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RESEARCH

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Exploration of the aptitude to alleviate oxidative impairment and curb colorectal cancer manifestation by *Nostoc calcicola* in HT-29 adenocarcinoma cells

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Abstract

Background Marine cyanobacteria have been known to contain several unique bioactive compounds which have different therapeutic potentials. The current research focuses to identify the efficacy of *Nostoc calcicola* to counteract the harmful effects of free radicals and testing its anticancer activity against colorectal adenocarcinoma cells (HT-29).

Results Methanol is used as a solvent for the extraction of bioactive metabolites from *Nostoc calcicola* followed by phytochemical screening representing the presence of flavonoids, phenols, tannins, saponins, and steroids to find out bioactive metabolites. Furthermore, evaluation of the extract efficacy revealed the profound ability of *Nostoc calcicola* to scavenge free radicals by neutralizing different reactive oxygen species. At 100 µg/mL concentration, it inhibited DPPH radicals (73.4%), enhanced phosphomolybdenum reduction (53.5%), displayed ferric-reducing power (55.1%), and finally the extract revealed remarkable hydroxyl radicals scavenging capacity (94.8%), compared to the standards. These compelling results emphasize the robust antioxidant potential of the *Nostoc calcicola* extract. In vitro, studies demonstrated the selective cytotoxic effects of methanol extracts of *Nostoc calcicola* on the HT-29 human colorectal cancer cell line, as indicated by IC_{50} values of 25 µg/mL for the extracts. Treatment with me *Nostoc calcicola* decreased the cell viability of HT-29 cells followed by consistent morphological changes leading to cytotoxicity. Nuclear condensation and DNA fragmentation were observed using AO/EtBr and DAPI staining. Flow cytometry analysis further confirmed the incidence of apoptosis during the S phase of the cell cycle. Furthermore, western blotting analysis confirmed the activation of caspase 9, a pivotal enzyme in the intrinsic apoptosis pathway, suggesting the ability of *Nostoc calcicola* to induce apoptosis in HT-29 colorectal cancer cells.

Conclusion These findings underscore the potential of *Nostoc calcicola* as a valuable source of bioactive compounds with antioxidant and anticancer properties, warranting further investigation for their potential therapeutic applications in colorectal cancer.

Keywords *Nostoc calcicola*, Colorectal cancer, HT-29 cells, Radical scavenging, Cytotoxicity, Apoptosis, Caspase 9, Bioactive compounds

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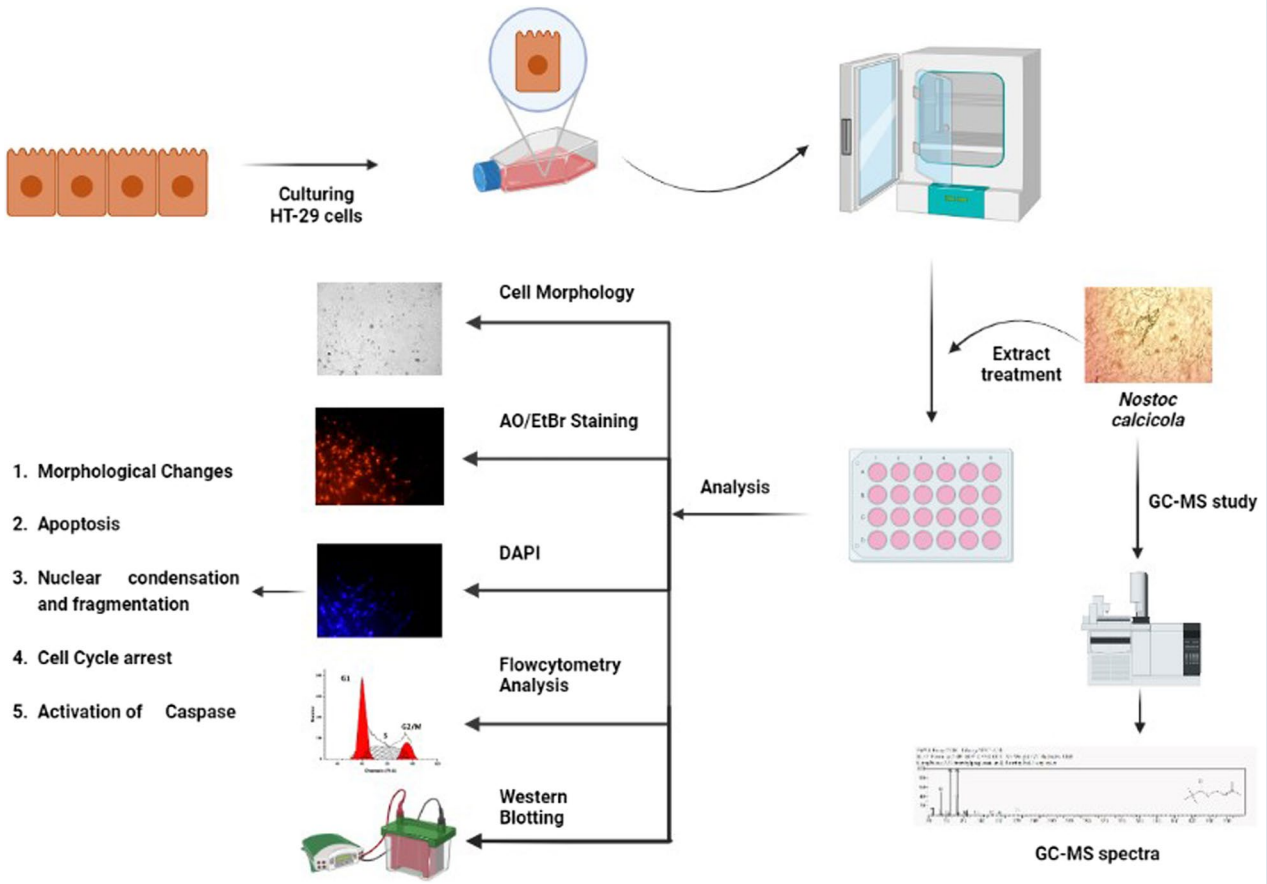
Graphical Abstract



Background

Among different cancer, colorectal adenocarcinoma is a highly prevalent malignancy arising from the glandular cells of the colon or rectum representing a significant global health burden, being the third most common cancer and a leading cause of cancer-related mortality worldwide. Colorectal cancer statistics 2023 revealed approximately 1,53,020 individuals are prone to be infected with colorectal cancer with a mortality rate of 52,550 individual due to this disease among which 3750 will be at an younger age [1]. Surgical resection remains the primary treatment, often accompanied by adjuvant chemotherapy or radiation therapy based on the stage and extent of the disease [2]. A common way to treat cancer is chemotherapy which uses different drugs that affect almost all parts of the body and act on both cancerous and normal cells. As a result these drugs can cause many side effects such as low blood count, loss of appetite, confusion, hair loss, nerve damage, and permanent harm to vital organs [3]. Besides these unwanted consequences of chemotherapy another problem is that cancer cells can become resistant to these drugs [4] and still, there is no effective cure for cancer without harming healthy cells with less or no side effects improving the patient's well-being.

Oxidative stress caused by an imbalance between the production of reactive oxygen species (ROS) and the body's ability to counteract their harmful effects is associated with various diseases such as cardiovascular disorders, neurodegenerative conditions, and cancer. Natural substances are a trustworthy source of finding such substances that can be useful for treating diseases [5, 6]. There is an urgent need for the development of new medicines with lesser or no side effects due to the resistance produced by the organisms against the available treatments [7, 8]. However, challenges remain in managing colorectal adenocarcinoma including the development of resistance to targeted therapies, tumor heterogeneity, and minimal residual disease. Ongoing research focuses on identifying novel herbal approaches unraveling the

role of the tumor microenvironment and exploring strategies to improve treatment outcomes with lesser side effects [9].

Studies have extensively investigated the antioxidant potential of cyanobacteria that can help combat oxidative stress by scavenging free radicals and reducing oxidative damage to cells and tissues and exploring their ability to neutralize ROS and their protective effects against oxidative stress-related diseases [5, 10]. Many research has established that certain algal species, such as cyanobacteria, possess phenolic compounds, flavonoids, antioxidant enzymes similar to those found in plants having the potential to scavenge free radicals and minimize the effects during diseases including cancer [6, 7, 10]. Among the marine bioactive compounds that have been examined so far, extracts or isolated compounds from marine algae have shown remarkable antioxidants, antimicrobials and even potential anticancer agent [11, 12]. Many reports has suggested that marine cyanobacteria are rich source of antineoplastic agents and identified potential anticancer compounds from cyanobacteria and availability of potent antiproliferative drugs derived from marine cyanobacteria like apratoxin, lyngbyabellin, and curacin [13, 14].

Cyanobacteria are valuable sources of natural bioactive compounds encompassing diverse secondary metabolites such as phenolic compounds, flavonoids, terpenoids, proteins, lipids, vitamins, polysaccharides, chlorophyll, phycobiliproteins, immunoactive products (e.g., insulin), and antioxidant enzymes, which possess significant nutritional and medicinal values [5, 10] offering great potential for the development of novel bioactive compounds in pharmaceutical industries. Many cyanobacterial species produce both intracellular and extracellular metabolites, exhibiting diverse biological activities, including antimicrobial properties [15]. These microorganisms can be easily cultivated and scaled up using simple culture media, allowing for precise adjustment of growth conditions to optimize the production of desired bioactive compounds [16, 17]. Cryptophycin 1 is an effective antifungal compound found in cyanobacteria and can act against many fungi that are important for agriculture [18].

Nostoc spp. has abundant bio-active compounds that exhibit various biological properties such as anticancer, anti-inflammatory, and antioxidant activities etc., [18]. Presence of polysaccharides, phenolic compounds, flavonoids, and sulfates in *Nostoc commune* are confirmed along with its ability to inhibit IL-6, and β-hexosaminidase while promoting collagen I secretion. *Nostoc commune* has an underlying wound-healing and anti-allergic potential that can be considered for the development of cosmetics [19]. *Nostoc* sp. N42 exhibited substantial anticancer activity against HepG2 (liver cancer) and A-549 (human non-small-cell lung carcinoma) cells, with an IC_{50} of 583.1 g mL^{-1} and 792.17 g mL^{-1}, respectively, highlighting its potential as an effective source of anticancer drugs [20]. In light of the medicinal value associated with cyanobacteria the present study focuses on investigating the anticancer properties of *Nostoc calcicola* against HT-29 colorectal adenocarcinoma cells.

Methods

Chemicals and solvents

All the chemicals and reagents used for this study were of standard analytical grade (NaOH, H_2SO_4, HCl, ferric chloride, ammonium molybdate, sodium phosphate, potassium ferricyanide, trichloroacetic acid, TCA, thiobarbituric acid, sodium pyruvate, ascorbic acid, Wagner reagent, Mayer's reagent were procured from Fisher Scientific (Chennai, India) and Hi Media Laboratories (Mumbai, India). Dulbecco's modified Eagle's medium (DMEM), 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES), DAPI (4',6-diamidino-2-phenylindole), beta-actin, MTT 3-(4, 5-dimethylthiozol-2-yl)-3, 5-diphenyl tetrazolium bromide) from Sigma Aldrich, protein assay kit from BioRad, India, nitrocellulose membrane (Millipore, Bangalore) solvents (methanol, benzene, chloroform were procured from Fisher Scientific (Chennai, India).

Collection, cultivation, and extraction of *Nostoc calcicola*

The *Nostoc calcicola* was obtained from NFMC (National Facility for Marine Cyanobacteria) in Tiruchirapalli and was cultivated in ASN III media to promote mass production. Freshly prepared media was sterilized, subjected to UV light exposure, and the media was replenished every 7 days to ensure the provision of essential minerals for optimal growth. Growth conditions included a controlled light/dark cycle of 16/8 h, a temperature of 22 ± 2 °C, and a light intensity of 3000 lx. Subculturing was conducted to sustain growth and attain substantial biomass production.

After 15 days, a significant quantity of *Nostoc calcicola* biomass was obtained, which was collected through centrifugation followed by drying, weighing, and extraction utilizing methanol. Filtration was employed to remove the extracted residue while the remaining solution was concentrated under reduced pressure using a rotary evaporator at a precisely controlled temperature of 60 ± 10 °C. The resulting concentrated extract enriched with bioactive substances served as the basis for further investigations [21].

Assessment of photochemical in *Nostoc calcicola*

Preliminary phytochemical screening of methanolic extract of *Nostoc calcicola* to identify the presence of bioactive secondary metabolites using standard methods as described by Uddin et al., in 2018 [22] with slight modification.

Flavonoids using alkaline reagent test where a mixture of 2 mL of 2.0% sodium hydroxide (NaOH) and the aqueous extract was prepared. This mixture displayed a concentrated yellow colour, which turned colourless upon the addition of dilute acid, confirming the presence of flavonoids.

Alkaloids using Wagner reagent—where two drops of Wagner reagent were added to 2 mL of the extract and thoroughly mixed. The development of a reddish colour indicated the presence of alkaloids. Further confirming using Mayer's Test by adding few drops of Mayer's reagent to 1 mL of the extract, resulting in the formation of a yellowish or white precipitate, confirming the presence of alkaloids.

Saponins by Froth's Test in which 2.0 mL of extract was shaken vigorously for 2 min. The appearance of frothing indicated the presence of saponins.

Terpenoids was identified using Salkowski's Test by adding 5.0 mL of extract with 2.0 mL of chloroform followed by addition of concentrated sulfuric acid to create a layered solution. A reddish-brown coloration at the interface confirmed the presence of terpenoids.

Steroids by Libbermann Burchard's Test in which 1.0 mL of extract along with 0.5 mL of acetic anhydride was taken and cooled using ice bath. Chloroform was added to the mixture followed by addition of 1.0 mL of concentrated sulfuric acid (H_2SO_4) at the sides of the tube. The presence of steroids was indicated by the formation of a reddish-brown ring at the junction of the two liquids.

Tannins were identified using ferric chloride test, where three drops of 5% ferric chloride was added to 1.0 mL of the extract, resulting in the formation of a greenish-black precipitate, indicating the presence of tannins. Further confirmed using lead acetate Test in which 5.0 mL of the extract and few drops of 1% lead acetate were added. The formation of a yellow precipitate indicated the presence of tannins.

Glycosides by Borntrager's Test—Ferric chloride was added to the extract, and the resulting solution was filtered. Dilute hydrochloric acid (HCl) and an organic solvent (benzene) were added to the filtrate, followed by dilute *ammonia*. The pink colour transitioned to red, indicating the presence of glycosides. Further confirmed using Killarkillani Test in which, this involved mixing 2 mL of the extract with glacial acetic acid, one drop of 5% ferric chloride (FeCl3), and concentrated sulphuric acid (H_2SO_4). A reddish-brown colour appeared at the interface of the two liquid layers, and the upper layer exhibited a bluish-green hue.

Phlobatannins was identified by adding 1% hydrochloric acid (HCl) to 10 mL of the extract, followed by heating in a boiling water bath. The formation of a red precipitate indicated the presence of phlobatannins.

Anthraquinones identification was done by taking equal volumes of extract and benzene were combined in a test tube and thoroughly mixed. Half the volume of 10% ammonia solution was added to this mixture. The presence of anthraquinones was confirmed by the development of red, pink, or violet coloration in the ammonia phase.

Phenol by Phthalein dye test—A mixture of 0.1 g of extract and 0.1 g of phthalic anhydride was heated with 1–2 drops of concentrated sulphuric acid. The resulting solution was then carefully poured into a beaker containing 15 mL of dilute sodium hydroxide solution. The presence of phenol groups was indicated by the appearance of a pink fluorescent colour.

Investigation of competence to attenuate oxidative damage by *Nostoc calcicola*

The radical scavenging capacity of the extracts was studied using.

DPPH radicals scavenging assay

The assessment of the ability of *Nostoc calcicola* extract to scavenge free radicals was conducted using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. A minor modification was employed following the method of Shimada et al. in 1992 [23]. The reaction mixture was prepared consisting of 1.0 mL of the Nostoc calcicola extract at various concentrations (ranging from 20 to 100 µg/mL) and 1.0 mL of a 0.8 mM/L DPPH solution. This mixture was then subjected to incubation under shaking conditions for 30 min, and the absorbance was recorded at 517 nm, using *ascorbic acid* as a control. The inhibition percentage, indicative of the DPPH radical scavenging capacity, was subsequently calculated.

$$\%\text{Decolourization} = \left[1 - \left(\frac{\text{ABS sample}}{\text{ABS control}}\right)\right] \times 100$$

Phosphomolybdenum assay

A solution was prepared by dissolving 10 mg of *Nostoc calcicola* extract in 1 mL of DMSO. From this solution,

100µL was extracted and combined with 1 mL of a reagent solution. The reagent solution was prepared by mixing 0.588 mL of sulphuric acid, 0.049 g of *ammonium molybdate*, and 0.036 g of *sodium phosphate*, and then adjusting the final volume to 10 mL with distilled water. This mixture was then incubated in a boiling water bath at 95 °C for 90 min. After this incubation period, the absorbance of the solution was measured at 695 nm. As a standard, ascorbic acid (prepared at a concentration of 10 mg/mL in DMSO) was used. The Phosphomolybdenum reduction potential (PRP) of the tested extracts was reported as a percentage. Prieto et al., (1999) [24].

Ferric ions (Fe3 +) reducing antioxidant power assay (FRAP)

The ferric ion reducing power of *Nostoc calcicola* was evaluated following the methodology of Oyaizu et al. from 1986 [25], with slight modifications. Specifically, 20–100 µg/mL of *Nostoc calcicola* extract was blended with 2.5 mL of a 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of a 1% potassium ferricyanide $[K_3Fe(CN)_6]$ solution, after which the mixture was incubated at 50 °C for a duration of 20 min. Following incubation, 2.5 mL of 10% trichloroacetic acid was added to the mixture, followed by centrifugation at 3000 rpm for 10 min. Subsequently, 2.5 mL of deionized water and 0.5 mL of a 0.1% FeCl3 solution were added to 2.5 mL of the resulting supernatant. The absorbance at 700 nm was measured in comparison to ascorbic acid, which served as a positive control.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was assessed using the method developed by Halliwell, Gutteridge, and Arouma in 1987, with slight adjustments [26]. To prepare stock solutions, EDTA (1 mM), $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM), and deoxyribose (10 mM) were dissolved in distilled deionized water. For the reaction mixture, the following components were combined sequentially: 0.1 mL of EDTA, 0.01 ml of $FeCl_3$, 0.1 mL of H_2O_2, 0.36 mL of deoxyribose, 1.0 mL of Nostoc calcicola extract (at concentrations of 20–100 µg/mL), 0.33 ml of phosphate buffer (50 mM, pH 7.4), and 0.1 mL of ascorbic acid. The resulting mixture was then incubated at 37 °C for 1 h. Subsequently, 1.0 mL of this mixture was extracted separately and mixed with 1.0 mL of 10% TCA (trichloroacetic acid) and 1.0 mL of 0.5% TBA (thiobarbituric acid). The absorbance of this mixture was measured at 532 nm.

$$\text{Hydroxyl scavenging activity}(\%) = (A0 - A1/A0) \times 100)$$

($A1$ is the absorbance of standards or reaction mixture, $A0$ is the absorbance of the negative control).

Determination of cytotoxicity

Cell lines and culturing

The Human colorectal adenocarcinoma (HT-29) cells were sourced from the National Center for Cell Sciences (NCCS) in Pune, India. The cells were maintained and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM l-glutamine and a balanced salt solution (BSS). The BSS composition was adjusted to incorporate 1.5 g/L Na_2CO_3, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES), 2 mM l-glutamine, 10% fetal bovine serum (GIBCO, USA) followed by the addition of 1 mL/L penicillin and streptomycin solution (100 IU/100 µg). The optimal growth and viability were ensured regularly providing 37 °C temperature with a 5% CO_2 concentration in a humidified CO_2 atmosphere throughout the experimental period.

Antiproliferative action against colorectal cancer

Cell viability of *Nostoc calcicola* was assessed using the MTT assay with slight modifications, as previously described by Khader et al. [27]. HT-29 cells were cultivated in a 96-well plate until reaching a confluence of 75–80% after 48 h. The medium was then replaced with a fresh complete medium along with *Nostoc calcicola* extract (10–50 µg/mL), and incubated for an additional 48 h. After the incubation period, the culture medium was removed, and each well was treated with 100µL of MTT (3-(4, 5-dimethylthiozol-2-yl)-3, 5-diphenyl tetrazolium bromide) solution (Hi-Media) and incubated again for 4 h at 37 °C to obtain formazan crystals. Further, the supernatant was discarded followed by the addition of 50µL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals in each well and incubated for 10 min. The optical density (OD) of each well was measured at 620 nm using an ELISA multiwell plate reader (Thermo Multiskan EX, USA). The results were compared with the standard control doxorubicin.

The OD value was used to calculate the percentage of viability using the following formula

$$\%\ \text{of viability} = \frac{\text{OD of experimental sample}}{\text{OD of experimental control}} \times 100$$

Morphological screening

The selected cells that were grown on coverslips (1×10^5 cells/coverslip) were incubated with *Nostoc calcicola* at different concentrations and then fixed in ethanol: acetic acid solution (3:1, v/v). The coverslips were gently mounted on glass slides for the morphometric analysis. Three monolayers per experimental group were micrographed and the morphological changes of the cells were

analyzed using Nikon (Japan) bright field inverted light microscopy at 10× magnification [28]

Fluorescence microscopy analysis

Acridine orange (AO) and ethidium bromide (EtBr) staining

To perform staining and visualization, a dye mixture consisting of acridine orange (AO) and ethidium bromide (EtBr) at concentrations of 100 mg/mL each in distilled water was prepared. In a clean microscope coverslip add 1 µL of this dye mixture with 90 µL of a cell suspension containing a concentration of 1×10^5 cells/mL. The cells were initially washed with phosphate-buffered saline (PBS) at pH 7.2, stained with 10 µL of the AO/EtBr dye mixture along with different concentrations (10, 25, 50 µg/mL) of *Nostoc calcicola* extract and incubated for 2 min. The cells were washed twice with PBS buffer to remove excess stain and visualized using a fluorescence microscope (Nikon Eclipse, Inc, Japan) at a magnification of × 400, with an excitation filter set at 480 nm [29].

DAPI staining

DAPI (4′,6-diamidino-2-phenylindole) assay was performed in a clean glass microscope coverslip seeded with cells, placed in a 24-well plate, and treated with different concentrations of the *Nostoc calcicola* extract (10, 25, 50 µg/mL) for a period of 24 h. Further, the fixed cells were permeabilized using 0.2% triton X-100 (50 µL) for 10 min at room temperature and incubated with 10 µL of DAPI stain for 3 min and another coverslip is placed over the cells to ensure the uniform spreading of the stain. The stained cells were observed under a fluorescent inverted microscope (× 400), as described by Phull et al. [30].

Cell cycle arrest analysis

Cell cycle analysis was conducted to assess the effects of the *Nostoc calcicola* extract on HT-29 cells. The cells were initially seeded in a 96-well plate and incubated at 37 °C with 5% CO_2 for 24 h. Subsequently, the medium in each well was replaced with fresh medium supplemented with varying concentrations of the *Nostoc calcicola* extract (10–50 µg/mL), while a control group received an unsupplemented medium and incubated for 24 h. The cells were harvested using trypsin, washed with PBS, treated with 70% ethanol to fix the cell, and stored at − 20 °C for 1 h. For staining the cellular nuclear DNA, propidium iodide (PI) was employed. The ethanol-fixed cells were washed following centrifugation and resuspended in 50 µg/mL PI, 100 µg/mL RNase in PBS, and incubated at 37 °C for 30 min. Flow cytometry analysis was performed using a BD FACS flow cytometer, with duplicate samples analyzed. A total of 10,000 events were collected from each sample, and the fluorescence signal intensity was recorded during the analysis. These experimental procedures closely follow the methodology outlined by Khader et al. [31].

Table 1 Preliminary phytochemical analysis of methanol extract of *Nostoc calcicola*

Phytochemicals	Methanol extract of *Nostoc calcicola*
Glycosides	–
Alkaloids	–
Saponins	+
Flavonoids	+
Tannins	+
Terpenoids	+
Phenol	+
Phlobatannins	–
Anthroquinones	–
Steroids	+

(+)Presence, (–) Absence

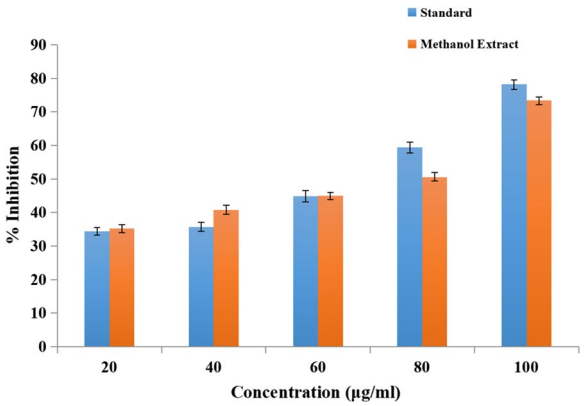


Fig. 1 Effect of *Nostoc calcicola* on DPPH (2,2-diphenyl-1-picrylhydrazyl)

Western blot assay of anticancer and apoptosis-related proteins

To assess the regulation of apoptotic and anti-apoptotic proteins in the treated cells, Western blotting was performed. HT-29 cells were seeded onto 100 mm culture dishes at a density of 1.5×10^6 cells and treated with *Nostoc calcicola* extract for 24 h. Following treatment, the medium was removed, and the cells were washed multiple times with PBS (0.01 M, pH 7.2). Subsequently, the cells were lysed with lysis buffer (0.1 mL per plate) for 20 min and the lysate was obtained by centrifugation at 10,000×*g* for 5 min at 4 °C, and the protein concentration in the harvested lysate was quantified using a protein assay kit. Equal amounts of protein (100 µg) from each lysate were loaded onto a 12% SDS polyacrylamide gel for

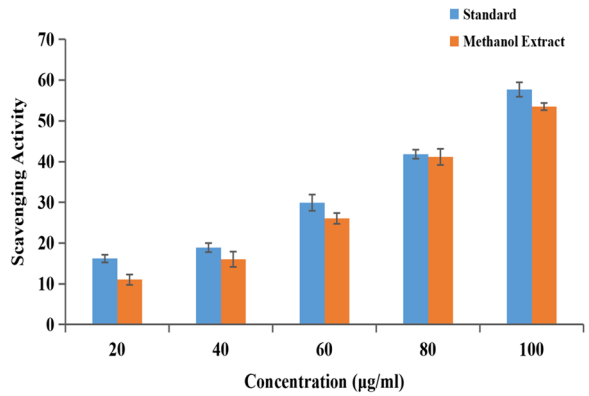


Fig. 2 Effect of *Nostoc calcicola* on Phosphomolybdenum Assay

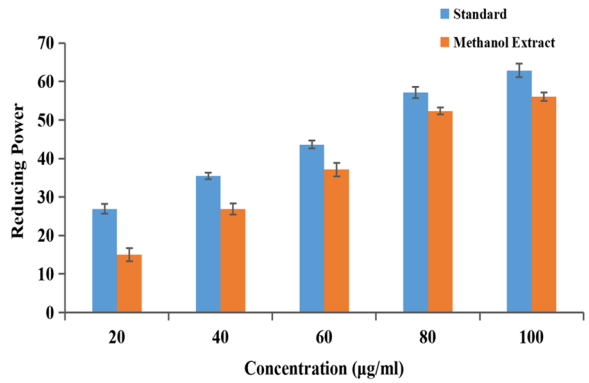


Fig. 3 Effect of *Nostoc calcicola* on FRAP (ferric ions (Fe3+) reducing antioxidant power assay)

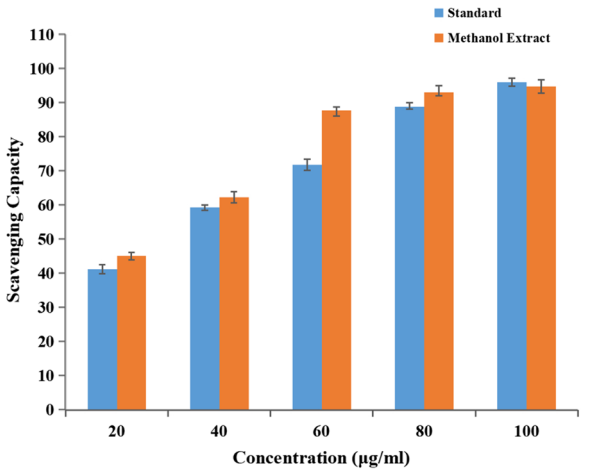


Fig. 4 Effect of *Nostoc calcicola* on Hydroxyl free radical scavenging capacity assay

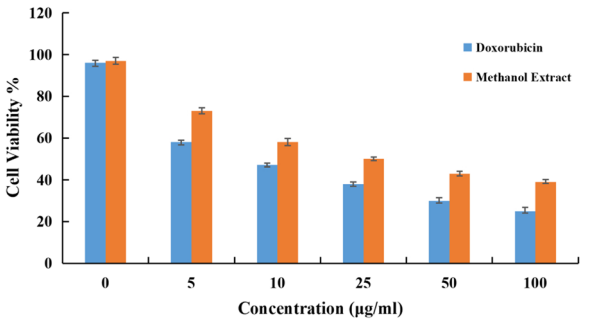


Fig. 5 Effect of *Nostoc calcicola* on cell viability (HT-29)

electrophoresis. The proteins were then transferred onto a nitrocellulose membrane (Millipore, Bangalore) and blocked with 10% skimmed milk in water for 1 h. After washing the membrane with PBS containing 0.1% Tween 20, primary antibodies against caspase-9 and beta-actin were added at a volume-to-volume ratio of 1:1000. The membrane was incubated overnight at 4 °C with the primary antibodies. Following overnight incubation, the primary antibodies were washed off, and secondary antibodies were added to the membrane for 1 h of incubation at room temperature. This step allowed for the detection of the primary antibodies bound to their respective target proteins [32]. The Intensity of blots were quantified with densitometric analysis and Amersham Image Quant™ 800, Image J software, instrument was used for densitometric analysis.

GC–MS analysis

The gas chromatography-mass spectrometry (GC–MS) profiling was performed using Clarus 680 GC fused with silica column packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250 µm df). Helium was employed as carrier gas at a constant flow of 1 mL/min and the injector temperature was set at 250 °C during the chromatographic run. The analysis was carried out by injecting 1µL of extract sample into the instrument and the oven temperature was maintained at 50 °C (1 min) followed by 280 °C at the rate of 10. The mass detector (transfer line temperature 280 °C; ion source temperature 200 °C and ionization mode electron impact at 70 eV) was conducted with a scan time 0.2 s and scan interval of 0.1 s [33, 34]. The spectrums

Table 2 Cytotoxic activity (IC_{50} µg/ml) of *Nostoc calcicola* against colorectal adenocarcinoma (HT29) cell line

Species	Cell line	Inhibitory concentration (IC_{50} µg/ml)	
		Extract	Doxorubicin
Nostoc calcicola. methanol extract	HT-29	25 ± 0.4	14 ± 0.5

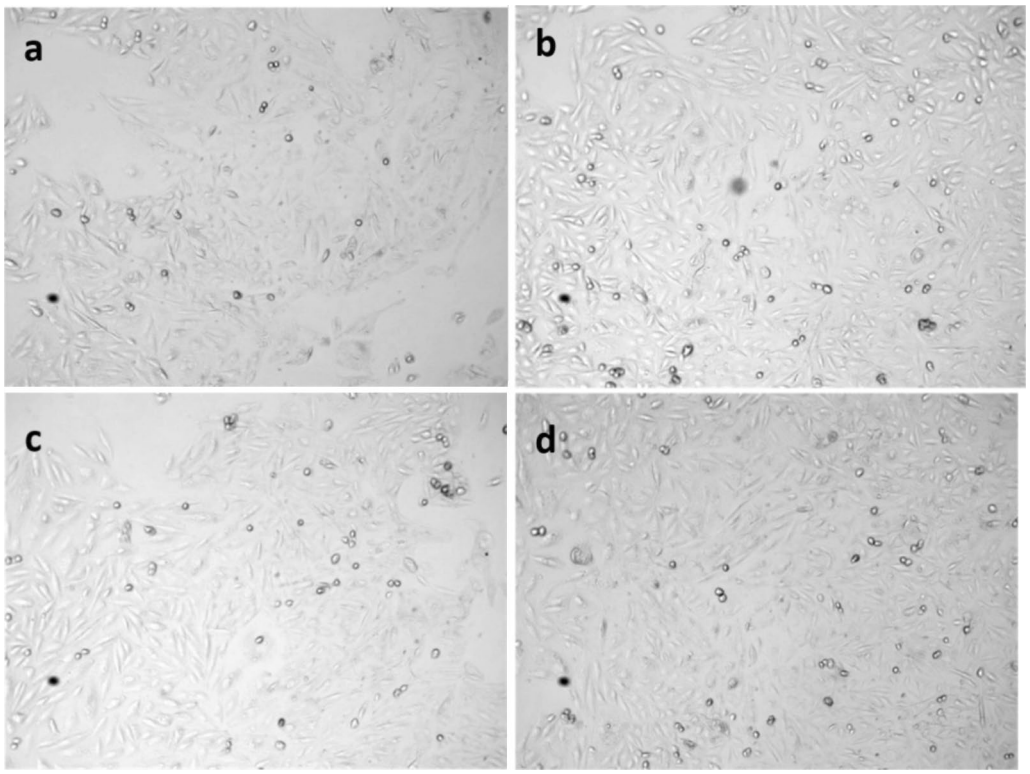


Fig. 6 Effect of *Nostoc calcicola* on morphology of human colorectal adenocarcinoma (HT-29) cell line magnification at 10×. **a** Control untreated cells (with no change), **b**–**d** *Nostoc calcicola* -treated cell at the concentrations of 10, 25, and 50 µg/mL (representing distinct changes in cell structure)

of the components were compared with the database of spectrum of known components in the GC–MS NIST (2008) library.

Statistics

All the in vitro experiments were carried out in triplicate, and the experiments were repeated at least thrice and expressed as the Mean ± S.D. and all the grouped data were statistically evaluated with SPSS\17.0 software.

Results

Phytochemical analysis

Qualitative phytochemical analysis of the methanol extract of *Nostoc calcicola* represented the presence of saponins, flavonoids, tannins, terpenoids, steroids, and phenols (Table 1).

Potential of *Nostoc calcicola* to counteract oxidative stress

The study aimed to assess the inhibitory effects of different concentrations of *Nostoc calcicola* extract revealing significant and dose-dependent inhibition, with a noticeable increase in inhibitory activity as the concentration of the extract increased. However, it is important to note that the extent of inhibition varied depending on the specific assay employed.

The antioxidant potential of the methanol extract of *Nostoc calcicola* was evaluated using four different methods. The extract was able to quench DPPH radicals in a dose-dependent manner, with inhibition percentages ranging from 35.2 to 73.4% (Fig. 1) at the concentration of 20–100 µg/mL with was on par with the standard (ascorbic acid) ($P < 0.01$). The extract also exhibited a significant ability to reduce the phosphomolybdenum complex to a green phosphate form at different concentrations (20–100 µg/mL), with inhibition percentages ranging from 11.0 to 53.5% (Fig. 2) with a significance ($P < 0.05$). Moreover, the extract demonstrated a considerable ferric-reducing power, which reflects its capacity to donate electrons, with inhibition percentages ranging from 15.1 to 56.2% at different concentration ranging from 20 to 100 µg/mL (Fig. 3). Furthermore, the extract showed a remarkable hydroxy radical scavenging activity at different concentrations (20–100 µg/mL), which indicates its ability to protect biomolecules from oxidative damage, with inhibition percentages ranging from 45.1 to 94.8% (Fig. 4) with a significance of ($P < 0.05$).

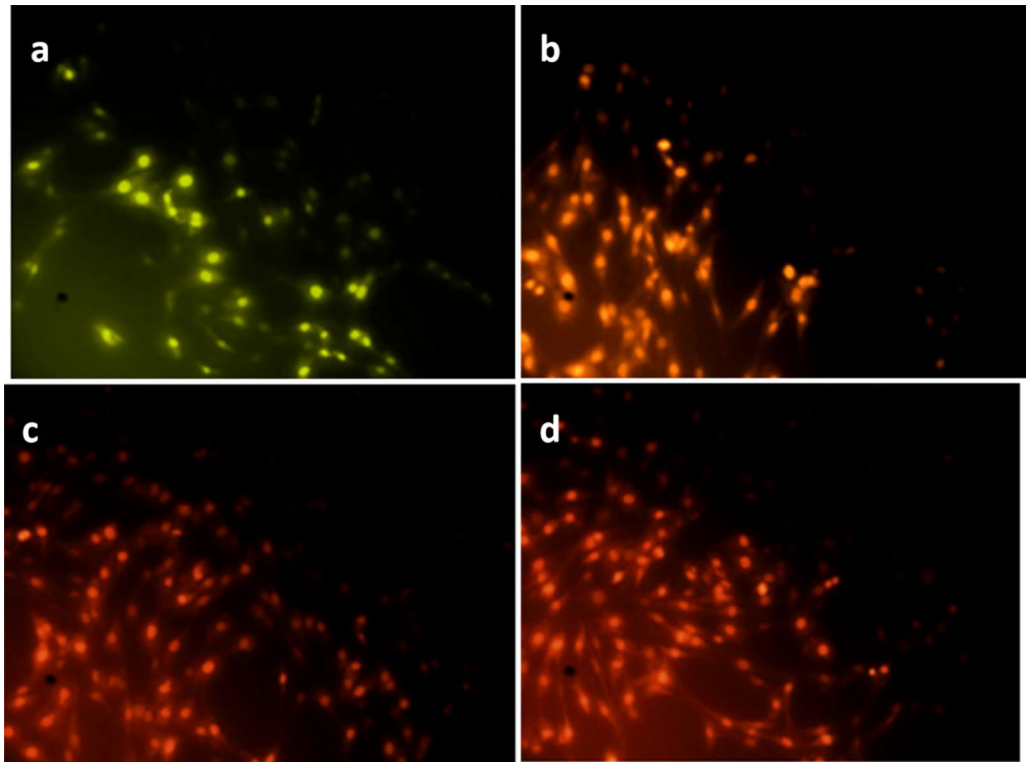


Fig. 7 Cellular morphology of *Nostoc calcicola*-treated HT-29 cancer cells following AO/EtBr staining using fluorescence microscope magnification at 400×. **a** Control untreated cells, **b**–**d** *Nostoc calcicola*-treated cell at different concentrations representing apoptotic cells, necrotic cells with orange/red colour

Assessment of anticancer activity of *Nostoc calcicola* on colorectal adenocarcinoma (HT-29) cell line

Antiproliferative potential of Nostoc calcicola

The colorectal adenocarcinoma cells were treated with different concentrations of extract of *Nostoc calcicola* showing its cytotoxicity effect on the HT-29 cell line tested using MTT assay where growth inhibition was examined comparing with the standard drug doxorubicin (Fig. 5). Treatment with *Nostoc calcicola* exhibited dose-dependent inhibition upon HT-29 adenocarcinoma cells and the observed IC_{50} value was found to be 25 µg/mL (Table 2).

Figure 6 illustrates the alterations in cell morphology of HT-29 cancer cells upon treatment with the methanol extract of *Nostoc calcicola.* In the absence of treatment (control), the cells exhibited a healthy cytoskeleton structure, indicating cell proliferation. However, when treated with different concentrations of the extract, notable changes in cell morphology were observed. These changes included cell shrinkage, irregular cell shape, cell clustering, and the formation of membrane protrusions known as blebs. These morphological alterations are characteristic signs of cell death and were found to be dependent on the dosage of the extract.

Fluorescence microscopy determination

Acridine orange (AO) and ethidium bromide (EtBr) staining activity

Fluorescence microscopy image of colorectal adenocarcinoma cells after being stained with A0/EtBr in the presence and absence of the extract is represented in Fig. 7. It is found that in Fig. 7a, the cells treated with the extract exhibited green fluorescence, indicating the presence of viable cells, and in Fig. 7b–d, the cells displayed a yellow-orange fluorescence at 10 µg/mL and 25 µg/mL, indicating the early stages of apoptosis. Subsequently, the cells transitioned to red fluorescence at 50 µg/mL, representing the late-stage process characterized by necrosis resulting from induced apoptosis. This transition was accompanied by nuclear condensation, as observed in the cells treated with the methanol extract of *Nostoc calcicola*.

DAPI staining activity

The nuclear condensation and fragmentation in colorectal cancer cells were visualized using DAPI staining with different concentrations of the extract of *Nostoc calcicola* illustrated in Fig. 8. The images captured both in the absence and presence of drugs demonstrate the

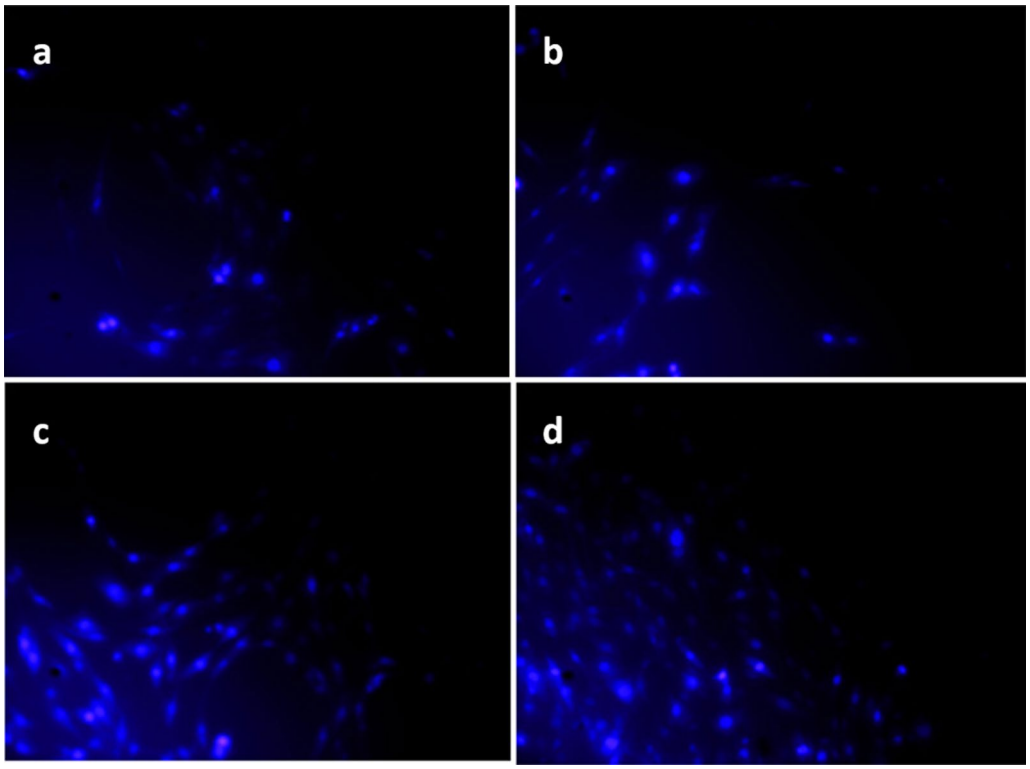


Fig. 8 Cellular morphology of *Nostoc calcicola*-treated HT-29 cancer cells-treated following DAPI staining using a fluorescence microscope magnification at 400×. **a** Control untreated cells, **b**–**d** *Nostoc calcicola*-treated cell at different concentrations representing nuclear condensation and DNA fragmentation

observed effects. In Fig. 8a, cells without treatment of the extract showed no significant changes in nuclear appearance, whereas *Nostoc calcicola* treated groups (varying concentrations ranging from 10 to 50 µg/mL) exhibited pronounced nuclear fragmentation and chromatins condensation when compared to the untreated cells indicated by bright spots (Fig. 8b–d).

Phase distribution by flow cytometry

To evaluate the potential anticancer activity of *Nostoc calcicola*, cell cycle analysis, and flow cytometry were employed. HT-29 cells were treated with the methanol extract of *Nostoc calcicola* at their respective IC_{50} concentrations for 24 h to investigate the inhibition of cell growth. The flow cytometry data, presented in Fig. 9, clearly demonstrate the impact of the compound on the cell cycle, resulting in inhibition at different phases. In comparison to the untreated control group, which shows an accumulation of cells in the G0–G1 phase, the treated cells display a significant reduction in the percentage of cells in the S phase.

Caspase study

In Fig. 10, the application of Western blotting is depicted, which enables the detection of cleaved caspase-9. Cleaved caspase-9 serves as a crucial indicator of caspase-9 activation, which plays a vital role in the intrinsic apoptosis pathway. This activation process relies on significant events such as mitochondrial membrane disruption and apoptosome formation.

GC–MS analysis

GC–MS analysis was performed to identify the presence of various bioactive compounds in *Nostoc calcicola* (Table 3). The analysis resulted in a distinctive chromatogram displayed in Fig. 11 illustrating the presence of various compounds among which four major molecules are represented. The compounds identified were Hexadecanoic acid, (2-pentadecyl-1,3-dioxolan-4-yl)methyl ester (Fig. 11a), 5-Amino-1-ethyl-1,2,3-triazole-4-carboxamide (Fig. 11b), 1,2,3-thiadiazole, propanoic acid derivative (Fig. 11c), 3,4-Dihydroxymandelic acid, ethyl ester, tri-TMS (Fig. 11d),. The spectrum of the components was

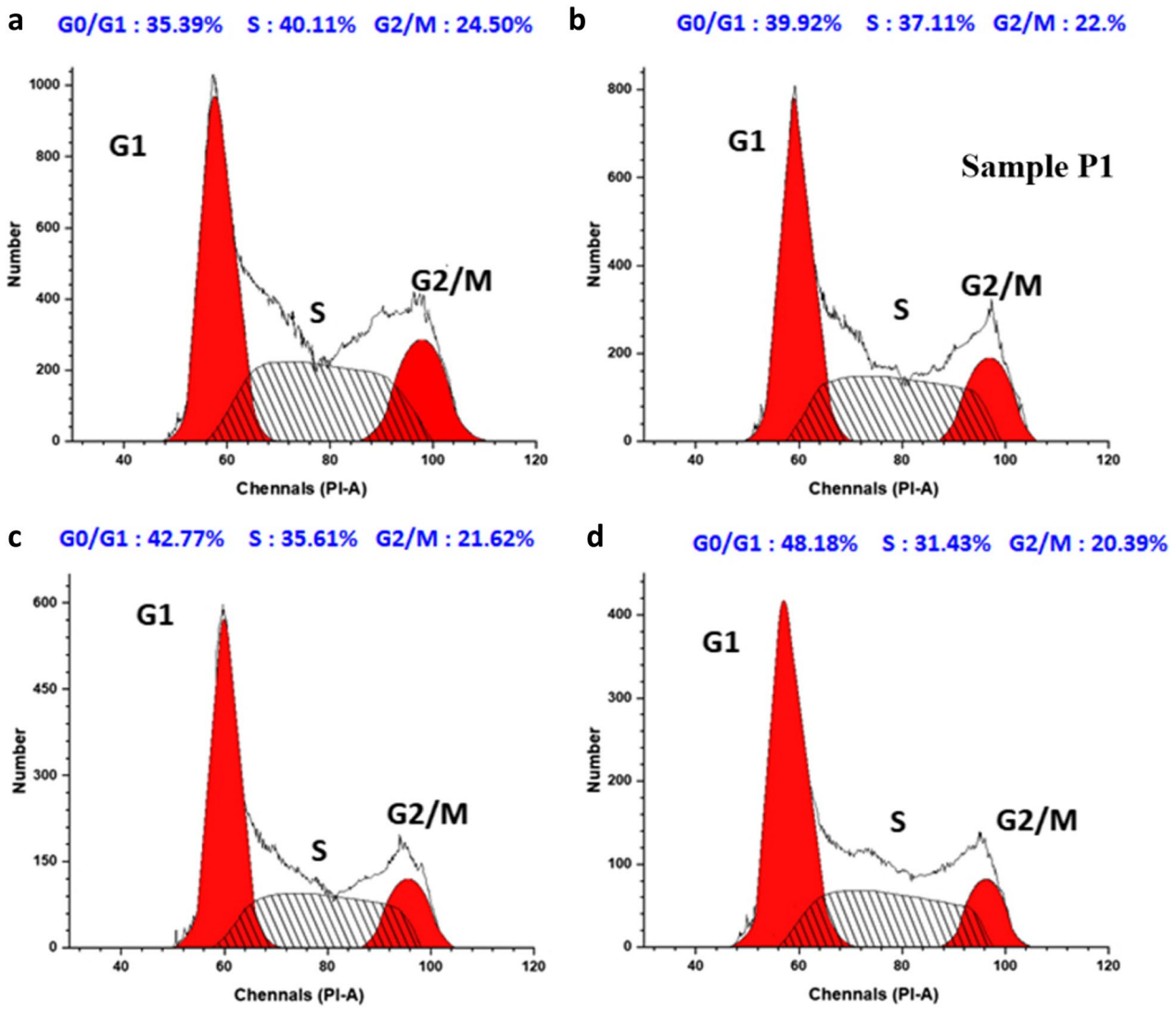


Fig. 9 Effect of *Nostoc calcicola* on cell cycle progression of HT-29 cells using flow cytometry. **a** Control cells, **b**–**d** *Nostoc calcicola*-treated cells at different concentrations representing cell cycle arrest in S phase

compared with the database of the spectrum of known components stored in the GC–MS NIST-WILEY' library search.

Discussion

Cyanobacterial were grown under standard laboratory conditions and extracted through cold extraction using methanol as a solvent the presence of phytochemicals was analyzed by following standard protocol and secondary metabolites were identified (Table 1). The phytochemical analysis revealed the presence of various compounds in the methanol extract of *Nostoc calcicola*, including saponins, flavonoids, tannins, terpenoids, steroids, and phenols where these compounds are known for their antioxidant properties. The results are in line with the previous research demonstrating the effect of *Nostac* species exhibiting the potent radical scavenging activity and effective antimicrobial activity tested against selected microbial species like *Escherichia coli* and *Salmonella bongori* [34, 35].

Research has proved that reactive oxygen species (ROS) are molecules that cause damage to the system and antioxidants are the ones that neutralize these ROS, protecting the system from further damage or disease. Some natural compounds, such as phenols, polyphenols, and flavonoids, have reported strong antioxidant

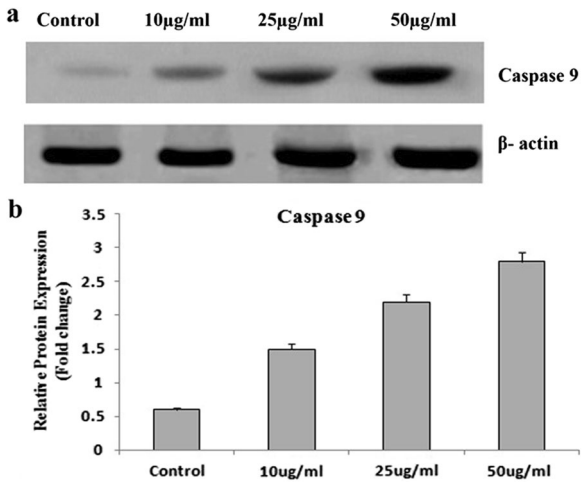


Fig. 10 **a**, **b** Effect of *Nostoc calcicola* on the protein expression of caspases through western blot using β-actin as the loading control and different concentration of *Nostoc calcicola*-treated HT-29 cancer cells

properties that can prevent reactive oxygen species from reacting with and destroying biomolecules like proteins, lipids, and carbohydrates. Overall, this can help prevent cell, tissue, and organ damage that can lead to cancer. The current study focuses and to evaluate the relationship between ROS and cancer.

DPPH is a nitrogen-centered free radical widely used to test the ability of compounds to function as free radical scavengers [36–38]. The antioxidant potential of the extract was evaluated using various assays that measured its ability to quench different reactive oxygen species, exhibiting significant scavenging activity against DPPH radicals, with a percentage inhibition of 73.4% at 100 µg/mL, compared to 78.2% for the standard shown in Fig. 1. Previous research has shown a similar effect of *Nostoc* sp. N42 illustrates 35.5% activity at the highest concentration [39] which shows *Nostoc calcicola* has a more significant activity to scavenge free radicals.

The extract also demonstrated considerable phosphomolybdenum reduction which is a widely employed method for assessing the antioxidant capacity of samples based on their ability to reduce molybdenum (Mo) from a Mo (VI) to Mo (V) state, where the results exhibited dose-dependent inhibition with the highest percentage of 53.5% at 100 µg/mL concentration (Fig. 2). The phosphomolybdenum reduction phenomenon is explored using cyanobacteria *firmicutes* and the study is on par with the current research revealing the capacity of *Nostoc calcicola* extract in reducing molybdenum [20].

Furthermore, the results of the ferric-reducing antioxidant power assay (FRAP) with *Nostoc calcicola* demonstrated potent inhibition activity with 55.1% at 100 µg/mL concentration (Fig. 3). The results are in line while comparing the results of other cyanobacteria found across the Odisha coast, India representing the capacity of cyanobacteria to combat free radicals [27]. Hydroxyl radical scavenging capacity was analyzed using the ability to neutralize hydroxyl radicals revealing 94.8% inhibition activity and the results are significantly similar to that of the results of phycocyanin pigment extracted from marine filamentous cyanobacteria [40, 41].

Research has proved that ROS are the by-products of cellular metabolism and have physiological functions, meanwhile, excessive ROS production and oxidative stress can lead to DNA damage, mutations, and cellular dysfunctioning [42] even leading to initiation, promotion, and progression of cancer. The interplay between ROS, cellular redox systems, and cancer involves various signaling pathways, including those related to cell survival, proliferation, and apoptosis. Targeting ROS and oxidative stress holds therapeutic potential, although the indiscriminate use of antioxidants may interfere with ROS-mediated signaling and accelerate cancer progression. Therefore, cancer cells are more prone to apoptosis in comparison to normal cells when treated with ROS-inducing agents [43]. These findings highlight the potent inhibitory properties of the methanol extract of *Nostoc calcicola*, which were evident to eliminate these radicals from the system.

The essential expression of the proliferation inhibition of extract against the cancer cell is shown by

Table 3 GCMS spectral data with major compounds from *Nostoc calcicola*

Peak	R.time	Area	Area%	Height	Name
55	24.920	10,833	0.76	5589	HEXADECANOIC ACID, (2-PENTADECYL-1,3-DIOXOLAN-4-YL)METHYL ESTER
20	22.284	2055	0.14	1419	5-AMINO-1-ETHYL-1,2,3-TRIAZOLE-4-CARBOXAMIDE
38	23.908	3122	0.22	2067	1,2,3-THIADIAZOLE, PROPANOIC ACID DERIVATIVE
56	24.984	19,485	1.36	7551	3,4-DIHYDROXYMANDELIC ACID, ETHYL ESTER

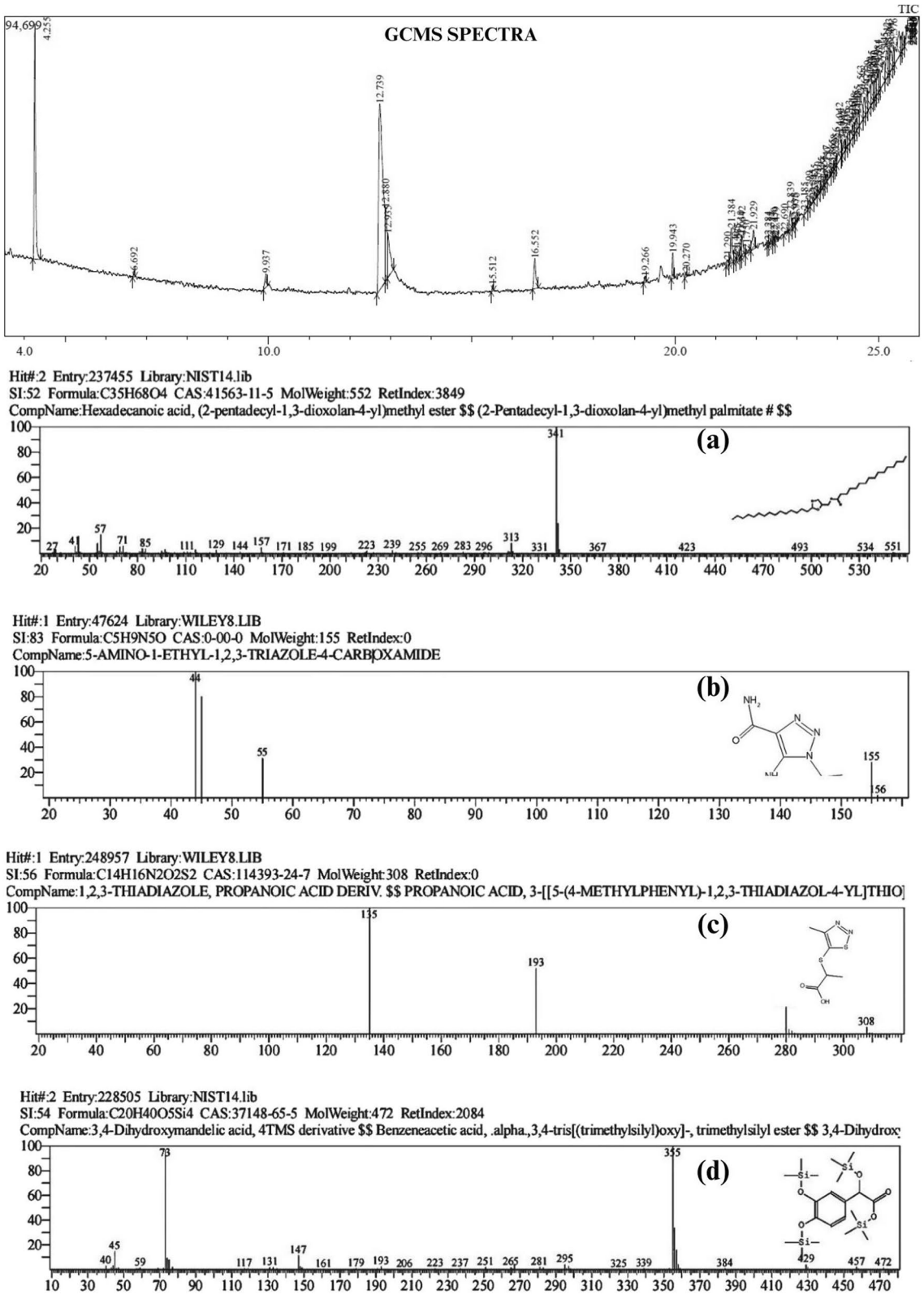


Fig. 11 GCMS profiling showing mass spectra with the structure of major compound in the *Nostoc calcicola* methanol extract **a** Hexadecanoic acid, (2-pentadecyl-1,3-dioxolan-4-yl)methyl ester, **b** 5-Amino-1-ethyl-1,2,3-triazole-4-carboxamide, **c** 1,2,3-thiadiazole, propanoic acid derivative and (d) 3,4-Dihydroxymandelic acid, ethyl ester, tri-TMS

morphological changes in the cell, which provides preliminary information regarding the mechanism of cytotoxicity. Inconsistent assembly of microtubules and disruption in cytoskeletal function were seen following treatment, as reported by other studies [28] whereas the essential expression of the proliferation inhibition of extract against the cancer cell is shown by morphological changes in the cell, which provides preliminary information regarding the mechanism of cytotoxicity. The untreated cells displaying cell proliferation were shown to have a healthy cytoskeleton structure. Additionally, extract-treated cells at various dosages displayed morphological alterations, such as cell shrinkage, uneven shape, clustering of cells, and blebbing, which are signs of cell death and are dose-dependent which was similar to the findings explored by previous studies [44, 45]. These findings suggest the observed morphological changes and potential cytotoxic effects of *Nostoc calcicola* on the HT-29 cell line and highlight its potential as an anti-cancer agent in a dose-dependent manner.

According to research, during cancer uncontrolled cell proliferation and tumor growth are observed, and the process of apoptosis is disrupted by uncontrolled cell growth. DNA damage is one of the triggers for apoptosis and is commonly observed in cancer cells due to various factors such as genetic mutations, exposure to carcinogens, or defects in DNA repair mechanisms [46]. AO/EtBr is a fluorescent dye that intercalates with DNA and can be used to visualize apoptotic cells. The results of apoptotic staining revealed *Nostoc calcicola* performed as a potential agent to induce apoptosis to stop the uncontrolled growth of cancer showing loss of the integrity of cell membranes by a change in the fluorescence from orange to red indicating the process of apoptosis or necrosis of cells. Whereas, in the control which typically consists of untreated cells, the AO dye can penetrate both live and dead cells so, AO dye stains the nuclei of all untreated cells green, indicating the intact cellular structure and their viability. While EtBr dye, on the other hand, cannot enter live cells, they do not show any red fluorescence indicating the absence of apoptosis and necrosis phenomena. Hence from the results it is observed that *Nostoc calcicola* exhibit and provokes apoptosis.

DAPI (4′,6-diamidino-2-phenylindole) is a fluorescent DNA-binding dye that specifically labels the nuclei of cells. After the treatment of the cells with different concentrations of extract of *Nostoc calcicola* alterations in nuclear morphology is seen, such as condensation or fragmentation, are indicative of cellular changes associated with apoptosis or other forms of cell death. As the concentration increases the nuclear fragmentation also increases (Fig. 7). These findings suggest that the *Nostoc calcicola* induces nuclear condensation and fragmentation in HT-29 cancer cells, highlighting their potential impact on cellular morphology and potentially indicating their cytotoxic effects. These nuclear condensations and fragmentation suggest the cells are arresting which can be accessed by flow cytometry. The phase distribution studies finding indicates that the *Nostoc calcicola* treatment suppresses cell progression through the S phase, supporting its potential as an anticancer agent. These results are consistent with a previous study, which reported strong inhibitory effects by causing DNA damage and loss of mitochondrial membrane potential, leading to cells with condensed chromatin and reduced size and arresting the cell cycle [30, 31, 47].

Caspase is one of the critical regulatory enzymes determining the proper execution of the apoptotic pathway leading to cell cycle arrest and the quantification of particular proteins (Caspase) in a complex protein mixture is carried out utilizing the Western blotting technique [48]. The expression and activation of caspase 9 at specific time points during cell cycle progression or in response to various stimuli are analyzed which exemplifies the process of apoptosome formation and mitochondrial membrane disruption. Hence from the results it is understood that there is a cessation in DNA duplication thereby inhibiting the growth of cancer cells upon treatment with *Nostoc calcicola*.

The GC–MS chromatogram obtained for *Nostoc calcicola* revealed various compounds using GC–MS NIST -WILEY' library search (Table 3) among which four major compounds are represented with potential anticancer activity. The bioactive compounds present in *Nostoc calcicola* have been identified for its nature, which revealed the compounds such as Hexadecanoic acid, (2-pentadecyl-1,3-dioxolan-4-yl)methyl ester, 5-Amino-1-ethyl-1,2,3-triazole-4-carboxamide, 1,2,3-thiadiazole, propanoic acid derivative, 3,4-Dihydroxymandelic acid, ethyl ester, tri-TMS. The compound 5-Amino-1-ethyl-1,2,3-triazole-4-carboxamide is a derivative of triazole which has the potential anticancer activity, 3,4-Dihydroxymandelic acid, ethyl ester, tri-TMS can be used as a biomarkers, especially for neuroendocrine tumors. The compound 1,2,3-thiadiazole, propanoic acid derivative was reported to have anticancer activity along with other biological activities. Hexadecanoic acid, (2-pentadecyl-1,3-dioxolan-4-yl)methyl ester was reported to have anticancer activity [49].

Conclusion

The current research investigated the potential of *Nostoc calcicola* articulating the presence of bioactive phytoconstituents responsible for its anticancer activity by initiating apoptotic pathway and inducing cell cycle arrest. Moreover, the harmful effect of free radicals and counteract oxidative stress is neutralized by the action of *Nostoc calcicola* reducing the complications of cancer associated with oxidative stress. The results emphasize the potential of *Nostoc calcicola* as a source of anti-cancer drugs after further exploration that holds promise for cancer management.

Abbreviations

HT-29	Human colorectal cancer cell line
DPPH α	α-Diphenyl-β-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
RPM	Revolutions per minute
TCA	Tri chloroacetic acid
DMSO	Dimethyl sulfoxide
NBT	Nitroblue tetrazolium
EDTA	Ethylenediamine tetraacetic acid

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Author contributions

Ms. PG and Ms. AKR carried out the entire experiment, Dr. SS and Dr. SZA hypothesized, drafted and coordinated all the experiments and represented to be a correspondence of the manuscript. Mr. SS and Dr. PP involved and helped anticancer activity. All authors read and approved the final manuscript.

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Availability of data and materials

The corresponding author can provide the data that were utilized to support the study's conclusions upon request.

Declarations

Ethical approval and consent of participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare competing interests.

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