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

Subramanian Velmurugan, Vijayaragavan Thanga Viji, Mariavincent Michael Babu, Mary Josephine Punitha, Thavasimuthu Citarasu*

Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Kanyakumari District, Tamilnadu, India

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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

*Corresponding author: Thavasimuthu Citarasu, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Kanyakumari District, Tamilnadu, India.
Tel: +91-4652-253078, +91-9994273822
Fax: +91-4652-253078
E-mail: citarasu@gmail.com
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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 (1×10^8 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