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Inhibition of Fish Pathogenic *Aeromonas hydrophila* and *Edwardsiella tarda* by *Centella asiatica In-vitro*

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Abstract

The present study assessed the *in-vitro* inhibition of fish pathogenic bacteria, viz., *Aeromonas hydrophila* and *Edwardsiella tarda* by aqueous, methanol and chloroform extracts of *Centella asiatica* by agar-disc diffusion, agar overlay well-diffusion, and broth dilution assays. The agar-disc diffusion assay with 10 µL of sterile crude *C. asiatica* extracts failed to inhibit *A. hydrophila;* while the crude chloroform extract inhibited *E. tarda* (11.25 ± 0.35 mm). In agar overlay well-diffusion assay, the methanol and chloroform extracts of *C. asiatica* (50 µL) inhibited *E. tarda* at varying levels exhibiting zones of 7.50 ± 0.70 mm and 30.50 ± 6.40 mm, respectively. With the increasing concentration of crude chloroform *C. asiatica* extract (0-10%/mL), an increased growth inhibition of *E. tarda* was noted in broth dilution assay. These results demonstrated that the chloroform extract of *C. asiatica* has the highest antibacterial activity against *E. tarda in-vitro*, which can be applied as an alternative to the commercial antibiotic to control *E. tarda* infection in aquaculture.

Keywords: *Centella asiatica*; Chloroform extract; Antimicrobial activity; *In-vitro* assay; Growth inhibition

Introduction

Fish diseases cause a huge economic loss in the aquaculture sector. Out of them, bacterial diseases are the most important cause of losses [1,2]. *Aeromonas hydrophila* is an opportunistic Gram-negative bacterial pathogen, which is prevalent in aquatic habitats with cosmopolitan distribution and has resulted in heavy mortalities in farmed and feral fish [1]. *Edwardsiella tarda* is a zoonotic Gram-negative bacterial pathogen, which can infect a variety of animals including mammals, amphibians, reptiles and fish. It has a worldwide distribution and can be found in pond water, mud, and the intestine of fish and other marine animals [2]. These pathogens are being controlled by antibiotics. However, considering the inherent negative effects of antibiotics, other alternative antimicrobials from plant origins are increasingly used in aquaculture [3]. Since prehistoric times, herbs were the basis for nearly all medicinal therapy until synthetic drugs were developed in the 19th century [4]. Medicinal plants have many traditionally reported properties including the treatment of ailments of infectious origin. Antimicrobial properties of medicinal herbs are increasingly reported from different parts of the world [5]. Among many herbs having antimicrobial activity, the members belonging to Apocynaceae and Lamiaceae are considered as the most effective against pathogens [6]. *Centella asiatica,* also known as Asiatic pennywort or *Gotu kola* or Indian pennywort, is a perennial herbaceous plant belonging to the family Apiaceae, subfamily Mackinlayoideae [7]. It is used as antibacterial, anti-inflammatory, antidiabetic, antioxidant and antifungal [8]. It is also recommended for wound healing, improving blood circulation, strengthening the veins, revitalising the nerve and brain cells, increasing the memory and concentration, bringing down the fever and treating dysentery, diarrhoea and skin diseases [9]. The active compounds in *C. asiatica* are many types of terpenes or terpenoids. It showed a promising antibacterial effect against *Bacillus cereus* and *Listeria monocytogenes* under normal and osmotic stress [10] and other human bacterial pathogens [11-13]. An earlier study has shown the potentialities of medicinal herbs in controlling certain fish pathogens [14]. This study assessed the antimicrobial activity of *C. asiatica* extracts against fish pathogenic bacteria like *Aeromonas hydrophila* and *Edwardsiella tarda.*

Materials and Methods

Fresh leaves (Figure 1a) of *C. asiatica* were collected from the Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, India (22°47'N, 88°40'E). The leaves were thoroughly washed in running water and air-dried. The leaves were then dried in hot-air oven at 50°C for 24 hours, ground to a fine powder and sieved (ϕ: 0.9 mm). The sieved samples (Figure 1b) were subsequently soaked separately in water and organic solvents, viz., methanol and chloroform for two days with continuous shaking at 200 rpm at 30°C. The extracts of each solvent were filtered twice using Whatman No.1 filter paper. The filtrates were concentrated, filter sterilized through a 0.45-µm membrane filter and stored at –20°C until further use. The fish pathogenic bacterial strains, viz., *Aeromonas hydrophila* (NCBI accession number KC914628) and *Edwardsiella tarda* (NCBI accession number KF853565) were from the collections of the Department of Aquatic Animal Health, Faculty of Fishery Sciences, Kolkata. Unless otherwise stated, the bacteria were routinely maintained aerobically on brain heart infusion agar [BHIA] or broth [BHIB] (HiMedia, India) at 30°C.

The agar-disc diffusion assay was performed using 18 h culture of *A. hydrophila* (2.35×10^9 CFU/mL) and *E. tarda* (1.28×10^9 CFU/mL) at 30°C. The bacterial lawn was prepared by spreading the cell suspension using a sterile cotton swab on Mueller-Hinton Agar [MHA] (HiMedia, India) in order to get a uniform bacterial growth [15]. Sterile discs

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(HiMedia, India) of 6 mm diameter were placed onto the seeded MHA and loaded with 10 µL of sterile crude aqueous, methanol and chloroform extracts, and their respective control (negative) separately. A chloramphenicol disc (30 µg/disc; HiMedia, India) was used as a positive control. The plates were incubated at 30°C for 24 hours and observed for the zones of inhibition (mm). The soft-agar overlay well-diffusion assay was performed as per Hockett and Baltrus [16]. Briefly, the wells of 5 mm diameter were made aseptically on deep MHA using a sterile well borer. The bottom of the wells were sealed-off using molten soft BHIA (BHIB + 0.7% agar). The filtered sterilized extracts (50 µL each) were added into the wells along with their respective solvents as control. The extracts were allowed to diffuse into the medium for 1 hour. The plates were then overlaid with 10 mL molten soft agar seeded with 10 µL of 20 h old culture of *A. hydrophila* and *E. tarda* so as to get a concentration of ~10^6 cells/mL. The plates were incubated for 24 h at 30°C and observed for the zones of inhibition. The growth inhibition of *E. tarda* by the chloroform extract of *C. asiatica* was determined by broth dilution assay [17]. The tubes containing BHIB were supplemented with sterile chloroform extract at concentrations of 0 (control), 0.5%, 1.0%, 1.5%, 2.5%, 5%, 7.5% and 10%. The broths were then inoculated with *E. tarda* at about 5 × 10^6/mL level. After incubation at 30°C for 24 h, the optical density (OD) was measured at 620 nm using a UV- visible spectrophotometer. The difference between the OD of the sample and control was interpreted as the inhibitory effect of *C. asiatica* on *E. tarda*. The growth inhibition of chloroform extract was determined by plotting the change in the OD against the concentration of *C. asiatica*. The experimental data, presented as the mean ± standard deviation, were statistically analyzed by one-way ANOVA followed by Duncan's Multiple Range Test using SPSS 16.0 software. The membrane filtered chloroform extract was adjusted to a concentration of 0.1 g/mL using sterile chloroform for UV-VIS spectrum analysis. The extract was scanned at wavelength ranging from 400 to 900 nm using LABINDIA® UV 3200 spectrophotometer and the characteristic peaks were detected using UV-Win spectrophotometer software Ver 5.2.0.1104. The peak values of the UV-VIS were recorded.

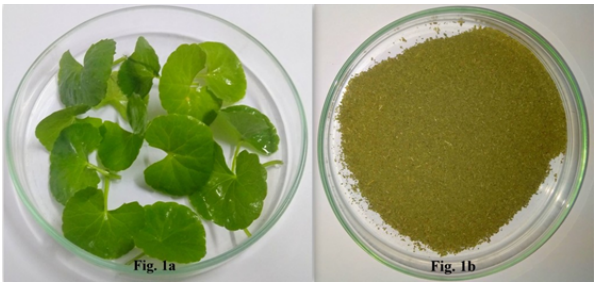

Figure 1: *Centella asiatica* leaves (a) fresh and (b) after drying, pulverizing and sieving.

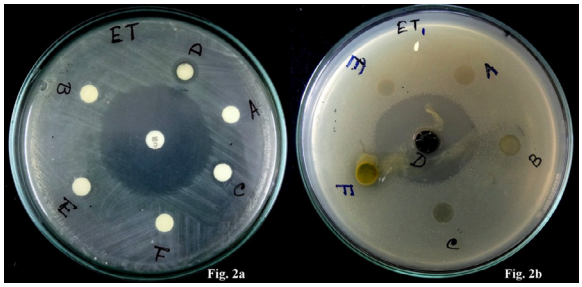

Figure 2: Inhibition of *Edwardsiella tarda* by (a) agar disc diffusion and (b) agar overlay well-diffusion assays. A: Aqueous control; B: Aqueous test; C: Chloroform control; D: Chloroform test; E: Methanol control; F: Methanol test; C 30: Chloramphenicol, 30 µg/disc. The volumes of extracts used were 10µL/disc and 50µL/well for agar disc diffusion and agar overlay well-diffusion assays, respectively.

Extracts	Zone of inhibition (mm)			
	Agar-disc diffusion assay		Agar overlay well-diffusion assay	
	Aeromonas hydrophila	*Edwardsiella tarda*	*Aeromonas hydrophila*	*Edwardsiella tarda*
Aqueous control	6.00 ± 0.00^a	6.00 ± 0.00^a	5.00 ± 0.00^a	5.00 ± 0.00^a
Aqueous test	6.00 ± 0.00^a	6.00 ± 0.00^a	5.00 ± 0.00^a	5.00 ± 0.00^a
Chloroform control	6.00 ± 0.00^a	6.00 ± 0.00^a	5.00 ± 0.00^a	5.00 ± 0.00^a
Chloroform test	6.00 ± 0.00^a	11.25 ± 0.35^b	5.00 ± 0.00^a	30.50 ± 6.40^b
Methanol control	6.00 ± 0.00^a	6.00 ± 0.00^a	5.00 ± 0.00^a	5.00 ± 0.00^a
Methanol test	6.00 ± 0.00^a	6.00 ± 0.00^a	5.00 ± 0.00^a	7.50 ± 0.70^a
Chloramphenicol	31.75 ± 1.06^b	40.75 ± 1.76^c	ND	ND
Data represent means ± Standard Deviation; ND: Not Done; $^{a-c}$ Values sharing uncommon superscripts within the column differ significantly (P<0.05)				

Table 1: *In-vitro* inhibition of *Aeromonas hydrophila* and *Edwardsiella tarda* by the crude extracts of *Centella asiatica* by agar-disc diffusion and agar overlay well-diffusion assays.

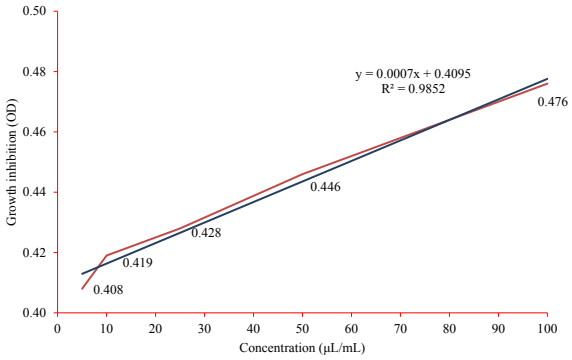

Figure 3: Growth inhibition of *Edwardsiella tarda* by the crude *Centella asiatica* chloroform extract in broth dilution assay. [OD: Optical Density].

Results and Discussion

The results of the *in-vitro* inhibition of *A. hydrophila* and *E. tarda* by the crude extracts of *C. asiatica* by agar-disc diffusion (Figure 2a) and agar overlay well-diffusion assays (Figure 2b) are presented in Table 1. There existed significant differences in the zones of inhibition among the extracts (P<0.05). The broth dilution assay was performed using the chloroform extract against *E. tarda* and the results of the growth inhibition in terms of optical density are represented in Figure 3. With the increasing concentration of crude chloroform *C. asiatica* extract, an increased growth inhibition was noted with a R^2 value of 0.9852 (P<0.05). Among the aqueous, methanol and chloroform extracts tried, only the crude chloroform extract of *C. asiatica* exhibited the maximum inhibitory activity *in-vitro*. The crude chloroform extract of *C. asiatica* showed antibacterial activity only against *E. tarda*. On the other hand, *A. hydrophila* was unaffected by any of the crude extracts even at a level of 50 µL. The agar overlay well-diffusion assay with crude chloroform extract of *C. asiatica* yielded the highest zone of inhibition (30.50 ± 6.40 mm), which was comparable to those of the chloramphenicol (40.75 ± 1.76 mm). This observation is particularly noteworthy because plants extracts are known to be more active against Gram-positive than Gram-negative bacteria possibly due to the structural difference in cell membrane [18]. The selective inhibitory effect of *C. asiatica* extract

among the bacterial strains tested could be attributed to the differences in outer membrane proteins of *A. hydrophila* and *E. tarda* [19]. In support of the present study, an earlier study observed significant zone of inhibition against *Proteus vulgaris, Staphylococcus aureus, B. subtilis and Escherichia coli* using the chloroform extract compared to aqueous extract [12]. Chloroform was found to be the most effective choice of solvent in inhibiting the growth of three strains of *Salmonella* compared to ethanol and hexane [13]. Similarly, the aqueous and acetone extracts were found to be less effective than the chloroform extract of leaf and callus of *C. asiatica* against the tested organisms [20]. They, however, noted that it was effective at the concentrations above 125 µg/mL against *S. aureus* and *E. coli*. The methanolic extract of *C. asiatica* of the present study had only weak inhibitory against *E. tarda* (7.50 ± 0.70 mm). Contrarily, in an earlier study, the methanolic extract of *C. asiatica* showed no antibacterial activity against *E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [5]. Besides, several other studies demonstrated that the hexane and ethyl acetate extracts of *C. asiatica* inhibited the Gram-positive *B. subtilis* and Gram-negative *P. aeruginosa, P. cichorii* and *E. coli* in the disc diffusion test [9,21]. The lack of antimicrobial activity in the aqueous and methanolic extracts may be due to the absence of antimicrobial components in these extracts or the interference of pigments and phenolics with the antimicrobial activity of these extracts. The chloroform extract of *C. asiatica* was reportedly had higher concentration of phenol and its derivatives, ester, sesquiterpene and other miscellaneous compounds compared to hexane and ethanolic extracts [13]. An earlier study established a good correlation between the antibacterial activity and total phenolic content of plant extracts [22]. It has also been demonstrated that the activity of *C. asiatica* extract against the microorganisms is mainly concentrated in the triterpene asiaticoside. The triterpenes weaken the membranous tissues, which results in dissolving the cell walls of the microorganisms so that they can be more efficiently eliminated [11]. The anti-*E. tarda* activity of the chloroform extract of *C. asiatica* may be related to the presence of phenolic compounds, possibly triterpene asiaticoside. The qualitative UV-VIS spectrum profile of the chloroform extract of *C. asiatica* at a wavelength from 400 to 900 nm showed peaks at 677, 542 and 443 nm with the absorption of 0.318, 0.303 and 0.301 respectively, which can serve as a baseline for future characterization of the extract. Considerable research has been carried out on the phytochemical properties of the plants, which have been amply reviewed [4,9,20,22]. It has been reported that the plant extracts consisted of triterpenoid glycosides, free acids, volatile oils and flavonoids [11]. The compounds such as alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and reducing sugars, etc have been identified in *C. asiatica,* which are known to possess antibacterial activities [20]. *Centella asiatica* has already been successfully used in controlling columnaris disease caused by *Flavobacterium columnare* without any inverse impact on fish [3]. Besides the antibacterial principles, *C. asiatica* reportedly possesses antifungal, anticancer, wound healing, neuroprotective, immuno-modulatory, anti-inflammatory, hepatoprotective, insecticidal and antioxidant activities [9], which can be exploited for use in aquaculture as immunomodulators or as an alternative to antibiotics. This would reduce the over-dependence on antibiotics, whose use in aquaculture became a major concern from the public health point of view. In general, the results of this *in-vitro* study demonstrated that the chloroform extract of *C. asiatica* has antibacterial activity against *E. tarda*, a potent fish and zoonotic bacterial pathogen.

Conclusion

The current study accentuates the need for further research to identify the active compound(s) responsible for such anti-*E. tarda* activity and the exact mechanism of inhibition. The use of such medicinal plants is eco-friendly and cost-effective, which can be used in aquaculture health management and to address the issue of growing antibacterial resistance.

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