Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-021-01509-7

ORIGINAL ARTICLE

Enhancement of active ingredients and biological activities of *Nostoc linckia* biomass cultivated under modified BG-11_0 medium composition

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Received: 26 February 2021 / Revised: 6 April 2021 / Accepted: 9 April 2021

Abstract

The current work aims to investigate the effect of abiotic stresses (nitrogen (N) and sulfur (S) [0.0 g/l, 1.5 g/l, 3 g/l, 6 g/l, and 12 g/l N and 0.0 g/l, 0.07 g/l, 0.15 g/l, 0.3 g/l, and 0.6 g/l S] and their combination [0.3 g/l S + 6 g/l N]) of axenic culture of *Nostoc linckia* on the production of secondary metabolites which induce different biological activities. Growth rate was measured by dry weight (DW) and optical density $(OD)_{550\ nm}$. Additionally, phytochemical compounds, defense enzymes as well as antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radical assays of crude extracts (methylene chloride:methanol, 1:1) were evaluated. Based on antioxidant activity, four promising extracts were subjected to different biological activities such as anticancer, antimicrobial, and antiviral activities. The obtained results revealed that supplementation of external nitrogen source in the form of sodium nitrate was found to increase the total phycobiliprotein content by fivefold. Also, nitrogen depletion provoked significantly highest quantities of phenolic and flavonoid content and this has effects on biological activities of *Nostoc linckia*. Moreover, 0.3 g/l S was found to be the most effective extract exhibiting a significant increase in antioxidant activity based on DPPH and ABTS assays, respectively (88.18 ± 0.64% and 84.20 ± 1.01%). Furthermore, it recorded anticancer activity against HCT 116 cell line with IC_{50} of 155 μg/ml. Moreover, this extract possessed a noticeable antibacterial potency (21.0 ± 1.0 as mm inhibition zone against *Staphylococcus aureus* and 19.3 ± 0.6 against *Streptococcus mutans*). In addition, its antiviral activity against H_5N_1 virus as a percentage of inhibition was 50% and 63.6% at a concentration of 7 μg/ml and 28 μg/ml, respectively, with cytotoxicity less than 7 μg/μl. GC-MS analysis recorded the presence of bioactive compounds exhibiting different biological activities. Therefore, the obtained results can represent valuable bioactive compounds with variable biological potencies.

Keywords Cyanobacteria · *Nostoc linckia* · Phytochemicals · Antioxidant · Anticancer · Antiviral · Defense enzymes · GC-MS analysis

1 Introduction

Cyanobacteria are a wide group including approximately 150 genera and 2000 species [1, 2]. A large heterogeneity among the genera provides the opportunity for the production of different secondary metabolites with applications in many aspects of people's lives. These applications vary from biofuels [3, 4], nutrition [5, 6], and biofertilizers [7] to cosmeceuticals and pharmaceuticals [8].

The potential of cyanobacteria as novel sources of valuable chemicals and other products is gaining interest worldwide. In the last few decades, microalgae have become the focus for extensive screening of novel compounds with interesting biological activities [9–12], in addition to the pharmacologically active algal secondary metabolites such as phenolics, terpenes, and carotenoids that are associated with their biological activities as antioxidant, anticancer, biofuel, and antiviral against influenza virus and could be promising as anti-coronavirus (SARS-CoV-2) as reported by Shalaby et al. [13], Carpine and Sieber [14], and Priya and Ramesh [15].

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Published online: 18 April 2021

Stress involves the disruption of homeostasis as a result of a stressor application. The stress responses are the changes in the cell metabolism as the cells acclimatize and attempt to restore the homeostasis. The different stages in stress responses are alarming stage, regulation, acclimation, and adaptation [16]. Stress strategies have been adopted to enhance high-value compound production using single stress factor such as the nutritional factors (e.g., carbon source, nitrogen, phosphorus) and environmental factors (high light intensities, temperature, pH, salinity, reactor configurations, and operating conditions) [17–19].

Nitrogen and sulfur represent, for all algal cells, essential macronutrients, so their deficiency triggers a variable range of metabolic responses in algae and higher plants. However, cyanobacteria are considered as a valuable support for investigating the effects of nutrient limitation due to their rapid growth, high reproduction rate, and simple culture system of experimentation.

Sulfur represents an essential macronutrient that is needed for growth and survival of all microalgae and cyanobacteria. Living organisms that depend on sulfur for synthesis and modification of different biomolecules including iron-sulfur proteins (ferredoxins), protein, and the cofactors thiamine, biotin, thiouridine, and lipoic acid [20]. Sulfur deficiency induces an imbalance between nitrogen and carbon. The dramatic reduction in phosphoenolpyruvate carboxylase activity proposed that carbon was diverted away from anaplerosis and possibly channeled into C3 metabolism [21]. Moreover, as a macroelement, N is very important mineral for microalgal metabolism and its deprivation is compensated by radical changes in the metabolic pathways. As a response to the increased ROS level, microalgae may accumulate antioxidant compounds such as polyphenols, flavonoids, and carotenoids to quench the free radicals [22, 23].

There is no evidence of how the manipulation of the growth conditions influences the phytochemical and biological activities. Hence, the current study focused on the effect of N and S concentrations and their combination on the phytochemical (phenolic, flavonoid, tannin, and proline) content as well as defense enzymes and pharmacological (antioxidant, anticancer, antimicrobial, and antiviral) activities of *Nostoc linckia*. Moreover, the chemical composition of promising extract (0.3 g/l S) was analyzed using gas chromatography-mass spectrometry (GC-MS) analysis.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals and reagents

Pure hexane, chloroform, ethanol, ether, acetone, methanol, and methylene chloride were purchased from E. Merck Co. (Darmstadt, Germany). Sulforhodamine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2′-azino-bis(ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid and other materials were of the highest available commercial grade. Gallic acid, butylated hydroxytoluene (BHT), rutin, tannic acid, L-proline, and vitamin C were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.2 Algal cultivation

The blue green microalga *Nostoc linckia* used in this study was kindly isolated in Phycology Lab. and identified by Dr. Sanaa Shanab, Professor of Phycology in the Department of Botany and Microbiology, Faculty of Science, Cairo University, according to Prescott [24].

In brief, alga was isolated from the soil at both sides of water stream of Helwan hot water spring in 2005. The ground air-dried soil was sieved (mesh no. 30). One gram of the soil was mixed with 99 ml of distilled water and shaken for 2 h, and 1 ml of the soil water extract was inoculated on autoclaved solid agar basal bold medium and incubated at 25 °C (37.5 μmol-1m2 sec-1, 16/8 L/D cycles).

The separated algal colonies were examined by a light microscope, and the process was repeated several times till the isolated spot was unialgal. Transfer to liquid nutritive medium, purified from bacteria by a mixture of antibiotics (for this study), was performed to obtain an axenic culture ready to be used in experiments according to the method described by Andersen [25].

Trichomes of *Nostoc linckia* (Roth) Bornet and Flah were characterized by firm outer tegument of homogenous mucilage that encloses numerous coiled threads of bead-like cells with intercalary heterocysts and terminal akinetes. It occurs as minute blue balls in soft water lakes or on the downstream side of rocks or on damp soils.

The alga was maintained in standard conditions at 25 ± 1 °C in BG-11_0 medium, under fluorescent white light (Philips, TLD18W/54-765) of intensity of 40 μmol/m^2/s and light duration of 16/8 L/D cycles, with constant bubbling of air (filtered through a 0.22-μm microporous filter) as reported by Elfayoumy et al. [10].

2.1.3 Antioxidant enzyme kits

Catalase enzyme (biodiagnostic) It is an antioxidant enzyme used against H_2O_2 which causes intracellular damage.

The kit include the following: (1) phosphate buffer of pH 7 (100 mM/l), (2) H_2O_2 substrate as standard (500 mM/l), (3) chromogen inhibitor, and (4) enzyme peroxidase (>2000/l) and 4-aminoantipyrine preservative (2 mM/l).

Glutathione *S*-transferase (biodiagnostic) They are multifunctional enzymes which play a key role in cellular detoxification. The enzymes protect cells against toxicants by conjugating them to glutathione and neutralizing their electrophilic sites.

Glutathione *S*-transferase (GST) kit measures total GST activity by recording the absorbance at 340 nm.

2.1.4 Lipid peroxidation (malondialdehyde) (biodiagnostic)

Thiobarbituric acid (TBA) react with malondialdehyde (MDA) in acidic medium at 95 °C for 30 min to form thiobarbituric acid–reactive product determined spectrophotometrically by absorbance at 534 nm (of the pink product).

The kit includes the following: (1) standard (10 nmol/ml) and (2) thiobarbituric acid detergent stabilizer (25 mmol/l).

2.1.5 Cell line cultures (for anticancer activity)

A human lung cancer cell line (A549) was propagated in Dulbecco's modified Eagle's medium (DMEM) high-glucose medium (DMEM High Glucose w/ stable Glutamine w/ Sodium Pyruvate, Biowest), and human breast adenocarcinoma (MCF-7), human colorectal carcinoma (HCT 116), and human cervical cancer (HeLa) were propagated in RPMI 1640 L-glutamine medium (Lonza Verviers SPRL, Belgium, cat# 12-604F); both media were supplemented with 10% fetal bovine serum (FBS) (Seralab, UK, cat# EU-000-H) and 1% antibiotic (Antibiotic antimycotic, Biowest, cat#L0010). The cells were incubated in 5% CO_2 humidified at 37 °C for growth. The cell lines were obtained and propagated in the center for genetic engineering (Faculty of Medicine, Al Azhar University, Egypt).

2.1.6 Antimicrobial activity

Bacterial and fungal cultures All experiments were conducted at Biochemistry Central Laboratory, Department of Chemistry, Faculty of Science, Cairo University, Giza, Egypt.

For determination of antimicrobial activity of different algal extracts, it was tested in vitro against G+ve and G−ve bacterial strains using agar well diffusion method. The Gram-positive bacterial strains (*Staphylococcus aureus* [ATCC: 6538] and *Streptococcus mutans* [ATCC: 25175]) and the Gram-negative strains (*Escherichia coli* [ATCC: 9637] and *Klebsiella pneumoniae* [ATCC: 10031]) were cultured using the Luria-Bertani agar medium and incubated at 37 °C for 24 h.

Fungal strain used in this study was *Aspergillus niger* (ATCC: 32856) and the yeast, *Candida albicans* (ATCC: 10231). Fungal species were cultured on the Sabouraud Dextrose Broth medium and incubated at 27 °C for 7 days.

2.1.7 Antiviral activity (cell line cultures)

For MTT (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) cytotoxicity assay (TC_{50}) modified Eagle's medium (DMEM), Madin-Darby canine kidney (MDCK) cells by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. For the determination of antiviral activity of different algal extracts, it was tested in vitro against A/CHICKEN/M7217B/1/2013 (H_5N_1) virus by plaque reduction assay and virus was diluted to give 10^4 PFU/well. The cell lines were obtained and propagated in Virology Laboratory, National Research Center (NRC), Giza, Egypt.

2.2 Methods

2.2.1 Modification of the chemical composition of the culture medium

Studying the effect of varying nutritive elements provided by the culture medium ($BG\text{-}11_0$), increasing or decreasing of certain element concentration was performed (as single-element stress). Nitrogen and sulfur concentrations used were 0 g/l, 1.5 g/l, 3 g/l, 6 g/l, and 12 g/l for nitrogen and 0 g/l, 0.07 g/l, 0.15 g/l, 0.3 g/l, and 0.6 g/l for sulfur, respectively.

A combination of the two major nutrients used was at a concentration of 6 g/l for nitrogen and 0.3 g/l for sulfur (as double-element stress).

2.2.2 Determination of algal growth rates

Growth of *Nostoc linckia* culture (free from bacteria) was determined by measuring the optical density at 550 nm at 5-day intervals though the incubation period of 30 days at the different culture conditions.

2.2.3 Determination of algal dry weight

Algal dry biomass was determined at 5-day intervals of the incubation period (using 20 ml algal suspension), filtration, washing, and drying at 105 °C for 24 h according to the method described by Talukdar [26].

2.2.4 Extraction and determination of water-soluble pigments (phycobiliproteins)

The quantification of total phycobiliproteins was made spectrophotometrically and expressed in mg/g dry cell weight basis using the following equations [27].

$$\text{Phycocyanin (PC)} = (A_{615} - (0.474 \times A_{652}))/5.34$$

$$\text{Allophycocyanin (APC)} = (A_{652} - (0.208 \times A_{615}))/5.09$$

$$\text{Phycoerythrin (PE)} = (A_{562} - (2.41 \times \text{PC}) - (0.849 \times \text{APC}))/9.62$$

$$\text{Total phycobiliproteins} = \text{PC} + \text{PE} + \text{APC}$$

where A_{615}, A_{652}, and A_{562} were the absorbance at 615 nm, 652 nm, and 562 nm, respectively. The crude extract was prepared by suspending the biomass in potassium phosphate buffer of pH 7 and ultrasonic cell disruption, followed by the clarification of the extract by high-speed centrifugation.

2.2.5 Extraction of algal biomass

The dried algal biomass was extracted three times with an organic solvent mixture of methanol and methylene chloride (1:1) for 40 min, followed by centrifugation, filtration, and evaporation of solvents using a rotary evaporator at 40–50 °C. The obtained crude extract was expressed as a percentage of the dried biomass weight used (mg extract/g dry biomass weight).

2.2.6 Determination of total phenolic contents

Phenolic contents in the crude extract was determined according to the method of Taga et al. [28] and expressed as gallic acid equivalent/gram (GAE/g) of the alga.

2.2.7 Determination of flavonoid contents

Flavonoid content in the algal extract was determined by the spectrophotometric method recorded by Quettier et al. [29] and expressed in terms of rutin equivalent (mg of Ru/g of extract)

2.2.8 Determination of tannins

Using vanillin hydrochloride method of Broadhurst and Jones [30], the amount of tannic acid in the sample was recorded from the standard curve and expressed as tannic equivalents.

2.2.9 Total protein determination

Extraction of protein was performed according the modified method of Rausch [31] and total protein concentration was determined using the method of Lowry et al. [32], and absorbance was recorded at 720 nm. Total protein was determined using the calibration curve of BSA.

2.2.10 Determination of antioxidant enzymes and lipid peroxidation

1. Catalase estimation

Algal homogenate + phosphate buffer + H_2O_2 at 25 °C was incubated for 1 min. Then, after incubation of chromogen inhibitor and enzyme peroxidase and preservative for 10 min at 37 °C, the absorbance of the sample against sample blank and the standard against standard blank was recorded using methods of [33]) and Fossati et al. [34] and the catalase activity was calculated as U/g = $A_{\text{standard}} - A_{\text{sample}}/A_{\text{standard}} \times 1$/g biomass used.

2. Glutathione *S*-transferase

Algal homogenate and phosphate buffer at pH 7.4 + reduced glutathione (GSH) was incubated at 37 °C for 5 min. Then, CDNB was added and incubated at 37 °C for 5 min and finally mixed with TCA and centrifuged at 3000 rpm for 5 min. The absorbance of the sample (A_{sample}) against blank at 340 nm was recorded, and the activity was calculated as U/g biomass = $A_{\text{sample}} \times 2.812$/g biomass sample according to the method reported by Habig et al. [35].

3. Lipid peroxidation (MDA determination method)

Algal homogenate + phosphate buffer (pH 7.5) was centrifuged at 4000 rpm for 15 min. Supernatant + chromogen was heated in boiling water bath for 30 min and allowed to cool, and the absorbance of the algal sample (A_{sample}) against blank and the standard against distilled water was determined to be at 354 nm.

MDA in algal sample (nmol/g algal biomass) = $A_{\text{sample}}/A_{\text{standard}} \times 10$/g tissue used. According the method described by Ohkawa et al. [36].

2.2.11 Biological activities of algal extract

For determination of biological activities of the crude extract produced from *Nostoc linckia* cultured in BG-11_0 growth medium (control) and altered (stressed) medium BG-11_0 with modified contents of nitrogen and sulfur, single or in combination, the following activities were performed:

A. Antioxidant activity

1. DPPH radical scavenging activity

The scavenging effects of crude methanol:methylene chloride (1:1) extract were determined by the method of Yen and Chen [37], where 2.0 ml of 0.16 mM DPPH solution (in methanol) was added to a test tube containing a 2.0-ml aliquot of the sample. The mixture was vortexed for 1 min and kept at room temperature for 30 min in the dark. The absorbance of all the sample solutions and BHT as synthetic standard were measured at 517 nm. The percentage (%) of scavenging activity was calculated as follows:

% Antioxidant activity = (Control − Sample × 100) / Control

where control in DPPH solution is 0.16 mM.

2. ABTS radical cation scavenging assay

This assay was based on the ability of different substances to scavenge $ABTS^{\cdot+}$ radical cation in comparison to a standard (BHT). The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 4–16 h until the reaction was completed and the absorbance was stable. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm for measurements according to Re et al. [38]. The photometric assay was conducted on 0.9 ml of $ABTS^{\cdot+}$ and 0.1 ml of tested samples and mixed for 45 s, and measurements were taken at 734 nm after 1 min. The antioxidant activity of the tested samples was calculated by determining the decrease in absorbance at different concentrations by using the following equation: $E = ((A_c - A_t) / A_c) \times 100$, where A_t and A_c are the absorbance of tested samples and $ABTS^{\cdot+}$, respectively.

B. Anticancer activity

1. Evaluation of cell proliferation by MTT assay

The cytotoxic effect of the crude extracts and fractions of *Nostoc linckia* on different cancer cell lines was evaluated by the MTT assay as reported previously with slight modification [39]. In brief, after evaluation of cell count and viability by Trypan blue dye, cancer cells (1×10^4 cells/well) were seeded in a 96-well plate in triplicate and were allowed to adhere for 24 h.

The crude extracts were weighted and dissolved in 1 ml dimethylsulfoxide (DMSO) to have a stock solution of 2000 μg/ml, as the final concentration of DMSO in the culture medium never exceeded 0.2% (v/v) [40] and then various concentrations of crude extracts were prepared by further diluting in complete medium to have a final concentration of 31.5 μg/ml, 62.5 μg/ml, 125 μg/ml, 250 μg/ml, and 500 μg/ml. In the next day, the medium was replaced with fresh medium with the indicated concentrations of tested compounds and cells were allowed to grow for 48 h. Four hours before completion of incubation, 10 μl of MTT (5 mg/ml in phosphate-buffered saline (PBS) w/o Ca and Mg; Lonza Verviers SPRL, Belgium, cat# 17-516F) was added in each well. After completing the incubation, 100 μl of DMSO was added to each well, and then the 96-well plates were centrifuged for 5 min at 4000 rpm to precipitate the formazan crystals. Color developed after the reaction was measured at 490 nm using a BioTek microplate reader.

The experiment was conducted in triplicate, and data were calculated as percent of cell viability by the following formula: % Cell viability = (Mean absorbance in test wells / Mean absorbance in control wells) × 100.

III. Antimicrobial assay

To investigate the antimicrobial activity of secondary metabolites produced under normal and stressed culture conditions of the selected alga for this study (*Nostoc linckia*), the agar well diffusion method [41] was used. Algal extracts were tested in vitro for their antibacterial activity against *Staphylococcus aureus* and *Streptococcus mutans* (Gram-positive bacteria), *Escherichia coli* and *Klebsiella pneumoniae* (Gram-negative bacteria) using the Luria-Bertani agar medium [42], and fungal strains (*Aspergillus niger* and *Candida albicans*) cultured and maintained on the Sabouraud dextrose medium [43]. Ampicillin (10 μg) and gentamicin (120 μg) were used as standard drugs for Gram-positive and Gram-negative bacteria, respectively, while nystatin (100 units) was used as standard antifungal activity agent (positive control) and DMSO was used as negative control. The extracts were tested at a concentration of 1.5 mg/ml against both bacterial strains. The activity was expressed as the diameter of inhibition zones in mm.

IV. Antiviral activity

1. MTT cytotoxicity assay (TC_{50})

Samples were diluted with DMEM. Stock solutions of the algal extracts were prepared in 10% DMSO in double-distilled H_2O. The cytotoxic activity of the extracts was tested in MDCK cells by using the MTT method [44] with minor modification. Briefly, the cells were seeded in 96-well plates (100 μl/well at a density of 3×10^5 cells/ml) and incubated for 24 h at 37 °C in 5% CO_2. After 24 h, cells were treated with various concentrations of the tested extracts in triplicates. After further 24 h, the supernatant was discarded and cell monolayers were washed with sterile PBS 3 times and MTT solution (20 μl of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for 4 h, followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 μl of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCl in 50 ml isopropanol). Absorbance of formazan solutions was measured at λ_{max} 540 nm with 620 nm as a reference wavelength using a multiwell plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation. The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (TC_{50}).

% Cytotoxicity = (Absorbance of cells without treatment − Absorbance of cells with treatment) × 100 / Absorbance of cells without treatment

2. Plaque reduction assay

Assay was carried out according to the method of Hayden et al. [45] in a six-well plate where MDCK cells (10^5 cells/ml) were cultivated for 24 h at 37 °C. A/CHICKEN/M7217B/1/2013 (H_5N_1) virus was diluted to give 10^4 PFU/well, mixed with the safe concentration of the algal extracts, and incubated for 1 h at 37 °C before being added to the cells. Growth medium was removed from the cell culture plates, and the cells were inoculated with 100 μl/well virus with the tested extracts, After 1 h of contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose and the tested extracts was added onto the cell monolayer, and plates were left to solidify and incubated at 37 °C till formation of viral plaques (3 to 4 days). Formalin (10%) was added for 2 h, then plates were stained with 0.1% crystal violet in distilled water. Control wells were included where untreated virus was incubated with MDCK cells, and finally, plaques were counted and percentage reduction in plaque formation in comparison to control wells was recorded as follows:

% Inhibition = Viral count (untreated) − Viral count (treated) / Viral count (untreated) × 100

2.2.12 GC-MS analysis

The chemical composition was determined using a Trace GC1300-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column (TG-5MS, 30 m × 0.25 mm × 0.25 μm film thickness). The column oven temperature was initially held at 60 °C and then increased by 5 °C/min to 200 °C, hold for 2 min, increased to the final temperature of 300 °C by 20 °C/min, and hold for 2 min. The injector and MS transfer line temperatures were kept at 250 °C and 260 °C, respectively. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min, and diluted samples of 1 μl were injected automatically using an AS1300 autosampler coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50–650 in full scan mode. The ion source temperature was set at 250 °C. The components were identified by comparison of their retention times and mass spectra with those of Wiley 09 and NIST 11 mass spectral database.

2.2.13 Statistical analysis

All the data are expressed as mean ± standard deviation of three determinations. Statistical comparison was performed via a one-way analysis of variance followed by Duncan's multiple range test (DMRT). P values of less than 0.05 ($P < 0.05$) were considered as significant.

3 Results and discussion

3.1 Effect of culture media composition (nitrogen and sulfur) on the growth of *Nostoc linckia*

The data in Fig. 1 shows the effect of different nitrogen concentrations (0.0 g/l, 1.5 g/l, 3 g/l, 6 g/l, and 12 g/l nitrogen), sulfur concentrations (0.0 g/l, 0.07 g/l, 0.15 g/l, 0.3 g/l, and 0.6 g/l sulfur), and a combination between 0.3 g/l sulfur and 6 g/l nitrogen on the growth of *N. linckia* during the cultivation period of 30 days on BG-11_0 medium. The results presented in Fig. 1a and b show a significant increment of growth (OD and dry weight (DW)) with elevation of nitrogen concentration and time of incubation especially at 1.5 g/l and 3 g/l nitrogen concentrations, while higher doses of nitrogen of 6 g/l and 12 g/l induced significantly marked retardation of growth during all the cultivation periods. Maximum growth rate of *N. linckia* was achieved by 3 g/l nitrate at the 25th day of cultivation (0.83 ± 0.008 and 0.88 ± 0.009 representing DW and OD, respectively) as nitrogen availability in optimum concentration is involved in different vital processes and metabolic pathways. Also, nitrogen is one of the most important elements for algal growth as it is a key constituent of amino acids, proteins, RNA, and DNA in all algal species.

In contrast, elevated nitrogen concentration (6 g/l and 12 g/l) induced an obvious, gradual and significant retardation in growth parameters during the incubation periods and alga stopped completely to grow on day 15 of cultivation. These inhibitory effects of higher nitrogen concentrations were due to its toxic influence to all vital processes and led to death of the alga.

This alga like all the heterocystous cyanobacteria is able to fix atmospheric nitrogen to satisfy its requirements of nitrogenous substances to grow and undergo primary and secondary metabolism.

Also, nitrogen fixation takes place by the activity of metalloprotein complex (known as nitrogenase enzyme) cofactor by seven metals (Fe, Mo, Ni, Mn, V, Cu, and Zn) and occurred in the absence of O_2 (or in microaerobic conditions) in the dark or even in the light. The addition of nitrogen source in the culture media of this alga induces inhibition of nitrogenase enzyme synthesis.

These results were in agreement with the results obtained by Solovchenko et al. [46]; Koksharova et al. [47] indicated that algal biomass productivity and its photochemical efficiency in addition to proteins and DNA repair enzymes are directly proportional to the nitrogen concentration.

The effect of sulfur concentration on the growth rate of *N. linckia* and sulfur concentrations (0.00 g/l, 0.075 g/l, 0.15 g/l, 0.3 g/l, and 0.6 g/l) were investigated. The data present in Fig. 1c and d show that, at 0.0 g/l sulfur (starvation), algal growth was slow, reached its maximum on the 15th day of cultivation, then started to decrease and stop completely after

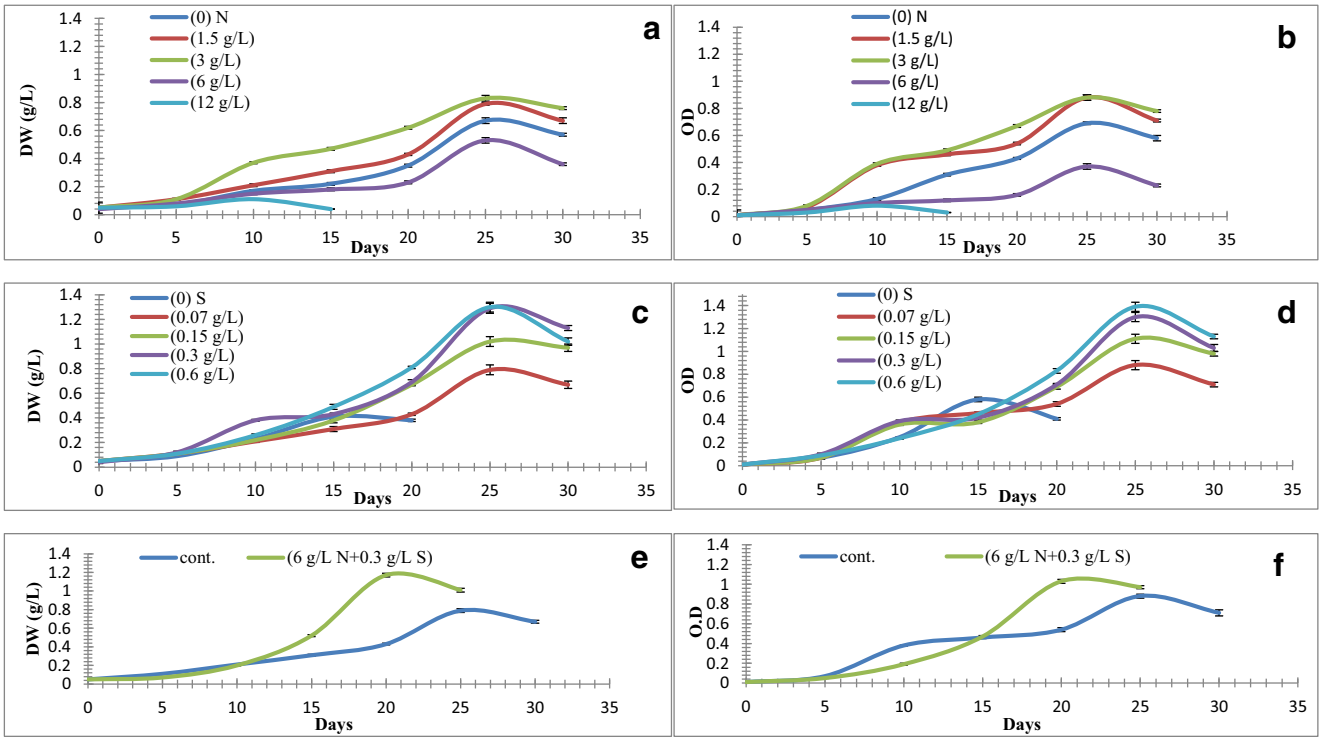

Fig. 1 **a**–**f** Growth curves of *N. linckia* (DW (g/l) and OD) during 30 days of cultivation under different nitrogen and sulfur concentrations and their combination

the 20th day of incubation. The obtained results coincided with the results reported by Zalutskaya et al. [48] on *Chlamydomonas reinhardtii*, where any stimulus (as sulfur starvation) causes the cytochrome-pathway complex dysfunction in mitochondrial electron transport chains, which led to the induction and expression of alternative oxidase 1 and 2 genes. Moreover, under sulfur starvation condition, photosynthetic activity and metabolism are affected [49].

On the contrary, increasing sulfur concentration led to an enhancement of algal growth in a time-dependent manner. Increasing sulfur concentrations (0.075–0.6 g/l) induced significant enhancement in growth parameters (DW and OD) recording 1.030 ± 0.005 and 1.39 ± 0.100, respectively, at the 25th day of cultivation of *N. linckia* at 0.6 g/l. Sulfur is one of the most essential macronutrients used for growth and survival of algae. It is included in the synthesis of biomolecules; iron-sulfur-proteins (as ferredoxin), as cofactors in enzymes; and vitamins as thiamine, biotin, and thiouridine, in various amino acids (as cysteine and methionine) and in sulfur-containing secondary metabolites [48].

The effect of combined N and S at concentrations of 6 g/l and 0.3 g/l, respectively, recorded in Fig. 1e and f illustrated that there was a significant and obvious enhancement of growth compared to that of the normal medium (1.17 ± 0.069 and 1.03 ± 0.060 of DW and OD, respectively) at the 20th day of growth, after which a relatively slight decrease in growth was observed at the combined N and S concentrations which is still higher than that obtained by the control at the 25th day of growth.

These findings were in agreement with Balaguer et al. [50], who studied the interactive effect of SO_2 and NO_3 on the growth of the green algal lichen *Ramalina farinacea*. They found that there was a synergistic effect in case of a combination between the two elements, leading to an increase in growth when compared to the effect of each element separately. As the dose increased, antagonistic effects were developed.

3.2 Pigment contents (phycobiliproteins) of *N. linckia* under different N and S stress conditions

Increasing N concentration from 0.0 to 1.5 g/l and 3 g/l led to a significant and pronounced increase in all phycobiliprotein pigments which reached its maximum values at 3 g/l (76.28 ± 4.61, 37.19 ± 2.44, and 146.82 ± 4.11 in phycocyanin, allophycocyanin, and phycoerythrin, respectively), as recorded in Table 1.

In cyanobacteria, the phycobiliproteins constitute major light-harvesting pigments, which are attached to the outer surface of the thylakoid membrane in an aggregated complex called the phycobilisomes [51]. Various environmental factors are known to affect the efficiency of energy transfer from PBSs to chlorophyll [52].

Phycobiliproteins serve as a nitrogen storage compound. They mobilize their intracellular nitrogen storage constituents

Table 1 Phycobiliprotein pigment contents as μg/g for *N. linckia* cultivated under different concentrations of nitrogen and sulfur

Stress conditions	Phycocyanin	Allophycocyanin	Phycoerythrin	Total phycobiliprotein
Nitrogen (g/l)				
0	60.64 ± 3.42^{b}	17.80 ± 2.15^{c}	123.05 ± 4.59^{b}	200.49 ± 5.361^{b}
1.5	90.32 ± 2.41^{c}	18.61 ± 2.14^{c}	123.05 ± 0.52^{d}	211.98 ± 4.71^{d}
3	76.28 ± 4.61^{a}	37.19 ± 2.44^{a}	146.82 ± 4.11^{a}	276.29 ± 5.45^{a}
6	27.36 ± 2.52^{d}	15.61 ± 1.09^{d}	35.66 ± 2.12^{e}	78.63 ± 3.14^{e}
12	7.63 ± 1.03^{e}	4.09 ± 1.05^{e}	10.09 ± 0.002^{f}	21.83 ± 2.02^{f}
6 g/l ($NaNO_3$) + 0.3 g/l ($MgSO_4{\cdot}7H_2O$)	67.37 ± 3.47^{b}	22.50 ± 2.40^{b}	88.01 ± 4.49^{c}	177.88 ± 7.48^{c}
Sulfur (g/l)				
0	14.52 ± 1.05^{d}	9.341 ± 1.04^{e}	16.39 ± 1.32^{f}	39.25 ± 2.40^{e}
0.07	60.64 ± 3.42^{c}	17.80 ± 2.15^{f}	123.05 ± 4.59^{a}	200.49 ± 5.36^{c}
0.15	66.47 ± 3.46^{b}	30.43 ± 2.39^{c}	102.30 ± 3.37^{d}	199.20 ± 6.39^{c}
0.3	62.37 ± 3.43^{c}	41.28 ± 2.48^{b}	105.62 ± 4.35^{c}	209.27 ± 7.36^{b}
0.6	71.60 ± 3.26^{a}	54.19 ± 3.53^{a}	113.95 ± 4.33^{b}	239.74 ± 7.18^{a}
6 g/l ($NaNO_3$) + 0.3 g/l ($MgSO_4{\cdot}7H_2O$)	67.37 ± 3.47^{b}	22.50 ± 2.40^{d}	88.01 ± 4.49^{e}	177.88 ± 7.48^{d}

Data are presented as means ± SD ($n = 3$) in each column, and for each concentration, means with different letters are significantly different ($p < 0.05$)

for other cellular activities during nitrogen shortage. Combined 6 g/l N and 0.3 g/l S was shown to inhibit the production of all phycobiliprotein pigments (67.37 ± 3.47 for phycocyanin, 22.50 ± 2.40 for allophycocyanin, and 88.01 ± 4.49 for phycoerythrin; total phycobiliprotein content 276.29 ± 5.45).

Our results were in agreement with Shanab et al. [12], who found that *Nostoc muscorum* and *Oscillatoria* sp. can be cultured under nitrogen stress conditions (by both increasing and decreasing the nitrate content of the normal BG-11 medium, 1.5 g/l). They found that, by increasing nitrate concentration (3 g/l, 6 g/l, and 9 g/l), a remarkable increase in phycobilin pigments was observed, followed by an increase in antioxidant and anticancer activities of both studied cyanobacterial species.

Johnson et al. [53] and Nayak et al. [54] proved that in *Nostoc* sp. (nitrogen-fixing cyanobacteria), supplementation of external nitrogen source in the form of sodium nitrate was found to increase the total phycobiliprotein content by five-fold. The most suitable concentration of sodium nitrate was 17.65 mM, yielding a total phycobiliprotein content of 0.10 g/g of dry cell weight compared to a control. Synthesis of phycobiliproteins was reduced at higher concentration of sodium nitrate. This may be because of substrate inhibition experienced by the cells.

More elevated nitrogen concentrations (6 g/l and 12 g/l) were found to be inhibitory. These results coincided with those of Loaiza et al. [55], who proved that synthesis of pigments, especially phycobiliproteins, is particularly susceptible to environmental influences. For *Nostoc*, nitrogen-limited cultures actually produce more phycobiliproteins than non-limited cultures. This can be explained through nitrogen fixation. Heterocystous cyanobacteria, such as *Nostoc* and *Anabaena*, are capable to fix the atmospheric nitrogen, to produce chlorophyll, carotenoids, and phycobiliproteins in significant quantities, and that can be seen in the results, especially with *Nostoc* strains.

On the contrary, elevated sulfur concentration (0.6 g/l) induced a significant enhancement in all pigments (71.60 ± 3.26 in case of phycocyanin, 54.19 ± 3.53 in allophycocyanin, and 113.95 ± 4.33 in phycoerythrin with a total phycobiliprotein content reaching 239.74 ± 7.18, an increment of phycobiliprotein as shown in Table 1).

Increasing sulfur concentration may be related to that pigmentation of light-harvesting phycobiliproteins of cyanobacteria may require covalent attachment of open-chain tetrapyrroles and bilins to the apoproteins. Formation of thioether bond via the addition of a cysteine residue to the 3-ethylidene substituent of bilins is interceded by lyases. T-type lyases are responsible for attachment to Cys-155 of phycobiliprotein β subunits [56, 57].

3.3 Effect of culture media composition on secondary metabolite production of *N. linckia*

Table 2 shows that nitrogen depletion provoked significantly highest quantities of phenolic content (27.00 ± 1.0), flavonoids (21.14 ± 0.80), and tannins (6.54 ± 0.43), which were in a good agreement with Li et al. [58], who proved that total phenolics were inversely proportional to nitrogen supply in two genotypes of leaf mustard (cvs. 'Xuelihong' and 'Zhujie'). Also, these results were in parallel with those reported by Al-Rashed et al. [59], who found that both phenolic and flavonoid contents increased significantly under nitrogen

Table 2 Phenolic, flavonoid, tannin, and proline contents of *N. linckia* cultivated under different concentrations of nitrogen and sulfur (and combined nitrogen and sulfur)

Stress conditions	Phenolic contents (mg gallic acid equivalent/g DW)	Flavonoids (mg of rutin/g DW)	Tannins (mg tannic acid equivalent/g DW)	Proline contents (mg/g)
Nitrogen (g/l)				
0	27.00 ± 1.0^{b}	21.14 ± 0.80^{a}	6.54 ± 0.43^{a}	4.77 ± 0.47^{cd}
1.5	$24.25 \pm 1.39b^{c}$	18.13 ± 1.02^{b}	5.45 ± 0.47^{b}	3.56 ± 0.40^{d}
3	22.35 ± 1.46^{c}	10.26 ± 0.64^{c}	2.17 ± 0.29^{d}	5.06 ± 0.30^{bc}
6	17.90 ± 1.15^{d}	6.10 ± 0.85^{d}	2.39 ± 0.44^{cd}	6.23 ± 0.58^{b}
12	13.00 ± 1.9^{e}	9.30 ± 1.08^{c}	1.73 ± 0.11^{d}	10.92 ± 0.61^{a}
6 g/l N + 0.3 g/l S	41.00 ± 1.0^{a}	2.90 ± 0.65^{e}	3.19 ± 0.35^{c}	4.43 ± 0.51^{cd}
Sulfur (g/l)				
0	34.74 ± 0.63^{c}	15.90 ± 0.39^{b}	1.81 ± 0.51^{f}	12.15 ± 0.58^{a}
0.07	24.25 ± 1.39^{d}	18.13 ± 1.03^{a}	5.45 ± 0.47^{d}	3.57 ± 0.40^{bc}
0.15	44.94 ± 0.96^{a}	14.56 ± 0.51^{b}	8.53 ± 0.41^{c}	4.52 ± 0.47^{b}
0.3	47.32 ± 0.58^{a}	10.91 ± 0.72^{c}	13.77 ± 0.58^{b}	2.95 ± 0.18^{c}
0.6	25.00 ± 1.00^{d}	15.22 ± 0.59^{b}	19.00 ± 0.99^{a}	3.50 ± 0.50^{bc}
6 g/l N + 0.3 g/l S	41.00 ± 1.00^{b}	2.90 ± 0.65^{e}	3.19 ± 0.35^{e}	4.20 ± 0.85^{bc}

Data are presented as means ± SD ($n = 3$) in each column, and for each concentration, means with different letters are significantly different ($p < 0.05$)

deprivation on the growth rate of *Spirulina platensis* and *Dunaliella salina*.

Short-term nitrogen starvation induced oxidative stress of the green microalga *Acutodesmus dimorphus* and accumulation of ROS, proline, and polyphenols as well as higher activities of the antioxidant enzymes (catalase, superoxide dismutase, and ascorbate peroxidase) and stress hormone ABA [60].

The obtained results coincided with Hamid and Sibi [61], who proved that the increment of proline contents reached eightfold, and total phenolic contents under nitrogen stress were induced by an antioxidant system in *Chlorococcopsis minuta*.

Moreover, Allahdadi and Farzane [62] reported that nitrogen application in higher levels improved the growth of artichoke but reduced total phenol, total flavonoid, and antioxidant activity.

However, Lauritano et al. [63] showed that the green alga *Tetraselmis suecica* was able to activate stress and antioxidant transcripts as well as signaling and solute transporter transcripts, indicating the activation of a series of defense and adaptation strategies to maintain cellular homeostasis and survival.

Secondary metabolites produced by *N. linckia* cultured on different concentrations of sulfur are recorded in Table 2. Maximum proline content (12.15 ± 0.58 mg/g) was recorded at 0.0 g/l sulfur (S depletion), while significantly great production of flavonoids was achieved at low sulfur concentrations (0 g/l and 0.079 g/l) (15.90 ± 0.39 and 18.13 ± 1.03 mg rutin/g DW).

On the other hand, phenolic contents reached significantly maximum production at 0.15 g/l and 0.3 g/l sulfur (44.94 ± 0.96 and 47.32 ± 0.58, respectively). At high sulfur concentration (0.6 g/l), the largest production of tannins (19.00 ± 0.99 mg tannic acid equivalent/g DW) was recorded. Regarding the combination N + S, phenolic content was increased twofold than the content produced by 0.3 g/l S.

Concerning flavonoids, the combination N + S highly decreased the flavonoid production (twofold to threefold decrease) which occurred by the same element concentration separately. In case of tannins, the combination effect slightly increased the quantity produced by 6 g/l N but largely decreased the tannin content produced at 0.3 g/l S (fourfold decrease). The proline produced in the combination was lower than that induced at 6 g/l N but, at the same time, higher (double) than that at 0.3 g/l S.

As sulfur is required for synthesis of important defense compounds as cysteine-rich peptides, phytochelations, metallothioneins as well as reduced GSH are involved in both metal chelation and scavenging of ROS. There was a positive correlation between increasing sulfur concentration and increasing phenolic contents which was in agreement with Li et al. [58], who determined the effect of sulfur on total phenolics and antioxidant activity in two genotypes of leaf mustard (cvs. 'Xuelihong' and 'Zhujie'). They found that by increasing sulfur concentration (0.5 mM, 1 mM, and 2 mM), total phenolic concentration increased, showing a positive correlation between antioxidant activity (by DPPH method) and total phenolics in the two genotypes. Additionally, Tóth et al. [64] proved that the highest contents of polyphenols were recorded in yellow cultivars. The highest values of antioxidant activity were achieved in all cultivars at the sulfur dose of 40.00 kg/ha, except for cultivars Boston and Diamant.

3.4 Effect of media composition on the production of antioxidant defense enzymes and lipid peroxidation (MDA) of *N. linckia*

The highest significant values of lipid peroxidation, enzyme glutathione *S*-transferase, and catalase were recorded at the most elevated nitrogen concentration used (12 g/l) (666.22 ± 9.07, 375.62 ± 7.35, and 72.99 ± 3.00, respectively), while protein content mostly produced was at 3 g/l nitrogen (287.30 ± 1.30 mg/g) as shown in Table 3.

Mixing 6 g/l N and 0.3 g/l S led to a significant decrease in all the parameters tested compared to their maximum production in the presence of nitrogen concentration only, as obviously seen in Table 3.

Cells exposed to stresses undergo changes in their metabolism in order to adapt with changes in their environment. Antioxidant enzymes and osmolytes are known to be produced in plants and other organisms in response to stress conditions.

Nitrogen limitation and starvation not only significantly decreased the photosynthetic activity and protein content but also affected the cell morphology of *Scenedesmus* sp. CCNM1077 as reported by Pancha et al. [65].

Our results were in parallel with those reported by Al-Rashed et al. [59], who investigated the effect of deprivation of nitrogen stresses on the growth rate of *Spirulina platensis* and *Dunaliella salina*, and the increment of some antioxidant compounds, antioxidant enzymes, superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, and glutathione reductase as well as the levels of total glutathione pool, UV-B-absorbing pigments, MDA, and H_2O_2 concentrations were studied in *Spirulina platensis* and *Dunaliella salina*.

The activities of SOD, CAT, and APX in green microalga *Chlorococcopsis minuta* were increased under nitrogen stress (deprivation), representing an enhancement in its antioxidant system response [61].

At elevated nitrogen concentrations, in this study, there was an increase in protein contents which may be due to excessive production of stress proteins which agreed with the results obtained by Menegol et al. [66], who found that *Heterochlorella luteoviridis* biomass production, composition, and protein contents were increased at 138 mg/g, the highest nitrogen concentration (60 mg/l N-NO_3). This value was 45% higher than that observed in the culture containing the lowest nitrogen concentration (12 mg/l N-NO_3).

Concerning sulfur stress, lipid peroxidation acquired the highest content at sulfur depletion condition (328.79 ± 7.90 MDA nmol/g). Significantly larger contents of protein, GST, and catalase activity were recorded at the highest sulfur concentration used as seen in Table 3.

Sulfur concentrations induced oxidative stress with the generation of a large quantity of ROS which, if not scavenged properly in time, induced damages to biomembranes and biomolecules with elevated lipid peroxidation (MDA), stress protein, and sulfur-containing proteins as well as elevation of the defensive antioxidant enzymes, GST, and catalase.

Glutathione is known to be a crucial sulfur-based molecule for resistance of oxidative stress, and many other intracellular antioxidant mechanisms depend on it and are limited by extracellular sulfur availability [67].

GSTs quench reactive molecules with the conjugation of GSH and detoxify cells from oxidative damage [68, 69]. GSTs are involved in the detoxification mechanisms, and 15 GST-

Table 3 Phenolic, flavonoid, tannin, and proline contents of *N. linckia* cultivated under different concentrations of nitrogen and sulfur (and combined N and S)

Stress conditions	Lipid peroxidation (MDA nmol/g)	Protein concentration as mg/g	Glutathione *S*-transferase in μ/g tissue	Catalase activity (%)
Nitrogen (g/l)				
0	195.85 ± 5.53^{c}	178.61 ± 1.33^{c}	89.32 ± 2.58^{d}	55.44 ± 2.86^{c}
1.5	109.67 ± 4.91^{f}	252.58 ± 1.23^{b}	81.04 ± 3.73^{e}	40.90 ± 2.52^{e}
3	128.10 ± 3.75^{e}	287.30 ± 1.30^{a}	92.66 ± 3.35^{c}	60.08 ± 3.18^{b}
6	227.65 ± 6.74^{b}	152.44 ± 0.97^{d}	101.56 ± 5.40^{b}	62.85 ± 3.54^{b}
12	666.22 ± 9.07^{a}	55.66 ± 1.52^{f}	375.62 ± 7.35^{a}	72.99 ± 3.00^{a}
6 g/l N + 0.3 g/l S	143.57 ± 5.50^{d}	140.06 ± 1.03^{e}	89.86 ± 3.80^{d}	44.77 ± 4.57^{d}
Sulfur (g/l)				
0	328.79 ± 7.90^{a}	207.78 ± 1.35^{e}	246.86 ± 0.51^{a}	40.84 ± 2.46^{c}
0.07	109.67 ± 4.91^{f}	252.58 ± 1.23^{d}	81.04 ± 3.73^{e}	40.90 ± 2.52^{c}
0.15	170.12 ± 5.42^{d}	270.24 ± 1.02^{c}	147.06 ± 4.40^{c}	41.10 ± 2.35^{c}
0.3	185.97 ± 5.53^{c}	310.84 ± 0.92^{b}	151.07 ± 5.59^{c}	42.22 ± 3.59^{c}
0.6	209.89 ± 7.37^{b}	319.80 ± 0.77^{a}	178.97 ± 5.54^{b}	51.04 ± 3.14^{a}
6 g/l N + 0.3 g/l S	143.57 ± 5.50^{e}	140.06 ± 1.03^{f}	89.86 ± 3.80^{d}	44.77 ± 4.57^{b}

Data are presented as means ± SD (n = 3) in each column, and for each concentration, means with different letters are significantly different ($p < 0.05$)

like isoenzymes (Cr GSTS) were found in the model species of green alga *Chlamydomonas reinhardtii* [70].

Moreover, Zahang et al. [71] found that sulfur significantly enhanced the tolerance of oil seed rape exposed to Cr stress by activating several detoxification mechanisms such as the ascorbate-glutathione (AsA-GSH) enzyme defense system and GSH production. Kumaresan et al. [72] studied sulfur stress on *Arthrospira* sp. and reported that the alga was able to cope the stress by altering the gene expression that involved in sulfur metabolism and different sulfur-dependent pathways.

The combination of N and S provoked a significant reduction in all the parameters tested, compared to the produced maximal quantities at the same sulfur concentration, singly. It seems that the addition of nitrogen (in the combination with sulfur) may ameliorate the adverse effect of sulfur and the lipid peroxidation (MDA), as well as the other parameters of antioxidant enzymes in algal species under investigation.

3.5 Effect of media compositions on the antioxidant activity of *N. linckia* under nitrogen and sulfur stress conditions

Table 4 shows the antioxidant activity (%) of *N. linckia* extracts (200 ppm) cultivated under different nitrogen and sulfur concentrations (and combined effect with sulfur) using DPPH radical scavenging activity (after 30 min and 60 min) and ABTS assay compared to synthetic antioxidant (BHT). Antioxidant activity was known to be concentration dependent. Nitrogen depletion (0.0 g/l) provoked the significantly higher antioxidant activity by DPPH (30 min) which reached 93.03 ± 1.00 and increased to 94.26 ± 0.80 at 1.5 g/l N (after 60 min). The extract 12 g/l N recorded the highest antioxidant activity that might be resulted from its contents of flavonoids, tannins, and phenolic compounds represented in Table 2, as reported by Shalaby and Shanab [73] and Gaber et al. [74].

Concerning sulfur stress, the highest antioxidant activity occurred at 0.07 g/l using DPPH assay (91.25 ± 1.09 and 94.26 ± 0.80 after 30 min and 60 min, respectively), whereas the activity using ABTS assay was 61 ± 1.00 at the concentration 0.3 g/l while the activity increased using ABTS assay (82.27 ± 1.01). The combination of 6 g/l N and 0.3 g/l S induced a decrease in antioxidant activity using DPPH (63.97 ± 1.00 and 69.19 ± 0.72 after 30 min and 60 min, respectively) and a slight decrease in antioxidant activity using ABTS (82.27 ± 0.82).

ROS are products of a normal cellular metabolism and play vital roles in the stimulation of signaling pathways in plant and animal cells in response to changes in intracellular and extracellular environmental conditions. Increased regeneration of ROS occurred under stress conditions, the defense system in the form of low molecular weight substances (as ascorbic acid, β-carotene, glutathione), and/or antioxidant enzymes (superoxide dismutase/catalase/peroxidase/glutathione *S*-transferase) were produced in order to scavenge the ROS and avoid their harmful action on biomolecules and biomembranes.

Proteins, nucleic acids, and lipids were also significant targets for oxidative attack, and modification of these molecules can increase the risk of mutagenesis. Therefore, antioxidants are good scavengers for ROS and free radicals. In other words, it defends and protects cells from their harmful action. The

Table 4 Antioxidant activity (%) of *N. linckia* extracts (200 ppm) cultivated under different nitrogen concentrations (and combined effect with sulfur), using DPPH radical scavenging activity (after 30 min and 60 min) and ABTS assay compared to synthetic antioxidant (BHT)

Stress conditions	DPPH (%), 30 min	DPPH (%), 60 min	ABTS (%)
Nitrogen (g/l)			
0	93.03 ± 1.00^{a}	91.18 ± 0.42^{b}	69.76 ± 0.68^{d}
1.5	91.25 ± 1.09^{b}	94.26 ± 0.80^{a}	61 ± 1.00^{f}
3	63.14 ± 0.79^{f}	65.51 ± 0.42^{f}	43.34 ± 0.65^{f}
6	71.64 ± 0.53^{e}	78.27 ± 0.54^{d}	67.42 ± 0.84^{e}
12	87.08 ± 0.77^{c}	89.10 ± 0.65^{c}	84.03 ± 0.94^{b}
6 g/l N + 0.3 g/l S	63.973 ± 1.00^{f}	69.19 ± 0.72^{e}	82.27 ± 0.82^{c}
BHT	85.63 ± 0.55^{d}	89.50 ± 0.55^{c}	90.82 ± 0.68^{a}
Sulfur (g/l)			
0	81.12 ± 0.82^{e}	89.02 ± 0.26^{b}	55.29 ± 0.61^{g}
0.07	91.25 ± 1.09^{a}	94.26 ± 0.80^{a}	61 ± 1.00^{f}
0.15	68.17 ± 0.65^{f}	69.04 ± 1.03^{d}	86.34 ± 0.83^{b}
0.3	89.063 ± 0.50^{b}	88.18 ± 0.64^{b}	84.20 ± 1.01^{c}
0.6	83.56 ± 0.51^{d}	83.18 ± 1.04^{c}	66.03 ± 0.84^{e}
6 g/l N + 0.3 g/l S	63.97 ± 1.00^{g}	69.19 ± 0.72^{d}	82.27 ± 0.82^{d}
BHT	85.63 ± 0.55^{c}	89.50 ± 0.55^{b}	90.82 ± 0.68^{a}

Data are presented as means ± SD ($n = 3$) in each column, and for each treatment, means with different letters are significantly different ($p < 0.05$)

antioxidants prevent damages in proteins, DNA (protect from mutation), and lipid peroxidation (protect plasma membrane) in living cells (normal cells).

In our results, the most effective extract that represented the highest antioxidant activity was 0.3 g/l S, and it also contains a considerable quantity of phytochemicals recorded in Table 2. Our results were in agreement with Deyab et al. [75] who performed extraction of *Dictyota dichotoma* using successive extraction with solvents of increasing polarity (hexane, ethyl acetate, acetone, and methanol), revealing the presence of 6, 9, 4, and 7 of phytochemical compounds, respectively. Flavonoids showed antimicrobial, antiviral, and antioxidant activities, while phenols have anticancer, antioxidant, antimicrobial, and antiviral activities, and quinones exhibited cytotoxic activity.

Additionally, our results were in parallel with those obtained by Jerez-Martel et al. [76] who screened the aqueous and methanolic extracts of several microalgae and cyanobacteria for their radical scavenging activity against the DPPH radical method. The methanol extracts showed compounds with a higher antioxidant activity. In addition, aqueous and methanolic extracts of microalgae *Euglena cantabrica* also exhibited the highest antioxidant activity, probably due to the presence of the high contents of phenolics.

From all the previous data, four promising extracts were selected (0.3 g/l S, 0.15 g/l S, 12 g/l N, 0.3 g/l S + 6 g/l N), and based on their antioxidant activity, these were tested for their anticancer, antimicrobial, and antiviral activities.

3.6 Anticancer activity of promising extracts of *N. linckia*

Anticancer activity of the four promising extracts (0.3 g/l S, 0.15 g/l S, 12 g/l N, 0.3 g/l S + 6 g/l N) was determined at different concentrations (31.25 μg/ml, 62.5 μg/ml, 125 μg/ml, 250 μg/ml, and 500 μg/ml) and tested against four cell line (lung, breast, colon, and cervical cancer cell lines) cytotoxicity. The obtained results revealed that % viability of cancer cells was inversely proportional to extract concentration as shown in Fig. 2.

The extract 0.3 g/l S showed the highest anticancer activity with the lowest IC_{50} (155 μg/ml) against HCT 116 cell line, whereas 12 g/l N recorded the highest activity with IC_{50} (193 μg/ml) against HeLa cell line. These results indicated that the effect of crude extracts on HCT 116 and HeLa cells is concentration dependent through the concentrations tested (31.25–500 μg/ml). This effect can be explained as receptor independent for these types of cells [77, 78]. The cytotoxic profile of the crude extract may be due to its content from bioactive compounds as recorded in Table 2 as there is a strong correlation between anticancer activity and phenolic, flavonoid, and tannin contents.

Anticancer results in this study were in parallel with those obtained by Alghazeer et al. [79], who evaluated the antioxidant activity and the potential inhibition of $CaCO_2$ cell proliferation of crude extracts of Chlorophyta (*Ulva lactuca* and *Codium tomentosum*), Phaeophyta (*Cystoseira crinita*, *Cystoseira stricta*, and *Sargassum vulgare*), and Rhodophyta (*Gelidium latifolium*, *Hypnea musciformis*, and *Jania rubens*). They found that the polyphenol- and flavonoid-rich extracts showed remarkable reducing power and antiradical properties, after exposure of $CaCO_2$ cells to various concentrations of extracts. The antiproliferative effect of algal extracts was correlated with their polyphenol and flavonoid contents.

3.7 Antimicrobial activity of promising extracts of *N. linckia*

The obtained results recorded in Table 5 revealed that the G −ve *K. pneumoniae* showed no activity (NA) with all the tested extracts while *E. coli* showed moderate activities only with 12 g/l N extract (16.6 ± 0.5 mm) and 0.3 g/l S + 6 g/l N (14.6 ± 0.6 mm) of *N. linckia*. The G+ve strains *S. aureus* and *S. mutans* showed variable activities (13.3–21.0 mm) with all the extracts except that of 12 g/l N where no activity was recorded. Sulfur-containing extracts (0.3 g/l and 0.15 g/l) showed higher activities with *S. aureus* and *S. mutans* (19.0 mm, 19.3 mm, and 21.0 mm).

Concerning antifungal, *A. niger* showed NA with any tested extract, while *C. albicans* yeast showed low to moderate activity (11.0–14.6 mm) with three extracts except that of 6 g/l N of *N. linckia* which showed no activity with the yeast.

The antimicrobial activity of *N. linckia* cultivated under S, N, and S combined with N concentration may be due to the types and content of secondary metabolites which were synthesized during algal cultivation under those stresses.

The most effective extract was 0.15 g/l S against the most susceptible bacterial strain (*S. aureus*), and this is may be due to high contents of phenolic, flavonoid, and tannin as represented in Table 2, which was in a good agreement with Seetharaman et al. [80], who evaluated antibacterial activity against some human clinically isolated pathogens (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Proteus vulgaris*, and *Bacillus subtilis*). The phytochemical analysis also showed the presence of alkaloids, saponin, phenols, terpenoids, coumarins, flavonoids, tannins, protein, and carbohydrates and absence of glycosides, steroids, and anthraquinone.

Our results were in parallel with those of Sujatha et al. [81], who investigated phytochemical screening and antibacterial efficacy of various solvent extracts of marine algae such as *Sargassum swartzii* against some selected human and fish pathogenic bacteria. The maximum bioactive compounds were present in the ethanolic extract, and the minimum bioactive compounds were present in aqueous extract. The ethanol

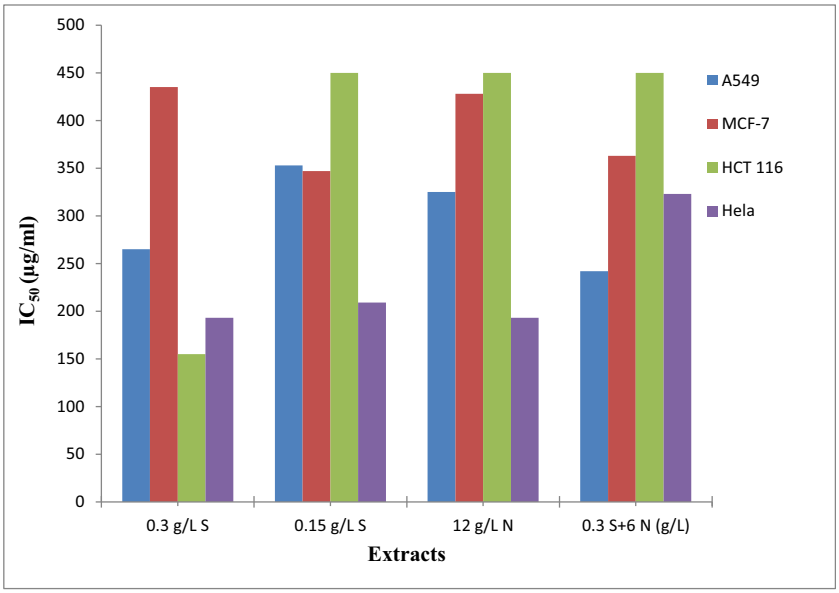

Fig. 2 IC_{50} as µg/ml of algal extracts against four cell lines

extract was significantly higher in phenolic content (15.35 ± 2.61 mg of GAE/g), and the methanolic extract was significantly higher in flavonoid content (26.92 ± 7.14 mg of QE/g). In human pathogen, the highest antibacterial activity was against *Pseudomonas aeruginosa* (18.00 ± 0.00 mm) and the fish pathogen was significantly higher against *Aeromonas hydrophila*.

3.8 Antiviral activity of *N. linckia* against (H_5N_1) influenza virus

Antiviral activity of the four promising extracts was tested against influenza virus (M7217B) 2013 (H_5N_1). Data recorded in Table 6 revealed that the extract 0.3 g/l S has the maximum inhibition of 50.0% and 63.6% at 7 µg/ml and 28 µg/ml, respectively, followed by 0.15 g/l S which induced 29.5% and 66% at 7 µg/ml and 28 µg/ml with cytotoxicity on MDCK cells, IC_{50}, less than 7 µg/µl and 29.5 µg/µl, respectively. The highest antiviral activity against H_5N_1 might be due to bioactive compounds such as high phenolic, flavonoid, and tannin contents in the extract as represented in Table 2, which were 47.32 mg/g and 44.94 mg/g for phenolic contents, 10.91 mg/g and 14.56 mg/g for flavonoid contents, and 13.77 mg/g and 8.53 mg/g for tannin contents.

Over the last few decades, natural products have been studied for anti-infective and, more specifically, antiviral activities. Basic researches in experimental models using various biological systems strongly suggested the protective role of plant-derived natural compounds against different viral infections [82]. Some natural and synthetic compounds can

Table 5 Antimicrobial activity as mm inhibition zone of the selected promising extracts (according to antioxidant activity result) of *N. linckia* against different bacterial and fungal strains using disc diffusion assay

Microorganism	Extracts				Standard antibiotic		
	0.3 g S	0.3 g S + 6 g N	0.15 g S	12 g N	Gentamicin	Ampicillin	Nystatin
Gram-negative bacteria							
Escherichia coli (ATCC: 9637)	NA	14.6 ± 0.6^{c}	NA	16.6 ± 0.5^{b}	27 ± 0.5^{a}	NT	NT
Klebsiella pneumoniae (ATCC: 10031)	NA	NA	NA	NA	25 ± 0.5^{a}	NT	NT
Gram-positive bacteria							
Staphylococcus aureus (ATCC: 6538)	21.0 ± 1.0^{b}	14.6 ± 0.5^{d}	19.0 ± 1.0^{c}	NA	NT	22 ± 0.1^{a}	NT
Streptococcus mutans (ATCC: 25175)	19.3 ± 0.6^{b}	19.6 ± 0.6^{b}	13.3 ± 0.5^{c}	NA	NT	30 ± 0.5^{a}	NT
Fungi							
Candida albicans (ATCC: 10231)	11.0 ± 1.0^{d}	13.6 ± 0.5^{c}	14.6 ± 0.6^{b}	NA	NT	NT	21 ± 0.5^{a}
Aspergillus niger (ATCC: 32856)	NA	NA	NA	NA	NT	NT	20 ± 0.5^{a}

Data are presented as means ± SD ($n = 3$) in each column, and for each extract, means with different letters are significantly different ($p < 0.05$)

NA no activity, *NT* not tested

Table 6 Antiviral activity (as % and IC_{50}) of different algal extracts against H_5N_1 virus using plaque reduction assay

Algal sample	Conc. (μg/ml)	Initial viral count (PFU/ml)	Viral count (PFU/ml)	% of inhibition	Cytotoxicity IC_{50} (μg/μl)
12 g/l N	7	4.4×10^8	2.7×10^8	38.6	19.5
	28	4.4×10^8	2.3×10^8	47.7	
0.3 g/l S	7	4.4×10^8	2.2×10^8	50	Less than 7 μg/μl
	28	4.4×10^8	1.6×10^8	63.6	
0.3 g/l S + 6 g/l N	7	4.4×10^8	3.7×10^8	16	Less than 7 μg/μl
	28	4.4×10^8	2.7×10^8	38.6	
0.15 g/l S	7	4.4×10^8	3.1×10^8	29.5	29.5
	28	4.4×10^8	1.5×10^8	66	

prevent, suppress, or reverse the progression of virus infection. Natural products have been proven to be the most effective in terms of their ability to act as an antiviral.

Our results were in agreement with those reported by Pereira et al. [83], who tested the antiviral effect of the CH_2Cl_2/MeOH-soluble fraction from the alga *Dictyota menstrualis* on HIV-1 replication in vitro. They found that the antiviral activity was attributed to two diterpenes: (6*R*)-6-hydroxydichotoma-3,14-diene-1,17-dial, named Da-1, and (6*R*)-6-acetoxidichotoma-3,14-diene-1,17-dial.

Additionally, our results were in parallel with those of Carpine and Sieber [14] who found that *Nostoc linckia* have high potential for use as an antiviral agent against influenza virus and could be promising as anti-coronavirus (SARS-CoV-2).

It was obviously clear that alga cultivated under sulfur stress (0.3 g/l S) recorded almost the promising biological activities as (i) antioxidant activity based on DPPH and ABTS assays, respectively (88.18 ± 0.64% and 84.20 ± 1.01%); (ii) anticancer activity with the lowest IC_{50} (155 μg/ml) against HCT 116 cell line; (iii) antibacterial activity (21.0 ± 1.0 as mm inhibition zone against *Staphylococcus aureus* and 19.3 ± 0.6 against *Streptococcus mutans*); and (iv) antiviral activity against H_5N_1 virus with a percentage of inhibition of 50% and 63.6% at a concentration of 7 μg/ml and 28 μg/ml, respectively, with cytotoxicity less than 7 μg/μl. These results may be correlated with the presence of bioactive compounds recorded in Table 7 as antioxidant, antimicrobial, anticancer, and antiviral compounds such as cyclohexasiloxane, dodecamethyl, cycloheptasiloxane, tetradecamethyl, 2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [r-[r*,r*-(e)]]-4h-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-, 2-hexadecanol, hexadecanoic acid, and methyl ester. These observations were in agreement with the previously published results [84, 88, 90, 91]. Also, the obtained results correlated with the data in Table 2; there is a strong correlation between the biological activity of the promising crude extract (0.3 g/l S) and the concentration of flavonoids (10.91 ± 0.72 mg of rutin/g DW), phenolic compounds (47.32 ± 0.58 mg gallic acid equivalent/g), and tannin (13.77 ± 0.58 mg tannic acid equivalent/g DW).

4 Conclusion

The cyanobacterium *Nostoc linckia* was investigated under N and S stress conditions. Different concentrations of nitrogen and sulfur were used depending on their content in the $BG\text{-}11_0$ nutritive medium. Four promising crude extracts (0.15 g/l S, 0.3 g/l S, 12 g/l N, and the combination of both elements [0.3 g/l S + 6 g/l N]) were selected based on their antioxidant activity. Growth of the cyanobacterium was monitored as OD and DW, algal pigments (chlorophylls, carotenoids, and phycobiliproteins), antioxidant enzymes, lipid peroxidation, and phytochemical active substances were screened spectrophotometrically and determined by GC-MS analysis. Biological activities were determined (antimicrobial, antioxidant, antiviral, and anticancer activities), as well as the IC_{50}. The obtained results generally illustrated that 0.3 g/l S extract showed the greatest biological activities. Enhancement in growth, pigment production, antioxidant enzymes, phytochemical screening, and the GC-MS analysis revealed that this extract contains several bioactive compounds which attributed all the recorded biological activities. A strong correlation was noticed between the promising sulfur extract (0.3 g/l) and the content of phenolic compounds, flavonoids, tannins, and phycobiliproteins which may have a role in the recorded biological activities. It was known from the literatures that algae and cyanobacteria cultured under abiotic stress as the nutrient stress conditions altered its normal metabolic pathways towards the production of important substances and secondary metabolites that exhibit pronounced biological activities and can be isolated and be used as active ingredients in pharmaceutical drugs and have a role in medicine in curing many serious diseases.

Table 7 GC-MS analysis of the most promising *N. linckia* extract (0.3 g/l S) as antioxidant, anticancer, antimicrobial, and antiviral as referred from available literatures

No.	Compound name	M.wt.	Relative percentage	Biological activity	References
1	Androstane-11,17-dione,3-[(trimethylsilyl)oxy]-, 17-[*o*-(phenylmethyl)oxime], (3à,5à)-	481	3.3	Antioxidant and antimicrobial activities; cancer enzyme inhibitors in pharmaceutical	Sayik et al. [84]
2	Cyclohexasiloxane, dodecamethyl-	444	2.00	Antibacterial, antifungal, and antitumor activities	Patil and Jadhav [85]
3	Cycloheptasiloxane, tetradecamethyl	518	3.77	Antibacterial, antifungal, and antitumor activities	Patil and Jadhav [85]
4	Cyclooctasiloxane, hexadecamethyl-	592	1.45	Antimicrobial activity	Mebude and Adeniyi [86]
5	Cyclononasiloxane, octadecamethyl-	578	0.98	Antioxidant activity	Kadri et al. [87]
6	6,7-Dimethoxy-2-methyl-3,4-dihydro[1-*d*]isoquinolinium ion	207	1.32		
7	Phenol, 2,4-bis(1,1-dimethylethyl)-	206	2.05	Antioxidant and antiviral activities	Prakash and Vuppu [88]
8	4h-1-Benzopyran-4-one,2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-	344	4.94	Antioxidant and antimicrobial activities; cancer enzyme inhibitors in pharmaceutical	Sayik et al. [84]
9	1-Nonadecene	266	0.99	Antimicrobial activity	Cherchi et al. [89]
10	1Monolinoleoylglyceroltrimethylsilyl ether	498	0.68		
11	2-Hexadecanol	242	3.13	Anticancer, anti-inflammatory, antimicrobial, and antioxidant activities	Kalaisezhiyen and Sasikumar [90]
12	Adgalactopyranoside,methyl-2,3-bis-*o*-(trimethylsilyl)-cyclicmethylboronate	362	0.95		
13	Dotriacontane	450	0.97	Antimicrobial activity	Cherchi et al. [89]
14	6-Ethyl-5-hydroxy-2,3,7-trimethoxynaphthoquinone	292	1.31		
15	Hexadecanoic acid, methyl ester	270	16.79	Antioxidant and antimicrobial activities; 5-alpha reductase inhibitor cancer enzyme inhibitors in pharmaceutical	Sayik et al. [84]
16	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[r-[r*,r*-(e)]]-	296	13.38	Anticancer, anti-inflammatory, antimicrobial, and antioxidant activities	Mohamed et al. [91]
17	9-Octadecenoic acid (z)-, methyl ester	296	17.2	Antioxidant and antimicrobial activities; cancer enzyme inhibitors in pharmaceutical	Sayik et al. [84]
18	4h-1-Benzopyran-4-one,2-(3,4-dihydroxyphenyl)-6,8-di-á-d-glucopyranosyl-5,7-dihydroxy-	610	1.16		
19	6,9,12,15-Docosatetraenoic acid, methyl ester	346	2.84	Phytopharmaceutical importance	Srivastava et al. [92]
20	9,12,15-Octadecatrienoic acid,2,3bis[(trimethylsilyl)oxy]propyl ester, (*z*,*z*,*z*)-	496	1.19		
21	1,2-Benzenedicarboxylic acid, diisooctyl ester	390	8.18	Antioxidant and antimicrobial activities; cancer enzyme inhibitors in pharmaceutical	Sayik et al. [84]
22	Decanedioic acid, bis(2-ethylhexyl) ester	426	5.47	Antioxidant and antimicrobial activities; cancer enzyme inhibitors in pharmaceutical	Sayik et al. [84]
23	.psI.,.psI.carotene,1,1′,2,2′-tetrahydro-1,1′-dimethoxy-	600	1.33	Antioxidant activity	Abd El-Aty et al. [93]
24	Tetrakis (dimethylsilylcarbodiimide)	392	2.61	Antioxidant and antibacterial activities	Abd El-Aty et al. [93]
25	Ergosta-14,22-dien-3-ol,acetate, (3á,5à)-	440	1.99		

Author contribution All authors read and approved the final manuscript.

Data availability The data used and analysed in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable in this section.

Competing interests The authors declare no competing interests.

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