Indian J. Fish., 71 (2): 98-104, 2024 doi:10.21077/ijf.2024.71.2.138810-13

Available online at: epubs.icar.org.in/index.php/IJF

Antibacterial activity of organic fractions from freshwater microalga *Chlorella vulgaris* against fish and shellfish pathogens

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Abstract

The ethanol-based crude extract and fractions partially purified from the freshwater microalga, *Chlorella vulgaris* underwent screening using disc diffusion assays. The tube dilution method was also employed to determine the minimum inhibitory concentration (MIC) values. The screening involved testing a variety of Gram negative bacterial fish and shellfish pathogens. The panel of pathogens included four strains of *Aeromonas hydrophila* (AH1, AH2, AH3, AH4), two strains of *Pseudomonas putida* (PP1, PP2), two strains of *Pseudomonas aeruginosa* (PA1, PA2), two strains of *Pseudomonas fluorescens* (PF1, PF2), *Escherichia coli* (O115, O1, O156, O164, O111 and O109), *Vibrio alginolyticus* (VA), *V. anguillarum* (VAN), *V. fluvialis* (VF), *V. parahaemolyticus* (VP), *V. harveyi* (VH), *V. fisheri* (VFS), and *Edwardsiella tarda*.The crude ethanolic extract underwent partial purification through silica gel column chromatography.The crude ethanolic extract was potentially active against all the selected bacterial pathogens, with the lowest MIC value (300 µg) against *P. aeruginosa* (PA2) and *E. coli* (O1, O156, O109). Among the nine chromatographic fractions, three exhibited higher activity with lower MIC values (40-50 µg). The results indicate that partially purified *C. vulgaris* extract has superior antibacterial activity compared to the crude extract. It could be a viable alternative for managing bacterial pathogens in aquaculture, potentially curbing the rise of antibiotic resistance.

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Keywords:
Antibacterial, *Chlorella vulgaris*, Minimum inhibitory concentration, Pathogen, *Vibrio*

Received : 05.07.2023
Accepted : 07.05.2024

Introduction

Microalgae, being a highly diverse group of organisms, have garnered considerable interest in recent years due to their promising potential as a valuable source of bioactive compounds for pharmaceutical development (Athbi, 2014; Kaur *et al.*, 2023). Although most research has concentrated on marine microalgae, there is a growing interest in freshwater microalgae as well, as they exhibit considerable promise and represent an emerging area of exploration. An inherent advantage of microalgae as a source of bioactive compounds lies in their capacity to biosynthesise intricate molecules that pose challenges for chemical synthesis (de Morais *et al.*, 2015). This makes them attractive for drug development, as these compounds often possess unique structures and biological activities that can be utilised in pharmaceutical applications (Lauritano and Ianora, 2016). Extensive investigations have revealed that freshwater microalgae possess a diverse array of bioactive compounds, exhibiting a multitude of medicinal properties (Athbi, 2014). These include pigments such as chlorophyll and carotenoids with anticancer and antioxidant activities (Patel *et al.*, 2022). Phycocyanin, a blue pigment present in certain microalgae, exhibits notable anti-inflammatory, antioxidant and neuroprotective properties (Pagels *et al.*, 2022; Patel *et al.*, 2022). The lipids and polyunsaturated fatty acids (PUFAs) derived from microalgae have gained substantial recognition owing to their well-established potential to promote various health benefits (Santin *et al.*, 2021). Other bioactive compounds obtained from microalgae include phlorotannins, polysaccharides, sulphated

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polysaccharides, peptides, amino acids, terpenes, polyacetylenes, aldehydes, alcohols, ketones, indole alkaloids, alkenes, sterols, halogenated furanones, aromatic organic acids and hydroquinones (Shannon *et al*., 2016; Vikneshan *et al*., 2020).

These compounds exhibit a wide spectrum of biological activities, encompassing antiviral, antioxidant, antifungal, antitumor, anti-inflammatory, antibacterial and various other beneficial effects (Patra *et al*., 2009; Lauritano and Ianora, 2016; Shannon *et al*., 2016).

Exploring the diversity of freshwater microalgae and their bioactive compounds can provide new opportunities for developing novel pharmaceuticals with diverse therapeutic applications. *Chlorella vulgaris* is a commonly found freshwater microalga with a diverse range of bioactive compounds. It is known to contain proteins, lipids, carbohydrates, minerals and beta-carotene, among other nutrients (Widjaja *et al*., 2009; Safi *et al*., 2014). *Chlorella* contains a significant component, the Chlorella growth factor (CGF), a complex mixture of substances including peptides, nucleic acids, vitamins and minerals (An *et al.,* 2016). These compounds exhibit a wide range of effects, including antibacterial (Pratt *et al*., 1944), antifungal (Sarkar *et al*., 2021), antiviral (Ibusuki *et al*., 1990), antitumor (Hasegawa et al., 2002), anti-oxidative, anti-inflammatory (Vijayavel *et al*., 2007) and immunomodulatory properties (Halperin *et al*., 2003; Pradhan *et al*., 2023). Research findings have indicated that organic extracts derived from *Chlorella* display substantial anti-proliferative activity against the MCF-7 breast cancer cell line. Additionally, *Chlorella* extracts have inhibited the growth of diverse human pathogens (Jayshree *et al*., 2016).

Drug-resistant antibiotics continue to pose a significant and escalating health concern, despite the development of numerous new antibiotics by pharmaceutical industries. Therefore, it is imperative and urgent to address this problem by discovering novel antibacterial compounds. Considerable efforts are underway to isolate and identify valuable active compounds derived from natural resources, aiming to combat drug resistance effectively. Green microalgae extracts have exhibited promising antibacterial activity against a broad spectrum of pathogenic bacteria. Among the notable compounds, chlorellin, an antibiotic synthesised by *Chlorella*, stands out for its inhibitory effects on both Gram positive and Gram negative bacteria (Alsenani *et al*., 2020). The free fatty acids derived from green microalgae can also effectively damage bacterial cell membranes. These properties make green microalgae a valuable source for developing novel antibacterial agents. One such example is the methanolic extract of *Chlorella* sp., which contains several major antibacterial compounds, including phytol, phenol, hexadecanoic acid and 9,12-octadecadienoic acid (Shaima *et al*., 2022). The primary objective of this study was to explore and evaluate the antimicrobial properties of the crude ethanolic extract and different organic fractions derived from *C. vulgaris*. The study emphasises the need for comprehensive research to purify the antimicrobial compounds present in crude ethanolic extracts of *Chlorella* that exhibit activity against bacterial pathogens in fish. The utilisation of microalgal-based bioactive compounds in aquaculture shows potential benefits, particularly in reducing the emergence of drug-resistant bacteria. By developing safe and effective alternatives, it is possible to minimise the reliance on traditional antibiotics in aquaculture practices.

Materials and methods

Culture of *C. vulgaris*

The axenic *C. vulgaris* strain obtained from the Algal Culture Unit of ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA), Bhubaneswar, India was cultured following established protocols (Das and Pradhan, 2010) with minor adaptations. The culture was cultivated in a glass cylinder with dimensions of 30 cm height and 10 cm diameter. The culture medium was constantly aerated and exposed to 6 h light (74 µmol m^{-2} s^{-}) and 6 h dark alternatively. After 20 days, the axenic *C. vulgaris* cultures reached the stationary growth phase. The culture was subsequently harvested by centrifugation at a 5000 *g* and 4°C, separating the biomass from the culture medium for further experimentation.

Preparation of algal extract

The algal samples were oven-dried at 60°C until a constant weight was reached. After drying, the samples were powdered using an electric grinder to obtain a fine powder. The resulting powder was extracted with ethanol (1:2 w/v) for 24 h and the ethanol extract was subsequently dried using a Büchii rotary evaporator-11 from Flawil, Switzerland, to remove the solvent and concentrate the desired components. The final concentrations of 10 mg ml^{-1} residual extracts were obtained by dissolving them in their respective solvents (Das and Pradhan, 2010).

Fractionation of crude ethanolic extract of *C. vulgaris*

Partial purification of the ethanolic extract of *Chlorella* (0.5 g) was performed using column chromatography with silica gel (SRL, 100-200 mesh size) (Motl and Novotny, 1979). The elution solvent system was determined by preliminary screening using thin-layer chromatography (TLC). The elution process involved sequential elutions using varying ratios of ethyl acetate (EA) and hexane (5, 10, 20, 40, 50, 70%), followed by 100% EA and finally 100% ethanol. Based on the TLC analysis, the eluents were combined into a total of nine fractions, namely: Ch1 (20:1 Hex/EA), Ch2, Ch3 (9:1 Hex/EA), Ch4 (4:1 Hex/EA), Ch5 (3:2 Hex/EA), Ch6 (1: 1 Hex/EA), Ch7 (7:3 Hex/EA) Ch8 (100% EA) and Ch9 (100% EtOH). Details of the total number of fractions, and the solvent system used with their R_f values are given in Table 1.

Test organisms

The antimicrobial efficacy of both the crude ethanolic extract and fractionated products of *Chlorella* was assessed against a range of Gram negative pathogenic bacterial strains, which included *Aeromonas hydrophila* (AH1, AH2, AH3 and AH4), *Pseudomonas putida* (PP1, PP2), *P. aeruginosa* (PA1, PA2), *P. fluorescens* (PF1, PF2), *Vibrio alginolyticus* (VA), *V. anguillarum* (VAN), *V. fluvialis* (VF), *V. parahaemolyticus* (VP), *V. harveyi* (VH) and *V. fischeri* (VFS), *E. coli* (O115, O1, O156, O164, O111 and O109) as well as *E. tarda*. These bacterial strains were isolated from diseased fish samples (Pradhan *et al*., 2011).

Table 1. Details of fractions and R_f values of silica gel (100-200 mesh) column chromatography fractionated *C. vulgaris*

Solvent used	Code of fractions	Weight of fractions (g)	Eluted solvents	TLC (R_f values)
Ethanol (EtOH)	Ch1	0.0012	Hex: EA (9.5: 0.5)	0.49, 0.66
	Ch2	0.0004	Hex: EA (9:1)	0.58
	Ch3	0.0120	Hex: EA (9:1)	0.35, 0.57
	Ch4	0.0045	Hex: EA (4:1)	0.56, 0.25
	Ch5	0.0015	Hex: EA (3:2)	0.46, 0.68
	Ch6	0.0210	Hex: EA (1:1)	0.55
	Ch7	0.0071	Hex: EA (7:3)	0.56, 0.68
	Ch8	0.0182	(EA)	0.65, 0.79
	Ch9	0.0179	EtOH	0.57, 0.66

Ch1 (20:1 Hex/EA), Ch2, Ch3 (9:1 Hex/EA), Ch4 (4:1 Hex/EA), Ch5 (3:2 Hex/EA), Ch6 (1: 1 Hex/EA), Ch7 (7:3 Hex/EA) Ch8 (100% EA) and Ch9 (100% EtOH)

Antibacterial activity by the disc diffusion method

The disc diffusion method (Das *et al*., 2005) was employed to conduct antibacterial sensitivity tests on the crude ethanolic extract and different fractions of *Chlorella*. Nutrient agar plates were used to subculture the chosen pathogenic bacteria, which were then incubated for 24 h at 37**°C.** A suspension of the test organism with a concentration of 10^7 CFU ml^{-1} was inoculated onto petri dishes containing Antibiotic Assay Medium from Hi-media. Subsequently, Hi-Media's sterile filter paper discs (6 mm diameter) were positioned on the plates. These paper discs were loaded with various fractions at concentrations of 10 mg ml^{-1} and incubated overnight at a temperature of 37°C. Each plate included a control disc containing only the solvent (10 µl). On the following day, the average diameter of the zone of inhibition was measured based on three experimental replicates (Izzo *et al*., 1995). Three reference commercial antibiotic discs, namely bacitracin (B) (10 mcg), tetracycline (T) (25 mcg) and streptomycin (S) (10 mcg), were utilised for comparing the antibacterial activity of *Chlorella* extracts.

Determination of minimum inhibitory concentration (MIC)

The tube dilution method was employed to determine the minimum inhibitory concentration (MIC) values of the crude ethanolic extract (CEtOH) and fractions of *C. vulgaris*, namely Ch2 (9:1 Hex/EA), Ch4 (4:1 Hex/EA) and Ch5 (3:2 Hex/EA) (Alderman *et al*., 2001). The fractions were subjected to serial dilutions in nutrient broth and 2 ml of the bacterial suspension containing the selected test organisms at a concentration of 10^7 CFU ml^{-1} was added to each test tube. Two control tubes were included, one with bacteria but without fractions and another without the test organism. The test tubes were then incubated overnight. The MIC, determined in duplicate, represents the lowest concentration of the fraction that effectively inhibited visible bacterial growth.

Statistical analysis

The results were presented as mean±standard deviation (SD) and the analysis of the difference among the mean doses was conducted using Student's t-test.

Results

Antibacterial sensitivity of organic fractions of *Chlorella*

The extracts and fractions exhibited activity against a diverse range of fish microbial pathogens isolated from diseased fish. After conducting column chromatography and thin layer chromatography, the process resulted in nine distinct fractions.

Out of these fractions, three chromatographic fractions derived from the ethanolic extracts exhibited enhanced activity against *Pseudomonas* and *A. hydrophila* (Fig. 1a, b, c and d). Among the fractions obtained using ethyl acetate:hexane eluents, Ch2 (9:1), Ch4 (4:1) and Ch5 (3:2), displayed activity against various strains of six pathogens, including *E. coli*. Notably, the polar fraction 4, Ch4 (4:1 Hex:EA), exhibited the highest activity against the selected *A. hydrophila* and *Pseudomonas* strains. Additionally, the fraction 2, Ch2, demonstrated the maximum zone of inhibition (15, 15.67±0.58 mm) against two strains of *A. hydrophila* (AH3 and AH4) (Fig. 1a). Except for *V. fluvialis* (VF) with the Ch4 fraction showing a moderate zone of inhibition, all the active polar fractions of *C. vulgaris* exhibited high antibacterial sensitivity against the selected *Vibrio* species. Interestingly, the organic fraction 2, Ch2, displayed significant activity (17 mm) specifically against *V. fluvialis* (VF). The results demonstrated that the antibacterial activity of two organic eluents, Ch2 and Ch5, was more potent (16.0±1.0, 16.67±0.58 mm) in inhibiting the growth of *E. coli* strains (O1, O156) compared to the crude extract. The effective fractions of *C. vulgaris* exhibited zone sizes ranging from moderate to high levels when tested against *E. tarda*. Notably, the hexane:ethyl acetate eluents Ch2 and Ch5 displayed the strongest antibacterial activity (14.67±0.58 mm) against *E. tarda* (Fig. 1d). The standard antibiotic discs, *e.g.,* bacitracin (B), streptomycin (S) and tetracyclines (T) showed similar zones of inhibitions (Table 3).

Minimum inhibitory concentration (MIC) values

The polar fraction Ch4 (4:1 hexane:ethyl acetate) exhibited the highest zone of inhibition against the tested strains of *Pseudomonas* and *A. hydrophila*, with MIC values ranging from 40-70 µg (Fig. 1a, 1b and Table 2). The polar fraction Ch5 (3:2 hexane:ethyl acetate) displayed the most significant activity, with minimum MIC values of 50 µg against *P. fluorescens* (PF2), whereas the crude extracts had MIC levels varying from 300-500 µg. Fraction 2 (Ch2)

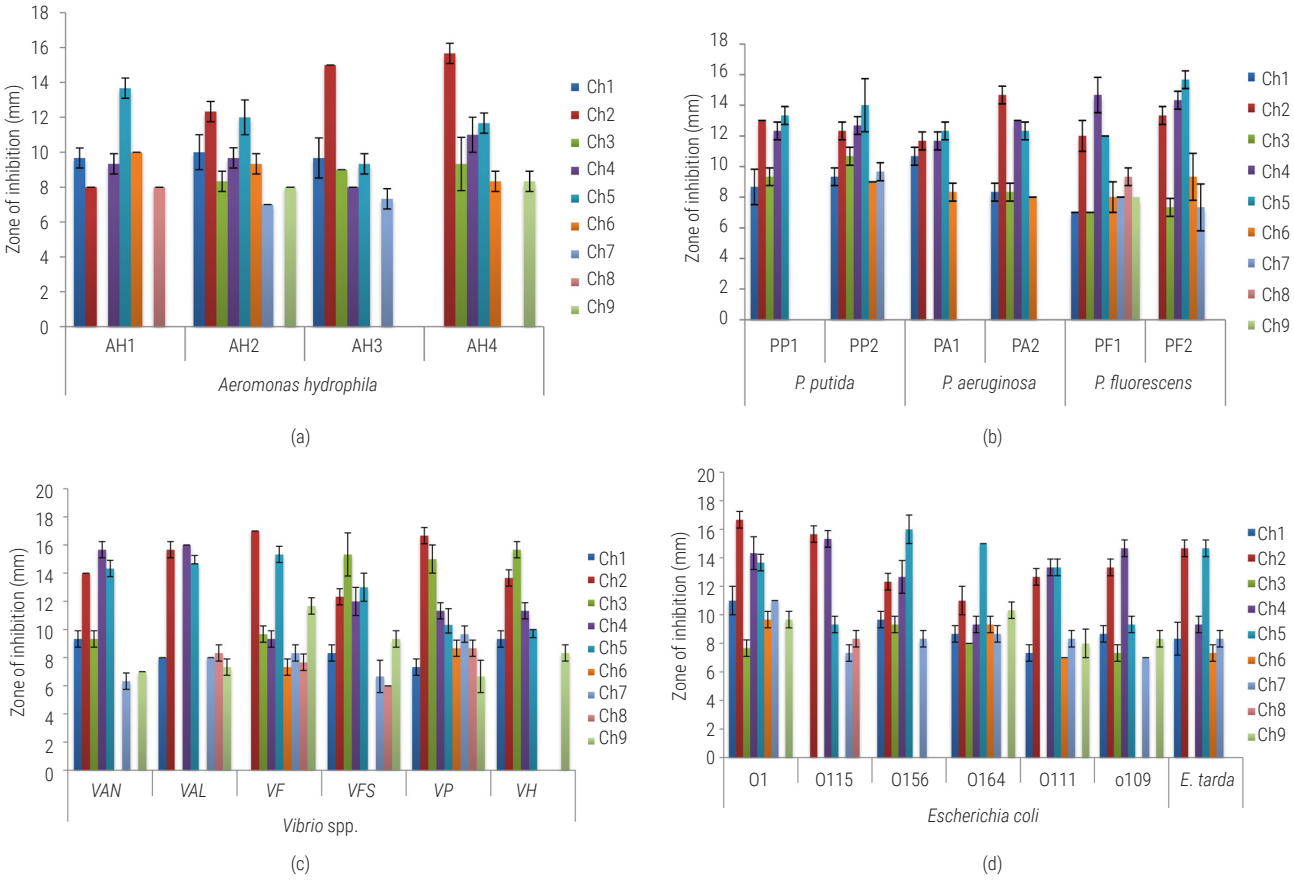

Fig. 1. Antibacterial activity of fractioned ethanol extracts of *C. vulgaris* against (a) *A. hydrophila,* (b) *Pseudomonas* spp. (c) *Vibrio* spp., (d) *E. coli* and *E. tarda*

exhibited the lowest MIC (40 µg) against *V. fluvialis* and for *E. coli*, the MIC levels ranged from 40 to 100 µg (Table 2). Furthermore, the eluents Ch2 and Ch5 demonstrated the maximum zone size against *E. tarda*, with the lowest MIC values of 70 and 50 µg, respectively, compared to the MIC value of 250 µg for the crude ethanolic extract of *C. vulgaris* (Table 2 and Fig. 1d).

Discussion

A previous study revealed that the crude extracts obtained from *C. vulgaris*, specifically the ethanol, methanol and aqueous extracts, exhibited activity against all the selected pathogens, except for four *Vibrio* species (VA, VP, VH and VFS) (Das and Pradhan, 2010)

Table.2 Minimum inhibitory concentration (MIC, µg ml^{-1}) of various organic fractions of ethanolic extract of *C. vulgaris* against different strains of *A. hydrophila*, *Pseudomonas* sp., *Vibrio*, *E. coli* and *E. tarda*

Extracts/ Organic fractions	MIC values (µg ml^{-1})									
	A. hydrophila				*P. putida*		*P. aeruginosa*		*P. fluorescens*	
	AH1	AH2	AH3	AH4	PP1	PP2	PA1	PA2	PF1	PF2
Ch2	50	100	70	100	100	50	100	70	70	50
Ch4	70	70	70	70	70	50	70	40	100	70
Ch5	70	100	70	100	100	70	70	50	70	50
Ethanolic extract	350	400	400	450	450	350	500	300	500	450

Extracts/ Organic fractions	MIC values (µg ml^{-1})												
	Vibrio sp.						*E. coli*						*E. tarda*
	VAN	VAL	VF	VFS	VP	VH	O1	O115	O156	O164	O111	O109	
Ch2	70	50	40	70	50	70	70	50	40	40	50	50	70
Ch4	50	40	75	70	70	70	50	100	40	40	50	50	100
Ch5	50	50	40	70	70	75	50	70	50	100	100	70	50
Ethanolic extract	350	300	300	300	400	350	300	500	300	350	350	300	250

Ch2 (9:1 Hex/EA), Ch4 (4:1 Hex/EA), Ch5 (3:2 Hex/EA)

Table 3. The antibacterial sensitivity of various antibiotics against fish bacterial pathogens showing zone of inhibition (mm)

Antibiotics	Bacterial strains										
	A. hydrophila				*P. putida*		*P. aeruginosa*		*P. fluorescens*		*E. tarda*
	AH1	AH2	AH3	AH4	PP1	PP2	PA1	PA2	PF1	PF2	
Tetracycline (T) 30 mcg	11	11	13	11	18	18	17	13	18	17	15
Streptomycin (S) 10 mcg	15	15	18	19	19	18	16	16	18	17	16
Bacitracin (B) 10 mcg	16	18	19	16	12	12	14	12	15		18

Additionally, Sampathkumar *et al*. (2017) stated that the ethanol extract of *Chlorella* demonstrated inhibitory effects against various clinical isolates, including *E. coli*, *Proteus mirabilis*, *P. aeruginosa, Staphylococcus aureus* and *Bacillus subtilis*, at a concentration of 100 mg ml^{-1}, with varying zone size from 15 to 24 mm. Other workers have documented similar findings (Ghasemi *et al*., 2007; Kokou *et al*., 2012; Syed *et al*., 2015). Furthermore, Jayshree *et al*. (2016) found that the methanolic extract of *Chlorella* not only retarded the growth of human pathogens but also inhibited the proliferation of the MCF-7 breast cancer cell line.

The study demonstrated that the specific fractions Ch2 (9:1 Hex:EA), Ch4 (4:1 Hex:EA), and Ch5 (3:2 Hex:EA) of *C. vulgaris* (ethanolic extract) exhibited significant antibacterial activity against all the tested bacterial fish and shellfish pathogens when compared to the crude extracts. Among these fractions, the polar fraction Ch2 (Hex:EA) displayed the maximum activity, with a MIC level of 50 µg against *P. fluorescens* (PF2). Importantly, the MIC values of this fraction were seven times more efficient compared to its respective crude extract (Table 2). Similar characteristic features were observed for other polar fractions (Ch4 and Ch5). The overall findings of the screening of these fractions suggest that the antibacterial activity was concentrated more in the low polar fractions. This suggests that the bioactive compounds accountable for the observed activity are likely polar or semi-polar. These findings highlight the potential of *C. vulgaris* extracts and their fractions as sources of antibacterial agents for controlling and treating bacterial fish and shellfish pathogens. Additional investigations can be carried out to isolate and identify the precise bioactive compounds responsible for the noted activity within these low-polarity fractions. The present study confirms that *C. vulgaris* possesses antibacterial properties, not only in its crude form but also in individual organic fractions. Previous studies have indicated that the Chlorellin pigments found in the algae exhibit activity against certain bacteria, as per the findings of Pratt *et al.* (1944) and Matusiak *et al.* (1965). In addition, the analysis of *Chlorella* sp. (UKM8) using Gas Chromatography-Mass Spectrometry (GC-MS) highlighted the presence of major compounds such as phenol, phytol, hexadecanoic acid and heptane, which were found to possess antibacterial activity (Shaima *et al.*, 2022). Phenolic compounds have been known to alter the permeability of microbial cell membranes and interact with proteins and enzymes, disrupting cellular functions. This property can contribute to the antibacterial activity observed in *C. vulgaris* extracts. The presence of phenolic compounds and other bioactive compounds may synergistically enhance the antibacterial effects of *Chlorella* extracts (Hanaa *et al*., 2008; Alshuniaber *et al*., 2021). In recent years, the emergence of therapeutic resistance among microorganisms towards commercial antibiotics has become a significant concern (Serwecinska, 2020; Urban-Chmiel *et al*., 2022). This situation necessitates the exploration of alternative antimicrobial agents, such as those derived from *C. vulgaris*. The potential antipathogenic actions of *C. vulgaris*, exhibited by its crude as well as organic and fractionated products, underscore the substantial contribution of algae in managing aquaculture diseases, particularly aeromoniasis, pseudomoniasis and vibriosis.

In addition to the *in vitro* studies conducted, a preliminary *in vivo* study (data not provided) demonstrated the potential efficacy of *C. vulgaris* in reducing the load of the aforementioned microbial pathogens without causing any stress to fish, specifically *Labeo rohita* (Pradhan *et al*., 2023). This suggests that *C. vulgaris* could potentially be used as a therapeutic approach in the future, such as by injecting brood fishes. The fractionated products of *C. vulgaris*, when used at the appropriate doses, hold potential for controlling microbial infections in fish. Additionally, the powdered form of the algae can be advocated as a preventive therapy food supplement to control bacterial fish diseases. This approach takes advantage of the inherent antimicrobial properties of *C. vulgaris* and its potential to boost the fish's immune system and overall health.

Another approach involves incorporating *C. vulgaris* into the culture water, where it can act as a natural food source while simultaneously inhibiting the growth of pathogenic bacteria. By providing a natural and nutritious food source, *C. vulgaris* can help prevent or protect fish from microbial infections, ultimately promoting their overall well-being and reducing the risk of disease outbreaks. Previous reports have highlighted the potential of *Chlorella* for antitumor and antiviral activities (Fukada *et al*., 1968; Ibusuki and Minamishima, 1990; Hasegawa *et al*., 2002). Although these specific properties have not been tested in the present study, it is possible that by releasing *Chlorella* into the culture environment, it may contribute to protecting animals from viral infections and tumor development. Comprehensive study on purification of active secondary metabolites and drug development are essential for pharmaceutics. Further research is warranted to fully understand the mechanisms underlying the antimicrobial activity of *C. vulgaris* and to optimise its application in aquaculture disease management.

Acknowledgements

The study received support from the Director, ICAR-CIFA, Bhubaneswar and financial assistance from the Department of Science and Technology (DST), Ministry of Science and Technology, New Delhi, India.

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