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Algae-Synthesized Bismuth Nanoparticles for Drug Delivery in A549 Lung Cancer Cells

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ABSTRACT

The increasing prevalence of lung cancer, compounded by the limitations of conventional therapies, necessitates the exploration of innovative drug delivery systems. This study presents a novel approach to synthesizing bismuth nanoparticles (BiNPs) using *Chlorella* sp. extracts, aimed at enhancing targeted drug delivery for the human lung cancer cell line (A549). An extract of *Chlorella* sp. and bismuth nitrate was used to prepare BiNPs under optimized conditions. The nano-solution was characterized by various techniques. Gas chromatography-mass spectrometry (GC-MS) analysis was employed to identify the active algal phytocompounds. The cytotoxic activity of the BiNPs was tested against A549, while the normal human fibroblast cell line (NHF) was used to evaluate the biosafety of the nano-solution. Characterization using UV-vis spectroscopy and X-ray diffraction confirmed the successful synthesis of BiNPs, indicating a relative size of 26 nm. Cytotoxicity assay demonstrated that BiNPs exert a dose-dependent effect on A549 cells, showing significant selective toxicity with an IC_{50} of 5.797 µg/mL, while minimizing affecting NHF cells, which had an IC_{50} of 17.68 µg/mL. Furthermore, morphological assessments via microscopy indicated that BiNPs induced distinct apoptotic features in A549 cells. Gas chromatography-mass spectrometry analysis of the algal extract revealed the presence of bioactive compounds, including terpenoids and fatty acids, known for their antioxidant and anticancer properties, which may synergistically enhance the therapeutic efficacy of BiNPs. The study highlighted *Chlorella*-synthesized BiNPs as a promising targeted drug delivery system, advancing cancer nanomedicine and addressing challenges in traditional chemotherapy for lung cancer treatment.

1. INTRODUCTION

Phyconanotechnology, a branch of bionanotechnology, is an emerging field within nanoscience focused on synthesizing nanoparticles of various sizes and shapes using algal metabolites. Algal-derived biomolecules act as reducing and capping agents, offering ease of processing and enhanced biosafety [1]. It is proposed that using plant extracts in nanosynthesis may yield a synergistic effect, enhancing medicinal efficacy by creating nanomaterials with stronger or novel biological activities [2].

Despite the best efforts of scientists, the development of creative cancer treatment options remains an obstacle. Thus, cancer is still the world's second-largest cause of death [3]. According to Jameel et al. [4], lung cancer is the largest reason for cancer mortality around the world. A549 cells are adenocarcinoma cells derived from human alveolar basal epithelial cells and have recently surpassed squamous cell carcinoma as the most common histological subtype across all races and genders. Chemotherapy has many drawbacks including weak specificity and dosage-limiting toxicity [5]. Despite promising preclinical findings, natural products often have physicochemical properties, such as low stability, bioavailability, and aqueous solubility that can limit their clinical application [6]. To eliminate these limitations, nano-based drug delivery systems (NDDSs) are pointed out as promising attempts.

Nanostructured drug delivery systems (NDDSs) are a rapidly advancing area of science, employing nanomaterials to transport drugs to targeted sites, significantly enhancing the efficacy of anticancer therapies. They can improve the bioavailability of poorly water-soluble drugs, enable the co-delivery of multiple medications, enhance targeted delivery, protect healthy cells from drug toxicity, and extend drug activity [7].

Bismuth (Bi) is a well-known element valued for its unique combination of diverse properties [8]. It is a non-toxic and cost-effective inorganic material that exhibits the unsettled effectiveness of photothermal transition and an expanded half-life [9]. Bismuth nanoparticles (BiNPs) have shown promising characteristics in combined tumor diagnosis and therapy, making bismuth oxide (Bi_2O_3) nanoparticles a potential option for cancer treatment. Bismuth-based compounds can be used to target gamma rays with high energy at cancer cells. This

property can be combined with existing chemotherapeutic technologies to enhance therapeutic outcomes [10].

The key gaps addressed by the present study included limited exploration of algal-derived BiNPs, particularly for lung cancer, and the need to understand their specific cellular interactions. The study sought to improve nanoparticle stability, bioavailability, and solubility, addressing essential limitations in phyconanotechnology for more effective targeted cancer therapies. The study introduced unique advancements in phyconanotechnology and cancer therapy. Using *Chlorella* sp. for BiNP synthesis provided an eco-friendly alternative, enhancing biocompatibility and minimizing chemical exposure. The study explored BiNPs in targeted cancer treatment, their selective toxicity toward A549 cells, and their potential for multi-modal therapies, incorporating cancer-fighting phytochemicals.

The study aimed to develop and assess the efficacy of BiNPs synthesized with *Chlorella* metabolites as a targeted, nano-based drug delivery system to enhance cytotoxicity against A549 lung cancer cells while minimizing toxicity in normal cells. This research hypothesized that algae-synthesized BiNPs using *Chlorella* metabolites offer a novel, selective drug delivery system for A549 lung cancer cells, providing significant anticancer effects with reduced toxicity to normal cells for safer lung cancer treatment.

2. Materials and Methods

2.1 Isolation and cultivation of the algal isolate

Water samples were collected in clean glass containers from an area along the Tigris River in Baghdad City, situated at longitudes 20°44'45.58"E and latitudes 20°33'33.55"N. The isolation and purification of algae used in this study were conducted using serial dilution and streaking methods on solid media with Chu-10 medium (HIMEDIA). Microscopically, the isolated alga was identified following the method described by Prescott [11]. The isolated alga was then cultivated in an illuminated incubator (Han Yang, Korea) at a light intensity of 200 µE/m²/s and a temperature of 26±2℃, using 100 ml of the same culture medium in liquid form to obtain biomass. After an incubation period of 14 days, the biomass was transferred to a 500 ml medium, followed by 100 ml transfers, each lasting 14 days. Finally, it was transferred to a glass basin and grown for an additional one month. Then the biomass of the cells was harvested, dried, and stored in the refrigerator until subsequent examinations were conducted.

2.2 Preparation and characterization of bismuth nanoparticles

The algal crude extract was obtained using a cold extraction protocol described by Al-Enazi et al. [12]. The algal biomass was ground into a fine powder using a mixer grinder. Two grams of the powder were soaked in 70% ethanol with continuous shaking for 24 hours. The mixture was filtered, and the remaining residue was dried using a rotary evaporator. For nanosynthesis, 2 grams of the *Chlorella* extract were diluted in 100 mL of deionized distilled water (DW), and the pH was adjusted to 8.5. One molar of bismuth nitrate pentahydrate ($Bi(NO_3)_3 \cdot 5H_2O$) solution, with a molecular weight of 485.07 (Sigma Aldrich, China), was gradually added to the extract solution while stirring continuously at 30℃ until a color change was observed. Three optical analyses were conducted to characterize the prepared BiNPs. Ultraviolet (UV)–visible spectroscopy was performed using a Shimadzu 1601 spectrophotometer. Additionally, X-ray diffraction (XRD) was conducted to determine structural properties, utilizing a Shimadzu 6000 XRD diffractometer (Japan).

2.3 Gas chromatography and mass spectrometry analysis (GC-MS) of algal extract

The main phytochemicals of the algal sample were analyzed by employing a high-temperature column (Shimadzu, Japan) with an initial temperature set at 100℃. The column properties included inert cap 1 MS; 30 m × 0.25 mm id × 0.25 µm film thickness. Using helium as the carrier gas, 5 µL of the algal sample was injected into the column, with temperature programming set for the total run time of 63.109 minutes. Quantitative determination was achieved by correlating the respective peak areas to the total ion current (TIC) areas obtained from GC-MS.

2.4 Maintenance of cell cultures

A549 lung cell line was maintained in RPMI (Capricorn, Germany) supplemented with 10% fetal bovine serum (Capricorn, Germany), 100 µg/mL each of penicillin and streptomycin. Cells were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA; Capricorn, Germany) and reseeded at 50% confluence twice a week, then incubated at 37℃ [13].

2.5 Cytotoxicity assay

To examine the cytotoxic effect of prepared BiNPs, the MTT cell viability assay was performed on 96-well plates [14]. The cell line was distributed at 1×10^4 cells/well. After 24 hrs, the monolayer was achieved, and then cells were treated with different concentrations (100, 50, 25, 12.5, 6.25, and 3.125 µg/mL) of BiNPs. The viability of cancer cells was assessed 72 hours post-treatment. The medium was removed, and 28 µL of a 2 mg/mL MTT stain solution (Bio-World, USA) was added. The cells were then incubated for 1.5 hours at 37℃. After removing the MTT stain, the residual crystals in the wells were solubilized by adding 130 µL of DMSO (Santa Cruz Biotechnology, USA) and incubating for 15 minutes at 37℃ with shaking [15]. The absorbency was measured on a microplate reader at a test wavelength of 492 nm. The assay was achieved in triplicate. The percentage of cytotoxicity was calculated according to a formula described by Carreño et al. [16]:

$$Cytotoxicity\ \% = 100 - Cell\ Viability$$

where, cell viability is calculated by:

$$Cell\ Valiability\ \% = \frac{Absorbance\ of\ Treated\ Cells}{Absorbance\ of\ Non\ Treated\ Cells} \times 100$$

2.6 Statistical analysis

The obtained data was statically analyzed using an unpaired t-test with GraphPad Prism 6. The values were presented as the mean ± SD of triplicate measurements.

3. RESULTS AND DISCUSSION

3.1 Description of the prepared BiNPs

The successful synthesis of nanoparticles was first indicated by a visible color change after a 30-minute reaction period, as illustrated in Figure 1(a). The algal extract solution was pale green, and the bismuth nitrate pentahydrate solution was relatively colorless. The color of the solution mixture was converted gradually during the reaction period to reach dark green with colloidal constancy, revealing the formation of BiNPs.

The UV-visible analysis results (Figure 1(b)) present the optical transmittance spectrum of the prepared BiNPs solution as a function of wavelength (200-1000 nm). Three distinct regions were observed. The figure indicated that transmittance remained stable at 0.14 in the first region (200-246 nm) before experiencing a sharp increase up to 0.64 between 480 nm and the beginning of the visible region. This is followed by a slight increase, reaching a maximum peak of 0.83 at a wavelength of 1,078 nm, indicating that the material is nearly transparent. Figure 2, Figure 3 and Table 1 represent the X-ray diffraction spectrum of BiNPs deposited on glass by the drop-casting method.

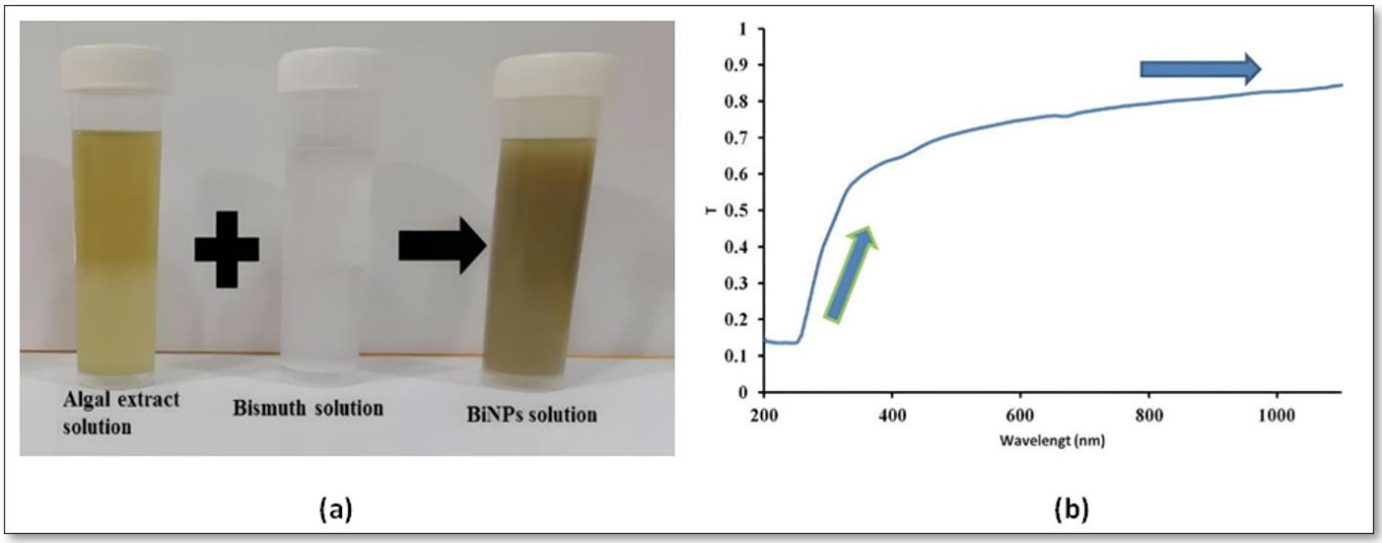

Figure 1. Preparation of bismuth nanoparticles (BiNPs). (a) Color alteration and manifestation of colloidal constancy during the reaction period; (b) Transmittance spectra of the prepared BiNPs using *Chlorella* sp. under spectra from 200 to 1000 nm

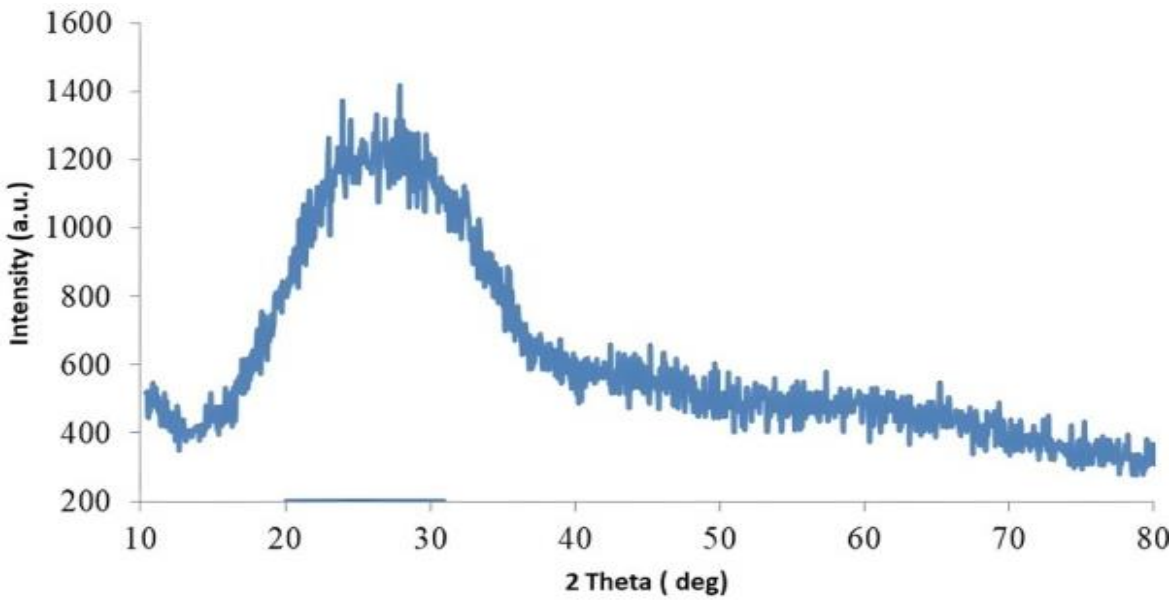

Figure 2. X-ray diffractometer pattern of the prepared bismuth nanoparticles

Table 1. Data obtained from X-ray diffractometer pattern of the prepared bismuth nanoparticles by green alga *Chlorella* species

Parameter	2 θ (deg)	FWHM (deg)	D (nm)	$\delta \times 10^{19}$ lines.m^{-2}	η lines^{-2}.m^{-4}
Value	26.29	14	0.57	3	0.6

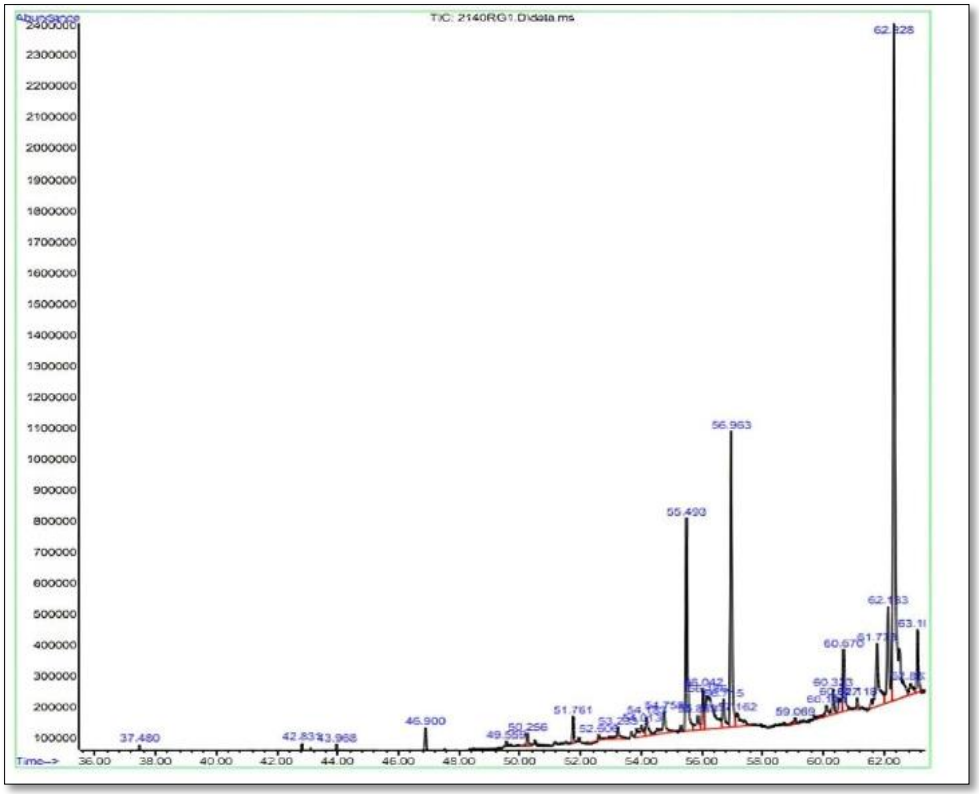

Figure 3. Gas chromatography-mass spectrometry chromatogram of the extract of *Chlorella* species

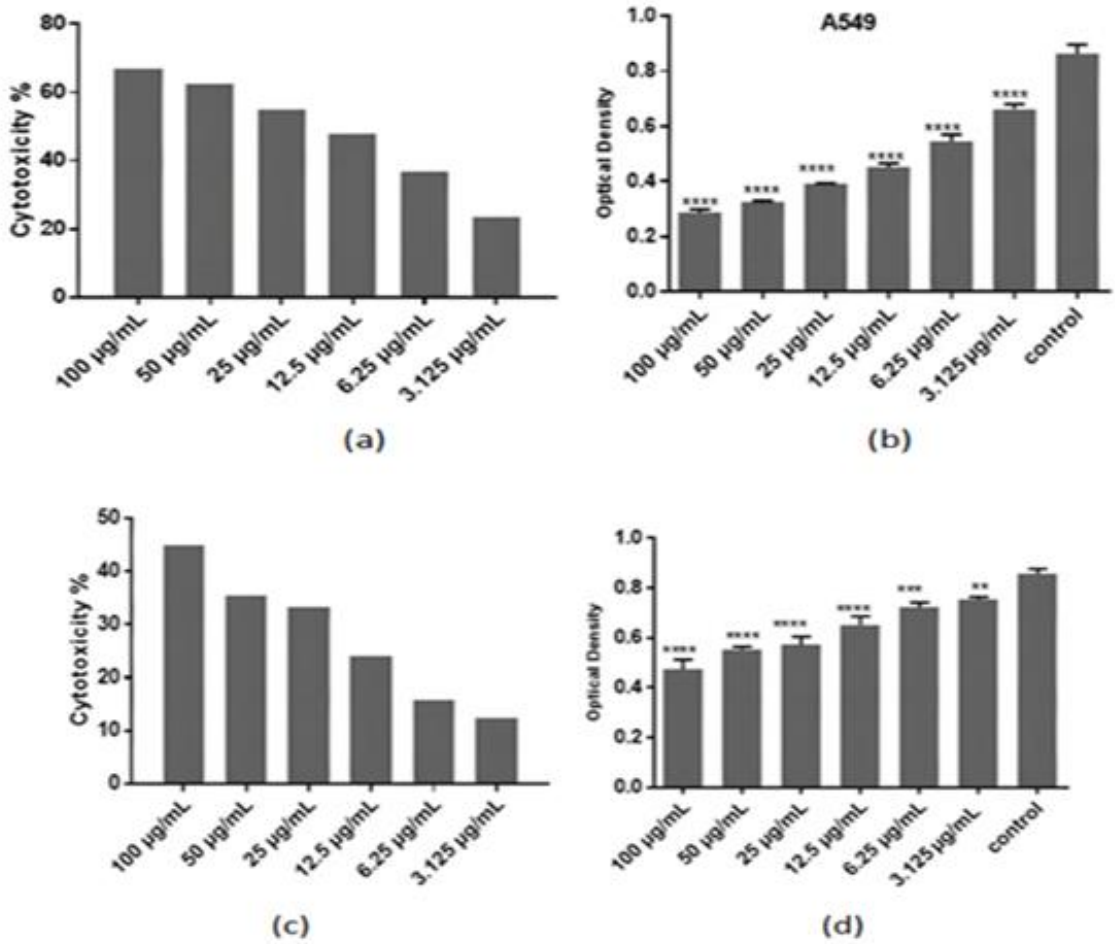

Figure 4. Cytotoxicity percentages and absorbance values (mean ± SD) of the test concentrations of bismuth nanoparticles synthesized using the green alga *Chlorella* species on A549 cancer cell line (a and b) and NHF cell line (c and d)

Table 2. Major anticancer phytochemicals detected within the extract of *Chlorella* species using gas chromatography-mass analysis, categorized according to their chemical nature

The Best-hit Phytochemical from Each Library	RT/min.	Peak Area %	Chemical Group
Hexadecanoic acid, methyl ester	54.759	1.90	Fatty acids and their esters
Hexadecanoic acid	56.188	6.29	
Oleic Acid	56.714	3.65	
Hexadecanoic acid, ethyl ester	56.965	12.48	
11-Octadecenoic acid, methyl ester	60.326	5.08	
9,12,15-Octadecatrien-1-ol	61.772	5.08	Terpenoids
2-Hexadecen-1-ol	60.669	3.22	
Squalene	54.182	1.20	
Citronellol	52.604	0.05	
Neophytadiene	51.758	2.01	
2-Pentanone, 4-hydroxy-4-methyl	9.574	0.89	others
Cycloheptasiloxane, tetradecamethyl	37.482	1.10	
1-Octadecene	42.831	0.54	

3.2 Characteristics of algal extract based on gas chromatography and mass spectrometry analysis

Many phytochemicals with varying proportions and biological activities were detected in the algal extract. Table 2 presents the key phytochemicals assumed to directly inhibit cancer cell proliferation or indirectly prevent cancer formation through their antioxidant properties.

3.3 Cytotoxic effect of bismuth nanoparticles

As illustrated in Figure 4, the cytotoxic effects of BiNPs were dose-dependent in both cancerous and normal cell lines. The maximum percentage of cytotoxicity was observed following treatment with 100 µg/mL, which decreased with lower concentrations of the tested BiNPs. Notably, the prepared BiNPs demonstrated reduced cytotoxicity in normal human fibroblasts (NHF), with an IC_{50} value of 5.797 µg/mL for cancer cell lines and 17.68 µg/mL for normal cell lines. Additionally, significant differences were observed across all test concentrations.

After incubation with the test concentrations of the prepared BiNPs for 72 h, morphological alterations in A549 cells were observed (Figure 5(a) and (b)). Compared to the control (untreated) cells, visualization of the control group revealed that cells maintained their original shape, with most remaining adherent to the tissue culture dishes. In contrast, A549 cancer cells treated for 72 hours displayed distinct apoptotic features, including cell rounding, shrinkage, membrane blebbing, and detachment from neighboring cells. In treated NHF cells, however, no significant visual alterations were observed (Figure 5(c) and (d)).

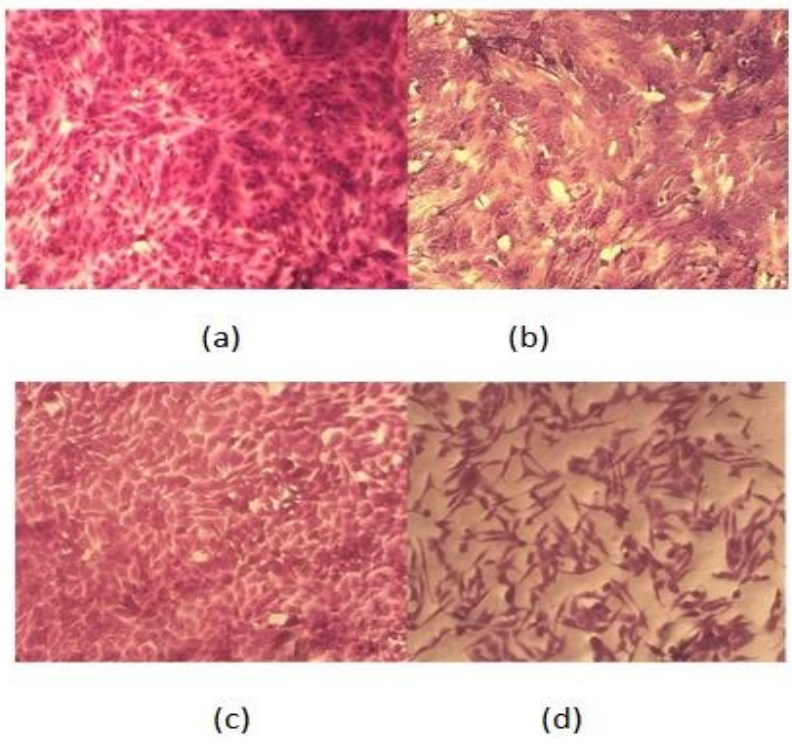

Figure 5. Microscopic views of treated normal and tumor cell lines. (a) Untreated NHF cell line (control); (b) Treated NHF cell line with prepared BiNPs showing no significant changes; (c) Untreated A549 cell line (control); (d) A549 cell line treated with prepared BiNPs showing typical apoptotic features (10X)

3.4 Characteristics of the biologically synthesized bismuth nanoparticles

The color of the synthesized nanoparticles depends on their shape and size. Additionally, nanoparticle coloration results from the metal reduction process, reflecting the progressive formation of nanoparticles either intra- or extracellularly [5]. This study demonstrated extracellular biogenic nanoparticle formation utilizing an extracted metabolite as the reducing agent rather than whole algal cells [17].

UV-vis spectroscopy is a valuable analysis that permits the evaluation of nanoparticles at shape, size, and aggregation levels, as well as for examining and studying plasmon resonance in metallic nanoparticles, including positions and forms of peak. Nanometals exhibit prominent spectral characteristics by surface plasmon resonance (SPR). This phenomenon is attributed to the resonant oscillation of free electrons in response to light waves, often influenced by the shape and size of the biosynthesized nanoparticles [17]. Blue and green light rays, with minimal intensity and maximum dispersion, are utilized within a broad spectral band ranging from 320 to 580 nm [18]. A single peak was observed at 26.29° in the XRD spectrum analyzed using Scherrer's equation. The diffraction pattern aligns with the findings by Das et al. [2], indicating that the biosynthesized BiNPs exhibit an amorphous morphology likely due to phytochemicals encapsulating these particles.

3.5 Gas chromatography and mass spectrometry analysis of algal extract

The findings of GC-MS revealed the dominance of several fatty acids and their esters. These compounds are reported in many studies to assess the potential for antioxidant and anti-cancer activities. Palmitic acid exhibited selective cytotoxicity against human leukemic cells [19]. Additionally, oleic acid and 9,12,15-octadecatrienoic acid have been recognized as potent antioxidant and anticancer phytochemicals [20]. Methyl ester derivatives of palmitic, hexadecanoic, and 11-octadecenoic acids have also been reported as antioxidant and anticancer agents by various researchers, including Wu and Papagiannakopoulos [21]. Also, several terpenes were detected in the algal sample including 2-hexadecen-1-ol and 9,12,15-octadecatrien-1-ol. Terpenoids, found primarily in plants, animals, and fungi, are known for their potential effectiveness against cancer. Their anticancer properties include the suppression of cell adhesion, migration, and proliferation [22].

Squalene is a well-known terpene studied as a cancer proliferation inhibitor against lung, breast, ovarian, and colon cancer. This terpene appears to alter the metabolic activation of carcinogenesis, neutralizing reactive oxygen species and free radicals that form mutagenic lesions in proteins, DNA, and lipid molecules, thereby helping to prevent carcinogenesis [23]. Citronellol was also detected in the algal sample. In the breast cancer MDA-MB-231 cell line, citronellol also decreased the expression of protein and Bcl-2 gene and increased Bax expression [24]. Another identified compound, 2-pentanone, 4-hydroxy-4-methyl (also known as diacetone alcohol), is the most abundant bioactive compound produced by *Oscillatoria* sp., demonstrating significant antioxidant and cytotoxic activity [25]. Cycloheptasiloxane, an organic compound, exhibits strong antioxidant effects. Additionally, the detected alkene, 1-octadecene, has been reported to possess anticancer and antioxidant properties [26].

3.6 Cytotoxicity assay

Numerous screening studies have been established over the past years for discovering new antibiotic or cytotoxic metabolites from different microalgae especially green algae and cyanobacteria [27]. Many previous studies have utilized *Chlorella* algae as a natural source for the reduction of various metal oxides, exploring the potential of these nanoparticles as inhibitors of different cancerous tumors. However, no previous study had addressed the reduction of bismuth oxide by the studied algae and the application of the resulting mixture on the A549 cell line. In a study presented by Hamouda et al. [17], *Chlorella* was successfully used to synthesize Au/cellulose nanocomposite. It was found that this nanocombination enhanced anticancer activity on A549 cells with an IC_{50} value of 4.67±0.17µg/µL. The suggested mechanism was signaling pathway regulation of the mitogen-activated protein kinases (MAPK). Furthermore, there was an increase in the relative expression of the tumor suppressor gene p53 compared to control cells. In another study, *Chlorella* algae were used to produce SnO_2 nanoparticles, which demonstrated inhibitory effects on A549 cells with an LD_{50} value of 188 µg/mL after 24 hours of incubation [12].

Phytochemicals from *Chlorella* sp., including terpenoids, palmitic acid, and squalene, inhibit cancer cell growth through antioxidant properties and mechanisms that reduce oxidative damage and mutagenesis. Combined with BiNPs, they enhance drug stability and targeted delivery, facilitating stress in cancer cells while preserving normal tissue. A study found that *Chlorella* vulgaris contains antioxidants like oleic and palmitic acids, which reduce oxidative stress and affect cell proliferation and apoptosis, highlighting their potential in cancer prevention and treatment [28]. Georgiopoulou et al. [29] studied *Chlorella* vulgaris extracts rich in bioactive compounds like squalene and terpenoids, which inhibit cancer cell adhesion and migration. Squalene modulates gene expression, reducing anti-apoptotic Bcl-2 while increasing pro-apoptotic Bax, promoting cancer cell apoptosis [29].

Recently, various therapeutic protocols have garnered significant interest in cancer treatment, including photothermal therapy, radiotherapy, photodynamic therapy, hyperthermia, and targeted drug delivery. In this context, Bi_2O nanoparticles (Bi_2ONPs) present a promising option for cancer diagnosis and treatment by releasing high-energy gamma rays directed at affected cells. This technology has the potential to enhance the efficacy of existing chemotherapy regimens [10].

In the current study, the researchers leveraged these findings to propose the potential use of the prepared BiNPs in conjunction with active compounds extracted from *Chlorella* as a co-treatment for lung cancer. This approach is particularly promising given the significant inhibition rates observed against the cancer cells compared to the relatively low inhibition rates against normal human fibroblast cells.

4. CONCLUSIONS

The current study demonstrated that the microalga can effectively synthesize bismuth nanoparticles without adversely affecting its internal cellular components. This finding supports the potential for using a nanoparticle mixture loaded with active compounds, including anticancer agents, against lung cancer cells. The mixture demonstrated effectiveness in inhibiting cell proliferation at acceptable levels compared to its inhibitory effects on normal cells used in the study and at much lower concentrations. This suggests that the prepared particles are relatively safe at the tested concentrations and could be considered for further research in the field of nano-cancer therapy. The present study advances cancer nanomedicine by leveraging *Chlorella*-derived BiNPs for targeted, eco-friendly drug delivery. Findings highlight their selective cytotoxicity, potential apoptosis induction, and promise for enhanced cancer therapies through multi-targeted treatment approaches, addressing key limitations in traditional chemotherapy with improved biosafety and therapeutic efficacy. Future studies may optimize algal extraction to boost active metabolite yield, enhancing bismuth nanoparticle stability and therapeutic efficacy. Exploring combined therapies, like photothermal techniques, could improve cancer cell targeting while protecting healthy cells.

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