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Antibacterial activity of *Chroococcus minutus* (Kützing) Nägeli isolated from Cochin estuary against selected pathogens

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Abstract
The antibacterial activity of different solvent fractions of *Chroococcus minutus*, isolated from Cochin estuary against a variety of aquaculture and human pathogens were tested. The diethyl ether extract was effective against nine pathogens, especially the vibrios. *V. parahaemolyticus* showed the maximum zone of inhibition (3.3cm). The methanolic and ethanolic extracts could inhibit only few pathogens whereas aqueous, dimethyl sulfoxide and acetone extracts were least effective.

Keywords: *Chroococcus minutus*, antimicrobial activity, Diethyl ether, *V. parahaemolyticus*

1. Introduction
Cyanobacteria, the Gram negative phototrophs produce biologically active molecules that are reported to have antibacterial, antiviral, antifungal, cytotoxic or antiplasmodial activities [1-5]. In the present scenario of emergence of antibiotic resistance among pathogenic bacteria there is a need for novel antibacterial substitutes in the pharmacological as well as in the aquaculture field. With the view of environmental acceptance and biodegradability researchers all over the world have turned their attention to algae that have enormous possibility for producing functional compounds [6].
Researchers have investigated the antimicrobial activities of *Chroococcus* sp. [7-10]. The studies reveal that the genus has got significant activity against a variety of Gram positive and Gram negative organisms and also exhibits antifungal properties.
The Cochin estuary (9º30' - 10º12' N and 76º10' - 76º29' E) has diverse forms of rich cyanophycean microflora [11] that are highly under exploited. The current study is an attempt to evaluate the antibacterial activity of *Chroococcus minutus* of order Chroococcales against a variety of human as well as aquaculture pathogens. We have also evaluated the efficiency of different solvents to extract the antibacterial principles from *C. minutus* against the different pathogens.

2. Materials and Methods
2.1 Isolation and maintenance of *C. minutus*
C. minutus was isolated from Cochin estuary by enrichment method [12]. The culture was purified on agar and identified morphologically based on standard keys [13]. The uni algal culture was maintained as batch culture in BG11 medium at 22±2 ºC, illuminated with 1500 lux at 12:12 light and dark period.

2.2 Preparation of algal extract
The late logarithmic phase culture of *C. minutus* was harvested by centrifugation at 5000 rpm for 15 minutes. The aqueous supernatant was collected and the algal pellet was weighed. 400 mg sample was submitted to extraction with 10 ml of different solvents such as acetone, ethanol, methanol, diethyl ether (DEE) and dimethyl sulfoxide (DMSO). This was again centrifuged at 5000 rpm for 15 minutes and the supernatants were collected and stored at 4 ºC for antimicrobial analysis.

2.3 Test organisms
A total of ten different Gram negative and Gram positive human and aquaculture pathogens were used for the test. The Gram negative strains of vibrios included *Vibrio alginolyticus* VKF44, *Vibrio Anguillarum* JWA3, *Vibrio harveyi* MTCC catalogue NO. 7954, *Vibrio mimicus* M9W1 and *V. parahaemolyticus* PMIS2, *Aeromonas hydrophila* SA7, *Pseudomonas aeruginosa* WV158 and *Escherichia coli* EW182. The Gram positive strains used were *Staphylococcus aureus* MTCC no. 3160 and *Bacillus* sp. AST55. All the strains were obtained from the culture collection maintained in the fish pathology laboratory of the institution.

2.4 Determination of antibacterial activity of the extracts
The antibacterial activity of the crude extract of *C. minutus* against various pathogens was determined by agar well diffusion method. Overnight cultures of bacterial strains grown in nutrient broth were plated onto nutrient agar plates using a sterile swab. For the vibrios and *A. hydrophila* plates were prepared with 1% salt. The bacterial count was adjusted to 10^7 CFU/ml. A total of five wells were made in the agar of diameter 9mm, and extract was added as 50, 100, 150, 200µl and centre well was added with 200µl of solvent alone which served as the control. The antimicrobial activity was determined after 24 hrs of incubation at 37 ºC by measuring the diameter of the inhibition zone with callipers.

3. Results and discussion
The diethyl ether extract of *C. minutus* inhibited nine pathogens except *S. aureus*. All vibrios were sensitive especially *V. parahaemolyticus* (plate1) had the maximum zone of inhibiton (3.3cm). The ethanolic extract of the alga could inhibit only two pathogens that are *V. parahaemolyticus* and *V. alginolyticus* and was more effective on *V. alginolyticus* than *V. parahaemolyticus*. Methanolic extract could inhibit only *V. anguillarum*. The aqueous extract, acetone and dimethyl sulfoxide extracts did not inhibit any of the pathogens tested. No zone of inhibition could be observed around the control well. This proves that the active principle present in the *C. minutus* was solely responsible for the antibacterial activity. *S. aureus* was found to be the least sensitive pathogen to the algal extract. The antimicrobial activity was highest in the 200µl concentration. For *V. parahaemolyticus*, *V. alginolyticus* and *E. coli* even 100µl of the extract were found to be inhibitory. However, least activity was recorded for 50µl of the extracts. The results also indicate that the activity varies with the solvent used (Table 1). The antibacterial activity of different freshwater and marine cyanobacteria such as *Spirulina platensis, Nostoc commune, Fischerella ambigua, Anabaena* sp, *Oscillatoria tenuis* etc. have been reported earlier [3, 14, 15]. Pradhan *et al.* (2012) [16] have reported that the ethanolic and methanolic extracts of *Spirulina platensis* was found to be effective against all the aquaculture pathogens tested. Similarly Rania and Hala (2008) [17] have reported the antibacterial activity of *S. platensis* on *P. aeruginosa*, *S. aureus*, and *E. coli* by different solvent extracts. The antibacterial activity of organic extracts of *Oscillatoria agardhii*, and *Anabaena sphaerica* was done by Azza *et al.* in 2014 [18]. They could get highest activity in the acetone extracts against *Salmonella senftenberg* and the aqueous extracts had no effect similar to our studies. Ghosh (2008) [19] has reported that aqueous extract were less potent than their organic counterparts.

Organic solvents with low polarity were most effective in extracting the antimicrobial compounds from the microalga reports Ranga Rao *et al.* (2010) [20] Hristo *et al.* (2013) [15] have analyzed the antibacterial activity of nine cyanobacteria against eight food borne pathogens including *E. coli, P. aeruginosa* and *Bacillus cereus*. *Gloeocapsa* sp., *Synechocystis* sp. and *Anabaena* sp. were found to inhibit the pathogens especially the exopolysaccharides (EPS) of Gloeocapsa and Synechocystis.

4. Conclusion
The crude extract of the *C. minutus* in diethyl ether was effective against vibrios which are the major pathogens in the aquaculture field. In future this could be utilized as an antimicrobial agent, however further detailed studies are required to identify the active principle present in the extract and also *in vivo* studies have to be conducted to analyze their effect in the cultured animal.

Table 1. Antibacterial activity of different solvent extracts of *C. minutus* against various pathogens

S.no	Pathogen	Solvent	Diameter of the zone (cm)			
			50 µL	100 µL	150 µL	200 µL
1.	*V. parahaemolyticus*	DEE	_	0.3	0.34	3.3
		Ethanol	_	1.1	1.6	2.3
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol	_	_	_	_
		Aqueous extract	_	_	_	_
2.	*V. mimicus*	DEE	_	_	_	1.8
		Ethanol	_	_	_	_
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol	_	_	_	_
		Aqueous extract	_	_	_	_
3.	*V. harveyi*	DEE	_	_	_	0.8
		Ethanol	_	_	_	_
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol	_	_	_	_
		Aqueous extract	_	_	_	_

4.	*V. anguillarum*	DEE	_	_	_	1.5
		Ethanol	_	_	_	_
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol	_	_	_	0.9
		Aqueous extract	_	_	_	_
5.	*V. alginolyticus*	DEE	_	1.1	1.3	1.6
		Ethanol	_	_	_	2.3
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol				
		Aqueous extract	_	_	_	_
6.	*A. hydrophila*	DEE	_	_	_	0.9
		Ethanol	_	_	_	_
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol	_	_	_	_
		Aqueous extract	_	_	_	_
7.	*Bacillus sp.*	DEE	_	_	_	0.6
		Ethanol	_	_	_	_
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol	_	_	_	_
		Aqueous extract	_	_	_	_
8.	*E. coli*	DEE	_	0.5	0.9	1.6
		Ethanol	_	_	_	_
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol	_	_	_	_
		Aqueous extract	_	_	_	_
9.	*P. aeruginosa*	DEE	_	_	_	1.1
		Ethanol	_	_	_	_
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol	_	_	_	_
		Aqueous extract	_	_	_	_
10	*S. aureus*	DEE	_	_	_	_
		Ethanol	_	_	_	_
		Acetone	_	_	_	_
		DMSO	_	_	_	_
		Methanol	_	_	_	_
		Aqueous extract	_	_	_	_

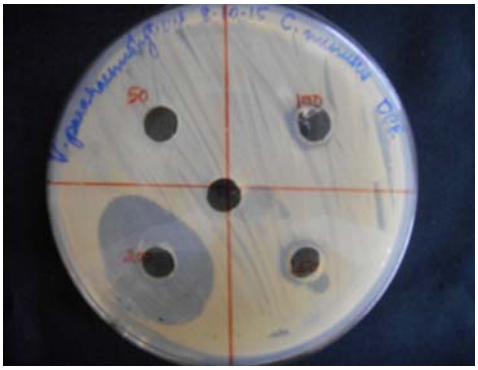

Plate 1: Antibacterial activity of *C. minutus* DEE extract on *V.parahaemolyticus*

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