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Antibacterial activity of microalgae and medicinal plants against common bacteria causing diseases in fish, shellfish, canine and poultry

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Rapid growth of antimicrobial resistance is a major threat to human and animal health, and development of new drugs with effective microbicidal properties are in demand to the scientific communities. Cyanobacteria and medicinal plants inherit antimicrobial, antioxidant, anti-cancerous and anti-inflammatory properties. In the current research, we investigated antibacterial potential of crude ethanolic extracts from marine microalgae (*Chlorella* sp. and *Spirulina* sp.) and medicinal plants (*Aloe vera* and *Clinacanthus nutans*) against pathogenic bacteria isolated from chicken, dog, fish, and shellfish. Bacteria including *Salmonella* sp., *Aeromonas* sp. and *E. coli*, *Vibrio* sp. *Streptococcus* sp. and *Staphylococcus* sp., were isolated through colony morphology, Gram staining and VITEK-2 tests. Antibacterial activity was determined by disc diffusion assay. *Spirulina* sp. showed the highest inhibition zone of 19.3±0.58 mm against *Vibrio* sp. compared to other treatments, whereas *Chlorella* sp. exhibited maximum inhibition zone of 11.41±0.65 mm against *Staphylococcus* sp., *C. nutans* had antibacterial activity against *Staphylococcus* sp. and *E. coli* with the maximum zone of inhibition (14.21±1.08 and 15.10±0.21 mm), whereas *Aloe vera* against *E. coli* and *Vibrio* sp. with significant inhibition zone of 14.04±0.90 and 15.36±1.11mm, respectively. Minimum inhibitory concentration (MIC) of *Chlorella* sp. was found to be 20 mg/mL against all the test bacteria. *Spirulina* sp., *Aloe vera*, and *C. nutans* had MIC values ranging from 20-40 mg/mL. These findings highlight the antibacterial potential of native microalgae and medicinal plants against virulent bacteria that poses significant threats in poultry, canine and aquaculture.

Keywords: *Aloe vera*, Antibacterial sensitivity, *Chlorella*, *Clinacanthus nutans*, Sabah snake grass, *Spirulina*

Aquaculture industries, and in particular the farming of fish and crustaceans, significantly drive the economies of numerous countries and play crucial roles in global food supply. In 2022, fish and aquaculture production reached an all-time peak of 223.2 million tonnes that is worth a record of USD 472 billion that contributes to an estimated 20.7 kg of aquatic animal foods per capita[1]. The primary constraint to the growth of the fish species in the field of aquaculture is disease[2]. Bacterial pathogens represent significant etiological agents within aquaculture, exerting pronounced influences on the management of fish health[3]. The approved widespread use of various antimicrobial drugs, particularly antibiotics, in numerous countries for treating bacterial diseases in aquaculture has led to a potent selection pressure favouring the emergence of antibiotic-resistant bacteria within the aquaculture systems[4,5]. Consequently, nearly 80% of antimicrobials used in aquaculture resulted in wastage of medicated feeds; unabsorbed antibiotics and excreta of culture organisms enter aquatic environments near aquaculture facilities, fostering the development of persistent aquatic antibiotic resistance genes (ARGs)[6].

Antimicrobials are used in animals to treat or prevent disease and to promote growth. The indiscriminate and improper use of antibiotics in diverse sectors contributes to the growing global threat of antibiotic resistance[7]. As a result, standard treatments become ineffective, infections persist and may spread to others. Hence, it is important to minimize the use of these drugs[8]. In Animal husbandry, alternative products play a crucial role in allowing farmers and veterinarians to reduce or largely phase out the use of antibiotics. Vaccines are among the most promising and widely used of these alternatives, but prebiotics, probiotics and other innovative products such as immune modulators, phages, phytochemicals, organic acid, antimicrobial peptides, etc. are also in use or currently being investigated[9]. Many alternative products enhance

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animal productivity and prevent infection at the same time, which could make them particularly attractive for commercial operations[10].

In this regard, microalgal species and medicinal plants have the potential to benefit health as they contain some bioactive components. Different microalgal species such *as Chondrus crispus*, *Mastocarpus stellatus*, *Ascophyllum nodosum*, *Alaria*, *esculenta*, *Spirulina platensis*, *Nannochloropsis oculata*, *Chlorella vulgaris* and *Dunaliella salina* which are used to make products such as anti-irritant, antibacterial, antifungal and antiviral agent[11]. Moreover, *Chlorella* sp., *Dunaliella* sp., *Scenedesmus* sp., *Nannochloropsis* sp., *Tetraselmis* sp., *Spirulina* sp. and *Aphanizomenonflos* aquae have been used as antimicrobial agent to cure aquatic animal disease[12]. It has also been reported that lupeol, β-sitosterol, terpenoids and flavonoids present in medicinal plants[13], *Clinacanthus nutans* possess antibacterial activity and Aloe vera contains six antiseptic agents: lupeol, salicylic acid, urea nitrogen, cinnamonic acid, phenols and sulfur, which causes *Aloe vera* to have inhibitory effects against fungi, bacteria and viruses[14,15]. However, there is only limited research is done on the native marine microalgae, medicinal plants and their effectiveness against microbial agents causing diseases in aquaculture and human. Thus, this study enumerates the antimicrobial effect of microalgae and medicinal plant extracts against bacteria including *Aeromonas hydrophila*, *Vibrio* sp., *Staphylococcus saprophyticus*, *Streptococcus* sp. and *Escherichia coli*, *Salmonella* sp. Precisely, we studied whether microalgae from the marine sources and medicinal plants have any suppressive effects on bacteria isolated from dog, chicken, fish and shellfish.

Materials and Methods

Experimental design

Bacterial samples were collected from dog (n=5) and chicken (n=5) supplied or visited to the S.A. Quadery Teaching Veterinary Hospital of Chattogram Veterinary and Animal Sciences University (CVASU). A small portion (1-3 cm^3) of liver was collected from dead chicken aseptically by sterile scissors and put into a sterile test tube containing buffer peptone water (BPW). The skin and anal mixed swabs from dogs were collected into BPW. Gill and intestinal samples were obtained from Tilapia (*Oreochromis niloticus*) as well as Bata fish (*Labeo bata*) (n=5) and abdominal and gill samples were collected from crab (*Scylla serrata*) and shrimp (*Penaeus monodon*) (n=5) from local market using sterile swabs. The samples in BPW were incubated at 37°C for 24 h to enrich bacterial population. The bacterial sample from BPW was smeared onto different selective media for isolation and identification by colony characteristics. At this stage, Gram staining was performed on single colonies and examined under light microscope. Bacterial species were confirmed by VITEK-2 tests. We tested *Chlorella* and *Spirulina* as these microalgae were found effective in our previous study[16]. The medicinal plants tested were *Aloe vera* and *Clinacanthus nutans* as these plants grow in our tropical climate and crude ethanol extracts were used to determine sensitivity against the isolated bacteria. Finally, minimum inhibitory concentration (MIC) values were determined to further asses the antibacterial potency.

Preparation of microalgae crude extracts

Pure microalgae isolate seeds of *Chlorella* and *Spirulina* were collected from Live Feed Research Corner of the Department of Aquaculture, Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. Three replicates of each species of microalgae were cultured in a 500 mL conical flask with the 150 mL of Conway Medium[17] at 28°C, 3000 Lux of illumination provided by warm-white fluorescent in 24 h light-dark cycle and aeration was supplied[18]. After that, *Spirulina* sp. and *Chlorella* sp. were harvested by centrifugation (5000 rpm for 5 min). Microalgal biomass was dried at 40°C for a day through a hot air oven (JSR Korea's Natural Convention Oven LNO-150). Ten milliliters of the ethanol used for the extraction of one gram of dried algal samples, and it was soaked in the ethanol in the sterile screw-capped bottles of 100 mL volume for two days at room temperature (20–25°C)[19]. Then, the extract was filtered through sterile Whatman no. 1 filter paper to remove all unextractable matter such as cellular material[20] and the filtrate was then concentrated with reduced pressure in a rotary evaporator[21]. The crude ethanolic extract was then stored at –20°C until tested.

Collection of medicinal plant

Clinacanthus nutans leaves were collected from Medicinal Plant Garden located at Mymensingh District of Bangladesh, and *Aloe vera* leaves were collected from a local herbal plant selling market, Chattogram, Bangladesh.

Preparation of medicinal plant crude extracts

The cleaned *Aloe vera* and *C. nutans* leaves were dried in the hot air oven (80℃ for two days). After that, the dried samples were blended to make fine powder. To get the crude extracts, 20 g of each sample was soaked in 100 mL of ethanol and put into a shaking incubator for a day. The solution was properly filtered and kept in the refrigerator until use[22].

Isolation and identification of bacteria from chicken, dog, fish and shellfish

Enriched bacterial samples from BPW were smeared onto selective media (MacConkey agar, Mannitol Salt Agar (MSA), Thiosulfate citrate bile salts sucrose (TCBS), Xylose Lysine Deoxycholate agar (XLD agar, Nutrient agar). After 24 h of incubation at 37°C, bacterial colonies were examined for morphological properties of size, shape, elevation, edges, surface and colour. Following that, single colonies were smeared on microscope slides and Gram staining was performed for microscopic identification of bacteria[23]. The VITEK 2 system was utilized to perform biochemical tests on bacterial samples in the Marine Biotechnology Laboratory at the University Malaysia Terengganu, Malaysia. Bacterial species, such as *Aeromonas*, *Staphylococcus*, *Streptococcus*, *Salmonella*, *E. coli*, were confirmed by using VITEK 2 system. Catalase test, triple Sugar Iron test, production of H_2S and motility tests were done to confirm *Vibrio* sp., at Disease and Microbiology lab, Chattogram Veterinary and Animal Sciences University, Bangladesh.

Antibacterial sensitivity test

Disk diffusion testing was performed[24] using Mueller-Hinton agar (MHA) plates and standard antibiotic disks. The bacterial suspension was adjusted to match the turbidity of 0.5 McFarland standard (1.5×10^8 CFU/mL)[25] by adding physiological saline (0.85%). Three replicates of each bacterial species were inoculated onto MHA plates to assess antimicrobial activity. Sterile cotton swabs were employed for inoculation, and sterile filter paper discs (6 mm) were immersed in the extraction solvents. Then, crude ethanolic extracts containing disks were air-dried and placed onto the MHA plate using sterilized forceps. The standard antimicrobial disks used for the *Aeromonas*, *Salmonella*, *E. coli*, *Vibrio* sp. was Colistin and for *Staphylococcus* sp. and *Streptococcus* sp., Ceftriaxone was utilized as positive control. A sterile blank Whatman filter paper was utilized as negative control. The plates were incubated invertedly at 37°C for 12 h. Clear and circular zones surrounding the discs (inhibition zones) were measured through digital slide calipers (Robotics BD shipment).

Determination of Minimal Inhibitory Concentration (MIC)

The MIC values of crude ethanolic extract of microalgae and medicinal plants against pathogenic bacteria were assessed using broth microdilution technique with slight modification[26,27]. For the preparation of *Chlorella*, *Spirulina*, *C. nutans* and *Aloe vera* stock solutions, 2.5 g powder of each treatment was taken into 15 mL PBS (phosphate buffer saline) solution in a centrifuge tube and mixed vigorously using vortex machine. The tubes were then placed in a sonicator machine for ten minutes using pulsed and high-frequency sound waves to agitate and lyse the microalgal cells. The bacterial suspension was prepared and matched the 0.5 McFarland standards. One hundred and fifty microliters (150 µL) of bacterial suspensions were placed in each well of a 96-well microtiter plate down the column (1-12). In two replicates, stock solutions of microalgae and medicinal plants were put in to make the concentrations as 10, 20, 30, 40 and 50 mg/mL. Bacterial suspensions with PBS act as the positive control and bacterial suspensions with antibiotic act as a negative control. The plates were incubated overnight at 37°C. The presence of bacteria in each well was assessed by color; turbidity and optical density value (630 nm) through a spectrophotometer.

Statistical analysis

All experiments were performed in triplicates and the data presented are the means of average of three independent experiments. Statistical analysis done using GraphPad Prism8 software. Comparison between the control and treatments was performed using 't' test. A P value of <0.05 was considered significant.

Results

Biomass of microalgae and medicinal plant

Microalgae species including *Chlorella* sp. and *Spirulina* sp., alongside medicinal plants *Aloe vera* and, *Clinacanthus nutans*, were specifically selected to ascertain their potential antimicrobial efficacy against pathogenic bacteria responsible for diseases in aquatic organisms such as fish and shellfish. The microalgae were mass cultured using Conway

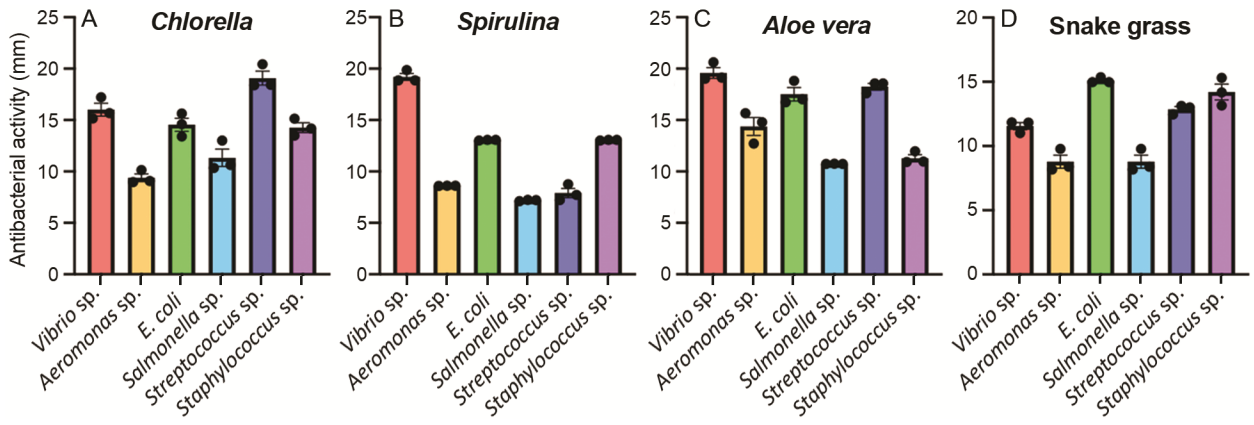

Fig. 1 — Antibacterial sensitivity of microalgae and medicinal plants against bacteria isolated in this study (mean±SD). (A) *Spirulina* has the maximum antibacterial potential against *Vibrio* sp. isolated from shrimp and crab. Antibacterial sensitivity against bacteria isolated from (B, C & F) Bata and Tilapia fish where *Aloe vera* and *C. nutans* have the maximum antibacterial potential; from the isolated bacteria of (D) chicken; and (E) dog. *Chlorella* showed highest inhibition zone compared to other treatments respectively. [Statistical analysis done using GraphPad Prism8 software. Comparison between the control and treatments was performed using t test. **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001]

medium, yielding 7.2 and 7.4 g of dry powder from a 20 L bulk culture for *Chlorella* sp. and *Spirulina* sp., respectively. Raw leaves of *Aloe vera* and *C. nutans* were collected and subsequently dried, resulting in 40.72 g and 46.5 g of dry powder, respectively.

Isolation of bacteria

It was observed that *Aeromonas hydrophila*, *E. coli*, *Staphylococcus saprophyticus*, and *Vibrio* sp. were present in various anatomical regions of fish (skin, gill, intestine), crab (abdominal flap, mouth part, claw, carapace region), and shrimp (joined appendages, uropod, endoskeleton, cephalothorax region).

Antibacterial activity

Ethanolic extracts of microalgae and medicinal plants were used to assess their antibacterial efficacy against bacterial strains isolated from fish, shrimp and crab. The data of zones of inhibition are presented in Fig. 1. The findings of antimicrobial activity test showed that *Aloe vera*, *Clinacanthus nutans*, and *Chlorella* sp. extracts exhibited antimicrobial properties against the identified bacteria. Additionally, *Spirulina* sp. demonstrated effective antimicrobial activity against *Vibrio* sp., *Staphylococcus saprophyticus*, and *Aeromonas hydrophila* except *E. coli*.

Clinacanthus nutans and *Chlorella* sp. displayed similar inhibition zones against *Vibrio* sp. (isolated from crab) measuring approximately 11.38±0.778 mm and 11.59±1.888 mm, respectively, while the control exhibited a zone of inhibition measuring 14.637±1.898 mm. *Spirulina* sp. and *Aloe vera* significantly inhibited *Vibrio* sp., isolated from crab

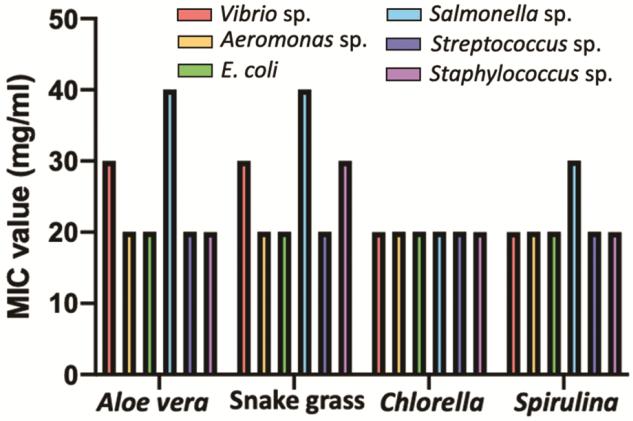

Fig. 2 — MIC values of microalgae and medicinal plants. The MIC values of *Aloe vera*, Sabah Snake grass, *Chlorella* and *Spirulina* indicated the lowest concentration of 20 mg/mL effective in preventing the growth of different bacteria.

displaying respective inhibition zones of 19.193±0.577 mm and 15.363±1.11 mm.

Minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration (MIC) assay was conducted to assess the degree of antimicrobial activity of the four extracts based on their notable inhibition zones against the test bacteria (Fig. 2). Almost all the test extracts exhibited MIC values at 20 mg/mL by effectively inhibiting all test bacterial growth (1.5×10^{-8} CFU/mL or 0.5 McFarland standards). Both *Aloe vera* and *C. nutans* showed antimicrobial potential against *Vibrio* sp. (crab and shrimp) and *Salmonella* sp. at 30 and 40 mg/mL concentration, respectively. *Clinacanthus nutans* demonstrated MIC value at 30 mg/mL concentration against *Staphylococcus* sp.

Discussion

Aeromonas hydrophila, *E. coli*, *Staphylococcus saprophyticus* and *Vibrio* sp. bacteria were isolated in this study. These isolated bacteria are significant in aquaculture and are associated with a spectrum of infectious diseases in fish such as motile aeromonas septicemia (MAS), vibriosis, streptococcosis, etc.[28].

Antibacterial activity was investigated using the ethanolic extracts of *C. nutans*, *Chlorella* sp. *Aloe vera* and *Spirulina* sp. against *Aeromonas hydrophila*, *E. coli*, *Vibrio* sp. and *Staphylococcus saprophyticus* pathogenic bacteria. *Spirulina* sp. and *Aloe vera* showed higher inhibition against *Vibrio* sp. about 19.19±0.58 mm and 15.36±1.11 mm, respectively. In a recent study, *Spirulina platensis* extracts were reported to be highly effective against Gram-positive *Bacillus cereus*, *Staphylococcus aureus* and Gram-negative *Listeria monocytogenes*, *E. coli* and *Salmonella typhi*[29]. The active component of *Spirulina* might interrupt bacterial cell integrity either by increasing cell permeability or by cell membrane disruption during cell division which is an intensive area of investigation. *Chlorella* sp. and *C. nutans* exhibited lower inhibition zone than antibiotic about 11.59±1.89 and 11.38±0.78 mm, respectively. Das & Pradhan[30] observed that ethanolic extracts of *S. platensis* exhibited maximum zone size, ranging from 15.6 to 16.3 mm while *Chlorella* displayed an inhibition zone of 12.0±1.0 mm against *Vibrio* sp. Thus, *Chlorella* sp. showed nearly similar results while *Spirulina* sp. showed higher inhibition zone than Das & Pradhan[30]. This can be due to species variability of both microalgae and bacteria. These findings explain the higher effectivity of *Spirulina* sp. against Gram-negative *Vibrio* sp. *Spirulina* sp. possesses higher phycobiliproteins mainly phycocyanin and allo-phycocyanin which may be responsible for the higher antibacterial activity against *Vibrio* sp.

Ethanolic extract of *C. nutans*, *Chlorella* sp. and *Spirulina* sp. significantly inhibited *S. saprophyticus* as well as from control isolated from fish with mean diameter of inhibition zone 14.21±1.075; 11.41±0.658; 13.05±0.035 mm, respectively. *Aloe vera* showed an inhibition zone of 9.043±0.474 mm where control showed 10.44±0.121 mm. From comparison, it is evident that all the extracts significantly inhibited *S. saprophyticus*. Yang *et al.*[15] discovered that *C. nutans* extracts exerted significant antibacterial effect against *S. aureus*. Velichkova *et al.*[31] found that *C. vulgaris* ethanol extract showed a zone of inhibition against *S. aureus* (10.0±1.0). Chakraborty *et al.*[32] also studied ethanolic extract of *S. platensis* against bacteria and reported the zone of inhibition of *S. aureus* (13 mm) which corresponds to our results although the bacterial species are not similar. The Gram-positive nature of *S. aureus* can be the possible reason behind the effectiveness of all the extracts against it. As Gram-positive bacteria lack the outer membrane covering the peptidoglycan layers, it is more susceptible to microalgal and plant extracts than Gram-negative bacteria.

Das & Pradhan[30] reported that ethanolic extract of *Chlorella* vulgaris and *Spirulina platensis* displayed inhibitory zones against *Aeromonas hydrophila*, measuring 11.03±0.03 and 10±0.05 mm, respectively. Correspondingly, Razak *et al*.[33] found a 6.00 mm inhibition zone with *C. nutans* methanolic extract against the same bacteria, aligning with our findings despite the differing nature of the extracts. Iqbal & Ahmed[34] also found that ethanolic extract of *Aloe vera* shows an inhibition zone against *Aeromonas hydrophila* about 9.6±1.06 mm. Among the treatments tested, chlorellin from *Chlorella* sp., bioactive components from *Spirulina* sp. and phytol from *C. nutans* are the core components for the antibacterial activity against Gram-negative bacteria such as *Aeromonas hydrophila*. *Aloe vera*, *C. nutans* and *Chlorella* sp. demonstrated antimicrobial activity against *E. coli*. *Aloe vera*, *C. nutans* significantly inhibited *E. coli* with mean inhibition zone diameters of 14.037±0.903 and 15.103±0.214 mm, respectively, compared to the control. Sekar & Rashid[35] found that *Clinacanthus nutans* methanolic leaf extract showed the maximum antibacterial activity against *E. coli* at 100 mg/mL (8.00±2.00 mm). *Spirulina* sp. extracts did not exhibit inhibition against *E. coli*. Ahmed[36] found that *S. platensis* crude extract have no effect against *Pseudomonas* and *E. coli*, corroborating our results. A study conducted by Parnomo & Pohan[37] demonstrated that *Aloe vera* extracts inhibited *E. coli* growth. Velichkova *et al.*[31] observed a 9.1±0.02 mm inhibition zone with *C. vulgaris* ethanol extract against *E. coli* (9.1±0.02 mm). As *C. nutans* is a good source of phenolics and flavonoids, it created strong antibiotic activity against *E. coli*. *Spirulina* sp. extracts did not show any inhibition against *E. coli* which can be due to the differential *Spirulina* species and the different solvents in the test extracts. The higher inhibition zone of *C. nutans* and *Aloe vera* suggests the future potentialities

of both species in antibacterial drug preparation for *E. coli.*

Minimum inhibitory concentration (MIC) was analyzed to find the effectivity of the antibacterial activity of four test extracts. These extracts exhibited MIC value in the range of 20-40 mg/mL. Ali *et al.*[38] reported that the MIC value of *Spirulina* sp. against *E. coli* is 250 mg/mL, which is much lower than our result for the same species (20 mg/mL). MIC value of *Chlorella* sp. was found to be 20 mg/mL for all the pathogenic bacteria tested. MIC values of *Chlorella vulgaris* against pathogenic bacteria determined by broth macrodilution were also reported between 125-1000 µg/mL[39] which is also lower than the findings of this study. Yang *et al.*[15] discovered that *C. nutans* extracts exerted significant antibacterial effect against *E. coli* and *S. aureus* with minimum inhibitory concentrations (MIC) of 12.5 mg/mL. Zone of inhibition of ethanolic *A. vera* and *C. nutans* extract against pathogenic bacteria found to be 9.56 mm at 30 mg/mL and 11.21 mm at 30 mg/mL, respectively[22] which corresponds to our result. The difference in species, methodology, chemical nature and quantity of bioactive metabolite compounds present in the extracts and their mode of action to test organism might be the reason behind the difference of MIC values.

Conclusion

Aloe vera, *Clinacanthus nutans*, *Chlorella* sp. and *Spirulina* sp. extracts have demonstrated potential antimicrobial properties against the selected bacteria (*Vibrio* sp., *Aeromonas* sp., *E. coli*, *Salmonella* sp., *Streptococcus* sp. and *Staphylococcus* sp.) in the current study. Microalgae and medicinal plants can be represented as a new source of antimicrobial drugs that can be used in the pharmaceutical industries. The bioactive compounds of the microalgae and medicinal plants might be free from side effects and resistance problems and are an area of further investigation. The use of microalgae and plant extracts with known antimicrobial properties can be of great significance in bacterial disease management in aquaculture. More detailed studies are required to identify the bioactive compounds using mass spectrometry, molecular mechanisms, their beneficial effects in inhibiting diverse pathogenic bacteria, viruses and fungus etc. Cytotoxicity and bacterial challenge tests can also provide real-time application potentiality of microalgae and medicinal plants in the aquaculture sector.

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Conflict of interest

Authors declare no competing interests.

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