

Symbiotic Synergies: A Comprehensive Analysis of Antibacterial Potency, Antioxidant Proficiency, and Phytochemical Composition in *Rhizoclonium hieroglyphicum* and *Spirogyra* variants from Distinct Aquatic Habitats

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Abstract

Terrestrial plants have been the main source of many active agents used in the pharmaceutical and cosmetic industries. Many research studies have focused on different marine organisms like algae, fish, sponges, and crustaceans to obtain bioactive products like proteins and peptides for use in the pharmaceutical and cosmeceutical industries. In contrast to terrestrial plants, marine algae are a very abundant and important source of different constituents that have established beneficial effects on human skin and have been used in the cosmeceutical industry. Samples of algae were collected from two different aquatic sites. Identification of samples was made based on microscopic morphological and other key characteristics of algae, and they were found to be *R. hieroglyphicum* and *S. varians*. Extraction was carried out in methanol and in a mixture of DCM (dichloromethane) and methanol. Both samples collected from the two different sites showed significant antibacterial effects in various assays performed using the Disc Diffusion Method and the Well Method. The antioxidant activities of the extracts were also detected using a 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging assay. Quantitative and qualitative analyses showed significant quantities of phenolic and flavonoid contents. The stressful climatic factors lead to the synthesis and production of compounds with secondary metabolites, which are anti-stress and important for their antioxidant and therapeutic effects, resulting in various biological activities.

Introduction

A large number of active compounds have been isolated from terrestrial plants and used in the pharmaceutical and cosmetic industries throughout history. During the last four decades, many drugs approved by the FDA were prepared from plant species with medicinal value [1]. But in the last decade, many research studies have also been focused on different marine organisms for the production and isolation of bioactive products like proteins and peptides. Algae are one of the most important and diverse groups of aquatic organisms and have significant physiological and biochemical properties. They have been used as the source of different active compounds, which include carotenoids, steroids, fatty acids, vitamins, proteins, lectins, polysaccharides, dietary minerals, diverse antioxidants, and halogenated compounds. In contrast to traditional plants, algae do not possess true stems, roots, or leaves. Algae exhibit complex taxonomy, and as new molecular information is discovered over time, they are subjected to rapid change [2]. Carbohydrates are one of the most abundant and important constituents of marine algae [3, 4]. The carbohydrates extracted and isolated from marine algae have many applications in pharmaceuticals and cosmetics due to their important physical and chemical characteristics [5, 6]. Shanura et al [7] screened *Gracilaria edulis* and its methanol extract to study their anti-inflammatory properties in contradiction to lipopolysaccharide-induced inflammatory response. Koneri and Jha conducted a study on *G. edulis* and its methanol extract in which they determined their anti-diabetic properties for the treatment of male rats with fructose-induced type 2 diabetes mellitus [8]. Another study was conducted by Patra and Muthuraman [9] on ethanol extracts of *G. edulis*, in which they revealed their anticancer properties in mice for the treatment of ascites tumors. Furthermore, *G. edulis* was screened to study its antioxidant and hypoglycemic properties in different in vitro

mechanisms for the first time in Sri Lanka. The results of the study showed that the evident hypoglycemic activity of *G. edulis* was produced by inhibiting glucose absorption. The antioxidant and hypoglycemic effects were further confirmed by GC-MS analysis [10]. In several Asian countries, seaweed has traditionally been used as a food. Important biologically active compounds like fatty acids, vitamins, polysaccharides, phycobilins, carotenoids, phycocyanins, and tocopherol is present in seaweeds. Seaweed has extensive applications in the food, pharmaceutical, and cosmetic industries [11]. The crude polysaccharides and derivatives obtained from them show various useful bioactivities, including anticoagulant, antitumor, immunomodulatory, and cancer-preventive activities. These also have powerful antibiotic, anti-inflammatory, and antioxidant properties. For instance, strong anticancer and immunomodulating characteristics have been shown by the sulfated polysaccharides (SP) from *Enteromorpha* and *Porphyridium* [12]. Algal species also show antibacterial activity against *Staphylococcus* and *Escherichia coli*. Sodium alginate and fucoidan obtained from *Laminaria japonica* show antibacterial activities against *E. coli* by adhering to bacteria and killing them [13]. The growth of *Staphylococcus aureus* was inhibited by an S-galactan obtained from *Chaetomorpha aerea*, which could not inhibit the growth of *Salmonella enteritidis* but restricted the propagation of *Staphylococcus aureus* [14] While against *S. enteritidis*, a notable inhibitory activity was shown by the carrageenans obtained from some seaweeds [15]. Raposo *et al* [16] reported the antibacterial activities of some species from microalgae and explained that these activities are based on the solvent used for the extraction of polymer. The study hypothesizes that marine algae, specifically *R. hieroglyphicum* and *S. varians*, contain significant bioactive compounds with antibacterial and antioxidant properties, which could be beneficial in these industries. These findings suggest that the stressful climatic conditions experienced by marine algae lead to the synthesis and production of secondary metabolites, which are crucial for their anti-stress, antioxidant, and therapeutic effects. Consequently, these compounds contribute to various beneficial biological activities, highlighting the potential of marine algae as a valuable resource for the development of pharmaceutical and cosmeceutical products.

Materials and Methods

Collection of Material

Algae samples were collected from two locations: Mohli waterfall in Kotli and Lahri Head in Mirpur, Azad Kashmir. The samples were manually gathered and placed in glass bottles which had been thoroughly washed, dried, and preserved with diluted formalin, and was also kept in a cooler with ice. The algae were identified as *R. hieroglyphicum* and *S. varians* through microscopic examination and by observing key characteristics like appearance, color, texture, and smell [17].

Extraction of Algal Sample

After washing and fully drying the algae samples, they were ground into powder using a mortar and pestle. Each sample was split into two equal parts. One part was extracted with methanol, and the other with a mixture of DCM and methanol (1:4). The samples were soaked in these solvents and kept in a

dark place at room temperature for 7 days. After 7 days, the mixtures were filtered, and the process was repeated twice more. After the final filtration, the filtrate was left to evaporate until the solvents were completely gone [18].

Total Flavonoid Content Determination

Total flavonoid contents were measured by the method used by Ordonez et al. (2006) with minor modifications [19]. The stock solution of the algal sample was taken in different concentrations of 10 µL, 7.5 µL, 5 µL, and 2.5 µL in a cuvette. Then, 2.5 ml of distilled, deionized water was added to the cuvette. After that, 180 µL of 5% sodium nitrate (NaNO_3) was added, followed by 180 µL of 10% aluminum chloride (AlCl_3). After one to two minutes, 1200 µL of one molar sodium hydroxide (NaOH) solution was added. The whole solution was mixed and remained at room temperature for about 20 to 30 minutes. The absorbance of the solution was determined by a spectrophotometer at 510 nm wavelength.

Total Phenolic Contents Determination

The total phenolic content of algal extracts was determined by the Folin-Ciocalteu assay method [20]. Different concentrations of the algal extract solution were added in a cuvette and labeled accordingly. Then, in each cuvette, 500 µL of Folin-Ciocalteu reagent and 1400 µL of 7% sodium bicarbonate were added and mixed. After mixing, 980 µL of distilled water was added to all cuvettes and left for 30 minutes to complete the reaction at room temperature. The absorbance was checked at 750 nm wavelength by spectrophotometer. The absorbance of the extract was measured three times, and its mean value was taken. The procedure was repeated for different concentrations of gallic acid used as standards, and their absorbance was measured.

Disk Diffusion Method

In the disk diffusion method, five different strains of bacteria were used: *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Bacillus pumilus*. Nutrient medium was used for bacterial colonization, which was autoclaved along with Petri plates [21]. In the laminar flow hood, all Petri plates were labeled, and autoclaved nutrient agar media were poured when the temperature of the media reached between 40°C and 45°C in petri plates with a desirable strain of bacterial suspension. Rifampicin (a synthetic antibiotic) was used as the standard by soaking in a disk and placing it at the midpoint of the petri dish. All plates were enclosed with parafilm to avoid impurities. The plates were placed in an incubator set at 37°C for 20 to 24 hours [22]. After 24 hours, zones of inhibition in millimeters were measured from the circumference and the area free from bacterial growth around disks [23]. Interpretations were noted three times for each type of bacterial species against each sample, and the mean value was then calculated. The antibiotic effects against selective bacterial strains were plotted in graphs.

Antioxidant Activity

Antioxidant activity was checked by using different quantities of algal extracts in DMSO, in separated and labeled test tubes. Then, 1440 μ L of freshly prepared DPPH solution, prepared by adding 0.012 grams of DPPH to 100 ml of methanol, was added to the plant extract in the test tube. The solution was mixed and left for about 30 minutes to complete the reaction between DPPH and plant extract. In another test tube, 1.5 mL of methanol and 1.4 mL of DPPH were mixed and used as blanks. Absorbance was measured after 30 minutes by a spectrophotometer at 517 nm, and as a positive control, ascorbic acid was used. The IC₅₀ values were then calculated by the method mentioned previously [24]. The scavenging activity was measured using the following formula:

$$\text{Percentage radical scavenging activity} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100$$

Data Statistics and Analysis

All experiments were performed in triplicate to ensure the reliability and accuracy of the results. Statistical analysis was conducted using SPSS software (Version 27). Data were expressed as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the means of different groups, and significant differences between means were determined using Tukey's post-hoc test at a significance level of $p < 0.05$. Graphical representations of the data were created using MS Excel to illustrate the differences in the antioxidant, antibacterial, and total phenolic and flavonoid content between the samples. Additionally, IC₅₀ values for antioxidant activity were calculated using non-linear regression analysis.

Results and Discussion

Sample Collection and Extraction

The samples of algae *R. hieroglyphicum* and *S. varians* were collected from two different sites of Azad Kashmir and extracted in two different solvents, methanol and a mixture of methanol and DCM, for the assessment of phytochemical and biological activities for the first time.

Phytochemical Analysis

Qualitative phytochemical analysis for different chemical compounds was performed on extracts of *S. varians* and *R. hieroglyphicum*. Qualitative analysis of phytochemicals showed that almost similar phytochemicals were detected in all the samples. Among the four different extracts, *R. hieroglyphicum* extracted in the DCM-methanol mixture showed the most phytochemicals. Saponins were not detected in any of the tested samples. Flavonoids were detected in all four extracts with equal intensity. Steroids were detected in both extracts of *R. hieroglyphicum*, but they didn't show their presence in the extracts of *S. varians* in methanol. Glycosides, alkaloids, and tannins also showed their presence in all of the tested

samples, while the tannins were more intensely detected in *R. hieroglyphicum* than in *S. varians*. Results were in concordance with previous reports where *S. varians* and *R. hieroglyphicum* extracts showed significant bioactivities in the form of antimicrobial activity and antioxidant properties, and these activities were related to the versatile group of phytochemicals present in these algal extracts.

Total Phenolic and Flavonoid Content

Following the procedure for the flavonoid and phenolic content of the extracts, plots were obtained against concentration vs. optical density. Flavonoid and phenolic content varied according to the algal species, i.e., *S. varians* or *R. hieroglyphicum*, and to the solvents used for extraction. Total phenolic content in the case of *R. hieroglyphicum* was higher in the DCM methanolic extract (297 mg GAE/g) than in the methanolic extract (191.2 mg GAE/g). Total flavonoid content, on the other hand, was nearly identical in both *R. hieroglyphicum* extracts: 34.59 mg RE/g in DCM: methanolic extract and 33.7 mg RE/g in methanol extract. Similarly, in the case of *S. varians*, total phenolic content was higher in DCM: methanolic extract (183.5 mg GAE/g extract) than in methanolic extract (60.4 mg GAE/g extract). Total flavonoid content, on the other hand, was higher in DCM: methanolic extract (35.38 mg RE/g) as compared to the methanolic extract (24.05 mg RE/g extract).

Antioxidant Activity

The graph of antioxidant activities showed that the dosage of algal extract is correlated with antioxidant activity. In the present study, it was recognized that the methanol extracts of *S. varians* and *R. hieroglyphicum* had favorable antioxidant potential and varied bioactive phytochemicals. It can be utilized as a source of a possible natural antioxidant for numerous determinations. Although many past studies are available on the bioactive chemical composition, antioxidant capacity, medicinal properties, cytotoxicity, etc. of the algae present in different parts of Pakistan, no scientific research still documents the algal species showing bioactive phytochemicals and therapeutic effects from Azad Kashmir as far as our information goes. Recent studies by other researchers have documented a strong positive association between the total phenolic content and antioxidant effects in algal species, which supports our results [25]. The resistance gained by algal cellular and physical compounds against such oxidative contest showed that the intracellular antioxidants are supposed to be responsible for the defensive role as an inherent mechanism for sustenance [26]. Both *S. varians* and *R. hieroglyphicum* contain a number of compounds having antioxidant potential. Antioxidant activities can be measured by different methods but in the present study, DPPH free radical scavenging activity method was used. The antioxidant activity of *S. varians* and *R. hieroglyphicum* was observed and considerable variations were observed between these two species [27, 28]. From the present study, it was recognized that the methanol extract of *S. varians* and *R. hieroglyphicum* had favorable antioxidant prospective and varied bioactive phytochemicals. It can be utilized as a source of a possible natural antioxidant for numerous determinations. Recent studies by other researchers have documented a strong positive association between the total phenolic content and antioxidant effects in algal species that give support to our results [29].

Antibacterial Activities

Regarding the application for bacterial infected skin, all four extracts were tested against five different bacterial strains, two of which are gram-negative strains, i.e., *S. aureus* and *E. coli*, and three gram-positive bacterial strains, i.e., *B. subtilis*, *B. pumilus*, and *P. aeruginosa* [30–32]. The methanolic extract of *S. varians* showed lower antibacterial activity compared to the methanolic extracts of *R. hieroglyphicum*, having a zone of inhibition of 13 mm against both gram-negative bacterial strains, i.e., *E. coli* and *P. aeruginosa*, and gram-positive strains, i.e., *B. subtilis*, *B. pumilus*, and *S. aureus*, with inhibition zones of 11 mm to 14 mm. *S. varians* extracted in a mixture of DCM and methanol shown the highest antibacterial activity against both gram-negative and gram-positive bacteria, with inhibition zones measured at 22 mm against *S. aureus*, 21 mm against *E. coli*, 16 mm against *B. subtilis*, 20 mm against *B. pumilus*, and 22 mm against *P. aeruginosa*. Both extracts of *R. hieroglyphicum*, i.e., methanolic extract (RM) and the one extracted in DCM and methanol (RDCM), showed similar activity against both gram-negative and gram-positive bacteria. The inhibition zone shown by the methanolic extract was measured at 17 mm against *E. coli*, 17.3 mm against *P. aeruginosa*, 16 mm against *S. aureus*, 17.2 mm against *B. pumilus*, and 16.9 mm against *B. subtilis*. Extracts of *S. varians* exhibited good antibacterial activity, but the results were less promising compared to *R. hieroglyphicum* extracts. The sterols extracted from *Chara wallichii* showed activity against various bacterial species [33], indicating that freshwater green algae may also show antibacterial effects. The results indicate that algae displayed antimicrobial activity depending on the solvents used for extraction, methods of extraction, bacterial strains, and culturing conditions.

Importance of Natural Antioxidants

Overall, algae exhibit complex taxonomy and are subjected to rapid change as new molecular information is discovered with time [34]. During the last few years, natural antioxidants have gained importance because oxidative stress production is ultimately a significant factor of disorder [35]. Antioxidants are important for the neutralization of free radicals by various methods and prevent the body from diseases such as aging, liver cirrhosis, cancer, and atherosclerosis, etc. [36]. Both natural and synthetic antioxidants are available in the market. Synthetic antioxidants like butylhydroxyanisole have shown harmful effects nowadays for the human body [37]. The natural antioxidants have gained attention for their minimal harmful effects [38].

Intracellular Antioxidant Mechanisms

The resistance gained by algal cellular and physical compounds against such oxidative contest showed that the intracellular antioxidants are supposed to be responsible for the defensive role as an inherent mechanism for sustenance [38]. Both *S. varians* and *R. hieroglyphicum* contain a number of compounds having antioxidant potential. Antioxidant activities can be measured by different methods but in the present study, DPPH free radical scavenging activity method was used. The antioxidant activity of *S. varians* and *R. hieroglyphicum* was observed and considerable variations were observed between these two species [40, 41]. From the present study, it was recognized that the methanol extract of *S. varians* and *R. hieroglyphicum* had favorable antioxidant prospective and varied bioactive phytochemicals. It can

be utilized as a source of a possible natural antioxidant for numerous determinations. Recent studies by other researchers have documented a strong positive association between the total phenolic content and antioxidant effects in algal species that give support to our results [42].

Our study contributes to the growing body of evidence on the bioactive potential of algae, particularly from regions like Azad Kashmir, which remain underexplored in scientific research. By showcasing the phytochemical, antioxidant, and antibacterial properties of *S. varians* and *R. hieroglyphicum*, we underscore the potential of these algae as sources of natural bioactive compounds for various applications. Future research should aim to isolate and characterize specific phytochemicals responsible for these activities, paving the way for their potential therapeutic and industrial applications.

Conclusion

The antioxidant activities of the algal extracts were reported in this study for the first time. The present study showed excellent antioxidant capacities, strong free radical scavenging capacity, and a significant quantity of total phenolic compounds in the samples, which could be utilized as an effective natural source of antioxidants. Moreover, it has gelling ability, so it could be useful in cosmetic, nutritional, and pharmaceutical products. However, the sulfate content and antioxidant activity relationship need to be investigated further. It is obvious from the current study that the *S. varians* and *R. hieroglyphicum* present in this region are reservoirs of novel bioactive compounds that could be used sustainably as a huge prosperity for the discovery and development of new drugs against various human diseases, especially oxidative stress-induced disorders. However, there is a need to investigate the cytoprotective properties of the algal extract in the cell culture system for proper authentication of the therapeutic and prophylactic potential and in-vitro antioxidant strength of this bio resource.

Declarations

Conflicts of Interest:

The authors declare no conflict of interest.

Author Contribution

Syed Mansoor Ahmed: Conceptualization, Data analysis, Writing-Reviewing & Editing. Hajra Hameed: Experimental design, Sample collection and identification, Writing-Original draft, Writing-Reviewing & Editing. Muhammad Tariq: Supervision, Writing-Reviewing & Editing. Afsa Hameed: Antibacterial and antioxidant assays, Data analysis, Writing-Original draft. Muhammad Shakeeb Sharif: Phytochemical analyses, Data visualization, Writing-Original draft. Dunia A Al Farraj: Funding, Writing-Reviewing & Editing. Reem A. Aljeidi: Project supervision, Resources. Afshan Afareen :Literature review, Background

research, Methodology, Writing-Original draft. Abdul Waheed: Experimental procedures, Phytochemical characterization, Statistical analysis, Writing-Original draft, Writing-Reviewing & Editing.

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Data Availability

The datasets underpinning the findings presented in this article are accessible upon request. Please feel free to reach out to the corresponding author for further information regarding data availability and access procedures.

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Tables

Table 1

Phytochemicals presence in the extracts of *S. varians* and *R. hieroglyphicum* are expressed as ++ detected strongly; + detected; - not detected. Whereas; SM: Spirogyra sample extracted in methanol; SDCM: Spirogyra sample extracted in methanol and dichloromethane mixture; RM: Rhizoclonium sample extracted in methanol; RDCM: Rhizoclonium sample extracted in methanol and dichloromethane mixture

	Samples	SM	SDCM	RM	RDCM
Total Phenolic Content (TPC)	Linear Regression equation	$Y = 0.0273x + 0.177$			
	Mean Absorbance of Sample Solution (y)	0.342 ± 0.007	0.678 ± 0.011	0.699 ± 0.003	0.990 ± 0.007
	Total Phenolic Content (TPC) mgGAE/g	60.4	183.5	191.2	297
Total Flavonoid Content (TFC)	Linear Regression equation	$y = 0.0503x - 0.019$			
	Mean Absorbance of Sample Solution (y)	0.102 ± 0.010	0.159 ± 0.006	0.152 ± 0.009	0.155 ± 0.004
	Total Flavonoid Content (TFC) mgRE/g	24.05	35.38	33.7	34.59
Phytochemicals	Alkaloids	++	+	++	+
	Glycosides	+	+	+	+
	Flavonoids	++	+	++	++
	Saponins	-	-	-	-
	Tannins	+	+	++	++
	Steroids	+	-	++	+
	Terpenoids	+	-	+	+

Figures

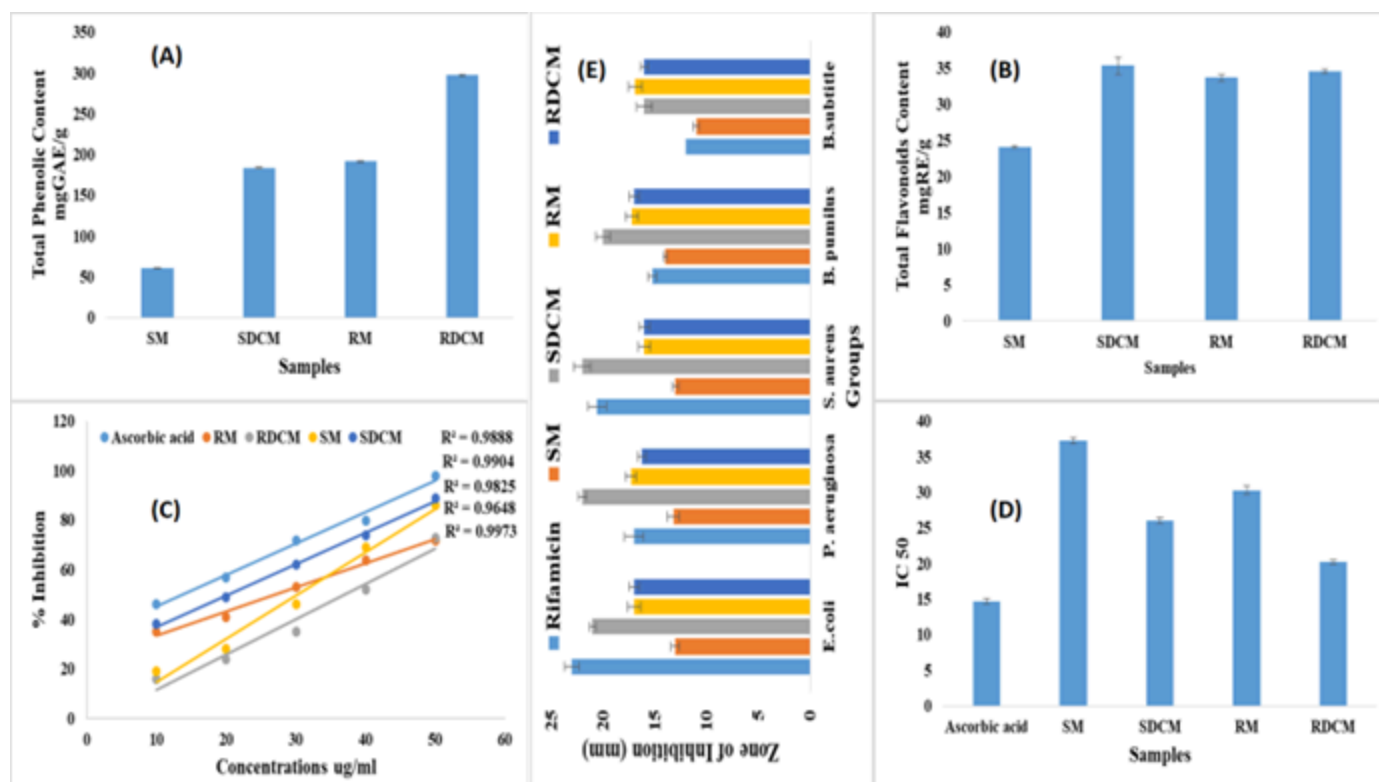


Figure 1

(A) Total phenolic contents of the samples determined by the Folin-Ciocalteu assay using Gallic acid as a standard; (B) Total flavonoid contents of the samples in mgRE/g. (C) Antioxidant activity (DPPH radical scavenging activity) of algal extracts and standard ascorbic acid Each data point represents the mean of three experiments with a standard error of the mean (mean $\pm$ SEM, $n = 3$). (D) Half the maximal inhibitory concentration (IC₅₀) of algal extracts. (E) The antibacterial activity of the positive control, SM, SDCM, RM, and RDCM was evaluated by the disc diffusion method. The results show the zones of inhibition of different samples against model Gram-positive and Gram-negative bacterial strains. All the activities were evaluated three times. All values are given as mean $\pm$ SEM.