

Antioxidant and antimicrobial activities in the four species of marine microalgae isolated from Arabian Sea of Karnataka Coast

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In the present study, four marine microalgae namely, *Chaetoceros calcitrans*, *Skeletonema costatum*, *Chroococcus turgidus* and *Nannochloropsis oceanica* were evaluated for their antioxidant and antimicrobial activities. Among the four microalgae, planktonic green alga, *Nannochloropsis oceanica* showed higher antioxidant potential (340.13 $\mu\text{mol AAE/g DW}$), radical scavenging activity (13.3 $\mu\text{mol GAE/g DW}$) and reducing power capacity (86.21 $\mu\text{mol GAE/g DW}$). Microalgae namely, *Skeletonema costatum* and *Chroococcus turgidus* showed chelating ability, whereas *Nannochloropsis oceanica* and *Chaetoceros calcitrans* did not. Total phenolic content of microalgae was highly correlated with the antioxidant activity. Methanol, ethanol and hexane extracts were screened for their antimicrobial activities against selective human pathogens. Extracts showed effective inhibitory activity against *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus subtilis*. Hexane extract of *Chroococcus turgidus* showed significant inhibition ($p < 0.01$) against *Escherichia coli* followed by ethanol extract of *Skeletonema costatum* against *Streptococcus pyogenes*. Antifungal activity was found only in *Skeletonema costatum* and *Chroococcus turgidus*. Findings have indicated promising antioxidant and antimicrobial activities of microalgae and they could be used to derive biologically active compounds of nutraceutical potential.

[**Keywords:** Marine microalgae, Antioxidant activity, Antimicrobial activity, Phenolic compounds]

Introduction

During the last decades, numerous novel compounds have been isolated from marine organisms and many of these substances have been demonstrated to possess interesting biological activities^{1&2}. Recently, much attention has been focused on the marine microalgae as sources of structurally novel and biologically active metabolites^{3&4}. They produce a wide range of bio-active substances with antimicrobial, enzyme inhibiting, immunostimulant, cytotoxic and antioxidant activities^{5,6&7}. Generally, microalgae are considered as an important component of primary productivity in the aquatic ecosystem and are employed in aquaculture industry.

Microalgae have developed some defiance system against photo-oxidative damage by oxidative mechanisms to detoxify and eliminate the highly reactive oxygen species. They represent an almost untapped resource of natural antioxidants, due to their enormous biodiversity, much more diverse than higher plants. Phenolic compounds are considered to be major contributors to the antioxidant capacity of plants⁸. These antioxidants also have diverse biological activities such as anti-inflammatory, antimicrobial and anticarcinogenic activities⁹. Reports on the antioxidant activity of microalgae are limited, especially concerning the relationship between their phenolic content

and antioxidant capacity³. The value of microalgae as a source of natural antioxidants is further enhanced by the relative ease of purification of target compounds¹⁰. Some studies have been made on antimicrobial activity in cell lysates or extracts of various microalgal species^{11,12,13&14}. Antimicrobial study is desirable not only to contribute towards an understanding of ecological interactions but also to assess the potentials of algal antimicrobial activity and their possible therapeutic value. Aim of the present study was to screen for the antioxidant and antimicrobial activities of the four species of marine microalgae and to determine the relationship between antioxidant capacity and phenolic content.

Materials and Methods

The four microalgae species namely, *Chaetoceros calcitrans* (MA 1), *Skeletonema costatum* (MA 2), *Chroococcus turgidus* (MA 3) and *Nannochloropsis oceanica* (MA 4) were isolated by serial dilution culture technique and agar plate method¹⁵. Axenic cultures were maintained in nutrient rich natural sea water media (Walne's and f/2 medium) under florescent light (1000 lux, 8 dark: 16 light) at room temperature¹⁶. Microalgal cells in stationary growth phase were harvested by centrifugation at 3000 rpm for 10 min and washed in physiological saline (0.85% NaCl).

The biomass were dried at 60°C to reach a fixed weight and extracted with three solvents, namely methanol, ethanol and hexane. The extracts were dried under reduced pressure and preserved at 4°C for further use.

Total phenolic content of the methanol extracts were estimated by the Folin-Ciocalteu method¹⁷. Hundred microlitres of diluted samples were added to 1 ml of 1:10 diluted Folin-Ciocalteu reagent. After 4 min, 800 µl of saturated sodium carbonate (75g/l) was added. After 2 hrs of incubation at room temperature, the absorbance at 765 nm was measured. Results were expressed as Gallic acid equivalent (GAE) /g dry weight of extract. Antioxidant activity of methanol crude extracts was determined by Phosphomolybdenum method¹⁸. Hundred microlitres of sample solution was mixed with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95°C for 90 min in a waterbath. Absorbance was measured at 695 nm and results were expressed as Ascorbic acid equivalent (AAE).

The free radical scavenging activity was determined by DPPH scavenging assay¹⁹. Aliquots of 100 µl methanol extract and 4 ml of freshly prepared 0.1 mM DPPH methanol solutions were thoroughly mixed, and kept for 20 min in the dark. Decrease of absorbance at 517 nm was read and expressed as Gallic acid equivalent (GAE). Reducing power of the extracts was determined by ferric reducing power assay²⁰. One ml of extract was mixed with 2.5 ml of 0.2 M phosphate buffer and 2.5 ml of 1% potassium ferricyanide. Reaction mixture was incubated at 50°C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. One ml of supernatant was mixed with 1 ml of distilled water and 0.2 ml of 0.1% ferric chloride. Absorbance at 700 nm was measured and expressed as Gallic acid equivalent (GAE). Chelating activity was determined by ferrous ion chelating assay¹⁹. One ml of extract was mixed with 0.1 ml of 2 mM FeCl₂.4H₂O and 0.2 ml of 5 mM ferrozine. Volume in all tubes made upto 5 ml with methanol and reacted for 10 min. Absorbance was read at 562 nm and expressed as EDTA equivalent (EDTAE).

The *in vitro* antimicrobial activity of microalgal extracts were tested against seven bacterial pathogens and four fungal pathogens by disc diffusion method⁵. Bacteria namely, *Staphylococcus aureus* MTCC 7443 (BP1),

Streptococcus pyogenes MTCC 1928 (BP2), *Bacillus subtilis* MTCC 441 (BP3), *Escherichia coli* MTCC 725 (BP4), *Proteus vulgaris* MTCC 426 (BP5), *Klebsiella pneumoniae* MTCC 3384 (BP6), *Salmonella typhi* MTCC 733 (BP7) and the yeast *Candida albicans* MTCC 183 (FP1), mycelial fungi *Fusarium moniliforme* MTCC 2088 (FP2), *Aspergillus flavus* MTCC 871 (FP3) and *Aspergillus niger* MTCC 281 (FP4) were the test organisms.

Hundred microliter suspensions of bacterial and fungal strains prepared in physiological saline (0.85% NaCl) were spread on Muller-Hinton agar (MHA) and Sabouraud dextrose agar (SDA) respectively. Sterilized filter paper discs (6 mm diameter) were impregnated with microalgal extracts (10 mg/ml) and placed on the inoculated agar plates. Negative controls were prepared using the respective solvents. Gentamycin and Ketoconazole were used as positive controls. The inoculated plates were kept at room temperature for 10 min and incubated at 37°C (24 hrs) for bacterial strains and at room temperature (48 hrs) for fungal strains. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microbial pathogens. Minimum inhibitory concentration (MIC) was determined by agar well method²¹. Six wells (5 mm) were made from the test microbial inoculated plates and filled with 50 µl of extracts in an increasing concentration (2 to 0.1 mg/ml). After incubation, inhibition of growth was observed around the well and the lowest concentration at which inhibitory zone was observed was taken as the MIC value.

All the results were calculated as mean value $\pm$ the standard deviation ($n = 3$). One way analysis of variance (ANOVA) was applied to test for significant differences at $p < 0.01$.

Results and Discussion

Phenolic compounds serve as important antioxidants because of their ability to donate a hydrogen atom or an electron in order to form stable radical intermediates²². Total phenolic contents of four marine microalgae were evaluated in order to make a comparison between the species and identify a natural source for phenolic compounds (Fig.1). Extract of *Nannochloropsis oceanica* showed highest phenolic content (17.01 mg GAE/g DW) followed by *Chaetoceros calcitrans* (11.23 mg GAE/g DW), *Skeletonema costatum* (10.11 mg

GAE/g DW) and *Chroococcus turgidus* (7.94 mg GAE/g DW).

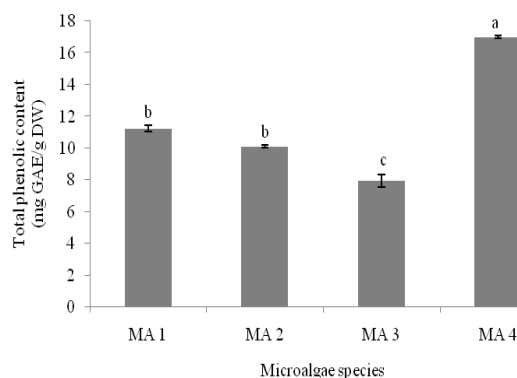


Fig. 1—Total phenolic content (mg GAE/g dry weight) in the four marine microalgae.

Algal biomass and algae derived compounds have a very wide range of potential applications, from animal feed and aquaculture to human nutrition and health products. Some algae are considered as rich sources of natural antioxidants^{3,23&24}. Microalgae showed diverse antioxidant capacity in the present study (Table 1). Extract of planktonic green alga, *Nannochloropsis oceanica* showed higher antioxidant potential (340.13 μ mol AAE/g DW).

Table 1—Antioxidant activities (μ mol) in the four marine microalgae.

Microalgae species	Total antioxidant (AAE/g DW)	Radical scavenging capacity (GAE/g DW)	Reducing power capacity (GAE/g DW)	Chelating capacity (EDTAE/g DW)
MA 1	204.88 $\pm$ 0.73	3.32 $\pm$ 0.33	48.40 $\pm$ 0.34	-
MA 2	170.68 $\pm$ 0.34	2.91 $\pm$ 0.03	16.61 $\pm$ 0.39	15.57 $\pm$ 0.17
MA 3	130.25 $\pm$ 0.34	3.91 $\pm$ 0.33	29.00 $\pm$ 0.34	8.78 $\pm$ 0.20
MA 4	340.13 $\pm$ 0.50	13.3 $\pm$ 0.32	86.21 $\pm$ 0.69	-

The minimum activity was observed in *Chroococcus turgidus* (130.25 μ mol AAE/g DW). Radical-scavenging power was determined by DPPH assay²⁵. Antioxidants in the extracts reduced DPPH radical to DPPH-H and that indicated the potential to be an H-atom donor²⁶. Among the four microalgae, *Nannochloropsis oceanica* exhibited higher radical scavenging activity (13.3 μ mol GAE/g DW). Diatom species showed minimum activity compared to cyanobacterium, *Chroococcus turgidus* (3.91 μ mol GAE/g DW). Reducing power is an ability to reduce or give electrons to free radicals and change them into the stabilized form, which can halt the free radical chain reaction²⁰. Higher reducing power capacity was observed in *Nannochloropsis* sp. (86.21 μ mol GAE/g DW) and least in *Skeletonema costatum* (16.61 μ mol GAE/g DW). Results of DPPH radical scavenging activity and reducing power proved that the crude extracts of microalgae

contained both the electron and hydrogen atom donating ability. Ferrous ion, commonly found in food systems, is well known as an effective pro-oxidant¹⁹. Ferrous ion may be released in the breakdown of red blood cells, causing the levels of ferrous ion in the body to increase²⁷. Antioxidant compounds present in the microalgae extract can chelate pro-oxidant metal ions, thus preventing the free radical formation from these pro-oxidants. The extract of *Skeletonema costatum* had the most chelating ability (15.57 μ mol EDTAE/g DW) than *Chroococcus turgidus* (8.78 μ mol EDTAE/g DW). The extracts of *Nannochloropsis oceanica* and *Chaetoceros calcitrans* did not show any chelating power.

The correlation coefficient (R^2) between the antioxidant capacity and the total phenolic content of the microalgae is shown in Fig. 2A-D. Total phenolic content in crude extracts of

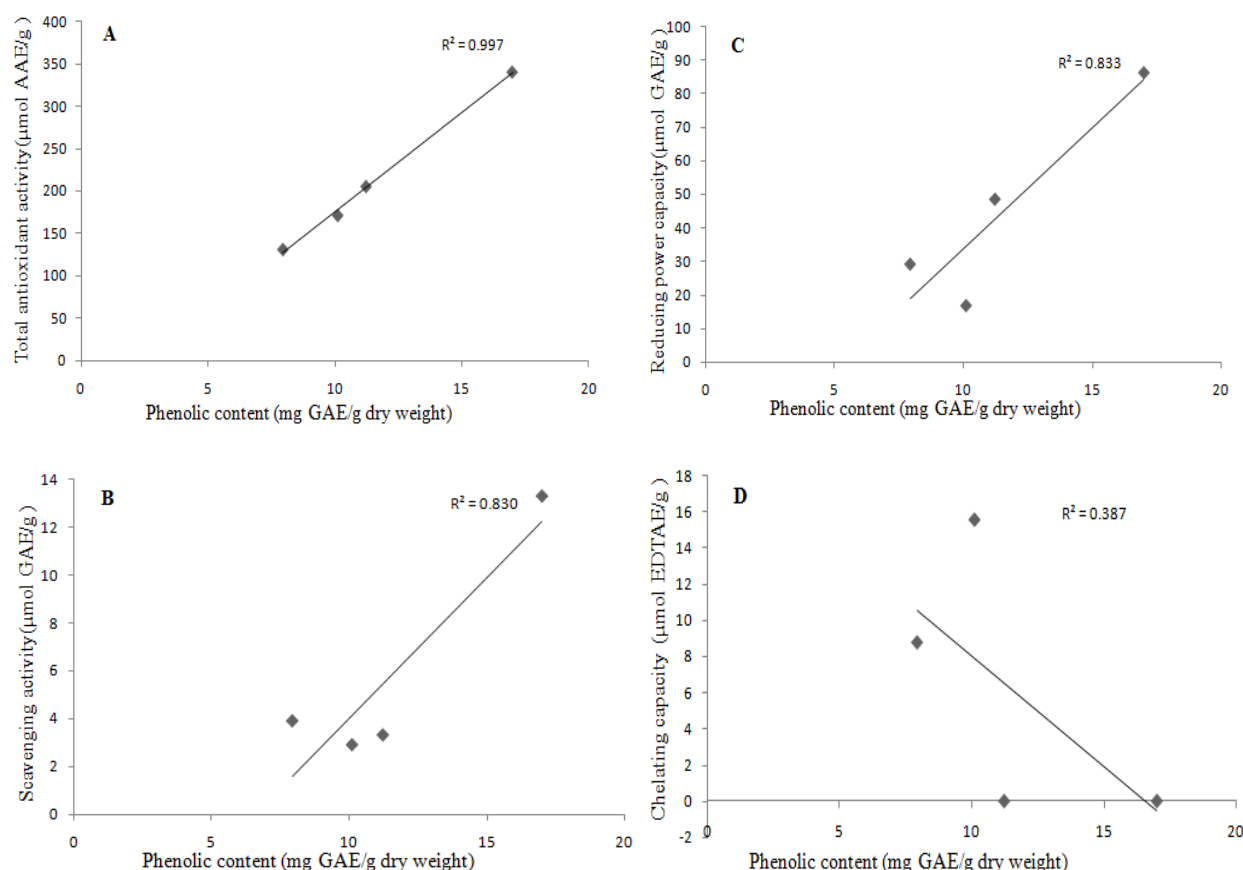


Fig. 2—Correlation between total phenolic content and total antioxidant activity (A), DPPH radical scavenging assay (B), Ferric reducing power assay (C) and Ferrous ion chelating capacity (D).

microalgae was highly correlated to the antioxidant activity (Total antioxidant activity, DPPH assay and reducing power assay) and the study agrees with the earlier studies^{28&29}. But the correlation coefficient between the ferrous ion chelating capacity and the phenolic contents was found to be very small ($R^2 = 0.387$). This indicates that only phenolic compounds were not contributing to the antioxidant properties of the microalgae. They could produce wide range of other antioxidant compounds such as pigments, polyunsaturated fatty acids and polysaccharides^{30&31}. Recent works indicate that a variety of phenolic classes are present in microalgae, but further identification of phenolic components is required to get a better idea about the relationship between the phenolics and the antioxidant activity^{32&33}.

Marine microalgae are a potential source of new antimicrobial compounds which can be attributed to their genetic diversity. They represent a yet untapped resource of novel natural products. Like all organisms, they also need to protect themselves against opportunistic attack or pathogenic damage^{34&35}. To date, many

chemically unique compounds of marine origin with various biological activities have been isolated, and some of them are under investigation and are being used to develop new pharmaceuticals³⁶. Cell extracts and active constituents of various algae have shown to possess antimicrobial activity *in vitro* against microbial pathogens. A wide range of results of *in vitro* antifungal activities of extracts of green algae, diatoms and dinoflagellates have also been reported³⁷. Results obtained from the study on the antimicrobial activity of four microalgae against different species of bacteria and fungi are shown in Table 2. Antimicrobial activity shown by the microalgae will be a good source of the new bioactive compounds from the pharmaceutical view point. Antimicrobial activity of microalgal extracts depends on the type of the algal species, type of the solvent used and the tested bacterial and fungal pathogens. On the study, microalgae were extracted with three solvents i.e., ethanol, methanol and hexane and tested against seven bacterial and four fungal pathogens.

Table 2—Antimicrobial activity of microalgal extracts against selected microbial pathogen.

Microalgae	Solvents used	Diameter of zone of inhibition (mm)										
		BP1	BP2	BP3	BP4	BP5	BP6	BP7	FP1	FP2	FP3	FP4
MA 1	Ethanol	9.1 ±0.12	10.3 ±0.20	9.5 ±0.15	-	-	-	-	-	-	-	-
	Methanol	8.4 ±0.31	10.1 ±0.17	8.2 ±0.15	-	-	-	-	-	-	-	-
	Hexane	9.5 ±0.20	13.4 ±0.26	14.5 ±0.06	9.4 ±0.12	-	-	-	-	-	-	-
MA 2	Ethanol	9.1 ±0.12	18.1 ±0.17	-	7.6 ±0.12	-	6.1 ±0.10	12.0 ±0.06	-	8.6 ±0.12	-	-
	Methanol	-	8.1 ±0.12	12.1 ±0.12	-	-	7.1 ±0.15	-	-	9.5 ±0.06	-	-
	Hexane	10.1 ±0.06	13.2 ±0.15	10.4 ±0.12	-	-	-	15.1 ±0.17	-	-	-	-
MA 3	Ethanol	6.5 ±0.06	6.6 ±0.06	-	-	-	6.5 ±0.15	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-
	Hexane	10.2 ±0.15	-	12.1 ±0.15	21.4 ±0.12	7.3 ±0.15	-	-	8.6 ±0.15	-	-	-
MA 4	Ethanol	6.6 ±0.10	-	-	-	7.1 ±0.12	-	-	-	-	-	-
	Methanol	-	8.1 ±0.15	-	-	-	-	8.2 ±0.15	-	-	-	-
	Hexane	9.7 ±0.15	17.4 ±0.10	9.3 ±0.15	7.2 ±0.20	-	-	7.5 ±0.20	-	-	-	-

*BP: Bacterial pathogens; FP: Fungal pathogens; -: No activity

The microalgal extracts showed effective inhibitory activity against three bacterial pathogens, namely, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus subtilis*. Hexane and ethanol extracts showed highest antibacterial activity, whereas methanol extracts showed moderate activity. Among four microalgae, hexane extract of *Chroococcus turgidus* showed significant growth inhibition against *Escherichia coli* (21.4 mm zone) followed by ethanol extract of *Skeletonema costatum* against *Streptococcus pyogenes* (18.1 mm zone) and hexane extract of *Nannochloropsis oceanica* against *Streptococcus pyogenes* (17.4 mm zone). In the study, the microalgae showed least antifungal activity; ethanol and methanol extracts of *Skeletonema costatum* against *Fusarium moniliforme* and hexane extracts of *Chroococcus turgidus* against *Candida albicans*. Fungi namely, *Aspergillus flavus* and *Aspergillus niger* showed resistance to all the four microalgae. Extracts which did not show any inhibition of growth for the tested concentrations might show inhibition if used in higher concentrations.

Table 3 summarizes the MIC values of the microalgal extracts. Among the tested

extracts, the ethanol extracts showed good MIC values against tested pathogens. One way analysis of variance (ANOVA) revealed significant difference in the antimicrobial activity of microalgae species ($p < 0.01$). Ethanol extracts of *Skeletonema costatum* exhibited significantly greater inhibitory activity than *Nannochloropsis oceanica* and *Chroococcus turgidus* against *Streptococcus pyogenes*. Methanol extracts of *Chaetoceros calcitrans* and *Nannochloropsis oceanica* showed high inhibitory activity against *Streptococcus pyogenes* than *Chroococcus turgidus*. A significant difference ($p < 0.01$) existed in the inhibitory activities between the hexane extracts of *Chaetoceros calcitrans* and other three microalgae species on *Streptococcus pyogenes* and *Bacillus subtilis*. Considerably high activity was observed in hexane extracts of *Chroococcus turgidus* than *Skeletonema costatum* and *Nannochloropsis oceanica* against *Escherichia coli*. Microalgal extracts screened for their antimicrobial activities could be more effective than antibiotics and fungicides. However, these microalgae are potential sources of bioactive compounds and should be investigated for natural antibiotics.

Table 3—Minimum Inhibitory concentration (MIC) of microalgal extracts (mg/ml).

Microalgae	Solvents used	BP1	BP2	BP3	BP4	BP5	BP6	BP7	FP1	FP2	FP3	FP4
MA 1	Ethanol	0.10	0.10	0.10	-	-	-	-	-	-	-	-
	Methanol	0.10	0.10	0.25	-	-	-	-	-	-	-	-
	Hexane	0.50	0.10	0.50	>2.00	-	-	-	-	-	-	-
MA 2	Ethanol	0.10	0.10	-	>2.00	-	0.50	2.00	-	0.10	-	-
	Methanol	-	0.10	1.00	-	-	0.75	-	-	2.00	-	-
	Hexane	1.00	0.75	0.50	-	-	-	1.00	-	-	-	-
MA 3	Ethanol	0.75	0.10	-	-	-	2.00	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-
	Hexane	>2.00	-	>2.00	>2.00	>2.00	-	-	>2.00	-	-	-
MA 4	Ethanol	0.10	-	-	-	>2.00	-	-	-	-	-	-
	Methanol	-	1.0	-	-	-	-	>2.0	-	-	-	-
	Hexane	>2.0	>2.0	>2.0	>2.0	-	-	>2.0	-	-	-	-

*BP: Bacterial pathogens; FP: Fungal pathogens; -: No activity

Conclusion

The present study demonstrates the *in-vitro* antioxidant and antimicrobial properties of four species of marine microalgae. It concludes that marine microalgae are a potential source of novel bioactive compounds. They have nutritionally potential applications as foods/feeds which are able to provide additional physiological and pharmacological benefits which was also stressed by other investigators^{22,25&29}. The characterization of these compounds and the techniques of evaluation employed need further investigations to give a clear picture about their biological activity.

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