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Evaluation of the anti-bacterial activity of methanolic extract of *Chlorella vulgaris* Beyerinck [Beijerinck] with special reference to antioxidant modulation

Biswajita Pradhan, Srimanta Patra, Soumya Ranjan Dash, Rabindra Nayak, Chhandashree Behera and Mrutyunjay Jena*

Abstract

Background: The natural antioxidants from *Chlorella* have potent therapeutic implication in several diseases. However, the anti-bacterial activity and their molecular mode of action have not been investigated yet. The present study focussed on the assessment of antioxidant potential as well as free radical scavenging activity such as DPPH, hydroxyl radical, hydrogen peroxide, and superoxide anion radical assay of *Chlorella vulgaris* Beyerinck [Beijerinck] (BUACC25) isolated from marine habitat. Furthermore, the anti-bacterial activity and their molecular mode of action have been evaluated.

Results: In the present study, the preliminary phytochemical screening of methanolic algal extract revealed the presence of alkaloids, glycosides, proteins, terpenoids, saponins, coumarin, phenols, and tannins, which was confirmed by in an UV-visible and FT-IR spectroscopy, indicated the distinct spectral peaks. The methanolic algal extract was found to be rich in phenolic content (45 ± 0.06 mg GAE g^{-1}) and flavonoid content (470 ± 0.25 mg of RUE g^{-1}). Furthermore, the methanolic extract was revealed potent antioxidant scavenging activity to scavenge various free radicals with minimum IC_{50} values of DPPH, hydroxyl, H_2O_2, superoxide 2.82 ± 0.30, 2.30 ± 0.25, 3.24 ± 0.32, and 3.15 ± 0.02 μg ml^{-1} respectively. Furthermore, the methanolic extract of *C. vulgaris* exhibited potent anti-bacterial activity which was evident with the reduction in cfu × 10^7/ml and % of cell viability. Mechanistically, reduction of SOD, CAT, and GSH activity provoked ROS-mediated cell death after drug treatment. Moreover, in combination with norfloxacin and ciprofloxacin, methanolic extract of *C. vulgaris* demonstrated enhanced anti-bacterial activity with an evident reduction in cfu/ml and % of cell viability.

Conclusion: This study advocates that *C. vulgaris* (BUACC25) has promising antioxidant activity owing to the presence of phenolic and flavonoids evidenced by scavenging of DPPH, hydroxyl, H_2O_2, and superoxide radicals. In addition to this, it sustained anti-microbial activity against *E. coli* through modulation of SOD, CAT, and GSH. This study carved a path for uncovering a better therapeutic agent against disease-causing bacterial pathogens.

Keywords: Antioxidants, Antimicrobial, *Chlorella vulgaris*, Drug synergism, ROS

* Correspondence: mrutyunjay.jena@gmail.com
Algal Biotechnology and Molecular Systematic Laboratory, Post Graduate Department of Botany, Berhampur University, Bhanja Bihar, Berhampur 760007, India

Statement of novelty

Methanolic extract of *C. vulgaris* demonstrates enhanced anti-bacterial activity via modulation of antioxidant defense enzymes. The antibiotic synergism with methanolic extract of *C. vulgaris* exerts enhanced anti-bacterial activity in *E. coli*.

Background

The marine environment is rich in biodiversity [1] and promising natural resources of anti-cancer, antioxidant, anti-inflammatory, and antibiotic compounds [2, 3]. Algae are the most promising sources of proteins, vitamins, omega-3-fatty acids, and antioxidants [4, 5]. Microalgae are reflected in numerous significant source of bioactive compounds such as antioxidants, carotenoids, phenolic and flavonoids [6]. Although many studies reported that several bioactive compounds from macroalgae and their effects on several free radical diseases [7–10] but antioxidant properties of microalgae and particularly of *Chlorella* spp. from the marine environment are poorly addressed. Microalgae are also potential candidates to produce a wide range of bioactive compounds under different stress conditions [11] with possible modifications in physiological as well as biochemical pathways to maintain cellular homeostasis [12]. The reactive oxygen species (ROS) and other free radicals generated by normal metabolism and enhance their production under environmental stresses, cause the deleterious effect to organisms [13]. Numerous studies have reported that free radical synthesis occurs due to oxidative stress that leads to cancer [14], early aging, Alzheimer's, Parkinson's [15], and cardiovascular diseases such as atherosclerosis and other harmful diseases [16]. The antioxidants act as free radical scavengers, as they are electrons or hydrogen donors and produce numerous stable intermediate radicals. They also prevent oxyradical formation to avoid oxidative stress [17]. Further, antioxidants at lower concentrations affect to delay the oxidation of oxidizable elements and inhibit further oxidation [17]. The antioxidants from algal sources scavenge the oxygenated free radicals more than the antioxidants of plants and seaweeds [18]. Generally, phenolic compounds are the biologically active secondary metabolites chiefly present in microalgae are also act as a strong antioxidant, which neutralizes the ROS before harmful physiological effects in the cell [19, 20]. Artificial antioxidants are generally phenolic compounds, cause adverse health effects on human health [21]. Therefore natural antioxidants are potent enough to substitute synthetic antioxidants [22]. The different species of *Chlorella* isolated from both freshwater and marine habitat have been generally used as single-cell protein (SCP) for nutritional supplements for a long [23]. Furthermore, the bioactive compounds from various *Chlorella* spp. are mostly used as anti-cancer, anti-aging, anti-inflammation, anti-bacterial, and anti-fungal agents [24]. Pharmaceutically value-added products, particularly antioxidant compounds (phenolic and flavonoids) from microalgae for commercialization are still in infancy stage to replace the synthetic phenolic and flavonoid compounds available in the current scenario.

Biologically active phytoconstituents from microalgae have proven as potent anti-bacterial, anti-fungal, and anti-viral agents against several disease-causing pathogens owing to their antioxidant and radical scavenging activity. *C. vulgaris* has been proven as an important source of bioactive compounds with several therapeutic implications [25]. Previously, several reports have demonstrated the presence of bioactive compounds in *C. vulgaris* that act as anti-microbial agents [25]. However, the mechanistic pathways underlying the exhibition of such activity have not been demonstrated yet. Therefore, keeping all the scenarios in mind, the present study was focused to evaluate the phytochemicals constituents, antioxidant potential as well as free radical scavenging activity that contribute towards the anti-bacterial activity. In addition to this, the pathways associated with such activity was investigated in connection with drug synergism for enhanced anti-bacterial efficacy. The findings of this investigation may deliver information of *C. vulgaris* as a potential source of future therapeutic agents for disease-causing microbes and can be represented as a dietary supplement during therapeutic intervention.

Methods

Reagents and chemicals used

1,1-Diphenyl-2-picrylhydrazyl (DPPH•), gallic acid, ascorbic acid, rutin, hydrogen peroxide (H_2O_2), sodium salicylate, ferrous sulfate ($FeSo_4$), nicotinamide adenine dinucleotide (NADH), Tris-HCl, nitro blue tetrazolium chloride (NBT), phenazine methosulfate (PMS), sodium phosphate, ammonium molybdate, sodium carbonate (NA_2CO_3), ferric chloride ($FeCl_3$), Folin–Ciocalteu's reagent, ammonia solution (NH_3), sulfuric acid (H_2SO_4), hydrochloric acid (HCl), chloroform, Fehling's solutions A and B, sodium carbonate, aluminum chloride ($AlCl_3$), mercury potassium iodide, sodium hydroxide (NaOH), isoamyl alcohol, glacial acetic acid, nitric acid, and methanol were purchased from Sigma-Aldrich, Merck and Himedia.

Sample collection and isolation of algal pure strain

The samples were collected by phytoplankton net (mesh size 25 μm) from Sonapur on the sea, Ganjam, Odisha (N′′ 19°09.606′ and E′′ 085°12.576′). The unialgal cell was isolated from the collected sample by the micro-capillary method and identified as *Chlorella vulgaris*

Beyerinck [Beijerinck]. The pure strain *Chlorella vulgaris* assigned with strain no. BUACC25.

Algal culture and preparation of crude extract

The pure algal strain was grown in BBM + salt (NaCl) medium for 15 days at 14 ± 10 day: night condition under 7.5 w.m^2 (flux) light intensity and temperature maintained at 23 ± 2 °C. Algal biomass was harvested by centrifugation and extracted in methanol. Then, methanolic crude extracts were centrifuged, filtered, and evaporated by rotary evaporator. The % of the yield of algal crude was calculated [26]. The algal crude extract was stored at − 20 °C for future analysis.

Qualitative screening of phytochemicals

The phytochemicals screening of algal crude extracts was carried out by the standard procedures [26–28].

Estimation of total phenol content (TPC)

The total phenolic content was determined by the Folin–Ciocalteu method [29, 30] with little modification as described in our previous report (OD; 765 nm taking gallic acid as standard) [26]. The results were interpreted after three individual experiments and TPC content in the extract was expressed in milligram gallic acid equivalent dry weight (mg GAE g^{-1} DW) of the sample.

Estimation of total flavonoid content (TFC)

The total flavonoid content of the algal crude extract was estimated by the spectrophotometric method (OD; 415 nm taking rutin as standard) [31]. The TFC in the extract was expressed in terms of milligram rutin equivalent dry weight (mg RUE g^{-1} DW) of the algal sample.

Free radical scavenging assay

The total antioxidant activity was determined by the phosphomolybdenum method [32] with minor modifications as described in our previous report (OD; 695 nm taking ascorbic acid as standard) [26, 33]. The standard curve calibration was plotted by taking ascorbic acid (10–500 μg ml^{-1}) as the positive control. The total antioxidant activity was expressed as the number of gram equivalent of ascorbic acid.

DPPH free radical scavenging activity of the algal crude extract was determined according to our previous report (OD; 517 nm taking ascorbic acid as standard) [26] and the percentage of DPPH decolorization of the extract was calculated using the following formula:

$$\%\text{of decolorization} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100$$

Hydrogen peroxide radical scavenging activity of the algal extract was estimated by following detailed protocol (OD; 230 nm taking ascorbic acid as standard) [29, 34]. Ascorbic acid was used as a positive control to plot the standard curve.

Hydroxyl radical scavenging activity of the algal extracts was estimated by following our previous method (OD; 562 nm taking ascorbic acid as standard) [29] taking ascorbic acid as standard and the percentage of scavenging effect was calculated by using the following formula:

$$\%\text{scavenging inhibition} = \left\{1 - \frac{A_1 - A_2}{A_0} \times 100\right\}$$

Where A_0 = absorbance of the control (without extract), A_1 = absorbance in the presence of the extract with sodium salicylate and A_2 = the absorbance without sodium salicylate.

Superoxide anion radical scavenging activity assay was determined based on the reduction of NBT in the presence of NADH and PMS under aerobic conditions (OD; 560 nm taking ascorbic acid as standard) [35]. Ascorbic acid was used as a standard. The experiment was carried out as per the protocol described in our previous study [29].

UV-visible spectroscopic spectral analysis

The UV-visible spectroscopic spectrum scan range was 200 to 800 nm on a UV-visible spectrophotometer to check the presence of phytochemicals compounds in the crude extracts, and spectral peaks were analyzed by comparing the spectrum.

FT-IR spectroscopic spectral analysis

The presence of various active phytochemicals present in the algal crude extract was crossed checked by FT-IR spectroscopy. The scanning of spectra of FT-IR was fixed within the signal region 4000 to 400 cm^{-1} of 32 scans with 4.0 resolutions. The functional group of compounds with reference to obtained spectra were determined.

Isolation, culture, and maintenance of bacterial strain

The pathogenic clinical strain of *E. coli* was isolated from the liquid medical waste by subsequent serial dilution followed by streaking on agar plate [36]. The bacterial strain was cultured in Luria Broth (LB) for further experiments. The strain was maintained in 50% sterile glycerol under − 20 °C for further use.

Colony-forming unit count and determination of cell viability

To determine the cfu/ml, 3 ml of LB was taken in different test tubes containing 100 μl of bacterial culture

attained the exponential phase of growth. 50, 250, and 500 μg/ml of methanolic extract of *C. vulgaris* were added. The test tubes were incubated for 18 h, and the absorbance was taken at 600 nm taking the drug in culture medium as blank [37, 38].

Determination of antioxidant enzymes of cultured bacterial strain

The bacterial protein was isolated as per the method described previously [39]. The bacterial SOD was estimated spectrophotometrically as per the method described by Scott et al. [40]. Similarly, for the determination of CAT activity, the previous method was described by Schwartz et al. [41]. The GSH activity was determined with little modification to Prins and Loos as described by Scott et al. [40].

Statistical analysis

Experiments were done by taking each sample in triplicate and results were expressed as mean ± standard deviation (SD). The IC_{50} value of phenolic content with antioxidant activity was calculated through linear regression and also linear regression coefficient (R^2). All the experiments were repeated in triplicate and the experimental data were analyzed by one-way analysis of variance (ANOVA) represented as values ± SD. The p value; $^{*}p \leq 0.05$ and $ was compared with the drug were considered as significant.

Results

Evaluation of active phytoconstituents in methanolic extract of *C. vulgaris* for effective antioxidant activity

After the methanolic extraction, the percentage of the yield of *Chlorella vulgaris* was calculated to be 4.93%. The phytochemical screening was done to screen bioactive compounds that were subsequently important for potent antioxidant activity. Phytochemical analysis of the crude extract of *C. vulgaris* was investigated. The results exhibited the presence of bioactive compounds and associated groups present (Table 1). The UV-visible spectral analysis revealed the presence of phenolic bioactive compounds and FT-IR spectral analysis informed the presence of functional groups and the bonding pattern of the compounds. Hence, the methanolic crude extracts were crossed checked by UV-visible and FT-IR spectral analysis. The UV-visible spectral peak value between 230–290, 300–360, and 234–676 nm have exhibited the presence of flavonoids and phenolic compounds in crude extract shown in Fig. 1a. Furthermore, the FT-IR spectroscopic spectral peaks were attributed to the presence of functional groups and bonding patterns in the compound. The characteristic wavenumbers of specific functional groups detected were shown in Fig. 1b. The chemical bonding pattern and functional groups present in the crude extract of *C. vulgaris* through spectral peak analysis were analyzed and represented (Table 2).

Table 1 Phytochemical screening for bioactive compounds of methanol extract of *C. vulgaris*

Bioactive compounds	Test	Present/absent
Alkaloids	Mayer's test	+
Glycosides	Keller-kiliani test	+
Reducing sugar	Fehling's test	−
Proteins	Xanthoproteic test	+
Terpenoids	Salkowski's test	+
Phenol and tannins	Ferric chloride test	+
Steroids	Salkowski's test	−
Saponin	Foam test	+
Anthocyanin	Hydrochloride test	−
Coumarin	Sodium hydroxide test	+

Methanolic extract of *C. vulgaris* exhibits potent in vitro radical scavenging activity

Phenolics and flavonoids play a very crucial role in antioxidant activity. The presence of TPC in the crude extract was measured by Folin–Ciocalteu's reagent and was represented in terms of gallic acid equivalent (R^2 = 0.9815). The value was estimated to be 45 ± 0.06 mg g^{-1} DW. Further, the TFC content algal crude extract was estimated and expressed in terms of rutin equivalent (R^2 = 0.9904). The concentration of flavonoid was found to be 470 ± 0.25 mg g^{-1} DW. The correlation between TPC and antioxidant activity was further estimated. High correlations between TPC and antioxidant scavenging capacity (DPPH, R^2 = 0.8191; hydroxyl radicals, R^2 = 0.8081) was observed (Fig. 2a). By comparing the correlation coefficients (R^2 = 0.9815), it was determined that phenolic groups were extremely responsible for antioxidant activity.

The total antioxidant activity was estimated by phosphomolybdenum assay and the results were estimated in terms of mg AAE g^{-1} and to be 411 ± 0.39 mg AAE g^{-1} (Table 3). The quantification of DPPH exhibited a concentration-dependent increase in radical scavenging capacity. The DPPH antioxidant scavenging activities of algal crude extract at various concentrations (10 to 500 μg ml^{-1}) were calculated against the standard ascorbic acid concentrations. The crude extracts results displayed 90.48% of inhibition of antioxidant radical scavenging activities against the standard ascorbic acid 89.68% at concentrations of 500 μg ml^{-1} (Fig. 2b). The IC_{50} value of DPPH radical scavenging activity of algal crude extract and ascorbic acid were 2.82 ± 0.30 μg ml^{-1} and (2.68 ± 0.29 μg ml^{-1}) respectively (Table 4). The OH scavenging activities of crude extract exhibited a similar inclination in a concentration-dependent manner as

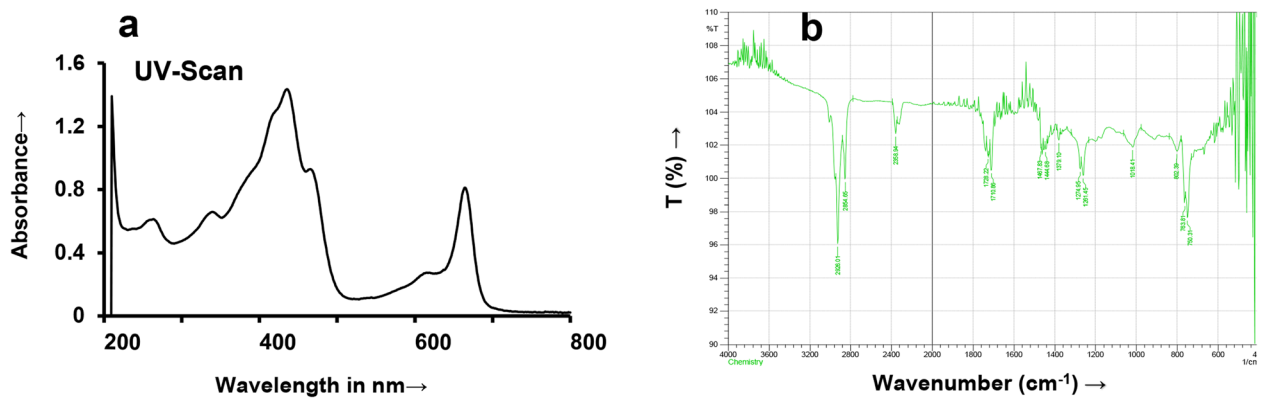

Fig. 1 UV-visible spectrum of methanol extract of *C. vulgaris* (**a**), FT-IR results from methanol extract of *C. vulgaris* (**b**)

compared to the standard ascorbic acid, and it was displayed the highest level of inhibition, i.e., 90.43 % in the crude extract and 91.85% in ascorbic acid respectively at 500 μg ml^{-1} concentrations (Fig. 2c). The extract exhibited an increase in hydroxyl radical scavenging properties with IC_{50} value (2.30 ± 0.25 μg ml^{-1}) over the ascorbic acid 1.87 ± 0.16 μg ml^{-1} (Table 4). The superoxide radical scavenging activity was quantified to know the potency of the extracts to reduce the NBT. On the other hand, the superoxide scavenging activity also exhibited a similar pattern at 10–500 μg ml^{-1} of crude extract. The result exhibited a concentration-dependent scavenging activity of the crude extract. The inhibition value of crude extract was showed maximum (89.12%) as compared to standard ascorbic acid was showed maximum (89.47%) (Fig. 2d) and IC_{50} value 3.15 ± 0.02 μg ml^{-1} compared to ascorbic acid 3.22 ± 0.03 μg ml^{-1} (Table 4). Furthermore, The H_2O_2 scavenging activity of the crude extract was investigated, and the results displayed a concentration-dependent increase in radical scavenging activity and the standard ascorbic acid was calculated to be 9.73% to 75.97% and 11.39% to 90.23% respectively at 10 to 500 μg ml^{-1} concentration (Fig. 2e). The IC_{50} value of H_2O_2 radical scavenging activity of crude extract and standard ascorbic acid showed 3.24 ± 0.32 μg ml^{-1} and 3.21 ± 0.38 μg ml^{-1} (Table 4). These results indicated that the methanolic algal extract exhibited a strong superoxide radical scavenging potency as compared to the standard ascorbic acid. The extract displayed higher hydroxyl and DPPH radical scavenging activity than other radical scavenging assays with IC_{50} values 2.30 ± 0.25 and 2.82 ± 0.30 μg ml^{-1} (Table 4).

Table 2 FT-IR spectroscopy of methanol extract of *C. vulgaris*

Peak values	Functional group	Bonding pattern
2926.01	Amine salts	N–H
2854.65	Amine salts	N–H
2358.94	Carbon dioxide	O=C=O
1728.22	Aldehyde	C=O stretching
1710.86	Aliphatic ketone	C=O stretching
1467.83	Alkene methylene group	C–H bending
1444.68	Alkene methyl group	C–H bending
1379.10	phenol	O–H bending
1274.94	Aromatic ester	C–O stretching
1261.45	Alkyl aryl ether	C–O stretching
1018.41	Sulphoxide	S=O
802.39	Alkene	C=C bending
763.91	Halo compound	C–Cl stretching
750.31	Mono-substituted	C–H bending

Methanolic extract of *C. vulgaris* mediates anti-bacterial activity against *E. coli* through downregulation of antioxidant enzymes

The anti-bacterial activity of methanolic extract of *C. vulgaris* was determined against *E. coli*. With increase in drug concentration of methanolic extract of *C. vulgaris*, a reduced cfu/ml was observed with values estimated (control 15.0 × 10^7, 50 μg/ml: 11.575 × 10^7, 250 μg/ml: 9.868 × 10^7, 500 μg/ml: 7.744 × 10^7) (Fig. 3a). Further, we evaluated the bacterial cell viability upon drug treatment and observed a reduction in cell viability with an increase in drug concentration. The % of cell viability was estimated to be 51.63% at 500 μg/ml (Fig. 3b). Furthermore, to delineate the effective role of antioxidant enzymes for bacterial cell viability, we evaluated the SOD, CAT and GSH activity. Post-drug treatment a subsequent decrease in SOD activity was observed with values (control: 13.9, 50 μg/ml: 9.78, 250 μg/ml: 7.5, 500 μg/ml: 5.78) represented in units/mg protein (Fig. 3c). A similar reduction in CAT activity was also evident with values (control: 15.0, 50 μg/ml: 11.49, 250 μg/ml: 9.8, 500 μg/ml: 7.1) represented in units/mg protein (Fig. 3d). Moreover, a similar reduction in GSH activity was observed upon drug treatment with values (control: 5.8,

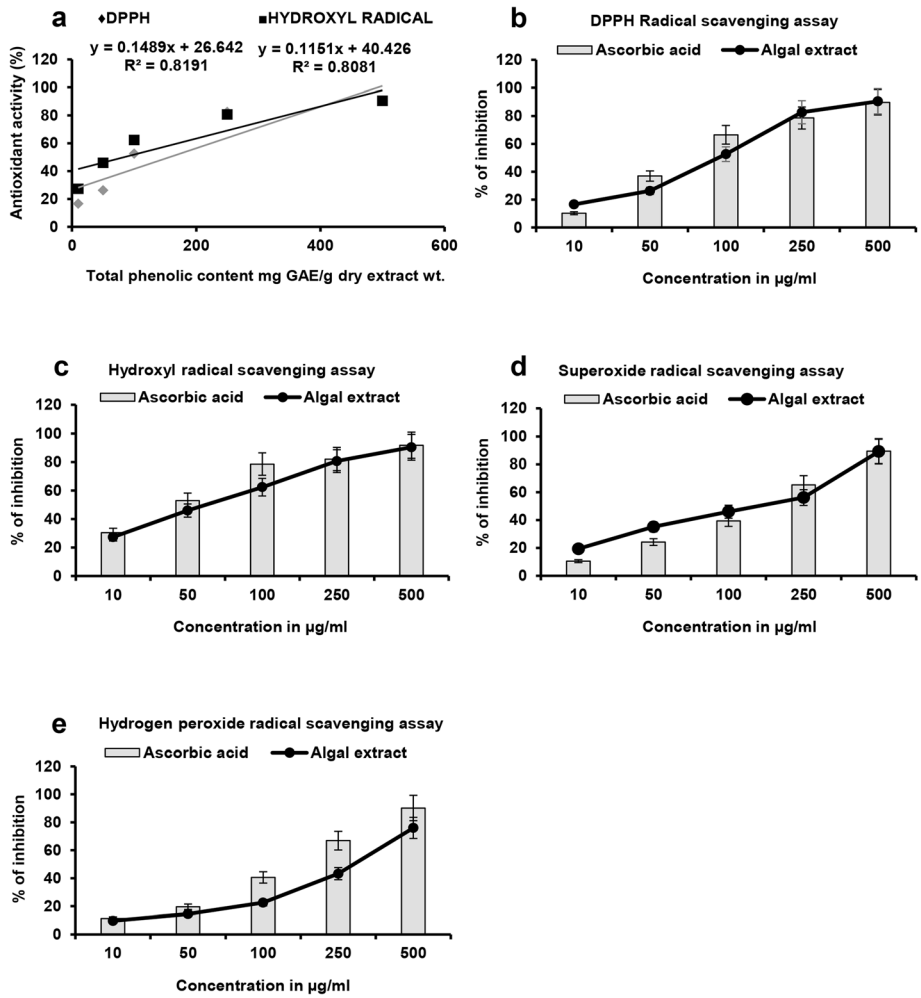

Fig. 2 Correlations between antioxidant capacity and total phenols of *C. vulgaris* extract (**a**). Different radical scavenging activity of methanol extract of the *C. vulgaris* such as DPPH radical scavenging activity (**b**), hydroxyl radical scavenging activity (**c**), superoxide radical scavenging activity (**d**), and hydrogen peroxide radical scavenging activity (**e**)

50 µg/ml: 4.25, 250 µg/ml: 3.39, 500 µg/ml: 2.3) represented in µmol/mg protein (Fig. 3e).

Drug synergism of methanolic extract of *C. vulgaris* in combination with norfloxacin and ciprofloxacin exert enhanced anti-bacterial potency against *E. coli*

To evaluate the enhanced anti-bacterial activity with drug synergism of methanolic extract of C. vulgaris with norfloxacin and ciprofloxacin, we evaluated the cfu/ml and % of cell viability in *E. coli.* In combination with norfloxacin, the cfu/ml was estimated to be 4.969×10^7 as compared to norfloxacin with a value of 7.303×10^7 cfu/ml (Fig. 4a). Similarly, a concurrent reduction of bacterial cell viability was observed in combination with norfloxacin 33.13% as compared to norfloxacin alone 48.69 % (Fig. 4b). Similarly, in combination with ciprofloxacin, the cfu/ml was estimated to be 4.0×10^7 as compared to ciprofloxacin with a value of 6.829×10^7 cfu/ml (Fig. 4c). Similarly, a concurrent reduction of bacterial cell viability was observed in combination with ciprofloxacin 26.69% as compared to ciprofloxacin alone 45.53% (Fig. 4d).

Table 3 Total phenolic, flavonoid, and antioxidant activity of *C. vulgaris*

Parameters	Extract
Total phenolic content (mg of GAE/g)	**45 ± 0.06**
Total flavonoid content (mg of RUE/g)	**470 ± 0.25**
Total antioxidant activity (mg of AAE/g)	**411 ± 0.39**

Discussion

Currently, the potential phytochemicals are given greater attention for the formulation of new pharmaceuticals for possible therapeutic use. These phytochemicals are usually categorized into primary metabolites such as

Table 4 IC_{50} Values of free radical scavenging activities of the methanol extracts of *C. vulgaris*

Parameters	IC_{50} value (μg/ml)	
	Ascorbic acid	*Chlorella* sp.
DPPH scavenging assay	2.68 ± 0.29	2.82 ± 0.30
Hydroxyl radical scavenging assay	1.87 ± 0.16	2.30 ± 0.25
Hydrogen peroxide Radical scavenging assay	3.21 ± 0.38	3.24 ± 0.32
Superoxide radical scavenging assay	3.22 ± 0.03	3.15 ± 0.02

carbohydrates, proteins, lipid, nucleic acid, various pigments, and secondary metabolites such as alkaloids, glycosides, flavonoids, phenols, saponins, tannins, coumarins, and terpenoids. The major of these bioactive compounds were reported in various algal genera [26, 42]. Phenolic and flavonoid compounds of the plant and algae displayed several biological activities and elicited substantial antioxidant activity [26, 29, 42, 43]. The present study on *C. vulgaris* demonstrated the presence of active phytochemicals and exhibited antioxidant properties due to the presence of phenolics, flavonoids, and tannins in the line of findings of the earlier report [42]. The present study results were displayed an abundance of phenolic constituents as well as an increase of free radical scavenging activity in the crude extracts. Consequently, the presence of phenolics was an authoritative antioxidant agent responsible for neutralizing free radicals by contributing hydrogen atoms to free radicals and

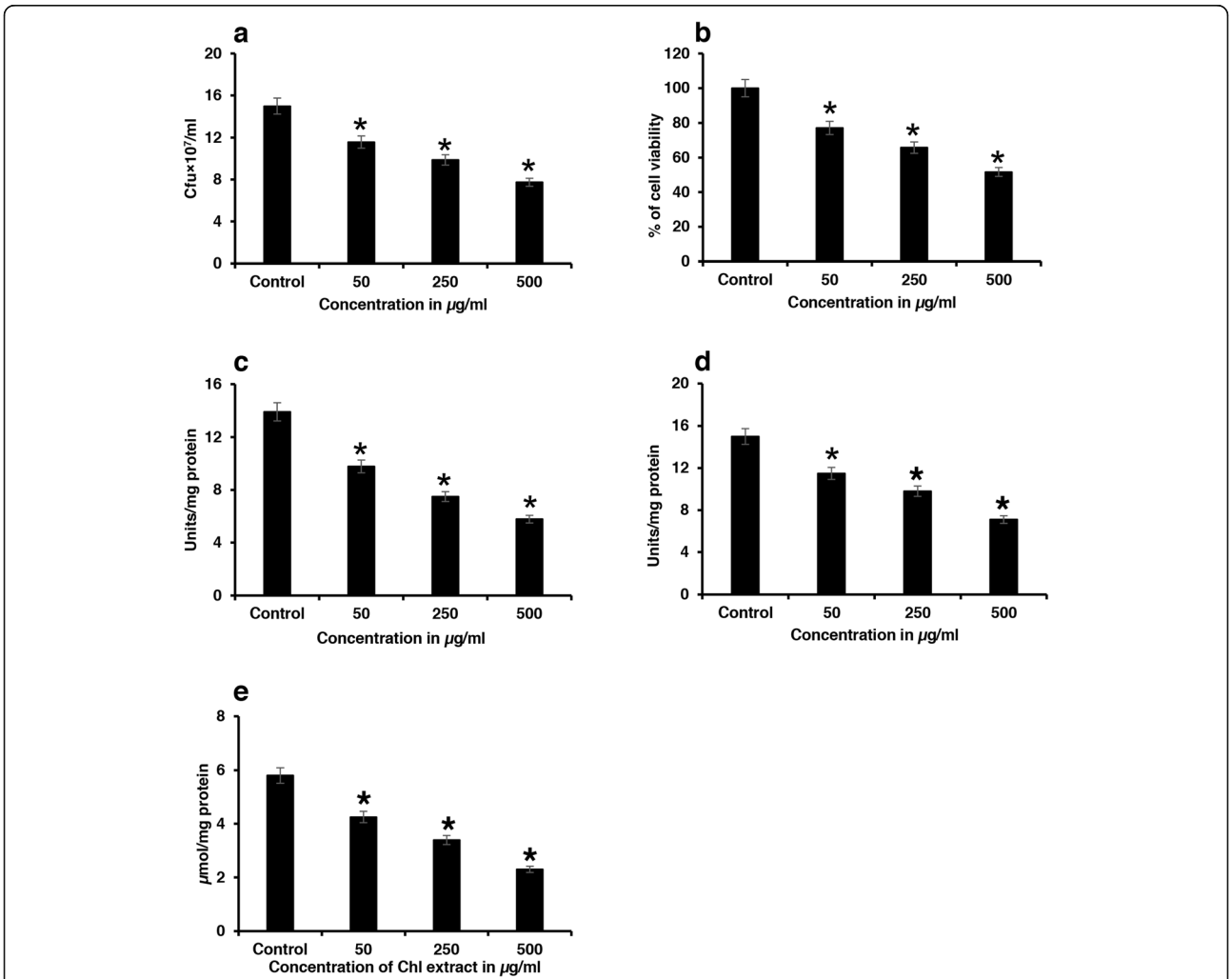

Fig. 3 The cfu/ml count in dose-dependent methanolic extract of *C. vulgaris* (**a**), % of cell viability (**b**). The SOD (**c**), CAT (**d**), and GSH (**e**) activity post-dose-dependent treatment of methanolic extract of the *C. vulgaris*

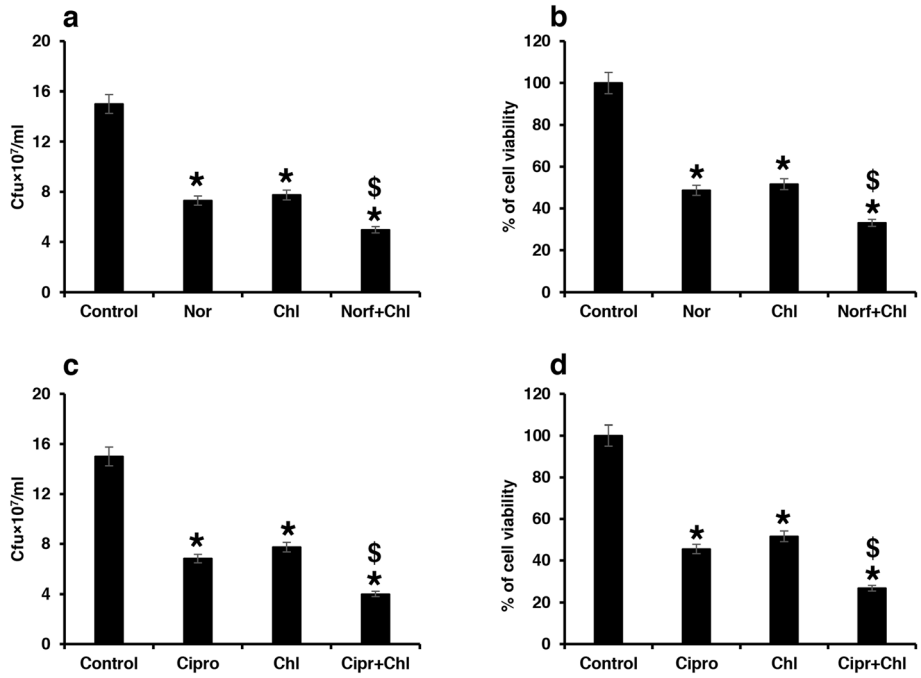

Fig. 4 The cfu/ml count (**a**) and % of cell viability (**b**) in the synergistic effect of methanol extract of the *C. vulgaris* with norfloxacin. A similar cfu/ml count (**c**) and % of cell viability (**d**) in the synergistic effect of methanol extract of the *C. vulgaris* with ciprofloxacin

reducing the reactive oxygen species. So, our findings in line with the previous report that phenolics are scavenging reactive oxygen species and reduce the production of singlet oxygen species and ROS [44]. The phenolic content of *Isochrysis galbana* Parke, *Tetraselmis chuii* Butcher, and *Dunaliella salina* (Dunal) Teodoresco were evaluated with maximum content signifying 17.798 mg GAE g^{-1} [45]. Further, *Euglena cantabrica* E.G. Pringsheim displayed the maximum phenolic compounds 5.87 mg GAE g^{-1} and 2.97 mg protocatechuic acids g^{-1} [46]. The phenolic content was evaluated in *Chlorella* sp. 43 ± 0.05 mg of GAE g^{-1} [26]. The present study signified with high TPC value (45 ± 0.06 mg GAE g^{-1}) compared to the previous report to date in *Chlorella* sp. The TFC value in microalgae *Euglena tuba* was evaluated to be 100.78 ± 2.114 mg g^{-1} extract quercetin equivalent [42] and flavonoids contents in *Chlorella* sp. was 501 ± 0.88 mg rutin g^{-1} in our previous report. However, interestingly in the current investigation elicited profusely, a less TFC, i.e., 470 ± 0.25 mg RUE g^{-1} compared to the previous report [26].

Different literature indicated a linear correlation between TPC and TFC with antioxidant activity [47]. Hence, various methods were used to evaluate the antioxidant activity in this study. The total antioxidant capacity (TAC) was measured based on the reduction of Mo (VI) to Mo (V) followed by later production of green phosphate Mo (V) compound in an acidic pH. The TAC of methanolic crude extract was elicited maximum in this strain *C. vulgaris* due to the presence of maximum TPC and TFC. The present study was in support of the published reports [26]. The UV-visible spectrophotometric spectral range from 200 to 800 nm, and resultant absorbance indicates the presence of phenolics compounds [48]. Therefore, the UV-visible spectroscopic spectral scan and FT-IR spectroscopy spectral scan was performed to cross-check the presence of phenolics and flavonoids in the *C. vulgaris* methanolic extract. Our findings exhibited the absorbance at various nm with signified spectral peak both in UV-visible spectroscopy and FT-IR spectroscopy displayed the presence of different functional groups of the compounds in the extract (Fig. 1a, b). The UV-visible spectral peak indicated the presence of phenolics and flavonoids, FT-IR spectral peak indicated the presence of different bioactive phytochemicals and their functional groups present (Table 2). Our present study results were in line with findings of a previous study in *Padina pavonica* (Linnaeus) extract and seaweed *Sargassum wightii* Greville ex J.Agardh extract [49]. In addition, the investigated results were also supported by spectral peaks obtained in seeds and flower extract that indicated the presence of alkaloids, flavonoids, and glycoside [27]. All these bioactive antioxidant agents hold high prevalence in therapeutic purposes [50]. So, in the present study, the microalga *Chlorella vulgaris* Beyerinck [Beijerinck] was attributed strong antioxidant activities due to the presence of an abundance of phenolic, flavonoids, and other active

phytochemicals which were in the line of findings of *Chlorella* strains antioxidant activities [51].

Furthermore, several biochemical assays have been employed to evaluate the free radicals scavenging capacity of antioxidants. Thus, the DPPH assay was used to estimate the competence of free radicals scavenging activity of active antioxidants present in the algal extracts. The presence of antioxidants in the algal extract reduces the deep violet color DPPH solution to fade yellow color by accepting an electron or hydrogen radical and convert to a diamagnetic stable molecule. Besides the IC_{50} values of the DPPH radical scavenging activity of the methanol extract against the standard ascorbic acid displayed significant inhibition as an increase in concentration confirmed the strong antioxidant activity. This finding is in line with the previous reports [50, 52–54]. The byproducts of immune action hydroxyl radicals can occasionally produce in the cell and are short-lived. However, these radicals are extremely reactive and can spoil various important macromolecules in the cells and are becoming dangerous to human health. Thus, the neutralizing redundant hydroxyl radical in the cell is essential. Hence, the hydroxyl radical scavenging activity of the algal extract was examined, and the result displayed an increase in inhibition with an increase of algal concentration and it was validated by IC_{50} values of the strain. The activity of hydroxyl ion scavenging activity perceived in this was in the line with the previous report [26, 29, 42, 43]. H_2O_2 is usually a fragile oxidizing agent, which can cross biological membranes and engage in the production of hydroxyl radicals. Further, this property has a major role to instigate cytotoxicity effects. Thus, diminishing H_2O_2 in the cells is very important for the protection of living systems [55]. Hence, the scavenging activities of the algal extract in the present study were examined and the scavenging activities displayed in a concentration-dependent manner compared to standard ascorbic acid. It was also observed from their respective IC_{50} values scavenging of H_2O_2 by the algal extract with ascorbic acid. It can be attributed to their phenolics, which can give electrons to H_2O_2 and neutralizing it to water [56]. Superoxide radical is produced by auto-oxidation and non-enzymatic electron transfers to reduce molecular oxygen in the cell. This is pretty toxic, causes oxidative upset in the cells, and may play a role in many oxidative diseases. The antioxidant properties of flavonoids are effective in scavenging the superoxide anion radicals [57]. The superoxide radical scavenging activity of the methanolic extract was measured by decreasing absorbance reflecting the consumption of superoxide radicals [58, 59]. From our result, the IC_{50} values of superoxide radical scavenging assay indicated that the extract possessed strong superoxide radical scavenging activity than the standard ascorbic acid. Our investigations are in the support of published reports [42, 53].

The anti-microbial compounds from algae include structurally devised phytoactive compounds that include phenolic and flavonoid groups. This study exhibited that methanolic extract of *C. vulgaris* was more effective against *E. coli* with the subsequent reduction in cfu/ml and % of bacterial cell viability. The results of the present investigation were well supported with the study of ethanolic extract of *Nostoc calcicola* exhibiting effective anti-bacterial activity against *E. coli* and *Staphylococcus aureus* [60]. The reduction in cell viability is possible due to the onset of oxidative imbalance post-drug treatment. After drug treatment, a subsequent reduction in cell viability was evident due to downregulation of SOD, CAT, and GSH activity which further lead to ROS-mediated cell death. The result of the current finding is in the line of previous study Scott et al. and Schwartz et al. [40, 41]. In the current therapeutic scenario, drug synergism has been implicated as a possible strategy for enhanced therapeutic efficacy. Interestingly, the results of the present investigation have demonstrated that methanolic extract of *C. vulgaris* in combination with norfloxacin and ciprofloxacin exhibited a reduction in cfu/ml and cell viability. The finding of this study is well supported by the previous investigation of Coronarin D in combination with antibiotics exhibit enhanced antimicrobial activity against *E. coli* [61].

Conclusion

Microalgal bioactive compounds act as potent scavengers of intracellular free radicals owing to the presence of phenolic and flavonoids constituents. The present study uncovered the effectiveness of methanolic extract of *Chlorella vulgaris* as a potent antioxidant in vitro. The effective reduction of the DPPH, hydrogen peroxide, hydroxyl, and superoxide radicals established the methanolic extract of *Chlorella vulgaris* as a potent ROS scavenger than standard ascorbic acid. Moreover, it also regulates bacterial diseases via the downregulation of antioxidant enzymes. In addition, it displays enhanced anti-bacterial efficacy in combination with known antibiotics. Further investigation for designing and developing potential ROS scavengers from this species will ensure the production of effective synthetic pharmacophores with enhanced bioavailability and greater efficacy in therapeutic implementations.

Abbreviations

DPPH: 1,1-Diphenyl-2-picrylhydrazyl; TPC: Total phenol content; TFC: Total flavonoid content; TAA: Total antioxidant activity; SOD: Superoxide dismutase; CAT: Catalase; GSH: Reduced glutathione; Cfu: Colony-forming unit; *E.coli*: *Escherichia coli*; *C. vulgaris*: *Chlorella vulgaris*; FT-IR: Fourier transform infrared; Nor: Norfloxacin; Cipro: Ciprofloxacin

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Plant authentication
The pure strain *Chlorella vulgaris* was collected from Sonapur on the sea, Ganjam, Odisha, and isolated by Biswajita Pradhan, authenticated by Dr. Mrutyunjay Jena. The strain was assigned with strain no. BUACC25 and submitted the algal culture collection of Berhampur University.

Authors' contributions
BP, SP, and MJ designed the work. BP, SP, and RN have done the experimental work. BP, SP, SRD, and CB have done data interpretation and graphics. BP, SP, and MJ have drafted the work or substantively revised and proof reading. All authors read and approved the final manuscript.

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