, phagocytosis, and respiratory burst activity in Nile tilapia (Srichaiyo *et al*., 2020).

As an effort to combat the growing challenge of multidrug resistant *Vibrio* spp. in aquaculture, the current study examines the antimicrobial activity of methanolic extract of *C. asiatica* leaves against *V. alginolyticus* in whiteleg shrimp.

2. Materials and Methods

2.1 Methanolic extraction and phytochemical analysis

Fresh *C. asiatica* plant, locally known as pegaga (Malay), ji xui chao (Chinese) or vallarai (Tamil), was purchased from a local wet market in Kuala Terengganu. Plant identity was verified with reference to Malaysian Herbal Monograph 2015, and the plant identification app PlantSnap (www.plantsnap.com). The leaves were rinsed with running tap water, air-dried (50°C, 24h), and powdered. Dry powder (200g) was soaked overnight in 2L 80% methanol (Ali, El-Sharkawy, Hamid, Ismail, & Lajis, 1995), and filtered (Whatman No. 1, 125mm). The filtrate of crude extract was subjected to rotary evaporation, and stored at -20°C prior to use. Phytochemical analyses for tannins, saponins, cardiac glycosides, terpenoids and steroids were conducted following Edeoga, Okwu, & Mbaebie (2005).

2.2 Antioxidant assay

Antioxidant activity of *C. asiatica* extract was determined by a quantitative 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Sadhu, Okuyama, Fujimoto, & Ishibashi, 2003). Ascorbic acid and 100% methanol were used as positive and negative controls respectively. Absorbance at 540nm was measured using a spectrophotometer.

2.3 Bacterial culture preparation

Vibrio alginolyticus isolate was recovered from -20°C glycerol stock from the culture collection of Fish Disease Laboratory, Universiti Malaysia Terengganu. It had been previously isolated and identified from diseased whiteleg shrimp at a farm in Pengkalan Gelap, Setiu, Terengganu. For comparison, *V. mimicus, V. fluvialis, V. vulnificus, V. cholerae,* and *Photobacterium damselae* isolates were also prepared similarly from the culture collection.

2.4 *In vitro* antimicrobial screening

2.4.1 Agar well diffusion method

Screening was performed using agar well diffusion testing against the six bacteria (Laith & Najiah, 2014; Najiah *et al*., 2011). Overnight cultures were harvested by centrifugation (5,000×*g*, 10min). The bacterial cell pellets were washed twice with 0.85% physiological saline, suspended, and adjusted to 0.5 McFarland standard for 1.5×10^8 CFU/mL (Aznan *et al*., 2018). The inocula were spread on Mueller-Hinton agar (MHA) using sterile cotton swabs, and wells were made with a sterile cork borer. The wells were loaded with 30µL of the extract solution (100 mg/mL) prepared with 80% methanol. Tetracycline disc (TE30, 30 µg/mL) and 80% methanol were used as positive and negative controls respectively. The plates were incubated at 35°C for 24h. The bacterial growth inhibition was quantified by the measured diameter of inhibition zone.

2.4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC were determined using sterile 96-well microtiter plates (Laith, Ambak, Abol-Munafi, Nurhafizah & Najiah, 2017; Laith & Najiah, 2014; Najiah *et al*., 2011). Each well was added with 100µL tryptic soy broth (1.5% NaCl). Wells in column 1 were loaded with 100µL of 100 mg/mL crude extract, followed by two-fold serial dilution until 0.098 mg/mL. Each well was inoculated with 10µL of the overnight culture (1.5×10^8 CFU/mL), and incubated at 35°C for 24h. The mixtures from wells that showed no turbidity were streaked on MHA, and further incubated at 35°C for 24h. The remaining mixtures in the wells were added with 10µL of 0.1% 2,3,5-triphenyltetrazolium chloride (TTC) (Merck, Germany), and incubated for 1h, for purple-to-pink colour change (due to reduction of TTC to formazan because of cellular respiration). The lowest concentration that prevented visible bacterial growth (no colour change) was recorded as MIC, whereas the lowest concentration that prevented bacterial growth on MHA was recorded as MBC.

2.5 Cell disruption test

Extract solutions of 0.79, 1.56, 3.13, 6.25 and 12.5 mg/mL (Najiah *et al*., 2011) were prepared for bacterial cell disruption analysis. Each extract solution (500µL) was mixed with 500µL nutrient broth in 1.5mL tube, followed by adding 100µL bacterial solution (1.5×10^8 CFU/mL). Bacterial growth in nutrient broth was used as a control. The tubes were incubated at 35°C for 24h.

2.6 Scanning electron microscopy (SEM)

The overnight mixtures were centrifuged (5,000 ×g, 10min). The cell pellets were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 1h, rinsed thrice with 0.1M sodium cacodylate buffer (pH 7.2), and centrifuged (5min). The samples were dehydrated 10min each

with 35, 50, 60, 70, 80, 90, 95% ethanol, and twice with 100% ethanol, then air-dried by CO_2 critical point drying, coated with gold, and examined using SEM (EIZO, UK) (Najiah, Nadirah, Ibrahim, Shariat, *et al*., 2011).

2.7 Determination of LD_{50} of *V. alginolyticus*

The pathogenicity of *V. alginolyticus* was determined by experimental challenge in juvenile whiteleg shrimp. The shrimp were acclimatised for two weeks (temperature 28 ± 0.5°C; dissolved oxygen 5 mg/L; pH 7.6 ± 1; salinity 24ppt) before challenge. Inocula at 1.5×10^7, 1.5×10^6, 1.5×10^5, 1.5×10^4 and 1.5×10^3 CFU/mL were diluted from 1.5×10^8 CFU/mL with sterile 0.85% saline. Inoculum (100µL) was injected into the shrimp's ventral sinus near cephalothorax (Harikrishnan, Balasundaram, Jawahar, & Heo, 2011). Control group was injected with 0.85% saline. The shrimp were observed for mortalities for 120h post-infection. Median lethal dose (LD_{50}) was determined based on Reed and Muench (1938).

2.8 *In vivo* antimicrobial screening

Shrimp grower feed No.1 (CP, Thailand) was used to prepare the control (T1) and treatment (T2, T3, T4) diets with 0, 10, 20 and 30 mg/kg extracts respectively by spraying (Selvin, Ninawe, & Lipton, 2011), and drying at 40°C in an oven. Acclimatised (as in 2.7) healthy juvenile shrimp (5.0-6.0g) were injected with LD_{50} of *V. alginolyticus*, followed by in-feed treatment at 3.2% body weight daily. Signs of infection and mortalities were observed. Hepatopancreas samples were collected 15 days post-infection, and fixed in Davidson's fixative for 24h for histopathology.

3. Results

3.1 Phytochemical and antioxidant screening

Phytochemical screening showed the presence of tannins, saponins, steroids, cardiac glycosides, and terpenoids in the extract (Table 1). Antioxidant test showed positive antioxidant activity, which conferred free radicals neutralising capacity nearly as potent as 0.8 mg/mL ascorbic acid (Figure 1).

3.2 *In vitro* antimicrobial screening

3.2.1 Preliminary screening

At 100 mg/mL, the extract demonstrated significant antibacterial activities against *V. alginolyticus* (17 mm), followed by *P. damselae* (15 mm), *V. cholerae* (14 mm), *V. mimicus* (13 mm), *V. vulnificus* (11 mm), and *V. fluvialis* (9 mm). The inhibitory activities were however weaker than those of tetracycline (30 µg/mL) (Figure 2).

3.2.2 MIC and MBC

Vibrio alginolyticus was most sensitive to *C. asiatica* extract with the lowest MIC and MBC of 0.79 mg/mL and 12.50 mg/mL, respectively (Table 2).

Table 1. Bioactive compounds of *C. asiatica* methanolic leaf extract

Bioactive compound type	Results
Steroid	+
Cardiac glycoside	+
Saponin	+
Terpenoid	+
Tannin	+

+ : Present
- : Absent

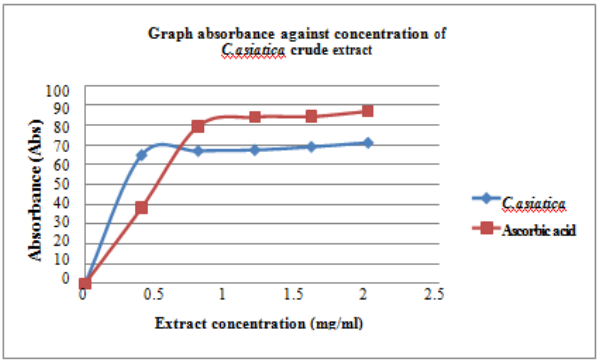

Figure 1. Antioxidant activity of *C. asiatica* leaf extract compared with ascorbic acid reference standard.

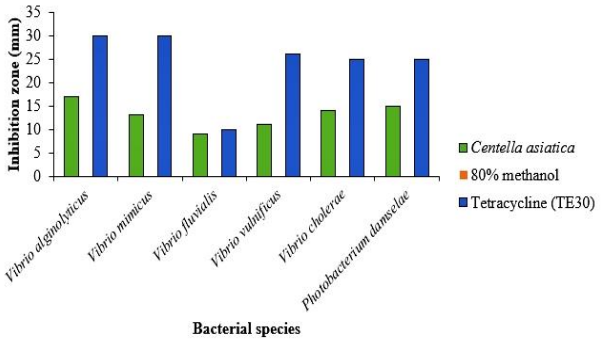

Figure 2. Preliminary antimicrobial screening of *C. asiatica* extract against different bacteria.

Table 2. The MIC and MBC of *C. asiatica* methanolic extract against different bacterial species

Bacteria	MIC (mg/mL)	MBC (mg/mL)
Vibrio alginolyticus	0.79	12.50
Vibrio mimicus	6.25	25.00
Vibrio fluvialis	6.25	12.50
Vibrio vulnificus	3.13	25.00
Vibrio cholerae	1.57	6.25
Photobacterium damselae	3.13	50.00

3.3 Scanning electron microscopy (SEM)

Morphological changes and cell disruption were observed by SEM. Figure 3A shows untreated cell with intact and smooth surface. Cells treated with 0.79 mg/mL (Figure 3B), 1.57 mg/mL (Figure 3C) and 3.13 mg/mL (Figure 3D) extract solutions showed increasing levels of surface roughening, shrinkage, wrinkling and cavitation. Rupture of bacterial cell wall and membrane began to be observed at 6.25 mg/mL (Figure 3E), and cell lysis was apparent at 12.50 mg/mL with released cell contents (Figure 3F).

3.4 Pathogenicity of *V. alginolyticus* against juvenile *P. vannamei*

Challenges from 1.5×10^3 to 1.5×10^7 CFU/mL of *V. alginolyticus* caused mortalities in juvenile *P. vannamei* (Table 3).

1.5×10^7 and 1.5×10^6 CFU/mL caused 100% and 92% mortalities respectively. Fifty percent mortality was observed at 1.5×10^4 CFU/mL, so this was the LD_{50} of *V. alginolyticus* for *in vivo* antimicrobial assay in juvenile *P. vannamei*.

3.5 *In vivo* antimicrobial assay

3.5.1 Cumulative mortality

The highest cumulative mortality was recorded in the control group (T1, 0 mg/kg) at 87%, followed by 67% in T2 (10 mg/kg), 50% in T3 (20 mg/kg), and 15% in T4 (30 mg/kg) (Figure 4).

3.5.2 Histopathological analysis

Figure 5A shows the normal histological structure of hepatopancreas of unchallenged shrimp with a complete star-shaped lumen (star), B-cells (dotted-arrow), E-cells (arrowhead), R-cells (arrow), and F-cells (red arrow). Group T1 (0 mg/kg) showed the most severe pathological changes including necrosis of hepatopancreatic tubule and intertubular connective tissue (arrow), which resulted in degeneration of tubule epithelium, and consequent loss of lumen shape, and enlarged lumen (star), as well as lack of B-, F- and R-cells (arrowhead) (Figure 5B). Groups T2 (10 mg/kg) and T3 (20 mg/kg) showed milder degree of tubule epithelial degeneration, characterised by enlargement of tubule lumen (star) and pyknotic nuclei (arrow) (Figure 5C-D).

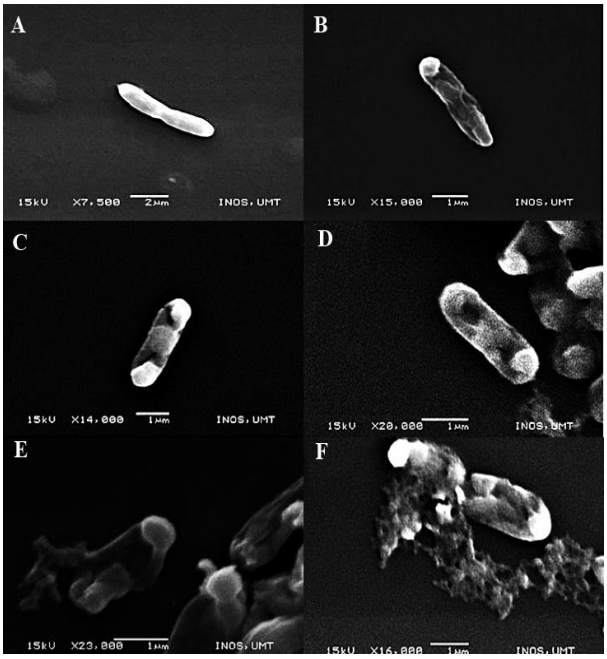

Figure 3. SEM of *V. alginolyticus*: (A) Control, (B-F) *V. alginolyicus* treated at (B) 0.79 mg/mL, (C) 1.56 mg/mL, (D) 3.13 mg/mL, (E) 6.25 mg/mL and (F) 12.50 mg/mL of *C. asiatica* extract. Magnification range from 7,500× to 23,000× at 15kV

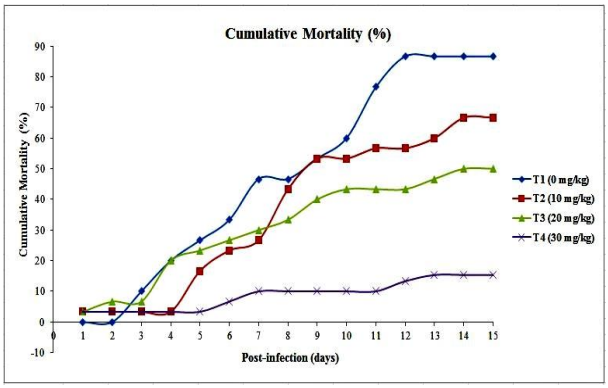

Figure 4. Cumulative mortalities of whiteleg shrimp challenged with *V. alginolyticus*.

Table 3. The LD_{50} of *V. alginolyticus* to whiteleg shrimp according to Reed and Muench method

Bacterial concentration (CFU/mL)	Initial number	Average mortality	Average survival	Cumulative total			Percent mortality
				Mortality	Survival	Mortality ratio	
1.5×10^7	5	5	0	17	0	17/17	100
1.5×10^6	5	4	1	12	1	12/13	92
1.5×10^5	5	3	2	8	3	8/11	73
*1.5×10^4	5	3	2	5	5	5/10	50
1.5×10^3	5	2	3	2	8	2/10	20
Control	5	0	5	0	13	0/13	0

*LD

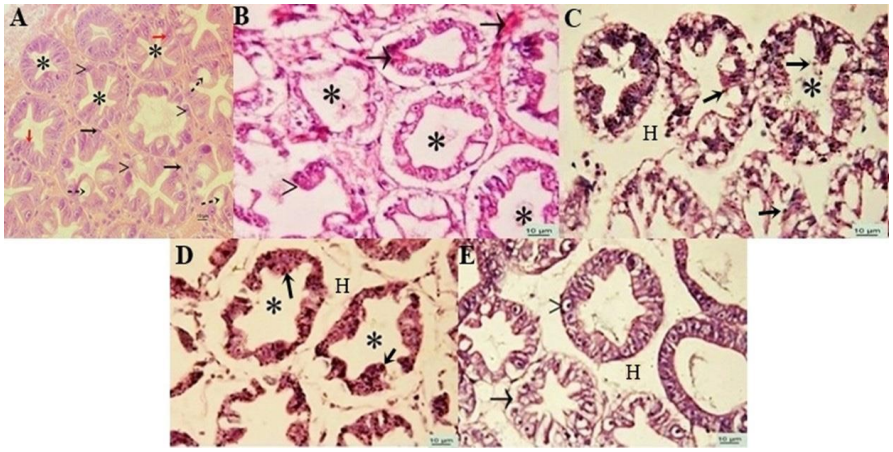

Figure 5. Transverse sections of hepatopancreas. (A) Normal hepatopancreas; (B) hepatopancreas of untreated shrimp (0 mg/kg); (C-E) hepatopancreases of shrimp treated with 10, 20, and 30 mg/kg of *C. asiatica* extract. Scale bar 10 µm

Degeneration and vacuolisation of tubule epithelium and basement membrane caused detachment of tubule from endothelium sheath, and increased distance between adjacent tubules. In general, the alterations of intertubular connective tissue resulted in apparent haemal sinuses (H) despite the dosages of extract given (Figure 5C-E). The shape of tubules was, however, generally retained, though some pyknotic nuclei were observed. The shrimp fed with 30 mg/kg of extract supplement showed relatively normal hepatopancreas with more star-shaped tubule lumen (arrow), as well as B-, F- and R-cells (Figure 5E).

4. Discussion

The misuse of antibiotics in shrimp aquaculture has promoted the emergence of antibiotic resistance, prompting the quest for natural remedies for controlling bacterial diseases. Herbal medicinal applications in aquaculture are evolving (Citarasu, 2010), and evaluation of antimicrobial activities is essential for subsequent compounds identification (Das, Tiwari, & Shrivastava, 2010). Due to its high organic content, methanolic herb extract is efficient against most bacteria (Chopra, 2007). Methanolic extracts of medicinal plants have demonstrated higher antibacterial activities than those extracted using aqueous and hexane solvents (Ahmad, Zaiba-Beg, & Mehmood, 1999), due to high polarity of the bioactive compounds (probably polyphenols or aldehydes) (Power, 1997). Methanolic plant extracts have been proven to be promising antimicrobials against pathogenic bacteria both *in vitro* (Laith *et al*., 2016; Najiah *et al*., 2011) and *in vivo* (Aznan *et al*., 2018; Laith *et al*., 2017).

Methanolic extract of *C. asiatica* contains saponins, tannins, steroids, terpenoids, and cardiac glycosides, having as well antioxidant properties that may be attributed to some of these bioactive substances. Steroidal compounds limit the microbial development by causing plasma membrane leakage and cell death (Harlina, Prajitno, Suprayitno, & Nursyam, 2013). Tannins inhibit microbial adhesion, enzymes, and cell envelope transport proteins (Cowan, 1999). The saponins, steroids, cardiac glycosides, and tannins in *C. asiatica* leaves are likely responsible for free radical scavenging. Putative active compounds are also present in sufficient quantities in crude extract with dose-dependent activities (Taylor, Rabe, McGaw, Jäger, & van Staden, 2001), as seen in the methanolic extract of *C. asiatica*. Antioxidant properties are crucial for defence against reactive oxygen species that cause pathophysiological conditions, and complement the endogenous radical scavenging mechanism (Naznin & Hassan, 2009). The present study demonstrated mitigation of *V. alginolyticus*-induced oxidative damage in *P. vannamei* by the antioxidant activity of *C. asiatica*.

Centella asiatica contains triterpenoids (asiaticoside, madecassoside, asiatic acid, madecassic acid), glycosides, flavonoids, alkaloids, steroids, volatiles, and fatty oils (James & Dubery, 2011; Subban, Veerakumar, Manimaran, Hashim, & Balachandran, 2008), of which triterpenoid saponins represent the most important active ingredient for wound healing (Irham, Tamrin, Marpaung, and Marpongahtum, 2019). Methanolic extracts of *C. asiatica* also possess antibacterial secondary metabolites, and variant metabolites. It has also been found that *C. asiatica* from different geographies varies considerably in active components, despite having identical phenotypes and growth conditions (James and Dubery, 2011; Aziz, Sarmidi, Kumaresan, and Foo, 2005).

The methanolic extract of *C. asiatica* exhibited a MIC of 0.79 mg/mL against *V. alginolyticus*. Previously, whole plant aqueous and methanolic extracts of *C. asiatica* demonstrated about the same level of inhibition strength against *V. alginolyticus* in disc diffusion assay (Lee *et al*., 2008). The morphological alteration and deformation of the treated bacterial cells in a dose-dependent manner as revealed by SEM re-affirm that *C. asiatica* extract acts by disrupting the bacterial cell wall and membrane, and eventually bursts the cells as the extract concentration increases. Previously, *C. asiatica* has been reported to contain asiatic acid (pentacyclic triterpenoid) (Venter *et al*., 2018), which inhibits gram-negative and gram-positive bacteria by disrupting the membranes, and by increasing potassium and nucleotide leaks (Chi *et al*., 2021; Sycz, Tichaczek-Goska & Wojnicz, 2022).

The extract supplement feeding trial significantly reduced shrimp mortality due to *V. alginolyticus* infection with the lowest mortality observed at 30 mg/kg. The improved shrimp survival with the increase of *C. asiatica* extract concentration in feed also suggests that the tested dosages are not at a toxic level. Previously, Phumkhachorn and

Rattanachaikunsopon (2010) demonstrated that *C. asiatica* bath treatment at 100 mg/L caused no adverse effects in *Flavobacterium columnare*-infected Nile tilapia. More recently, Deshpande *et al*. (2019) determined the acute oral toxicity and 90-day repeated dosage toxicity (LD_{50}) of *C. asiatica* in Sprague-Dawley rats to be >2000 and 1000 mg/kg, respectively. These studies help estimate the safe dose levels for short and long-term repeated use of the extract. OECD guideline 425 states that a substance is regarded as safe if the maximum dose causes no deaths or clinical symptoms in the acute oral toxicity investigation.

Hepatopancreas is a very sensitive and important organ in shrimp, which indicates metabolic level, ecdysis phase, nutritional and disease status (Esteve & Herrera, 2000; Iswarya *et al*., 2022). The present study conducted histopathological evaluation of the protective effect of *C. asiatica* extract on hepatopancreas against *V. alginolyticus* infection. Compared with the untreated group (0 mg/kg), the treated groups demonstrated increasingly fewer hepatopancreatic alterations due to *V. alginolyticus* infection from 10, 20 to 30 mg/kg supplement. In other words, 30 mg/kg extract supplement helped preserve more of the healthy hepatopancreatic tissues. The protective effects are attributed to the antibacterial, antioxidant, and anti-inflammatory activities of *C. asiatica* methanolic extract as previously reported (Krishnaiah, Devi, Bono, & Sarbatly, 2009).

5. Conclusions

Our results highlight the potential of methanolic leaf extract of *C. asiatica* as an alternative antimicrobial against *V. alginolyticus* infection in *P. vannamei*. The mechanism of action that breaks cell wall and membrane implies that *C. asiatica* extract may also work on other *Vibrio* spp. not tested in this study, as well as on other gram-negative bacteria. These extracts may serve as a tool for treatment, prevention and control of infections in aquaculture.

Acknowledgements

The research was funded by Fundamental Research Grant Scheme (FRGS/1/2014/STWN03/UMT/02/6), Ministry of Higher Education (MOHE), Malaysia.

References

Ahmad, I., Zaiba-Beg, A. Z., & Mehmood, Z. (1999). Antimicrobial potency of selected medicinal plants with special interest in activity against phyto pathogenic fungi. *Indian Veterinary Medical Journal*, *23*(4), 299–306.

Ali, A. M., El-Sharkawy, S. H., Hamid, J. A., Ismail, N. H., & Lajis, N. H. (1995). Antimicrobial activity of selected Malaysian plants. *Pertanika Journal of Tropical Agricultural Science*, *18*(1), 57–61.

Aziz, R. A., Sarmidi, M. R., Kumaresan, S., & Foo, D. C. Y. (2005). Engineering aspects of herbal and phytochemical processing:A Malaysian perspective. *Herbal and Phyto, December 2005*, 10–19.

Aznan, A. S., Lee, K. L., Low, C. F., Iberahim, N. A., Wan Ibrahim, W. N., Musa, N., . . . Musa, N. (2018). Protective effect of apple mangrove *Sonneratia caseolaris* extract in *Edwardsiella tarda*-infected African catfish, *Clarias gariepinus*. *Fish and Shellfish Immunology*, *78*, 338–345. Retreived from https://doi.org/10.1016/j.fsi.2018.04.033

Chi, J., Sun, L., Cai, L., Fan, L., Shao, C., Shang, L., & Zhao, Y. (2021). Chinese herb microneedle patch for wound healing. *Bioactive Materials*, *6*(10), 3507–3514. Retreived from https://doi.org/10.1016/J.BIOACTMAT.2021.03.023

Chopra, A. K. (2007). Medicinal plants: Conservation, cultivation and utilization. *Medicinal Plants*: *Conservation, Cultivation and Utilization*. Delhi, India: Daya Publishing House.

Citarasu, T. (2010). Herbal biomedicines: a new opportunity for aquaculture industry. *Aquaculture International*, *18*(3), 403–414. Retreived from https://doi.org/10.1007/s10499-009-9253-7

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, *12*(4), 564–582.

D'Arienzo, R., Maurano, F., Mazzarella, G., Luongo, D., Stefanile, R., Ricca, E., & Rossi, M. (2006). *Bacillus subtilis* spores reduce susceptibility to *Citrobacter rodentium*-mediated enteropathy in a mouse model. *Research in Microbiology*, *157*(9), 891–897. Retreived from https://doi.org/10.1016/j.resmic.2006.06.001

Das, K., Tiwari, R. K. S., & Shrivastava, D. K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research*, *4*(2), 104–111. Retreived from https://doi.org/10.5897/JMPR09.030

Deshpande, N. G. J., Saxena, J., Pesaresi, T. G., Carrell, C. D., Ashby, G. B., Liao, M. K., & Freeman, L. R. (2019). High fat diet alters gut microbiota but not spatial working memory in early middle-aged Sprague Dawley rats. *PloS One*, *14*(5), 1–14. Retreived from https://doi.org/10.1371/JOURNAL.PONE.0217553

Edeoga, H. O., Okwu, D. E., & Mbaebie, B. O. (2005). Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology*, *4*(7), 685–688.

Esteve, M., & Herrera, F. C. (2000). Hepatopancreatic alterations in *Litopenaeus vannamei* (Boone, 1939) (Crustacea: Decapoda: Penaeidae) experimentally infected with a *Vibrio alginolyticus* strain. *Journal of Invertebrate Pathology*, *76*(1), 1–5. Retreived from https://doi.org/10.1006/jipa.2000.4951

Harikrishnan, R., Balasundaram, C., Jawahar, S., & Heo, M. (2011). *Solanum nigrum* enhancement of the immune response and disease resistance of tiger shrimp, *Penaeus monodon* against *Vibrio harveyi*. *Aquaculture*, *318*(1–2), 67–73. Retreived from https://doi.org/10.1016/j.aquaculture.2011.05.024

Harlina, Prajitno, A., Suprayitno, E., & Nursyam, H. (2013). The identification of chemical compound and antibacterial activity test of Kopasanda (*Chromolaena Odorata* L.) leaf extract against Vibriosis-causing *Vibrio harveyi* (MR 275 Rif) on Tiger shrimp. *Aquatic Science and Technology*, *1*(2), 15–29. Retreived from https://doi.org/10.5296/

ast.v1i2.3558

Idris, F. N., & Nadzir, M. M. (2021). Comparative studies on different extraction methods of *Centella asiatica* and extracts bioactive compounds effects on antimicrobial activities. *Antibiotics*, *10*(4), 1–24. Retreived from https://doi.org/10.3390/ANTIBIOTICS10040457

Irham, W. H., Tamrin, Marpaung, L., & Marpongahtun. (2019). Bioactive compounds in Pegagan leaf (*Centella asiatica* L. Urban) for wound healing. *Journal of Physics*: *Conference Series*, *1232*(1), 1–6. Retreived from https://doi.org/10.1088/1742-6596/1232/1/012019

Iswarya, A., Marudhupandhi, T., Vaseeharan, B., Ibrahim, W. N. W., Lee, K. L., & Najiah, M. (2022). Chapter 27: Shrimp vibriosis. *Aquaculture pathophysiology*: *Volume II*. *Crustacean and molluscan diseases* (pp. 191–206). Amsterdam, Netherlands: Elsevier.

James, J., & Dubery, I. (2011). Identification and quantification of triterpenoid centelloids in *Centella asiatica* (L.) Urban by densitometric TLC. *Journal of Planar Chromatography - Modern TLC*, *24*(1), 82–87. Retreived from https://doi.org/10.1556/JPC.24.2011.1.16

Krishnaiah, D., Devi, T., Bono, A., & Sarbatly, R. (2009). Studies on phytochemical constituents of six Malaysian medicinal plants. *Journal of Medicinal Plants Research*, *3*(2), 67–72.

Laith, A. A. A., Mazlan, A. G. G., Effendy, A. W. W., Ambak, M. A. A., Nurhafizah, W. W. I., Alia, A. S. S., . . . Najiah, M. (2017). Effect of *Excoecaria agallocha* on non-specific immune responses and disease resistance of *Oreochromis niloticus* against *Streptococcus agalactiae*. *Research in Veterinary Science*, *112*, 192–200.

Laith, A. A., Mazlan, A. G., Effendy, A. W., Ambak, M. A., Nadirah, M., Muhammad, T. S., Zain, S. M., . . . Najiah, M. (2016). Phytochemical composition and *in vitro* antimicrobial, antioxidant activites of methanolic leaf extractsfrom *Excoecaria agallocha*. *Biosciences Biotechnology Research Asia*, *13*(1), 599–608.

Laith, A. A., & Najiah, M. (2014). Antimicrobial activities of blinding tree, *Excoecaria agallocha* against selected bacterial pathogens. *Journal of Microbiology and Antimicrobials*, *6*(2), 29–36. Retreived from https://doi.org/10.5897/jma2013.0291

Lee, S. W., Musa, N., Tse Sengm, C., Wee, W., & Azhar Mohd Shazili, N. (2008). Antimicrobial properties of tropical plants against 12 pathogenic bacteria isolated from aquatic organisms. *African Journal of Biotechnology*, *7*(13), 2275–2278.

Najiah, M., Nadirah, M., Ibrahim, W. W. N., Zahrol, M., Shariat, A., Zamani, A. I., . . . Sakhawi, A. A. S. (2011). Methanolic activities of selected weeds on bacteria isolated from *Macrobrachium rosenbergii* larvae. *The Thai Journal of Veterinary Medicine*, *41*(4), 535–540.

Naznin, A., & Hassan, N. (2009). In vitro antioxidant activity of methanolic leaves and fowers extracts of *Lippia alba*. *Research Journal of Medicine and Medical Sciences*, *4*(1), 107.

Noor Uddin, G. M., Larsen, M. H., Christensen, H., Aarestrup, F. M., Phu, T. M., & Dalsgaard, A. (2015). Identification and antimicrobial resistance of bacteria isolated from probiotic products used in shrimp culture. *PloS One*, *10*(7), 1-21. Retreived from https://doi.org/10.1371/journal.pone.0132338

Nurhafizah, W. W. I., Aznan, A. S., Saari, N. A., Leong, L. K., Musa, N., Razzak, L. A., . . . Din, M. (2017). In-vitro characterization of lytic bacteriophage PhVh6 as potential biocontrol agent against pathogenic *Vibrio harveyi*. *Aquaculture, Aquarium, Conservation and Legislation - International Journal of the Bioflux Society*, *10*(1), 64–76.

Nurhafizah, W. W. I., Lee, K. L., Laith, A. R., Nadirah, M., Danish-daniel, M., Zainathan, S. C., & Najiah, M. (2021). Virulence properties and pathogenicity of multidrug-resistant *Vibrio harveyi* associated with luminescent vibriosis in pacific white shrimp, *Penaeus vannamei*. *Journal of Invertebrate Pathology*, *186*(2021), 1–13. Retreived from https://doi.org/https://doi.org/10.1016/j.jip.2021.107594

Nuwansi, K. K. T., Verma, A. K., Chandrakant, M. H., Prabhath, G. P. W. A., & Peter, R. M. (2021). Optimization of stocking density of koi carp (*Cyprinus carpio* var. koi) with gotukola (*Centella asiatica*) in an aquaponic system using phyto remediated aquaculture wastewater. *Aquaculture*, *532*(2021), 1–9. Retreived https://doi.org/10.1016/j.aquaculture.2020.735993

Phumkhachorn, P., & Rattanachaikunsopon, P. (2010). Isolation and partial characterization of a bacteriophage infecting the shrimp pathogen *Vibrio harveyi*. *African Journal of Microbiology*, *4*(16), 1794–1800.

Plaza, N., Castillo, D., Pérez-Reytor, D., Higuera, G., García, K., & Bastías, R. (2018). Bacteriophages in the control of pathogenic vibrios. *Electronic Journal of Biotechnology*, *31*, 24–33. Retreived from https://doi.org/10.1016/j.ejbt.2017.10.012

Power, E. G. M. (1997). 4 aldehydes as biocides. In G. P. Ellis, & D. K. Luscombe (Eds.), *Progress in Medicinal Chemistry* (pp. 149-201). Amsterdam, Netherlands: Elsevier.

Rattanachaikunsopon, P., & Phumkhachorn, P. (2010). Use of Asiatic pennywort *Centella asiatica* aqueous extract as a bath treatment to control columnaris in Nile tilapia. *Journal of Aquatic Animal Health*, *22*(1), 14–20. Retreived from https://doi.org/10.1577/H09-021.1

Reed, L. J., & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology*, *27*(3), 493–497.

Rico, A., Phu, T. M., Satapornvanit, K., Min, J., Shahabuddin, A. M., Henriksson, P. J. G., . . . Van den Brink, P. J. (2013). Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. *Aquaculture*, *412*, 231–243. Retreived from https://doi.org/10.1016/j.aquaculture.2013.07.028

Sadhu, S. S., Okuyama, E., Fujimoto, H., & Ishibashi, M. (2003). Separation of *Leucas aspera*, a medicinal plant of Bangladesh, guided by prostaglandin

inhibtory and antioxidant activities. *Chemical and Pharmaceutical Bulletin*, *51*(5), 595–598.

Selvin, J., Ninawe, A. S., & Lipton, A. P. (2009). *Shrimp disease management*: *Prospective approaches*. New Delhi, India: Ane Books.

Srichaiyo, N., Tongsiri, S., Hoseinifar, S. H., Dawood, M. A. O., Jaturasitha, S., Esteban, M. Á., . . . Van Doan, H. (2020). The effects gotu kola (*Centella asiatica*) powder on growth performance, skin mucus, and serum immunity of Nile tilapia (*Oreochromis niloticus*) fingerlings. *Aquaculture Reports*, *16*, 1–8. Retreived from https://doi.org/10.1016/J.AQREP.2019.100239

Subban, R., Veerakumar, A., Manimaran, R., Hashim, K. M., & Balachandran, I. (2008). Two new flavonoids from *Centella asiatica* (Linn.). *Journal of Natural Medicines*, *62*(3), 369–373. Retreived from https://doi.org/10.1007/S11418-008-0229-0

Sycz, Z., Tichaczek-Goska, D., & Wojnicz, D. (2022). Anti-planktonic and anti-biofilm properties of Pentacyclic Triterpenes—Asiatic acid and ursolic acid as promising antibacterial future pharma ceuticals. *Biomolecules*, *12*(98), 1–29. Retreived from https://doi.org/10.3390/biom12010098

Taylor, J. L. S., Rabe, T., McGaw, L. J., Jäger, A. K., & van Staden, J. (2001). Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulation*, *34*(1), 23–37. Retreived from https://doi.org/10.1023/A:1013310809275

Venter, H., Tharmalingam, N., Janvilisri, T., Harn voravongchai, P., Chankhamhaengdecha, S., Ounjai, P., Singhakaew, S., & Boonthaworn, K. (2018). Antimicrobial effect of Asiatic acid against *Clostridium difficile* is associated with disruption of membrane permeability. *Frontiers in Microbiology*, *9*(2125), 1–11. Retreived from https://doi.org/10.3389/fmicb.2018.02125

Zhang, X.-H., & Austin, B. (2000). Pathogenicity of *Vibrio harveyi* to salmonids. *Journal of Fish Diseases*, *23*(2), 93–102. Retreived from https://doi.org/10.1046/j.1365-2761.2000.00214.x