Original Article

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

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Abstract

Methanolic leaf extract of 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 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 disruption of treated bacterial cells. 30 mg/kg extract supplementation achieved 85% survival of juvenile shrimp in 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 star-shaped tubule lumen, and various cell types. The present findings suggest the potential of *C. asiatica* as an alternative source of antimicrobial 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 antibiotic use in aquaculture has caused emergence of antibiotic resistance, and prompted a shift to alternatives like phage therapy

(Nurhafizah et al., 2017), which is however disputable due to obscure health effect on consumer, and lack of 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 effect against shrimp pathogens (D'Arienzo et al., 2006). Probiotics are either applied to feed or directly into pond water to improve water quality, and reduce environmental stress (Rico et al., 2013). Probiotic bacteria recovered from shrimp aquaculture were nevertheless found to harbour 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 from the aspect 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 medication to improve the health of aquatic animals. Commonly known as Asiatic pennywort, Indian pennywort, and Gotu kola, *Centella asiatica* is a low-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 extract of *C. asiatica* has been found to be more inhibitory to bacteria than those extracted using acetone, chloroform, and water (aqueous) because it 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 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) and vallarai (Tamil), was purchased from a local wet market in Kuala Terengganu. Plant identity was verified with reference to Malaysian Herbal Monograph 2015, and 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 property of *C. asiatica* extract was determined by 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 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. The isolate was previously isolated and identified from diseased whiteleg shrimp from a farm at 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 technique 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 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 is measured by the 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 microtitre 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 was 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 mixture in the wells that showed no turbidity was streaked on MHA, and further incubated at 35°C for 24h. The remaining mixture 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 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 technique, 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 dried at 40°C (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).

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

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

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).

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

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

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) of 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 release of cell contents (Figure 3F).

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 ranged from 7,500× to 23,000× at 15kV.

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).

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

1.5×10^7 and 1.5×10^6 CFU/mL caused 100% and 92% mortalities respectively. Fifty percent mortalities were observed at 1.5×10^4 CFU/mL, thus 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).

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

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), 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). Group 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). 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).

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.

4. Discussions

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 by 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, as well as 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 adhesions, 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, volatile 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 extract 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

284 treated bacterial cells in a dose-dependent manner as revealed by SEM re-affirm that *C.*
285 *asiatica* extract acts by disrupting the bacterial cell wall and membrane, and eventually
286 bursts the cells as the extract concentration increases. Previously, *C. asiatica* has been
287 reported to contain asiatic acid (pentacyclic triterpenoid) (Venter et al., 2018), which
288 inhibits gram-negative and gram-positive bacteria by disrupting the membranes, and
289 increasing potassium and nucleotide leaks (Chi et al., 2021; Sycz, Tichaczek-Goska &
290 Wojnicz, 2022).

291 The extract supplement feeding trial significantly reduced shrimp mortality due
292 to *V. alginolyticus* infection with the lowest mortality observed at 30 mg/kg. The
293 improved shrimp survival with the increase of *C. asiatica* extract concentration in feed
294 also suggests that the tested dosages are not at a toxic level. Previously, Phumkhachorn
295 and Rattanachaikunsopon (2010) demonstrated that *C. asiatica* bath treatment at 100
296 mg/L caused no adverse effects in *Flavobacterium columnare*-infected Nile tilapia. More
297 recently, Deshpande et al. (2019) determined the acute oral toxicity and 90-day repeated
298 dosage toxicity (LD_{50}) of *C. asiatica* in Sprague-Dawley rats to be >2000 and 1000 mg/kg,
299 respectively. These studies help estimate the safe dose levels for short and long-term
300 repeated use of the extract. OECD guideline 425 states that a substance is regarded as
301 safe if the maximum dose causes no deaths or clinical symptoms in the acute oral toxicity
302 investigation.

303 Hepatopancreas is a very sensitive and important organ in shrimp, which indicates
304 metabolic level, ecdysis phase, nutritional and disease status (Esteve & Herrera, 2000;
305 Iswarya et al., 2022). The present study conducted histopathological evaluation of the
306 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 preserving 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. Conclusion

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 other gram-negative bacteria, as a tool for treatment, prevention and control of infection in aquaculture.

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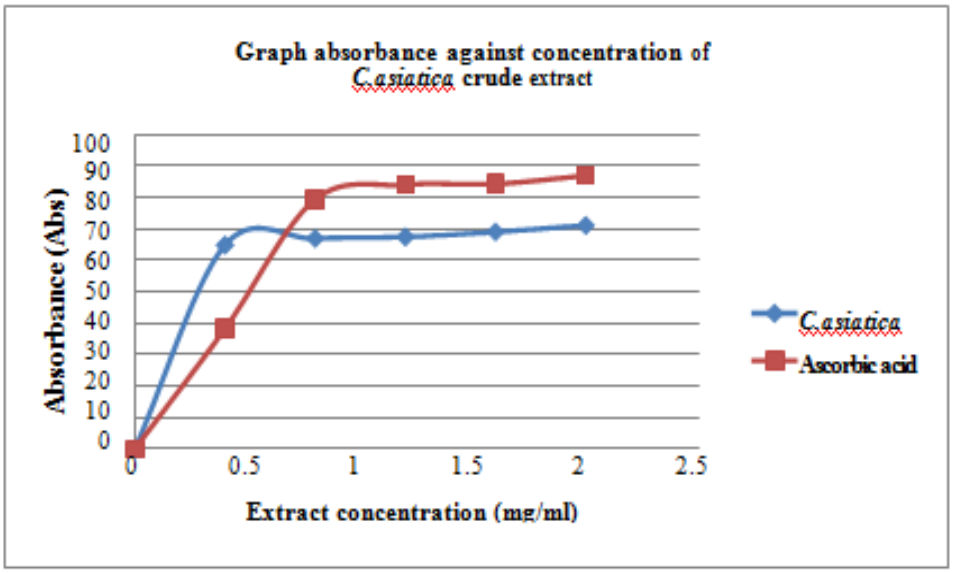
1

FIGURE FILE

2



3

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

5 reference standard.

6

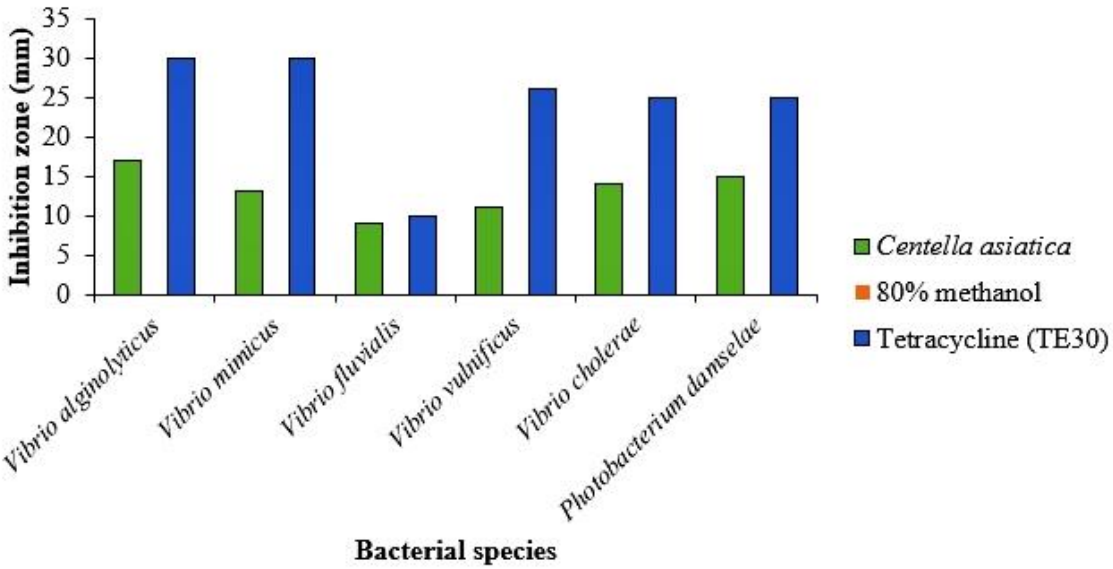


Figure 2 Preliminary antimicrobial screening of *C. asiatica* extract against different bacteria.

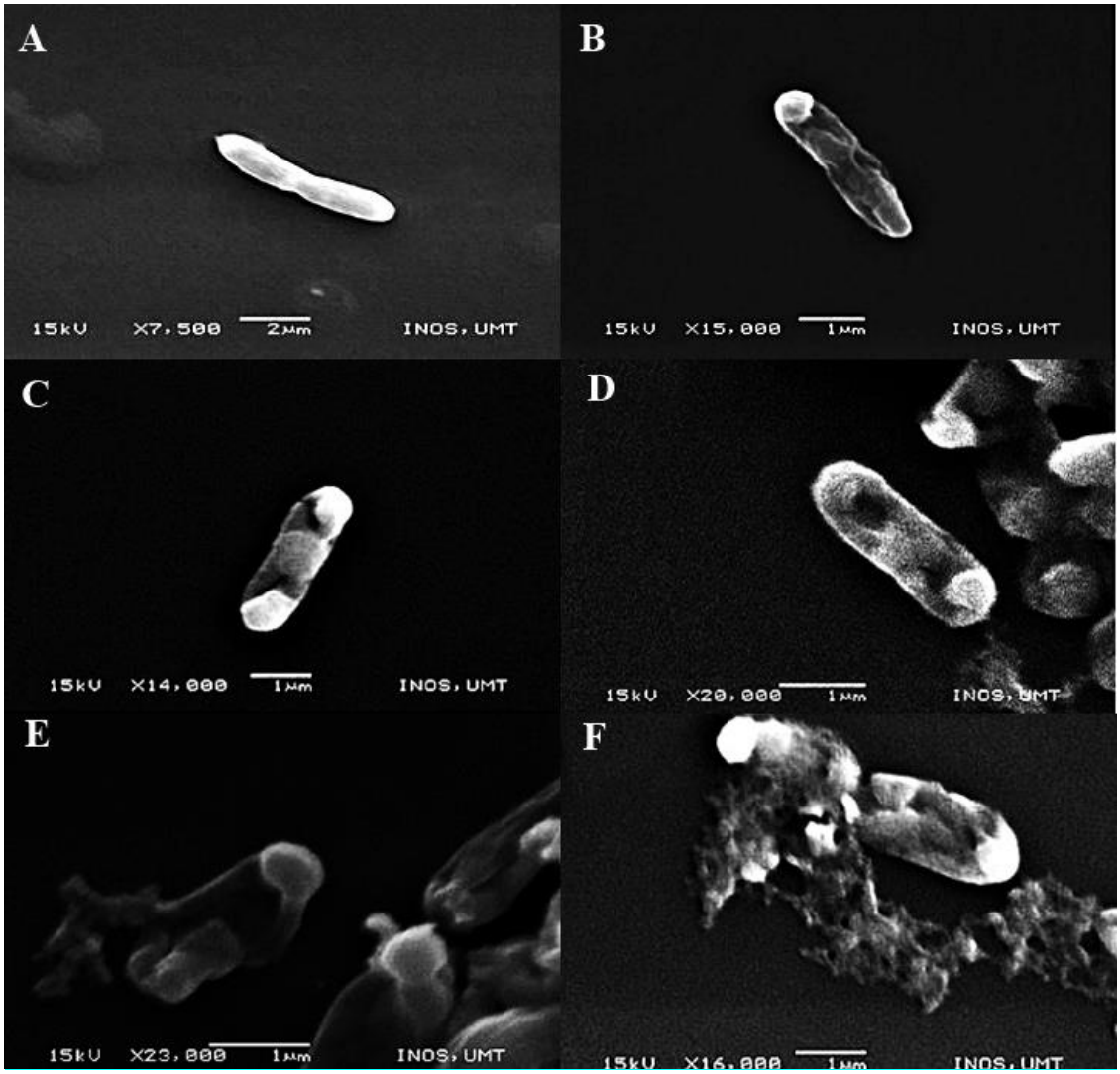


Figure 3 SEM of *V. alginolyticus*: (A) Control, (B-F) *V. alginolyicus* treated at (B) 0.79 mg/mL, (C) 1.56 mg/mL, (D) 3.13 mg/mL, (E) 6.25 mg/mL and (F) 12.50 mg/mL of *C. asiatica* extract. Magnification ranged from 7,500× to 23,000× at 15kV.

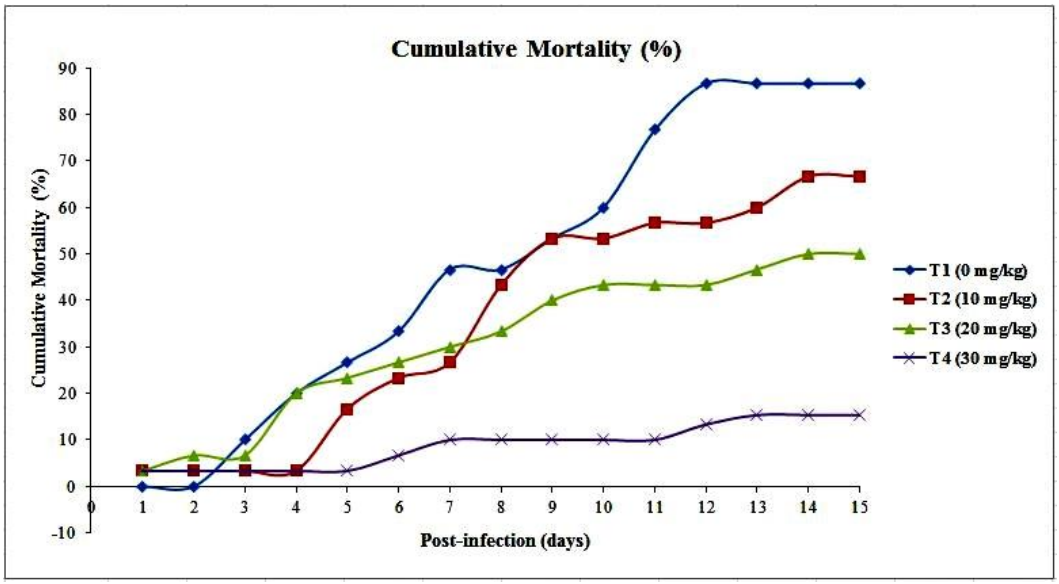


Figure 4 Cumulative mortalities of whiteleg shrimp challenged with *V. alginolyticus*.

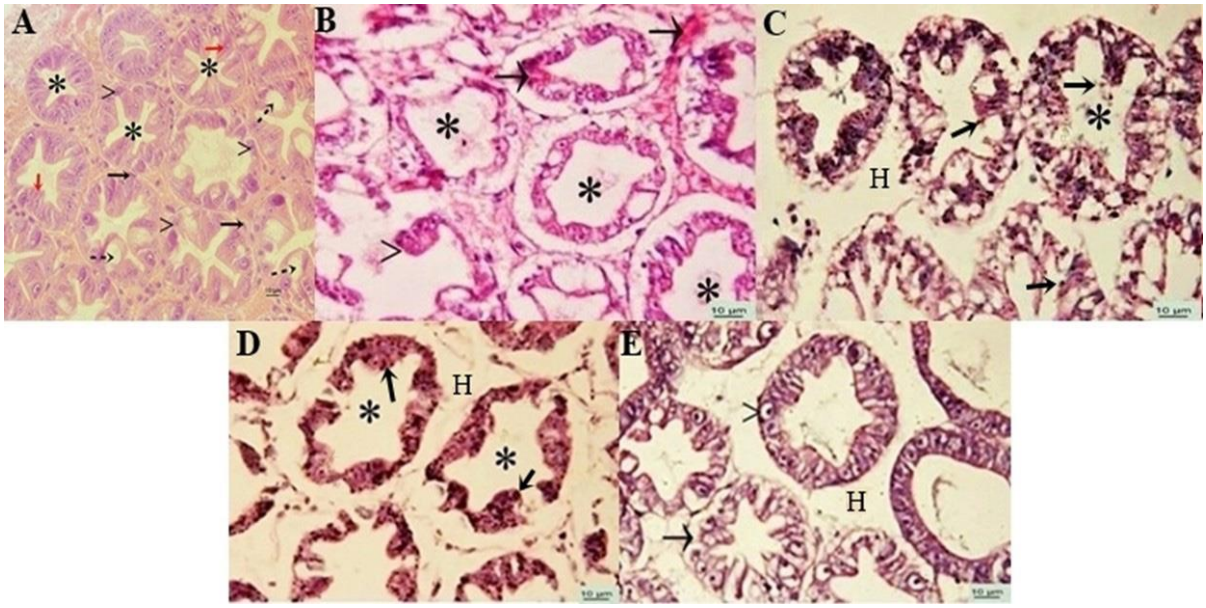


Figure 5 Transverse sections of hepatopancreas. (A) Normal hepatopancreas; (B) hepatopancreas of untreated shrimp (0 mg/kg); (C-E) Hepatopancreases of shrimp treated with 10, 20, and 30 mg/kg of *C. asiatica* extract. Scale bar 10 µm.

1

TABLE FILE

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

Bioactive compounds	**Results**
Steroid	+
Cardiac glycoside	+
Saponin	+
Terpenoid	+
Tannin	+

+ : Present

- : Absent

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

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