RESEARCH ARTICLE

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Phycosynthesis of Silver Nanoparticles Using *Cladophora Glomerata* and Evaluation of Their Ability to Inhibit the Proliferation of MCF-7 and L20B Cell Lines

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Abstract

Background: Nanotechnology is receiving greater attention these days as a result of its applications in numerous industrial, medical, and environmental fields. Objective: To synthesize silver nanoparticles with a green alga, *Cladophora glomerata*, and determine their inhibitory activity against tumor cell (MCF-7) and transgenic mouse cell (L20B) lines. **Materials and Methods:** Methanol extract was prepared from *Cladophora glomerata* and used as a safe factory for the synthesis of silver nanoparticles (AgNPs). UV-visible spectrophotometer, X-ray diffraction, scanning electron microscopy, and EDX analyses were used to characterize the biosynthesized AgNPs. The anti-tumor activity of the phycosynthesized AgNPs was tested against the MCF-7 and L20B cell lines. Furthermore, the bioactive compounds in the algal extract were determined by gas chromatography-mass spectroscopy (GC-MS). **Results:** The phycosynthesis produced clusters of spherical and polydispersed cuboidal pure AgNPs with an average size of 32 nm. The phycosynthesized AgNPs possess anti-cancer and anti-tumor activities on the MCF-7 and L20B cell lines, with significant anti-proliferation percentages of 52.8 and 65.8%, respectively, after 48 hours of treatment with 100 µg/ml AgNPs. Both treated cell lines showed a significant change in cellular shape and tissue detachment. The GC-MS analysis revealed the presence of a high proportion of octadecanoic acid (47.59%) and hexadecanoic acid (14.97%). **Conclusion:** *Cladophora glomerata* contains chemicals that improve the stabilization and reduction properties of the nanoparticles. It can be used as a safe, local, and natural source for the synthesis of AgNPs and can also be used as a benign factory for many other metal nanoparticles. The phycosynthesized AgNPs have anti-cancer and anti-tumor activities on the test cell lines and provide an insight into the potential for using them as a trend in cancer nanotherapy.

Keywords: Eco-friendly- Macro-algae- Nanotechnology- Reducing agents- Sustainable sources

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Introduction

One of the most challenging diseases, cancer, progresses as uncontrollably multiplying mutant cells. Its pathogenesis may result from genetic mutations or deregulation, chronic or acute exposure to various groups of mutagenic materials, or both. Through the metastasis process, cancerous cells can spread to various organs (Bharadwaj et al., 2021). Nanoparticles offer a novel and developmental perspective for the treatment of malignancies, as well as for their detection and prevention. The mortality caused by cancer has been significantly reduced as a result of the quick development of numerous diagnostic tools and therapeutic methods. Unfortunately, no one is now capable of selecting and binding tumor cells in therapeutic procedures to reduce toxicity and other side effects in patients. The preparation of nanoparticles offers a fresh solution to this problem (Hamouda et al., 2019). In the late 1800s, silver nanoparticles (AgNPs) were first used as a consumer health product. They were later incorporated into modern medicine because of their distinctive properties, such as their small size compared to their large surface area, qualified low toxicity, and significant stability, which make them useful as drug delivery carriers (Mulenos et al., 2020).

Algae are a reliable source of primary and secondary active metabolic compounds. Among the many types of algae, aquatic algae include a large concentration of phytochemicals that have been used to reduce silver ions into silver nanoparticles. These compounds include protein, carbohydrates, steroids, alkaloids, phenols, and flavonoids (Elumalai et al., 2021). *Cladophora glomerata* is a macro-alga in the chlorophyte class. The primary distinguishing characteristics of this green alga are a true branched thread with a hair-like appearance, with its hairs typically connected to form a net-like structure, frequent in

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the benthic region, attached to submerged solid particles, and a wide distribution in both fresh and marine water. Its filament is small and extremely rigid, and it is typically stable in a single spot, making it easy to harvest (Auer et al., 2010; Lefta et al., 2022). *Cladophora glomerata* is found in both freshwater and marine environments worldwide. Its external morphology is a dark green, moss-like structure with a few branches and a filamentous true branched form, with cross walls separating their filaments into multi-nucleated segments (Figure 1). The threads of *C. glomerata* are frequently longer, creating "streamers" that can reach lengths of up to 1 m (Aziz and Maulood 2016; Michalak and Messyasz, 2021). It has been demonstrated that the production of nanoparticles using algae is generally stable in solutions, environmentally friendly, and widely available. The suggested green preparation process also combines cutting-edge bioscience with low cost, high yield, and vertebrate-safe technology. In aquatic ecosystems, algae are abundant resources that can be exploited for food, feed, and medication (Ghareeb et al., 2020).

The aim of the study was to employ a novel method of AgNP synthesis utilizing a local isolate of the green alga, *C. glomerata* as a potential candidate for controlling the proliferation of two cancer and tumor cell lines.

Materials and Methods

Sources of cell lines

The breast cancer cell line (MCF7) and the transgenic mouse cell line (L20B) were obtained from the Center for Biotechnology Research of Al-Nahraine University/ Baghdad/Iraq.

Collection and preparation of algal samples

An algal sample was collected by hand in March 2021 from a rock inside a water stream in Samawah city, southern Iraq, at 31.32°N and 45.28°E (Figure 1). Plastic bags were used to store and transport the macro-alga to the laboratory after it had been harvested. To identify the species, the specimens were pressed and preserved in a 4% formalin solution. The algal biomass was then manually cleaned, rinsed with tap water, washed several times with distilled water, maintained in a ventilated area, and then heated to 50°C to produce around 2 grams of dry biomass, before being ground to a fine powder (Mohammed et al., 2018; Nutautait 2022).

Phycosynthesis of silver nanoparticles

Methanol extract from *C. glomerata* was prepared using the Soxhlet extraction method (Fayyad et al., 2019). Algal biomass was extracted for 24 hours with 70% methanol, and then the liquid phase of the extracted algal material was collected following centrifugation for 15 minutes at 10,000 rpm. Ten milliliters (10 ml) of algal extract and 90 ml of silver nitrate aqueous solution (1mM) were combined to synthesize silver nanoparticles (NPs), which resulted in a greenish yellowish solution. After three hours, a visible color change in the solution was observed at 50°C and pH9 conditions confirming the successful synthesis of AgNPs.

Purification and characterization of phycosynthesized silver nanoparticles

Bio-reduced silver ions were purified by spinning for 15 minutes at 10,000 rpm. The resulting pellet was rinsed using sterile double-distilled water to ensure that there were no residual biological molecules in the algal extract. Until further analysis, the obtained molecules were kept in a refrigerator at 4°C. The purified AgNPs were examined using a scanning electron microscope (AIS2300C, Oxford) and characterized with a UV-vis spectrophotometer (Shimadzu 1601, Japan) at various wavelengths between 320 and 700 nm. The X-ray diffraction (XRD) spectrum was used to assess the nanoparticles' size (Schimadzu6000, Japan). Additionally, an energy-dispersive X-ray (EDX; Quantax 400, Bruker, Billerica, MA, USA) analysis was performed to identify the elements and crystal structure of the nanoparticles (Rajeshkumar et al., 2017).

Determination of the anti-tumor activity of the phycosynthesized silver nanoparticles

An MTT assay was carried out in the Center for Biotechnology Research of Al-Nahraine University/ Baghdad/Iraq to determine if the biologically synthesized AgNPs were active against breast cancer cell (MCF7) and transgenic mouse cell (L20B) lines, which express the human poliovirus receptor on the surface. Purified AgNPs were serially diluted four times to obtain concentrations of 12.5, 25, 50, and 100µg/ml to determine the inhibition percentage of cancerous cell growth as described by Carreño et al., (2021).

Determination of bioactive compounds by GC-Mass Spectroscopy

A GC-MS (gas chromatography-mass spectroscopy) analysis was conducted on the algal extract to identify the main phytochemicals that make them up, particularly those that supported the synthesis of the NPs. After injection with 5 mL of algal extract, a column with the following characteristics was used: Inert cap 1MS; 30 m of 0.25 mm id; and 0.25 m of film thickness. A high temperature of 280 °C was used. Utilizing post-run software and (SHIMADZU, Japan), the complete process has been described by (Nutautait, 2022). Finally, the phytochemicals were identified by comparing their mass to the NIST library search and reporting standards.

Results

Physical characteristics of the phycosynthesized silver nanoparticles

After 20 minutes of green synthesis, the translucent solution in the reaction mixture changed color to a dark brown color. This observation was the primary indication that silver nanoparticle formation has occurred. The reduction process in Ag+ to AgO is what causes the color change (Kaushik et al., 2022). The UV-visible spectra of the Ag-NPs mixture revealed a single maximum absorbance at 440 nm, as shown in Figure 2. There was no significant change in the peaks for the reaction mixture. This observation was due to the excitations of the SPR (Surface Plasmon Resonance) of the NPs, which indicates

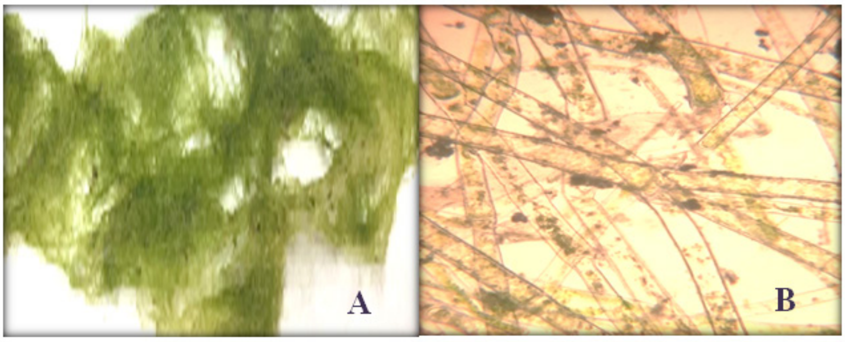

Figure 1. Structure of *Cladophora glomerata*. A, Hair-like shape; B, Branched filaments (40X)

Table 1. X-ray Diffraction Data of Phycosynthesized Silver Nanoparticles

Planes (hk£)	220	200	111
2 theta(deg)	32.207	46.208	27.792
FWHM (deg)	0.202	0.203	0.201
D (nm)	41	43	40
η x 10^{-4} lines^{-2} .m^{-4})	0.0008	0.0008	0.0084

that the biologically synthesized AgNPs are spherical, and this was confirmed by the SEM image (Sosa et al., 2003).

Characteristics of the phycosynthesized silver nanoparticles according to the SEM, EDX, and XRD analyses

The structure of the biosynthesized AgNPs is depicted in the image of the SEM (Figure 3). The NPs are spherical and polydispersed cuboidal as well as appearing as strongly agglomerated grains forming a cluster. Additionally, EDX analysis was performed to confirm the synthesis of the AgNPs. As shown in Figure 4, elemental silver exhibits a significant optical absorption band at energies around 3 keV. Furthermore, a weak signal from “O” appeared as a result of the X-ray emission from the Cladophora extract mixture. The EDX spectrum confirms the purity of the biosynthesized silver NPs with a weight percentage of 67.05. Three noticeable peaks were detected during the XRD analysis, and they were 2-theta 27.792°, 32.207°, and 46.208°. Meanwhile, the average crystallite size of AgNPs, which resulted from Scherer’s equation, was 32 nm (Figure 5 and Table 1).

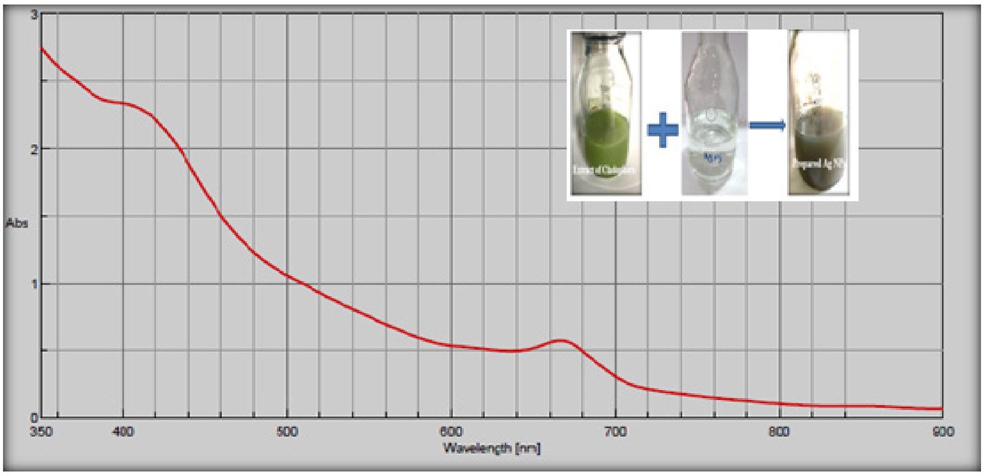

Figure 2. Ultraviolet-Visible Spectra Depict the Synthesis of Silver Nanoparticles in the Reaction Mixture of Cladophora Extract and $AgNO_3$

Table 2. Percentage Inhibition of Proliferation of Phycosynthesized Silver Nanoparticles on Two Tumor Cell Lines

Treated cell lines	MCF7		L20B	
Concentrations of generated Ag-NPs (µg/ml)	Growth inhibition % After 24 hr.	Growth inhibition % After 48 hr.	Growth inhibition % After 24 hr.	Growth inhibition % After 48 hr.
6.25	15.0	19.7	12.5	22.7
12.5	22.1	25.8	25.7	29.4
25	28.0	30.1	33.9	38.4
50	32.0	32.2	40.1	55.0
100	44.5	52.8	45.8	65.8

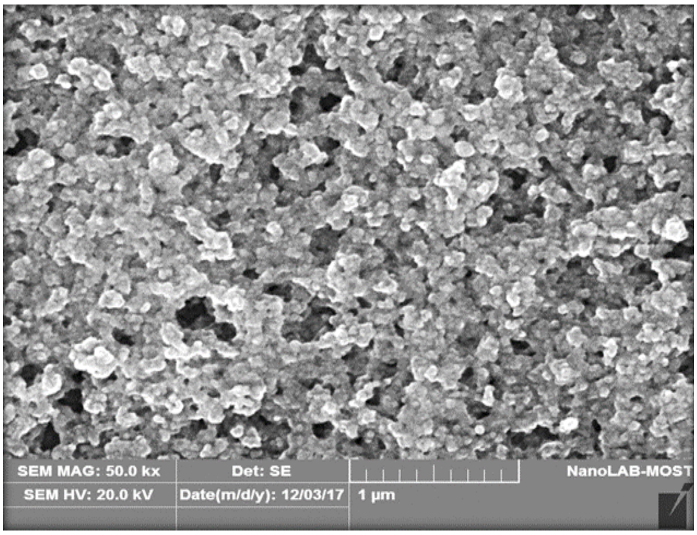

Figure 3. Scanning Electron Microscope Image of Phycosynthesized Silver Nanoparticles

Table 3. The Major Detected Phytoconstituents of Hot Methanol Extract of Cladophora Glomerata Alga Using GC-Mass Spectrophotometer

No.	Rt.	Area%	Name of compound
1	2.153	2.16	Tridecyne
2	15.57	10.51	Hexadecanoic acid
3	16.8211	1.06	Octadecadienoic acid
4	17.436	47.59	Octadecanoic acid
5	18.693	4.46	Hexadecanoic acid
6	20.299	25.59	Pentadecadien-1-o
7	22.058	4.16	Tetradecenal l

Anti-tumor activity of phycosynthesized silver nanoparticles

The two test cell lines (MCF7 and L20B) in this study were observed to be affected by the phycosynthesized AgNPs in a dose- and exposure-period-dependent manner. The most effective concentration of the AgNPs under investigation was 100 μg/ml, which resulted in 44.5 and 45.8 % inhibition rates of tumor cell growth after 24 hours and 52.8 and 65%, inhibition rates after 48 hours for both MCF7 and L20B, respectively (Table 2). A decrease in cellular viability was observed, as highlighted in Figure 6. Also, there was a significant change in cellular morphology and cellular detachment after treatment of cell lines with biosynthesized AgNPs. The anti-tumor impact of AgNPs was determined by essential properties, such as the shape, size, and charge of their surfaces. Furthermore, exposure time and material concentrations are important factors to consider; thus, longer exposure times and higher concentrations produced greater anticancer effects (Xu et al., 2020).

Discussion

The potent anti-proliferative capacity of AgNPs resulted in significant attention in the field of cancer treatment. This fact was proven by numerous in vitro studies and was supported by numerous findings to clarify the precise processes underlying their anti-tumor action on cell lines. Khan et al., (2021) discovered that AgNPs produced by green synthesis might interfere with the cytoskeleton reorganization of MCF-7 cells, which

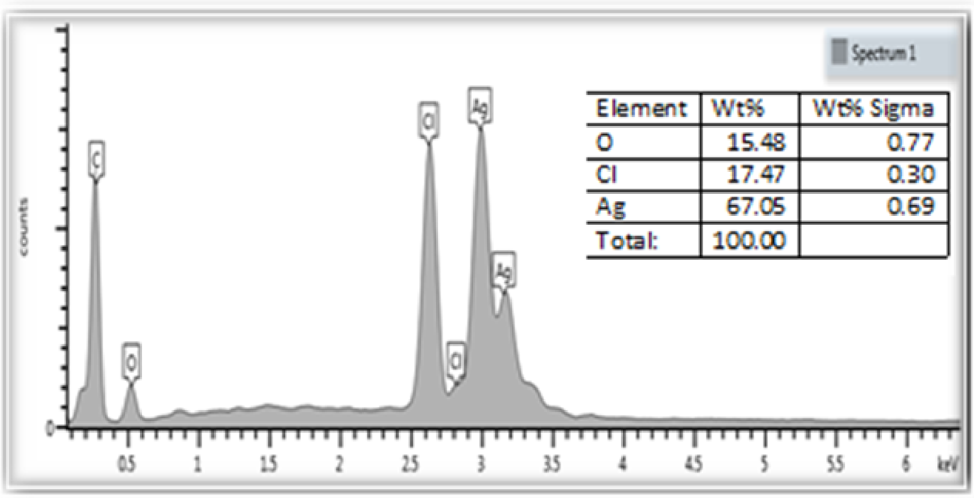

Figure 4. Energy-Dispersive X-ray Analysis of Phycosynthesized Silver Nanoparticles

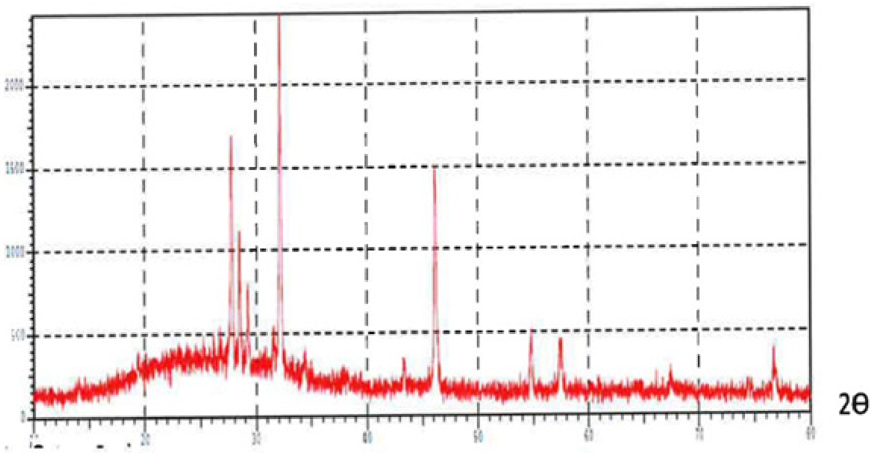

Figure 5. X-ray Diffraction Patterns of Phycosynthesized Silver Nanoparticles

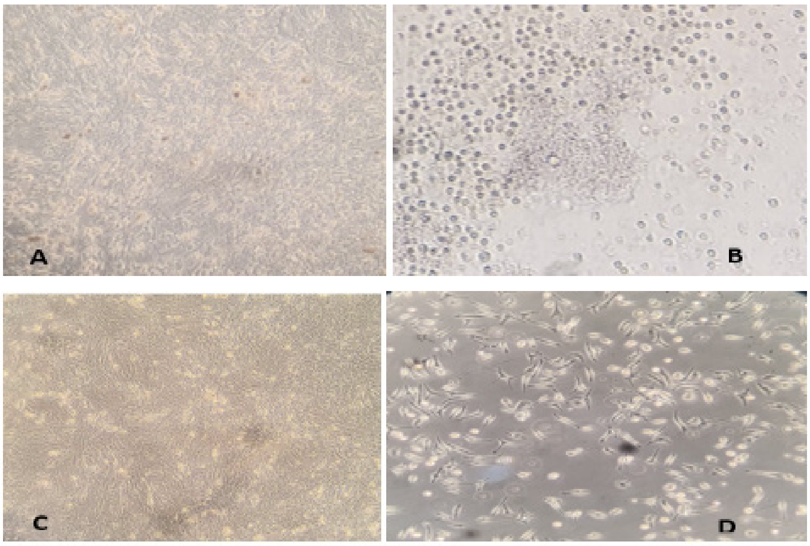

Figure 6. Microscopic Views of Treated Tumor Cell Lines. A, Untreated L20B cell line (control); B, L20B cell line treated with phycosynthesized silver nanoparticles; C, Untreated MCF7 cell line (control); D, MCF7 cell line treated with phycosynthesized silver nanoparticles (10X). Significant changes in cellular morphology and decrease in cellular attachment were noticed for both treated cell lines.

prevented the cells from migrating. This was required for the cell division and migratory processes to be optimized. It has been confirmed that one of the anticipated mechanisms of AgNPs' cytotoxicity on MCF-7 cells is the disruption of these NPs' interactions with cell cycle-engaged genes, which led to DNA damage and ultimately apoptosis. Additionally, specific chemical components of AgNPs, such as the amino, alcoholic, and aldehyde groups, have

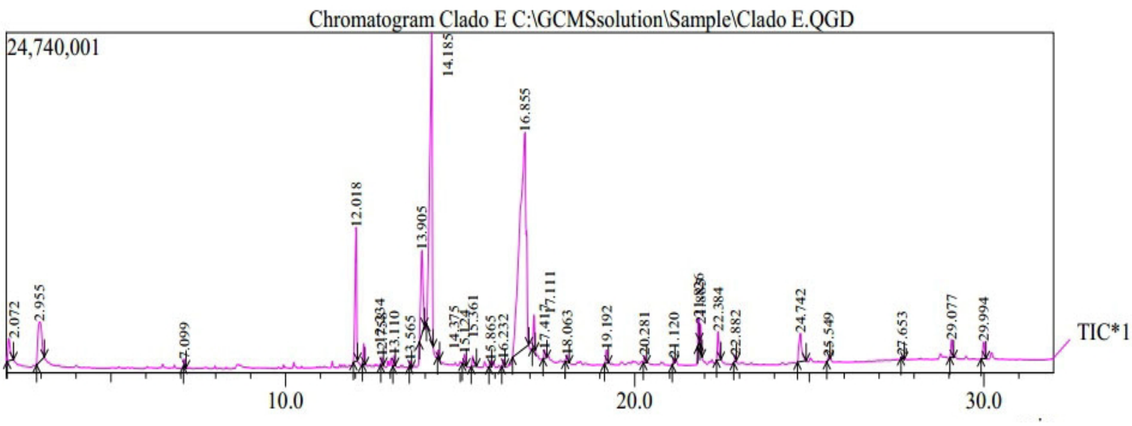

Figure 7. The Chromatogram of GC-Mass Spectrophotometry Showing the Phytoconstituents of the Hot Methanol Extract of Cladophora Glomerata

been linked to the inhibition of cancer cell growth. The cellular membrane, nuclear membrane, and intracellular interactions are the three most common entry points for NPs into the human body (Sathishkumar et al., 2019).

Macromolecules, such as proteins and nucleic acids, may ultimately induce the apoptotic effect of the cancerous cells with no recorded inflammatory response (Owaid et al., 2020). The surface charge and size of these NPs have a significant impact on the efficiency of this process; typically, nanoparticles with a size range of 30 to 100 nm have the lowest efficiency to penetrate cancerous cells. Silver nanoparticles are dispersed into the cells of mammals in one of two ways: endocytosis or phagocytosis (Netala et al., 2016). It has been demonstrated that the inactivation of the drug resistance property in malignant cells may be generated by inactivating the efflux activity of multidrug resistance transporters in cancer cells. This is another proposed mechanism regarding the damage to cancer hallmarks that may be induced by AgNPs, particularly glucose metabolism and drug resistance (Rank et al., 2020).

Bioactive compounds in Cladophora glomerata methanol extract

The GC-MS chromatogram of the *C. glomerata* methanol extract revealed seven noticeable peaks, indicating the presence of seven phytochemicals (Table 3). These active constituents were detected in the following proportions of the total area: Octadecanoic acid (47.59%), pentadecadien-1-ol (25.59%), hexadecanoic acid (10.97%), hexadecanoic acid (4.46%), tetradecane (4.16%), tridecyne (2.16%), and octadecadienoic acid (1.06%). As displayed in Table 3, Cladophora alga is presented as a new effective biological factory for AgNP synthesis. This assumption is supported by the presence of phytochemicals, particularly fatty acids like octadecanoic and hexadecanoic acids, in the algal extract. In the methanol extract of C. glomerata, these two compounds were detected at high percentages, 47.59 and 14.97 for octadecanoic acid and hexadecanoic acid, respectively. According to Bhuyar et al. (2020), fatty acids play a role in the synthesis of NPs and effective storage. It has been reported that fatty acids function as a stabilizing and protective agent, preventing the buildup of AgNPs. The biological molecules generated from plants undergo highly regulated assembly, which makes them a good candidate for the synthesis of metal nanoparticles (Monisha et al., 2016). Furthermore, plant extracts play a significant role in the synthesis of metal NPs. When compared to chemical and physical synthesizing techniques, eliminating their toxicity is as important as stabilizing and capping them (Shareef and Al-mussawi 2022).

Many researchers have employed algae as a safe factory to create AgNPs. Elumalai et al., (2021) have used the cyanophyte, Oscilatoria sancta to synthesize NPs. These NPs were also synthesized by Ghareeb et al. (2020) utilizing Cladophora species with a 13 nm size. Also, with the help of secondary metabolites from the marine alga, Padinapavonica species (Torabfam and Yüce, 2020) and the green alga, Chlorella vulgaris (Sahayaraj et al., 2012), AgNPs were successfully synthesized. Algae are a prime choice for the biosynthesis of NPs because they are abundant in the secondary metabolites necessary for reducing and capping NPs. As a result, NPs generated from algae are employed in a wide range of applications, including bioremediation, antifouling, and biosensing. Despite this, the use of algae in the early stages of research was less than that of green synthesis of NPs using plant extracts (Hano and Abbasi, 2021). In general, the present research offers an insight into the dependence on *C. glomerata* to produce not only AgNPs but other NPs as well. In particular, given that this green alga is widely spread in the water streams of Iraq and has been verified as a rich source of several metabolites. Additionally, researchers explored the effects of Cladophora-based AgNPs as an anti-proliferative drug on various tumor cell lines, particularly those that pose a concern to human beings.

In conclusion, the findings of this study reveal that the green alga, *Cladophora glomerata* can be used as a benign factory for the synthesis of AgNPs due to the presence of several chemicals that facilitate their reduction and stabilization. The results of the anti-tumor or anti-cytotoxic effect suggest that this approach may also be helpful for tumor therapy by preventing the growth of cancer and tumor cells as well as overcoming the problem of drug resistance. The proposed method can be readily adapted to many other applications other than tumor therapy. The future directive of the study is to prepare silver nanoparticles with smaller sizes and more attractive properties to obtain the highest possible penetration into the cellular membranes of cancerous, tumor, or transformed cells (not normal).

Author Contribution Statement

Raghad J. Fayyad (RF), and Alaa Naseer Mohammed Ali (AA), Noor Al-Huda Ali A. H. Saeed (NS), carry out the lab work, and follow up the results while Israa Hussein Hamzah (IH) and Ahmed S. Dwaish (AD) contributed to conception, design, interpretation, and writing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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