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# Antimicrobial effect of *Calotropis procera* active principles against aquatic microbial pathogens isolated from shrimp and fishes

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ABSTRACT

**Objective:** To study the influence of *Calotropis procera* (*C. procera*) active principles against aquatic microbial pathogens isolated from shrimp and fishes. **Methods:** *C. procera* leaf powder was serially extracted with hexane, ethyl acetate and methanol and screened by antibacterial, antifungal and antiviral activity against aquatic pathogens which isolated from shrimp/fish. After initial screening, the active extract was purified through column chromatography and again screened. Finally the active fractions were characterized by phytochemical analysis and GC-MS analysis. **Results:** *In vitro* antibacterial, antifungal and antiviral screening revealed that, the ethyl acetate extracts were effectively suppressed the bacterial pathogens *Pseudomonas aeruginosa* (*P. aeruginosa*), *Vibrio harveyi* (*V. harveyi*) and *Aeromons hydrophila* (*A. hydrophila*) of more than 20 mm zone of inhibition; the fungi Fusarium sp and the killer virus WSSV. The ethyl acetate extracts of *C. procera* incubated WSSV was failed to multiply its progeny in the *in vivo* system of shrimp *P. monodon*. The shrimp had 80% survival after WSSV challenge from the control group significantly ($P<0.001$) and also PCR detection confirmed that no WSSV transcription found in shrimp haemolymph. After purified the ethyl acetate extracts again antimicrobial screening performed and it concluded that the fraction namely F-II was effectively suppressed the bacterial growth and WSSV due to its enriched active principles such as cardiac glycosides, Phenols, alkaloids, Tannin and quinines. Surprisingly this fraction, F-II was effectively controlled the WSSV at 90% level at a highest significant level ($P<0.001$). Finally the structural characterization by GC-MS analysis revealed that, the F-II fraction contained Phenols including several other compounds such as 2,4-bis(1,1-dimethylethyl)-, Methyl tetradecanoate, Bicyclo[3.1.1] heptane, 2,6,6-trimethyl-, (1 α ,2 β ,5 α )-and Hexadecanoic acid *etc.* **Conclusions:** The present study revealed that there is a possibility for developing new eco-friendly antibacterial and antiviral drugs from *C. procera* against aquatic important pathogens.

## 1. Introduction

Diseases problems are currently an important constraint to the growth of aquaculture industry, which has impacted both socio-economic development and rural livelihoods in some countries[1]. As aquaculture production becomes more intensive, the incidence of disease including various infectious diseases has increased as a result of it leading to significant economic losses. Among the microbial diseases, pathogenic bacteria, virus and fungus cause severe damages and economic losses in the hatchery as well as grow out ponds. Bacterial diseases are the major problem affecting shrimp hatcheries and mass mortalities in shrimp hatchery are associated with luminous bacterial disease[2]. Among the viral diseases, White spot syndrome virus or WSSV is a highly lethal, stress-dependent and causes high mortalities and severe damages to shrimp cultures[3]. Generally, fungus is the secondary invaders in wounds, lesions, or abrasions caused by bacterial pathogens, parasitic organisms, abusive handling, or unfavourable environmental conditions[4].

In aquaculture industry, currently applied disease treatment protocols are rather difficult, non-effective, costly and environmentally hazardous. Antibiotics and several other chemicals have been tested as various purposes in aquatic food production. Even though the above chemicals have positive effects on the fishes and shrimps, they cannot be recommended due to their residual effects in

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the muscle of fishes and prawns. Considering the potential threat of diseases on the one hand and the environmental issues on the other hand, aquatic food productoion should concentrate on environment friendly method such as herbal treatment[5]. Natural plant products have been reported to promote various biological activities[5,6]. Citarasu[5] describe antibacterial, antiviral, immunostimulant and anti-stress effect of herbal product the significantly influenced in shrimp aquaculture. Prevention and control of diseases are now the priority for the durability of aquaculture industry.

*Calotropis procera* (*C. procera*) is well known for its ability to produce several biologically active compounds[7]. The broad pharmacological profile of *C. procera* could be interesting for the pharmaceutical industry to develop new drugs[7]. The plant parts have been used as antibacterial[8], antifungal[9], antiviral[10], anticancer[7], anti inflammation[11], antioxidant[12] and wound healing properties[13]. Natural plant products accordingly provide a continual inspiration of bioactive antimicrobial agents with low toxicity, a broad spectrum and good pharmacokinetics to be clinically used without chemical modification[14]. The present work investigates that, anti microbial effect of *C. procera* against the aquatic bacterial, fungal and viral pathogens.

## 2. Materials and methods

### 2.1. Aquatic pathogens

Bacterial pathogens such as *Pseudomonas aeruginosa*, *Salmonella typhi*[15], *Vibrio harveyi*[6], *Photobacterium* sp[16] and *Aeromonas hydrophila*[17] were used this study which isolated from infected shrimps and gold fish respectively. Fungal strain, *Fusarium* sp and the virus White Spot Syndrome Virus (WSSV) were used this study which isolated from infected shrimps.

### 2.2. Plant material and extraction

*C. procera* leaves powders were serially extracted with hexane, ethyl acetate and methanol by percolation method at 48 h. The extracts were filtered by Whatman no.1 filter paper and condense the filtrate by rotary evaporator under reduced pressure at a temperature of 45 to 50 ℃.

### 2.3. In vitro antibacterial and antifungal activity

The various condensed extracts were screened against bacterial pathogens which isolated from infected shrimps/fishes by agar disc diffusion method. For antifungal assay, Fusarium spores were ($1\times10^8$ CFU/mL) inoculated onto sabouraud dextrose agar. Sterile cork borer was used to bore 5 holes on the agar plates and then the extracts introduced aseptically and incubated at 30 ℃ for 5 d.

### 2.4. Minimum inhibitory concentration (MIC)

Different concentrations of plant extracts (10 μg-100 μg) were introduced on wells onto agar plates inoculated with the various pathogenic cultures. Minimum inhibitory concentration (MIC) values were taken as the lowest concentration of extract that inhibited the growth of the pathogen after 24 h of incubation at 37 ℃. Microbial growth was determined by measuring the diameter of the inhibition zone area.

### 2.5. In vitro antiviral screening

Five hundred milligram of condensed plant extracts was dissolved in 100 mL of NTE buffer (0.2 M NaCl, 0.02 M Tris-HCl and 0.02 M EDTA, pH 7.4) as stock for further bioassay studies. Five micro litre of WSSV suspension (300 μg of total protein) was mixed with 10 μL of individual extracts and incubated at 29 ℃ for 3 h. After incubation period, the mixture was injected intramuscularly to *Penaeus monodon* had the average weight of (10 ± 1) g. Three replicates were ($n$= 10×3= 30) maintained in all treatments. Mortalities were recorded daily and the experiment was carried out up to 10 days. Control shrimps were injected with a mixture of 10 μL NTE buffer and 5 μL viral suspensions. Haemolymph samples were collected from all injected shrimps and checked by WSSV diagnostic PCR using VP 28 primer designed by Namita *et al*[18]. The DNA extraction and PCR amplification were carried out by following the method described by Chang *et al*[19]. Haemolymph samples of experimental and control shrimps were tested by the first step PCR. The negative samples detected in the first step were further subjected for second step PCR analysis.

### 2.6. Purification of ethyl acetate extract of C. procera

Based on the primary screening, the active extract, ethyl acetate were purified through preparative silica column chromatography (mesh size 50-80 μm, 30 cm length, 0.5 mL flow rate, 3 bed volume elution) with hexane/ethyl acetate and ethyl acetate/methanol at various proportions as mobile phase, fractions were collected, condensed in a rotary evaporator and stored. Fractions were spotted on silica gel plates GF254 (Merck), 20 cm×20 cm, 1 mm thick and the chromatogram developed using, hexane: ethyl acetate (7:3) and n-butanol: acetic acid: water (5:1:4) as mobile phase. The plates were visualized under short UV light and also the spots were developed using two different spray reagents such as 10% Ammonium molybdic acid containing 1 g of ceric sulphate and 10% of $H_2SO_4$ in MeOH according to Wang *et al*[20].

### 2.7. Secondary antibacterial and antiviral screening

All fractions which eluted from the column chromatography were screened again the respective methodology mentioned earlier.

### 2.8. Phytochemical screening for C. procera active fraction

The phytochemical analysis of the column active fraction of *C. procera* was determined for Alkaloids, terpenoids, cardiac glycosides, steroids, phenols, resins and carboxylic acids, as described by Chatterjee *et al*[21].

### 2.9. GCMS analysis for ethyl acetate active fraction

GC-MS analysis of active fraction of *C. procera* were analysed individually using Agilent GC-MS 5975 Inert XL MSD (United States) gas chromatography equipped with J&W 122 - 5532G DB-5 ms 30 × 0.25 mm × 0.25 μm and mass detector (EM with replaceable horn) was operated in EMV mode. Helium was used as carrier gas with the flow rate of

1.0 mL/min. The injection port temperature was operated at 250 ℃. The column oven temperature was held at 80 ℃ for 2 min then programmed at 10 ℃/min to 250 ℃, which was held for 0 min, and then at 5 ℃/min to 280 ℃ which was held for 9 min. Electron impact spectra in positive ionization mode were acquired between m/z 40 and 450.

### 2.10. Data analysis

One way and two ways Analysis of Variance (ANOVA) were carried out using the software PASW statistics data editor and Ky plot respectively. Means were compared at 0.05 and 0.001% level for One Way ANOVA and Two Way ANOVA respectively.

## 3. Results

### 3.1. In vitro antibacterial and antifungal activity

The antibacterial and antifungal activity of hexane, ethyl acetate and methanolic extracts of *C. procera* leaves against shrimp pathogenic bacteria and fungi were presented in Table 1. From the results, it's evident that the ethyl acetate extract of *C. procera* leaves showed a maximum activity of 24.8, 24.3 and 23.3 mm of zone of inhibition against *V. harveyi*, *A. hydrophila* and *P. aeruginosa* respectively and against the fungi *Fusarium* sp had 15.1 mm of zone of inhibition.

### 3.2. Minimum inhibitory concentration (MIC)

MICs are considered the "gold standard" for determining the susceptibility of microorganisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. Results of MIC and MBC showed that *A. hydrophila* and *V. harveyi* had highest MIC (60 μg/mL) while the lowest MIC and MBC of *S. typhi* had (120 μg/mL) (Table 2).

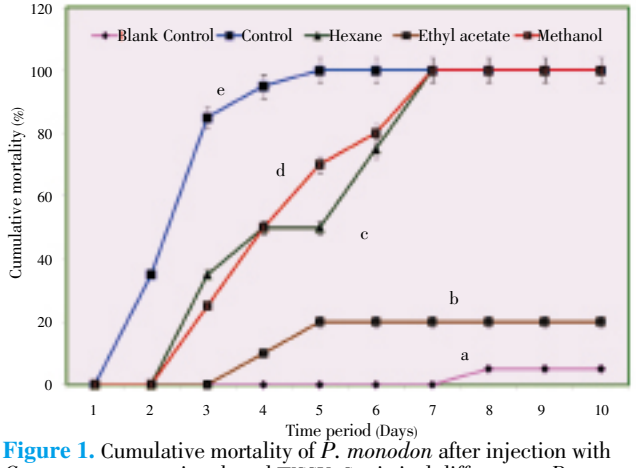


**Figure 1.** Cumulative mortality of *P. monodon* after injection with *C. procera* extracts incubated WSSV. Statistical differences ($P<0.001$; $F=28.97$) between treated and control groups are indicated by a–d; error bars are standard errors (two way ANOVA).

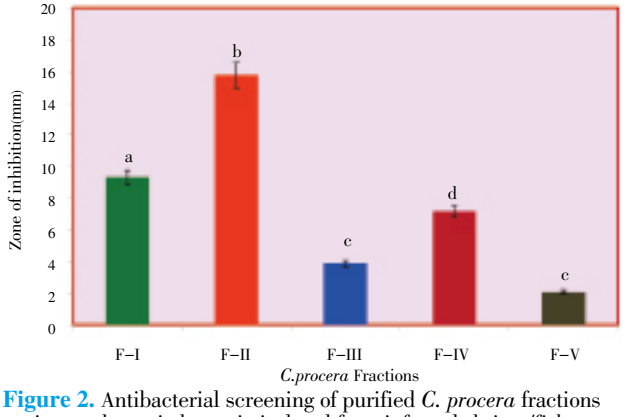


**Figure 2.** Antibacterial screening of purified *C. procera* fractions against pathogenic bacteria isolated from infected shrimp/fish. Statistical differences ($P<0.001$) between groups are indicated by a–c; error bars are standard errors (one way ANOVA).

**Table 1.**
*In vitro* antibacterial and antifungal activity of the *C. procera* extracts against aquatic pathogens.

| Aquatic pathogens | Antimicrobial activity (mm of zone of inhibition) | | |
|---|---|---|---|
| | Hexane extract | Ethyl acetate extract | Methanolic extract |
| *Pseudomonas aeruginosa** | 8.30 ± 2.86 | 23.30 ± 3.29 | 11.30 ± 2.94 |
| *Salmonella typhi** | 6.00 ± 1.32 | 17.60 ± 2.05 | 8.25 ± 2.16 |
| *Vibrio harveyi** | 3.23 ± 0.52 | 24.80 ± 3.29 | 9.50 ± 1.16 |
| *Photobacterium* sp* | 1.55 ± 0.52 | 16.30 ± 1.24 | 4.30 ± 1.69 |
| *Aeromonas hydrophila** | 3.30 ± 1.24 | 24.30 ± 1.24 | 5.30 ± 2.05 |
| *Fusarium* sp** | 8.09 ± 1.24 | 15.10 ± 2.86 | 5.30 ± 2.05 |

*Bacterial pathogens isolated from infected shrimp/Fish; **Fungal pathogen isolated from infected shrimp hatchery tanks.

**Table 2.**
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethyl acetate extract of *C. procera* against bacterial pathogens.

| Concentration (μg) | *P. aruginosa* | | *S. typhi* | | *V. harveyi* | | *Photobacterium* sp | | *A. hydrophila* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | MIC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| 10 | | ++++ | | ++++ | | ++++ | | ++++ | | ++++ |
| 20 | | +++ | | ++++ | | +++ | | ++++ | | +++ |
| 40 | | +++ | | +++ | | ++ | | +++ | | ++ |
| 60 | | ++ | | +++ | 60 | + | | +++ | 60 | + |
| 80 | 80 | + | | ++ | | − | | ++ | | − |
| 100 | | − | | ++ | | − | 100 | + | | − |
| 120 | | − | 120 | + | | − | | − | | − |
| 140 | | − | | − | | − | | − | | − |

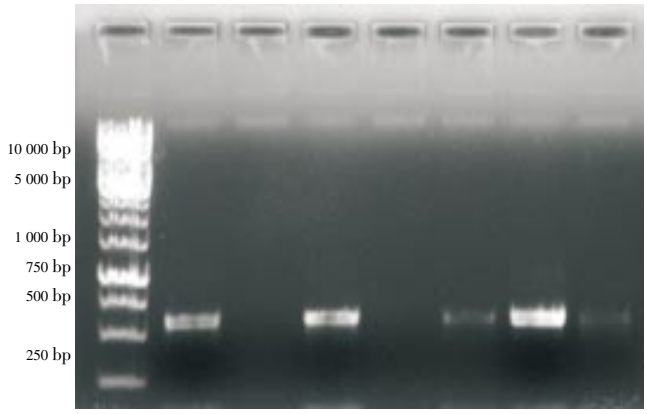


**Figure 3.** Antiviral screening of purified ethyl acetate extract of *C. procera* against WSSV by PCR detection. M-Marker; 1- Positive control; 2-Negetive control; 3: F-I; 4: F-II; 5: F-III; 6: F-IV; 7: F-V.

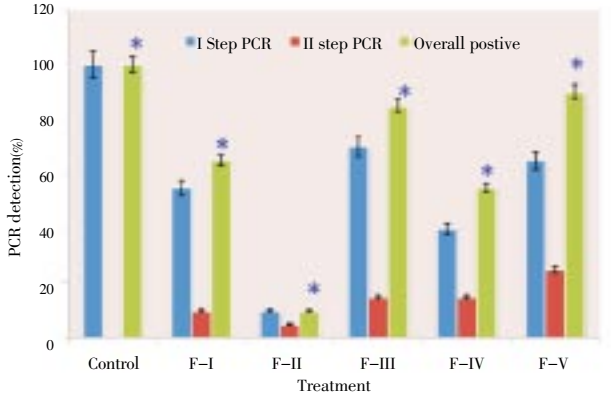


**Figure 4.** PCR detection of haemolymph samples of *P. monodon* after injection with *C. procera* ethyl extracts fractions incubated with WSSV. Statistical differences ($P$<0.001) between treated and control groups are indicated by asterisks; error bars are standard errors.

### 3.3. In vitro antiviral activity

Cumulative mortality data of *P. monodon* reflected that the antiviral activity of *C. procera* extracts (Figure 1). The ethyl acetate extract was effectively suppressed the WSSV during incubation. All *P. monodon* were succumbed to death within 5 d by WSSV challenge in the control groups. Surprisingly, the *P. monodon* had 80 % survival in the ethyl acetate extract treated group and two way ANOVA revealed that, there is a significant ($P$<0.001; $F$=28.97) differences between and control groups. The other extracts hexane and methanol treated groups had only little activity.

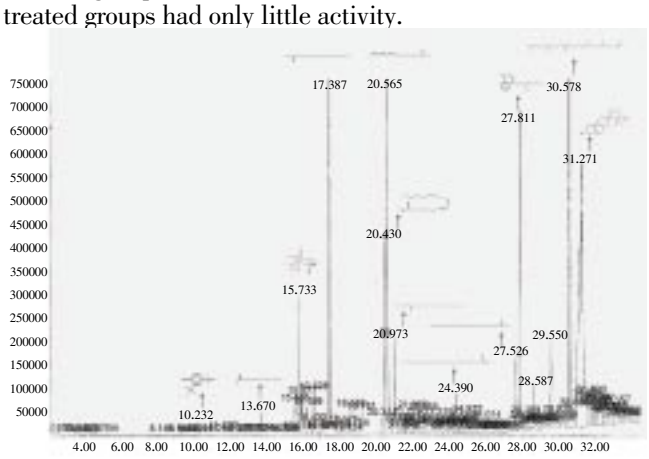


**Figure 5.** GCMS analysis and identified major compounds of the active fraction of ethyl acetate extract of *C. procera*.

**Table 3.**
Phytochemical analysis of the *C. procera* extracts.

| Phytochemical tests | *C. procera extracts* | | |
|---|---|---|---|
| | Hexane | Ethyl acetate | Methanol |
| Steroids -I | + | - | - |
| Steroids -II | - | ++ | + |
| Saponins | - | + | + |
| Flavonoids | + | + | + |
| Coumarin | - | - | - |
| Carboxylic acids | - | + | - |
| Cardiac glycosides | - | ++ | + |
| Phenols | - | ++ | - |
| Quinone | - | + | + |
| Resins | - | + | + |
| Terpenoids | - | - | + |
| Alkaloids | - | ++ | + |
| Tannins | - | + | - |

### 3.4. Secondary antimicrobial screening

Purified ethyl acetate extracts had five distinct fractions with antimicrobial activities. Among the five fractions, fraction (F-II) had higher activity (17.77 mm of zone of

**Table 4.**
Major chemical compounds identified from the active fraction of ethyl acetate extract of *C. procera* leaves by GCMS analysis.

| Sl. No | Retention time | Name of the compounds | Molecular formula | Molecular weight | Quality % |
|---|---|---|---|---|---|
| 1 | 10.232 | Phenol,2,4-bis (1,1-dimethylethyl) | $C_{14}H_{22}O$ | 206 | 96 |
| 2 | 13.670 | Methyl tetradecanoate | $C_{15}H_{30}O_2$ | 242 | 98 |
| 3 | 15.733 | Bicyclo [3.1.1] heptane, 2,6,6-trimethyl-, (1 α ,2 β ,5 α )- | $C_{10}H_{18}$ | 138 | 64 |
| 4 | 17.387 | Hexadecanoic acid,methyl ester | $C_{17}H_{34}O_2$ | 270 | 99 |
| 5 | 20.430 | 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | $C_{19}H_{34}O_2$ | 294 | 99 |
| 6 | 20.565 | 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- | $C_{19}H_{32}O_2$ | 292 | 99 |
| 7 | 20.973 | Octadecanoic acid, methyl ester | $C_{19}H_{38}O_2$ | 298 | 96 |
| 8 | 24.390 | Eicosanoic acid, methyl ester | $C_{21}H_{42}O_2$ | 326 | 99 |
| 9 | 27.528 | Docosanoic acid, methyl ester | $C_{23}H_{46}O_2$ | 354 | 99 |
| 10 | 27.811 | 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester | $C_{16}H_{22}O_4$ | 278 | 91 |
| 11 | 28.587 | Tricosanoic acid, methyl ester | $C_{24}H_{48}O_2$ | 368 | 99 |
| 12 | 29.550 | Tetracosanoic acid, methyl ester | $C_{25}H_{50}O_2$ | 382 | 99 |
| 13 | 30.578 | Squalene | $C_{30}H_{50}$ | 410 | 98 |
| 14 | 31.271 | Gamma-sitosterol | $C_{29}H_{50}O$ | 414 | 99 |

inhibition) against *V. harveyi*, followed by F-I (9.33 mm) and 7.17 of F-IV respectively. One Way ANOVA revealed that, the fractions (F-I, II and IV) had differed significantly ($P<0.001$) (Figure 2). PCR detection proved the antiviral activity against WSSV by different fractions. There is no PCR positive signals obtained in the F-II (Figure 3). Also the two step detections confirmed the strong viral activity of the F-II fraction. After the two step detections, only 10% of the *P. monodon* from F-II treated group had PCR positive followed 55 % in F-IV; 65 % in F-I. The fractions 3 and 5 had more than 80% PCR positive revealed that no antiviral activity in this fractions. Also statistical analysis revealed that between treated and control groups had significantly differed ($P<0.001$) (Figure 4).

### 3.5. Phytochemical screening

The phytochemical studies of the F-II column elution of the ethyl acetate extract, *C. procera* showed rich in Steroid-II, cardiac glycosides, phenols, alkaloids, Tannin and quinines. Also the fraction contains saponins, flavonoids, carboxylic acids and resins (Table 3).

### 3.6. Structural characterization

GCMS analysis revealed that, the Fraction-II of *C. procera* ethyl acetate extract had six identified phytochemical compounds. The higher intensity peaks and its quality revealed that the identified compounds are phenol, 2,4-bis(1,1-dimethylethyl)-, methyl tetradecanoate, bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (1α,2β,5α)-, hexadecanoic acid, methyl ester, 9,12-octadecadienoic acid (Z,Z)-, methyl ester, 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)-, eicosanoic acid, methyl ester, 1,2-benzenedicarboxylic acid mono(2-ethylhexyl) ester, tricosanoic acid, methyl ester, tetracosanoic acid, methyl ester, squalene and gamma-sitosterol (Table 4 & Figure 5).

## 4. Discussion

*C. procera* is widely used in folk medicine as a rich source of biologically active compounds capable of promoting diverse benefits such as control of dermal fungal infections, antimicrobial activities and pain relief among other useful properties. The present study revealed that, the organic solvent extracts of *C. procera* leaves showed considerable antibacterial, antifungal and anti viral activities against *P. aeruginosa*, *V. harveyi*, *A. hydrophila*, Fusarium sp and WSSV. Organic solvent extract of *Calotropis gigantia* of effectively suppressed the cariogenic bacteria, *Actinomyces viscosus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Streptococcus mitis* and *Streptococcus mutans*. The chloroform extracted fraction of latex showed inhibitory effect against *S. mutans* and *L. acidophilus* with MIC value of 0.032 and 0.52/mL respectively[8]. The present study, among the three different extracts, ethyl acetate had highest activities against the majority of the pathogens due to the mid polar compound present in the extracts. The hexane extract had very little activities against the pathogens because of the non polar compounds present in this extracts with no antimicrobial effects. Also the lowest MIC was recorded at 60 μg/mL of the ethyl extract against the three pathogens, *P. aeruginosa*, *V. harveyi* and *A. hydrophila*. Osmotin a purified protein from the latex of *C. procera* had effective antifungal activity against *Fusarium solani*, *Neurospora* sp and *Colletotrichum gloeosporioides*[22]. Also the water extracts of *C. procera* effectively suppressed the various *Fusarium* sp including *F. oxysporum*, *F. lupine* and *F. oxysporum*[23].

Our previous work[24], *P. guajava* ethyl acetate extract was effectively suppressed the WSSV during the *in vitro* and *in vivo* delivery. Twenty species of Indian traditional medicinal plants such as *Aegle marmelos*, *Allium sativum*, *Aristolochia indica*, *Azadirachta indica*, *Cassia fistula*, *Catharanthus roseus*, *Curcuma longa*, *Cynodon dactylon*, *Lantana camara*, *Melia azedarach*, *Mimosa pudica*, *Momordica charantia*, *Morus alba*, *Ocimum americanum*, *Phyllanthus amarus*, *Phyllanthus emblica*, *Psidium guajava*, *Solanum nigrum*, *Tridax procumban* and *Tylophora indica* were tested for their antiviral activity against WSSV[25]. The present study, the ethyl acetate extract of *C. procera* helps to enhance the survival at 80% against WSSV challenge from the control and other experimental groups due to the antimicrobial factors in the extracts. Also molecular detection evident that, there is no signal obtained from the Fraction-II treated shrimps. Citarasu *et al*[26], fed with the antiviral and immunostimulant herbal extracts incorporated diets to the WSSV infected Shrimp, *P. monodon* juvenile and the impact of the herbals were accessed with PCR diagnosis. The plants like *Cyanodon dactylon*, *Aegle marmelos*, *Tinospora cordifolia*, *Picrorhiza kurooa* and *Eclipta alba* were effectively controlled the WSSV, *in vivo* system.

The phytochemical screening of the crude extract of *C. procera* revealed that presence of saponin, tannins, sequiterpene and alkaloids. This leads to a conclusion that the presence of these compounds could have lead to the anti HIV-1 activity of *C. procera*[27]. The F-II of ethyl extracts of *C. procera* enriched with alkaloids, phenols, quinines and cardiac glycosides leading to suppress or inhibit the WSSV transcription. In India some of the shrimp farmers have been using the raw *C. procera* herbs for applying in the shrimp ponds against the prevention of WSSV (Palanikumar, pers. com). Recently three new metabolites, 5-hydroxy-3,7-dimethoxyflavone-40-O-β-glucopyranoside, 2b,19-epoxy-3b,14b-dihydroxy-19-methoxy-5a-card-20 (22)-enolide and β-anhydroepidigitoxigenin-3b-Oglucopyranoside were isolated from *C. procera* had potent antimicrobial and cytotoxic activities[28]. Mueen Ahmed *et al*[29] showed that the leaves and latex of *C. gigantea* species were found to have cardiac glycosides. The cardiac glycosides were identified as calotropogenin and calotropin. *C. procera* received special attention, with lots of publications describing the biological activities of molecules and aqueous and organic extracts obtained from its distinct tissues. The root bark of *C. procera* is reported to contain numerous chemical constituents including akundarol isovalerate, mundarol isovalerate and quercetin-3-rutinoside. It has been reported that the ethyl acetate phase of the methanolic extract of *C. procera* roots contain oxypregnaneoligoglycosides named Calotroposides[30]. The present study the GC-MS analysis revealed that, the F-II extracts enriched with phenolic and other related compounds and it may be suppress the transcription of virus and bacterial pathogens. The present study revealed that *C. procera* ethyl acetate extract had the antimicrobial activity against aquatic pathogens. It concludes that, there is a positive approach to develop safe antibacterial and antiviral drugs against aquatic pathogens and save the huge economic loss and severe damages in the aquaculture industry.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

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