Evaluation of Multitudinous Potentials of Photosynthetic Microalga, Neochloris aquatica RDS02 Derived Silver Nanoparticles

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ABSTRACT
The present study pertains to the silver nanoparticles (Ag NPs) synthesis from cell free supernatant of freshwater microalga, Neochloris aquatica RDS02 (KJ700475.1). The synthesized Ag NPs was confirmed with the characteristic features viz., UV–vis Spec: the surface plasmon resonance at 420 nm; XRD: the h k l values with 4 prominent peaks and the average size of NPs (~20 nm); FTIR: functional group involved in bio-reduction; HRTEM: well distributed spherical morphology with size ranged from 15 to 20 nm. The microalga (N. aquatica) mediated Ag NPs possesses enhanced antibacterial activity on Escherichia coli, Klebsiella pneumoniae, Bacillus subtilis and Staphylococcus aureus. The anti-proliferative activity on HeLa cell line by MTT and the antiviral activity on Herpes simplex viruses (HSV1 and HSV2) by plaque assays were evaluated using Ag NPs. The Ag NPs caused morphological changes, cytoplasmic content leakage, and membrane permeabilization in a dose-dependent manner. The IC_{50} values (μg mL^{-1}) of Ag NPs were found to be 39.5 for HeLa and 100 for HEp2 viral cells. Hence, the Ag NPs would be considered as a promising biological agent and emerge as an alternative for drug development to combat the problem over the existing pharmaceuticals.

1. Introduction
Nanoscience and nanotechnology represent one of the major revolutions of modern science and engineering, enabling materials of distinctive size, shape, and composition to be formed. Nanoparticles (NPs) from noble metals viz., platinum (Pt), gold (Au), silver (Ag), copper (Cu) and iron (Fe) are extensively used in consumer products, medical and pharmaceutical applications.
The NPs are usually small in diameter and huge in surface area, also possesses several important high-class medical and industrial applications i.e. bio-engineering, catalysts and electronic devices [2]. Of late, numerous physical, chemical and biological methods are reported for the syntheses of different types of NPs [3]. Though the physico-chemical methods are familiar, the practice of compounds may limit their applications and are cost-effective one. The silver nanoparticles (Ag NPs) synthesis using eco-friendly green chemistry approach is considered to be an important aspects in the field of nanobiotechnology [4]. Ag NPs possesses unique physical, chemical, thermo-dynamical and optical properties at nano-regime [3,4]. This would leads their significance in hetero and homogeneous catalysis because of their huge surface area to volume ratio [5,6].

There are numerous reports are available for the biosynthesis of NPs using several materials as reducing agents, viz., marine organisms, micro-fluids, plant materials, etc., [6–8]. Among the reducing agents, plant and algal extracts are effective bio-reductants due to the abundance of metabolites. Also, they are relatively ease, low cost, nontoxic, eco-friendly, commercially available and are well known for the green approach of the synthesized NPs [9]. The antimicrobial potentials of Ag NPs give rise to various medicinal nano-Ag products such as surgical instruments, wound dressings, implants and contraceptive devices [10,11]. Besides antimicrobials, Ag NPs are well-known to possess antiviral, antiinflammatory, antiplatelet, larvicidal and antiangiogenesis activities [12,13]. In addition, developments were made recently in the functionalized Ag NPs and utilized in laundry detergent, wallpaper gloves, wall paint and room spray preparations, also in the textile industry for the fabrications [14,15].

Microalgae are photosynthetic microorganisms which are present in aquatic and terrestrial ecosystems, representing a wide variety of species. They are used in several biotechnological areas including nutrition, food additives, cosmetics, aquaculture, and pharmaceuticals [16,17]. Microalgae are commonly used as a functional food due to their rich contents of protein, lipids, carbohydrates vitamins and minerals, as well, biologically active compounds with impending therapeutic applications towards oxidative stress, lipidemia, cancer, thrombosis, allergy, etc. [18–21].

Among the blue-green algae (Cyanobacteria), Calothrix, Nostoc ellipsosporum, Anabaena and Leptolyngbya were reported earlier for their intracellular biosynthesis of Au, Pt and Ag NPs [22]. Also, the green microalga, C. vulgaris was reported for the Au, ruthenium (Ru), Pt, rhodium (Rh), palladium (Pd), and iridium (Ir) NPs syntheses [23], whereas, the cell free extract efficiently produces silver and gold nano-plates [24,25]. The present study pertains to the synthesis and characterization of Ag NPs from the green oleaginous microalga, Neochloris aquatica RDS02 and also to evaluate their antibacterial, cytotoxic, and antiviral activities.

2. Materials and Methods

2.1. Material

Silver nitrate (Ag NO₃), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and all other chemicals were procured from Sigma-Aldrich (U.S.A.). Where the solvents are purchased from Merck, India, which were of analytical grade with maximum purity. The pathogenic bacteria from Microbial Type Culture Collection (MTCC) centre, Chandigarh, India; HeLa cell line (ATCC-CCL-2.2) and HEP2 (Herpes Simplex Viruses type I & II) (HSV-I & II) (ATCC- VR-2019) were procured from the National Centre for Cell Sciences (NCCS), Pune, India

2.2. Methods

2.2.1. Microalgal Isolation, Identification, and Culture Conditions

Microalgae species was isolated from the freshwater body, Kuppanur (11.7447° N, 78.2804° E). The water samples were transported to fermentation lab, Department of Microbiology (11.7184° N, 78.0773° E). The collected water samples were filtered through phytoplankton net with mesh size 20 μm for removal of phytoplankton and debris. The filtrate was then transferred to sterile bold basal medium (BBM) using pasteur pipette. The presence of microalgal species in the BBM medium was confirmed by microscopic observation. Further, a small amount of the sample was spread across the plate containing BBM medium for isolation of pure colony, which was then cultured on both solid and liquid BBM for further studies. The BBM composed of NaNO₃, K₂HPO₄, CaCl₂·2H₂O-20mgL⁻¹, MgSO₄·7H₂O-60mgL⁻¹, K₂HPO₄- 60mgL⁻¹, NaCl- 20mgL⁻¹, Na₃EDTA- 50mgL⁻¹, KOH- 31mgL⁻¹, FeSO₄·7H₂O- 45μgL⁻¹, H₃BO₃- 10μgL⁻¹, H₂BO₃- 10μgL⁻¹, MnCl₂·4H₂O- 14μgL⁻¹, ZnSO₄·7H₂O- 8μgL⁻¹, MoO₃- 7 μgL⁻¹, CuSO₄·5H₂O- 15 μgL⁻¹ and Co (NO₃)₂·6H₂O- 5 μgL⁻¹ at an ambient temperature of 25 ± 1°C and 12/12 light/dark cycle under light with an intensity of 100 μM m⁻²s⁻¹ in a shaker incubator agitated at 150 rpm.

2.2.1.1. Determination of Algal Growth. The Neochloris aquatica RDS02 microalgal culture was withdrawn every day (up to 15th day) and the optical density (OD) value of microalgal growth was measured
at 680 nm using UV spectrophotometer (Thermo fisher UV–Visible Spectrophotometers, Evolution 201). The microalgal growth rate was calculated.

2.2.2. Preparation of Algal Extract and Silver Nitrate Solution

Twenty gram of dry algal biomass was suspended in deionized water (100 mL) and heated at 100 °C for 20 min. The boiled algal extract was filtered using Whatman filter paper No.1 and the filtrate was then centrifuged at 8,000 rpm for 10 min to obtain the supernatant. The 100 mM AgNO₃ stock solution was prepared and the diluted concentration of 5 mM was used for the synthesis.

2.2.3. Synthesis of Silver Nanoparticles (Ag NPs)

The fresh supernatant (as reducing agent) collected from centrifuged algal extract was used for NPs synthesis. A 20 mL of supernatant and 80 mL of 5 mM Ag NO₃ solution were mixed and incubated at room temperature for 60 min. The synthesis of Ag NPs was initially confirmed by the change in color of reaction mixture. Then the synthesized Ag NPs solution was centrifuged and the pellet was washed thrice with distilled water to remove unbound Ag ions and stored at −20 °C until further characterization.

2.2.4. Silver Nanoparticles Reduction Kinetics

The reduction kinetics of the NPs synthesis was optimized using different time (10–60 min) intervals for size and calculated. The reduction kinetics calculated by modified Mie method as given:

\[ \Upsilon(R) = \Upsilon_0 + \left( A_{vf} \right) / R \]  

(1)

where, \( \Upsilon(R) \) - Size reduction resonance broadening, \( A \) - Scattering process (3/4 of Ag), \( \Upsilon_0 \) - Velocity of bulk scattering \( (5 \times 10^{-12} \text{ s}^{-1} \text{ for Ag}) \) and \( v_F \) - Fermi velocity \( (1.39 \times 10^6 \text{ ms}^{-1}) \).

2.3. Characterization of Ag NPs

The bio-reduction of Ag ions from AgNO₃ was monitored using UV–visible spectrophotometer, at the wavelength of 200–700 nm (Shimadzu UV–Visible Spectrophotometer, model UV-1800). The X-ray diffraction (XRD) analysis for the synthesized Ag NPs was performed in a Bruker Kappa APEXII instrument operated at a voltage of 50 kV and a current of 40 mA with Cu-Kα radiations \( \lambda = 1.54060 \text{ Å} \) in 2θ range of 30–80° C. The functional group responsible for the bio-reduction of Ag NPs was analyzed using FTIR. The FTIR spectrum values were recorded in the mid-infrared region \( (4000–400 \text{ cm}^{-1}) \) at 4 cm⁻¹ room resolution (Thermo Nicolet FTIR Nexus spectrometer). The size and shapes of the Ag NPs were analyzed by drop coating the NPs onto transmission electron microscopic (TEM) grids. The film on the TEM grids was left for 2 min and the solution remained was removed using a blotting paper. The grid was dried and morphological characters of the NPs were recorded. High resolution transmission electron microscopic (HR-TEM) analysis was employed to characterize the morphology of the synthesized Ag NPs using JEOL 3010 instrument operated at an accelerating voltage at 100 keV. The elemental composition of the synthesized Ag NPs was determined by energy disperse X-ray analysis (EDX). Atomic force microscopic (AFM) analysis was done to measure the height and roughness of the synthesized Ag NPs. The sample volume of 1 μL was dispersed on a mica-based substrate into a thin layer and dried using sonicator at 37 °C for 15 min and visualized under AFM (Nanosurf, v1.3R0).

2.4. Antibacterial Activity of Ag NPs

The antibacterial activity was carried out through well-diffusion method. Mueller–Hinton (MH) broth was used to culture the four clinical pathogens; E. coli ATCC 8739, K. pneumoniae ATCC 2719, B. subtilis ATCC 6633 and S. aureus ATCC 29736, respectively and incubated overnight at 37 °C. The grown bacterial strains were swabbed on MH agar plate and left for 30 min to dry and then, wells were made at 6 mm in diameter using the cork-borer. Different concentrations (15, 30 and 45 μL) of the freshly prepared Ag NPs (1 mg mL⁻¹) were added to the respective well and incubated at 37 °C. The antibiotics, Cefotaxime (1 mg mL⁻¹) and Ampicillin (1 mg mL⁻¹) solution were also loaded separately on respective agar plates for the purpose of comparison. After the incubation, the zone of inhibitions were measured. The experiment was performed in triplicates [26].

2.5. In vitro Cytotoxicity Assay or Anti-Proliferative Assay

2.5.1. Cell culture Condition

HeLa cells were cultured in Eagle’s minimum essential medium (EMEM) supplemented with 1% L-glutamine, 1% penicillin-streptomycin, and 10% fetal bovine serum. The grown cultures were maintained at 37 °C with 5% CO₂. For MTT assay, the cells were seeded into a 96-well microtiter plate and left overnight until attachment, then the culture was treated with varied concentrations of Ag NPs (10–100 μg mL⁻¹).

2.5.2. Cell Viability Assay

Cell viability assay was carried out using the MTT. Approximately, \( 2 \times 10^3 \) cells (in 6 replicates) were exposed to Ag NPs for the treatment. After 6 h of incubation,
cells were washed twice with 0.1 M phosphate buffer saline (PBS) and then incubated with MTT salt solution (4 mg mL\(^{-1}\) in PBS). Further, the total culture medium was incubated at 37 °C for 4 h. Thereafter, 100 μL of dimethyl sulfoxide (DMSO) was added to each well and incubated further at 37 °C for 1 h. The OD values of the formazan product was recorded at 570 nm. The results were calculated as percentage cell viability vs. concentration of Ag NPs, from which the half maximal inhibitory concentration at 50% of cells (IC\(_{50}\)) was determined [27].

2.5.3. Apoptotic Assay
The apoptotic effect of Ag NPs on HeLa cells were examined by nuclear DNA staining assay. Ag NPs treated and untreated cells were fixed in 4% paraformaldehyde for 20 min and washed with PBS, then subjected to propidium iodide (1 μg mL\(^{-1}\)) staining for 15 min. Further, the stained cells were washed twice with PBS. The morphological changes in the nuclei were observed under UV fluorescent microscope.

2.5.4. Acridine Orange and Ethidium Bromide Staining
Acridine orange and ethidium bromide (AO/EB) staining was performed to notice the morphological evidence of apoptosis in Ag NPs treated cells. About 25 μL of treated and untreated cell suspension (5 × 10\(^7\) cells mL\(^{-1}\)) were stained with 900 μL of AO/EB dye mix (100 mg mL\(^{-1}\) of each prepared in PBS, respectively) [28]. Then, the samples were examined under fluorescent microscope (Nikon Eclipse TS 100).

2.5.5. DNA Fragmentation Assay
The fragmentation of cancerous cell’s DNA was quantitatively determined using diphenylamine reagent. The cells treated with different concentrations of Ag NPs were harvested at 12 and 24 h of post-treatment. 200 μL of 5 M perchloric acid was added to the samples and the heat treatment was done at 70 °C for 15 min. Then, a double volume of diphenylamine reagent was added to the samples and stored at 4 °C for 48 h. The treated cell samples were measured at 575 nm. The treated and untreated DNA of the cells were isolated and subjected to agarose gel electrophoresis for fragmentation assay.

2.6. In vitro Antiviral Assay
2.6.1. Viral Cell Culture Conditions
HEp2 (Herpes simplex Virus type I and II (HSV- I and II)) and Vero cells obtained earlier were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 25 μg mL\(^{-1}\) gentamicin and 200 mM L-glutamine.

2.6.2. Virus Yield Reduction Assay
The effect of Ag NPs on the protection of infectious virus was assessed through yield reduction assay. Where, HEp2 cells were infected with the virus count of 0.6 pfu/cell and exposed to the Ag NPs for 72 h. When the HEp2 virus cells get adsorbed, the cultures were triplicated to serial half-log dilutions of the test sample prepared in assay medium composed of EMEM containing 5% infant calf serum [29]. The supernatants were collected after 72 h of infection and the cell-free virus infectivity (control) was determined by plaque assay on Vero cell monolayers. The endpoint of 24 h assay was expressed as the effective concentration (EC\(_{50}\)), which reduce virus yield by 99% in comparison with the control.

2.6.3. Plaque Reduction Assay
MRC-5 cell monolayers were prepared in 96-well plates and infected with HSV in each wells. Then, the plates were exposed to Ag NPs as described by Boyd et al. [29]. The assay was performed in triplicates for different dilution.

3. Results and Discussion
3.1. Microalgae Isolation, Identification and Culture Growth Conditions
The isolated microalgae species were identified based on the morphological features. They are single-celled and spherical shaped light green cells, identified as Neochloris sp. (Figure 1). The identified microalg, Neochloris sp. was further confirmed with molecular characterization and it was found to be Neochloris aquatica RDS02. The sequence of the microalg was submitted to GenBank and obtained its accession number (KJ700475.1).

The initial microalgal growth (at lag phase) was measured at 680 nm and it was calculated as 0.6 × 10\(^6\) cells mL\(^{-1}\). Further, the growth rate at every day was measured up to 15th day. The growth rate at first day was calculated
as $0.9 \times 10^9$ and 15th day as $5.18 \times 10^9$ cells mL$^{-1}$. After 15 days, the microalgal biomass was harvested at the stationary phase and dried at 70 °C for 2 h in hot air oven. The dried algal-biomass was used for further studies.

### 3.2. Characterization of Ag NPs

#### 3.2.1 Silver Nanoparticles Reduction Kinetics

In the present investigation, the biological reduction of nanoparticles was determined based on time different time interval using modified Mie equation method. When the Ag NO$_3$ solution and algal aqueous extract (8:2) mixed, the reduction through color change at different incubation times (10–60 min) were noticed. From equation (1), the Ag NPs reduction was significantly observed at 60 min time incubation, where the UV peak intensity and reduction kinetic $R^2$ value-0.996, abruptly increased, respectively (Figure 2(A)). The Ag NPs reduction was confirmed through a strong surface plasmon resonance (SPR) at 428 nm. Similarly, Khan et al. [30] have reported that the *Pulicaria glutinosa* plant extract have bio-reduced the Ag NPs at 60 min and their SRP peaks were recorded at 422–459 nm.

#### 3.2.1.1. UV–vis Spectrophotometric Analysis

From the reaction mixture, the initial color change from light yellow to brown was observed and the bio-reduction reaction was completed within 1 h. The overall time elapsed for the reduction of silver ions was reported earlier by Chanda [31], which confirmed the presently bio-reduced Ag NPs from min to h. The NPs might be synthesized by the use of specific proteins secreted by the microalgae or the possibility of specific reducing bio-molecules and capping agents present in the algal extract. The concentration of bio-reduced Ag NPs was confirmed through a strong surface plasmon resonance (SPR) at 428 nm (Figure 2(B)). The corresponding shift in the SPR might be due to the excitation of ions [32]. Similarly, the Ag NPs synthesized was comparably confirmed through a strong SPR at around 430 nm from the chemical based one [33].

#### 3.2.2. X-ray Diffraction Analysis

The XRD pattern of the synthesized Ag NPs revealed the crystalline nature of the NPs and the anatase form of the Ag NPs was confirmed by the 2 theta peak values at 38.20°, 44.38°, 64.10° and 77.80° indexed the Bragg diffraction planes (1 1 1), (2 0 0), (2 2 0) and (3 1 1), respectively (Figure 3). All obtained peaks were determined by the Joint Committee on Powder Diffraction Standards chart (JCPDS No. 040921). The crystallite size of NPs was calculated by the width of the XRD peaks using the Scherrer formula, $D = k\lambda/β\cosθ$ and the average size of the synthesized Ag NPs was found to be approximately 20 nm. The presently calculated size of the Ag NPs was in accordance with earlier report of Jayashree and Thangaraju, [34].

![Figure 2a. UV–vis absorption spectra and reduction kinetics of Ag NPs using *N. aquatica* RDS02.](image-url)
stretching vibration of –NH band of amino groups or indication of bonded –OH hydroxyl group. The absorption peaks at 2924 cm$^{-1}$ could be consigned to the stretching vibrations of –CH$_2$ and CH$_3$ functional groups. Where peaks at 1651 and 1543 cm$^{-1}$ indicate the region of CO, C–O, and O–H groups. The intense peak at 1026 cm$^{-1}$ corresponds to the C–N stretching vibrations of aliphatic amines. The FTIR spectrum also shows bands at 1543 and 1446 cm$^{-1}$ identified as amides I and II, which arouse due to carbonyl (C=O) and amine (–NH) stretching vibrations, respectively. The absorption band at 1446 cm$^{-1}$ could be consigned to methylene scissoring vibrations from Ag NPs. The presence of the carboxyl (–C=O), hydroxyl (–OH), and amine (N–H) groups in N. aquatica RDS02 extract was

Suriati et al. [35] reported that the chemical method synthesized Ag NPs were exhibits XRD pattern (1 1 1), (2 0 0) and (3 1 1). Muzamil et al. [36] examined the X-ray Diffraction of chemically synthesized Ag NPs were 1 1 1, 2 0 0 and 3 1 1, respectively.

### 3.2.3. FTIR Analysis

The FTIR analysis was employed to identify the responsible functional group (as capping agents) of Ag NPs synthesized by the microalga, Neochloris aquatica RDS02. The FTIR spectrum of N. aquatica RDS02 extract (Figure 4) shows a number of absorption peaks, depicting its complex nature. Some of the absorption peaks at 3394 and 3448 cm$^{-1}$ corresponds to the strong stretching vibration of –NH band of amino groups or indication of bonded –OH hydroxyl group. The absorption peaks at 2924 cm$^{-1}$ could be consigned to the stretching vibrations of –CH$_2$ and CH$_3$ functional groups. Where peaks at 1651 and 1543 cm$^{-1}$ indicate the region of CO, C–O, and O–H groups. The intense peak at 1026 cm$^{-1}$ corresponds to the C–N stretching vibrations of aliphatic amines. The FTIR spectrum also shows bands at 1543 and 1446 cm$^{-1}$ identified as amides I and II, which arouse due to carbonyl (C=O) and amine (–NH) stretching vibrations, respectively. The absorption band at 1446 cm$^{-1}$ could be consigned to methylene scissoring vibrations from Ag NPs. The presence of the carboxyl (–C=O), hydroxyl (–OH), and amine (N–H) groups in N. aquatica RDS02 extract was
3.2.5. EDX and AFM Analyses

The energy dispersive spectrum clearly represented the elemental compositions of the synthesized NPs, and confirmed the presence of Ag as the major element. The Ag NPs resulted an optical absorption peak at 3 keV owing to its SPR [39]. Also, there are other elemental signals recorded along with Ag, which are due to carbon coated copper grid (Figure 6). Guzmán et al. [40] have revealed the optical absorption around 22 keV and 25 keV for the chemically synthesized Ag NPs. Where, Mostafa et al. [41] have recorded the optical absorption of intense signal at 3 keV. From the earlier findings, it was clearly witnessed that such a high fluctuated optical absorption could not be readily utilized for the biological activity when compared to the present Ag NPs. Further, the roughness of the Ag NPs synthesized in extracellular from microalgal extract was determined using AFM. Figure 7 shows the bright spots on the surface that represents the synthesized Ag NPs. The dark spots indicated the dispersion of the nanoparticles (15–45 nm in height). The 3D images showed the peaks with higher to lower sized Ag NPs and the AFM histogram provides the average size of the Ag NPs. Previously, many researchers have reported the size of Ag NPs through AFM analysis i.e. Trichosanthes tricuspidata mediated Ag NPs with the size ranges approximately from 20 to 60 nm in height [42], Centella asiatica mediated Ag NPs with the determined size of 60 nm [43]. In connection

3.2.4. HRTEM Analysis

The HRTEM micrograph of synthesized Ag NPs showed mostly in spherical shape. Similarly, the spherical shaped Ag NPs were reported earlier by Balakumaran et al. [37]. The size of the Ag NPs ranged between 15 and 20 nm with well dispersed manner (Figure 5). Where in earlier findings of Muzamil et al. [36], who have obtained the chemical based Ag NPs sizes ranged between 70 and 350 nm. In support, Kummara et al. [38] have observed the sizes ranged from 60 to 90 nm for the chemically synthesized Ag NPs, respectively. Therefore, the presently synthesized microalga derived Ag NPs could be of smaller size when compared to the chemical-based ones.
of preferable one due to the size attributed for the effective biological activity.

3.3. Antibacterial Activity of Ag NPs

The test samples (algae extract, Ag NPs, AgNO₃, and antibiotics) evaluated on four pathogenic bacteria produced the zones of inhibitions and were measured in millimeter (mm) (Table 1 and Figure 8). The maximum inhibitory-zones were recorded from Ag NPs on K. pneumoniae and B. subtilis at the concentration of 45 μL, respectively, and followed by S. aureus and E. coli. These inhibitory effect could be due to smaller size of Ag NPs that reaches the nuclear content of bacteria easily and collapse the actual mechanism and also for its large and impressive surface area [44]. Therefore, the higher activity at 45 μL of Ag NPs would related to the
large surface area to volume ratio. Presently, the obtained antibacterial results are in good accordance with the earlier reports of Ag NPs synthesized from unicellular green microalga *Scenedesmus sp.* [45]. In addition, Cox et al. [46] have reported some secondary metabolites like terpenes, polyphenols, and aromatic compounds produced by microalgae being strongly supported the antibacterial potentials of Ag NPs in the present findings.

### 3.4. In vitro Anti-Proliferative Activity of Ag NPs

Presently, the normal Vero cell line maintained was served as control cells and the cancerous HeLa cells are the subject of target. The cells shape and their morphological changes were observed under an inverted microscope.

![Figure 8. Antibacterial activity of synthesized Ag NPs. (A) E.coli; (B) K. pneumoniae; (C) B. subtilis; (D) S. aureus.](image)

The control cells showed usual polygonal, short antennae and few round cells, whereas the cells treated with algal extract and Ag NPs showed enormous morphological changes, including the cell shrinkage, loss of adhesion, a decrease of cell volume, rounding, and sporadic distribution (Figure 9(A–C)). When compared to algal extract, Ag NPs showed drastic morphological changes like more spherical shaped with shrinkages in cells [47]. Similarly, Prabhu et al. [48] have observed the morphology of Human colon carcinoma (HCT15) cells treated with Ag NPs synthesized from *Vitex negundo*. Presently, the cell viability of Ag NP treated cancerous HeLa cell line found to be decreased with an increased concentration. The IC\(_{50}\) values of algal extract and Ag NPs were 116.1 and 34.81 μg mL\(^{-1}\), respectively. The recorded IC\(_{50}\) value of Ag NPs were found to be more potential against HeLa cells with a strong cell growth inhibition at lower concentration (Figure 10). Previously, there are several researchers reported the cytotoxic effects of different algal and plants mediated Ag NPs like *Scenedesmus sp.* [39], *Cassia auriculata* [49], grape seed [50], *Gelsemium sempervirens* [51] and *Areca catechu* [52]. Hence, the present finding also correlated well with the cytotoxic activity of *Gelidiella* sp. mediated Ag NPs. Where, 50% of cell death was observed at IC\(_{50}\) value of 39.5 μg mL\(^{-1}\) [53] and profoundly the present Ag NPs also revealed significant cytotoxic property.

### 3.5. Morphological Evidence of Apoptosis by Acridine Orange/Ethidium Bromide (AO/EB) Dual Staining

The AO/EB staining was to identify the apoptotic morphological changes occurred in the algal extract and Ag

![Figure 9. Cytotoxicity activity of synthesized Ag NPs. (A) HeLa control cell line; (B) Algal extract treated HeLa cell line; (C) Ag NPs treated HeLa cell line; (D) Vero control cell line; (E) IC\(_{50}\) image of algal extract treated HeLa cell line; (F) IC\(_{50}\) image of Ag NPs treated HeLa cell line.](image)
3.6. Evaluation of Antiviral Activity of Ag NPs

The synthesized Ag NPs was evaluated for their antiviral activity using different assays. When the virus incubated with Ag NPs for 1 h prior to infecting Vero cells, it showed excellent inhibition of HSV-1 and II replications. While viruses along with Ag NPs treated on cells at the same time, the inhibition activity was decreased. After being infected by HSV-1 and HSV-II for 48 h, a noticeable cytopathic effect was seen from Vero cells, and their morphological changes significantly resulted in rounding of the cell outer layer, wrinkled cell walls, and loss of contact between cells by separation. On the other hand, the morphology of the cells incubated with Ag NPs remains unchanged. When increasing the Ag NPs concentration, the inhibitory effect on HSV-1 and HSV-2 became more prominent. The inhibition rate on HSV-1 and HSV-2 reached up to 100%, at 100 μg mL\(^{-1}\) of Ag NPs, where the replication was completely inhibited (Figure 12). The earlier finding of Hu et al. [58] strongly supports the present findings, which relates the 100% of incubation at the maximum concentration.

With these consideration of the present findings, the laboratory analyses of the prescribed procedures on micro algal derived NPs synthesis would be developed and considered for the specific properties of nanomaterials. In addition, the environment is substantially more complexes than nanoscale objects, which limiting the
of present Ag NPs. Apart from their uses in several dis
ciplinary, only the chemical based NPs could cause some
hazards to the natural ecosystem from their production
to disposal \[ 60 \].

The effect of particle size on the behavior and reactivity of
the present micro algal derived Ag NPs remains unclear.
Auspiciously, no major impacts would be found in releases

Figure 12. HSV-1 and HSV-2 inhibition effect of synthesized Ag NPs (A) Normal HEP-2 cells. The monolayer consists of short, polygonal-
shaped epithelial cells. Many rounded cells in mitosis are present. (B) HEP-2 cells with herpes simplex virus 1 Cytopathic Effect – Many
round swollen epithelial cells are present in several focal areas. Extensive piling of round epithelial cells into clusters. (C) The absence of
Herpes Simplex Virus 1 Cytopathic Effect – An increased number of rounded cells and numerous areas of cellular overgrowth. (D) Normal
HEP-2 cells. The monolayer consists of short, polygonal-shaped epithelial cells. Many rounded cells in mitosis are present. (E) HEP2 Cells
with Herpes Simplex Virus 2 Cytopathic Effect – The extensive piling of round swollen epithelial cells into clusters. (F) The absence of
Herpes Simplex Virus 2 Cytopathic Effect – An increased number of rounded cells and numerous areas of cellular overgrowth.

Scheme 1. The possible bio-reduction process involved in the Ag NPs synthesis.

Table 1. Measurement of inhibitory zone by synthesized Ag NPs, antibiotic and DMSO against bacterial pathogens.

| Micro organisms            | Concentration of Ag NPs (μg mL\(^{-1}\))^* | 15          | 30          | 45          | Antibiotic* | DMSO* |
|----------------------------|-------------------------------------------|-------------|-------------|-------------|-------------|-------|
| E. coli                    |                                           | 13.38±1.22  | 14.19±0.34  | 18.73±0.06  | 20.19±0.36  | 0.0±0.0 |
| K. pneumoniae              |                                           | 16.69±0.04  | 23.86±0.46  | 24.88±0.07  | 25.35±0.78  | 0.0±0.0 |
| B. subtilis                |                                           | 16.91±0.07  | 23.35±2.69  | 24.00±0.03  | 25.86±0.46  | 0.0±0.0 |
| S. aureus                  |                                           | 13.48±0.04  | 15.55±2.43  | 15.98±0.14  | 17.22±0.56  | 0.0±0.0 |

*indicates the zone of inhibition measured in mm (Mean ± SE).

good interpretability results of synthesized NPs [59]. The
effect of particle size on the behavior and reactivity of
the present micro algal derived Ag NPs remains unclear.
Auspiciously, no major impacts would be found in releases
of present Ag NPs. Apart from their uses in several dis-
ciplinary, only the chemical based NPs could cause some
hazards to the natural ecosystem from their production
to disposal [60].
4. Conclusion
To screw up the present investigation, the Ag NPs was successfully synthesized using Neochloris aquatica RDS02 extract. The micro algal extract could be one the potent biological resource for the eco-friendly and single step synthesis of Ag NPs, which provides simple, low cost, nontoxic, and efficient methodology. The physical properties of the synthesized Ag NPs were analyzed using HRTEM that revealed the spherical shaped NPs with an average size of 20 nm. In view of literature, this is the first report from this oleaginous microalga, N. aquatica derived Ag NPs on evaluating significant antibacterial, anticancer, and antiviral activities. Overall, the presently synthesized Ag NPs could be used as effective alternatives to control the prevalence of bacteria, cancer and viral replication. Therefore, the micro-algal derivatives could build a new arena in the nano-drug formulation for this various biological applications in near future.

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No potential conflict of interest was reported by the authors.

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