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Evaluation of Anticancer Potential of Silver Chloride Nanoparticles Biosynthesized by *Penicillium chrysogenum*

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ABSTRACT

Backgrounds: Green synthesis of nanoparticles (NPs) is a simple, fast, and eco-friendly method which could be performed by various microorganisms or plant extracts. Silver NPs are well-known as antimicrobial and anti-fungal materials. They play an essential role in the control of tumors via their cytotoxic effects. Therefore, they have attracted significant attention for developing an effective treatment solution for cancer cells. This study aimed to investigate the potential of *Penicillium chrysogenum* for the synthesis of silver NPs and to evaluate their toxicity on liver cancer cell line (HepG2).

Materials & Methods: After synthesis of NPs using *P. chrysogenum*, characterization of the synthesized NPs was performed by UV–Vis spectroscopy, X-ray diffraction (XRD), and transmission electron microscopy (TEM). Fourier transform infrared spectroscopy (FTIR) was carried out to detect biomolecules that may be responsible for the synthesis and stabilization of NPs. The cytotoxic activity of the synthesized AgclNPs on HepG2 cell line was evaluated using MTT assay.

Findings: UV–Vis spectroscopy and XRD analysis confirmed the synthesis of AgclNPs using *P. chrysogenum*. TEM analysis revealed the spherical shape of AgclNPs with an average crystalline size of 15 to 45 nm. FTIR spectroscopy indicated the possible functional groups that could be responsible for the reduction of metal ions and the capping process. These nanoparticles showed a dose-dependent anticancer activity against HepG2 cells.

Conclusion: The results suggest that biosynthesized silver chloride nanoparticles could offer potential applications in cancer therapy.

Keywords: Silver chloride nanoparticles, Green synthesis, *Penicillium chrysogenum*, Liver cancer, Cytotoxic activity

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Introduction

Cancer is one of the leading causes of mortality, and its treatment is very challenging. Surgery, radiation therapy, chemotherapy, and hormone therapy are the most common treatment methods used for cancer [1]. In recent years, nanotechnology-based therapeutic and diagnostic approaches have shown good potential for improving cancer therapy [2].

Recently, nanotechnology has attracted the attention of many scientists, and its products are applied in many branches of industry. Nanoparticles (NPs) are materials with at least one dimension under 100 nm. Due to their large surface area-to-volume ratio, NPs have considerably unique optical, magnetic, catalytic, and electronic properties [2].

The synthesis of nanoparticles is the most important part of nanotechnology. The most conventional methods employed to produce NPs are chemical and physical synthesis. Nevertheless, the production of NPs by these methods poses numerous challenges; in addition, they are often extremely expensive, hazardous, non-eco-friendly, and comparatively complex [3]. For medicinal applications, NPs synthesis method must be eco-friendly and non-toxic or at best with low toxicity. Recently, green synthesis of NPs using biological agents such as microorganisms (bacteria and fungi) or plant extracts has gained attention in nanotechnology as an eco-friendly, non-toxic, relatively inexpensive, and easy method for large-scale production [4]. In this method, biomolecules such as amino acids, proteins, polysaccharides, enzymes, and vitamins act as reducing agents. Thus, in this biological method, the usage of expensive and toxic materials is avoided [5].

Due to the unique properties of silver nanoparticles, such as conductivity, stability, as well as optical and catalytic properties, this group of nanoparticles has received considerable attention in recent years. These properties are strongly influenced by their size, surface properties, distribution, and morphology. Silver NPs are well-known as antimicrobial and anti-fungal materials and widely used in many commercial products against pathogenic microorganisms [6]. In addition, silver NPs play an essential role in the control of tumors via their cytotoxic effects. Thus, they have attracted significant attention for developing an effective treatment solution for cancer cells. Their cytotoxic activity against cancer cell lines is mediated through their destructive effects on various cellular systems, such as deregulating cell cycle, decreasing mitochondrial function, producing reactive oxygen species (ROS), releasing lactate dehydrogenase (LDH), inducing apoptotic genes, inducing chromosomal aberrations, and DNA damage [7]. In recent years, many studies have been performed on various types of cancer cells, human liver cancer [8, 9], cervical carcinoma [10, 11], lung cancer [12, 13], colon carcinoma [14, 15], and breast cancer [16, 17].

Objectives: This study aimed to investigate the potential of *Penicillium chrysogenum* for the synthesis of silver NPs and to assess their toxicity on HepG2 (liver cancer cell line).

Materials and Methods

Source of fungal strains: The fungal strain used in this study was isolated from historical paintings in the storeroom of Mouze Makhsus of Iran. This isolate was identified as *Penicillium* species by staining method and showed high ability to produce cellulase enzyme.

Molecular identification: Total DNA of the isolates was extracted using AddPrep Genomic DNA Extraction Kit (Korea). Molecular identification of the fungal strain producing silver nanoparticles was carried out by PCR (Eppendorf, Germany) using specific primers previously designed by

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White et al. (1990) [18] to amplify the ITS region (ITS1: 5´- TCCGTAGGTGAACCTGCGG-3´ and ITS4: 5´- TCCTCCGCTTATTGATATGC-3´). PCR amplification was performed under the following conditions: a pre-denaturation step at 95°C for 5 minutes; followed by 30 cycles of denaturation at 95°C for 30 seconds, 59°C for 45 seconds, and 72°C for 30 seconds; and a final extension step at 72°C for 6 minutes. PCR products were sequenced by the Codon Genetic group and analyzed in the Genbank using NCBI nucleotide database and the BLAST algorithm to assess the similarity with other reported sequences of fungal species.

Fungal extract preparation: In order to produce AgNPs, the fungal strain was inoculated in a flask containing 50 mL of sterile Sabouraud dextrose broth medium (Merck, Germany) and incubated at 28 °C for 72 hours while shaking at 150 rpm. After incubation, fungal biomass was separated from the growth medium using a filter paper (Whatman No.1). In order to remove any medium component, the separated biomass was washed twice with distilled water. Then 5 g of biomass was re-suspended in 150 mL of sterile deionized water and incubated at 25°C for 24 hours while shaking at 150 rpm. After incubation, the biomass was filtered again, and cell-free filtrate was collected and stored at 4 °C for further analysis [19].

Biosynthesis of silver NPs: In order to synthesize AgNPs, 5 mL of 100 mM $AgNO_3$ (Merck, Germany) solution was mixed with 45 mL of cell-free filtrate (final concentration, 10 mM). After stirring the solution on a magnetic heater at 50°C for 2 hours, it was incubated at 37°C for 24 hours while shaking at 150 rpm. Cell-free filtrate without silver ions was used as a control and run along with the experimental flask [20].

Purification of silver nanoparticles: In order to perform further studies on the produced nanoparticles, it was necessary to remove excess materials in the reaction mixture, such as salt ions, proteins, and other impurities of fungal extract. The supernatant containing AgNPs was transferred into a sterile microtube and centrifuged at 10000 rpm for 30 minutes. The supernatant was removed, and the pellet was re-suspended in 1 mL of normal saline. This process was repeated three times [20].

Nanoparticles characterization UV-Vis spectroscopy: The synthesis of AgNPs changes the color of the solution to brown, which could be detected by naked eye. Brown solutions were examined by a UV-Vis spectrophotometer (SHIMADZU UV-1240, Germany) from 250 to 700 nm.

Transmission electron microscopy (TEM): The size and shape of AgNPs were investigated using TEM. The liquid sample of the produced AgNPs was poured on a carbon-coated copper grid and analyzed using a transmission electron microscope (Zeiss EM900, Germany).

X-ray diffraction (XRD): XRD analysis was used to detect the crystallographic structure of the synthesized silver nanoparticles. The freeze-dried sample was analyzed by a PANalytical device made in the Netherlands with CuKa lamp radiation and θ2 angle within 0 to 80 degrees.

Fourier transform infrared spectroscopy (FTIR): FT-IR analysis was performed by Spectrum Two model of Perkin Elmer (USA).

In vitro cytotoxicity assay: The cytotoxicity effect of AgNPs was evaluated against HepG2 cancer cells using MTT(3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. The cells were grown in Dulbecco's Modified Eagle's medium (DMEM, Sigma–Aldrich, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin–streptomycin (Sigma–Aldrich, USA) and incubated at 37°C in the presence of 5% CO_2. Specifically, 200 µL of the medium containing 1×10^4 cells/well in

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their exponential growth phase was seeded in a 96-well microtiter plate and incubated at 37°C for 24 hours. After the incubation period, the medium of each well was replaced with 100 µL of the medium containing 0.5 to 100 µg/mL of AgNPs and incubated for 24 hours. After the incubation, the medium was removed, and the cells were washed using phosphate-buffered saline (PBS). Briefly, 20 µL of MTT dye (Sigma–Aldrich, USA) solution (5 mg/mL in PBS) was added to each well. After incubation for 4 hours, excess MTT dye was removed. For stabilizing the formed formazan crystals, 100 µL of DMSO (Sigma–Aldrich, USA) was added to each well and placed on a shaker for 20 minutes. The optical density of the wells was measured using an ELISA plate reader (Anthos, UK) at 570 nm. Finally, cell toxicity percentage and IC_{50} value were calculated [21, 22].

Findings

Molecular Characterization: Fungal strain producing AgclNPs was analyzed by molecular method. Based on the ITS region sequences, the isolate showed 99.81% similarity index with *P. chrysogenum* (Table 1).

UV-Visible Spectroscopy: The initial detection of silver nanoparticles was performed visually based on the conversion of colorless $AgNO_3$ solution to brown after incubation. Under the same conditions, no color change was observed in the control solution. The absorption spectrum of the green synthesized AgNPs is illustrated in Figure 1. Due to the surface plasmon resonance of AgNPs, a significant peak was observed at 420 nm, indicating reduction of $AgNO_3$.

XRD Analysis: XRD patterns of AgclNPs synthesized by *P. chrysogenum* are demonstrated in Figure 2. This figure shows diffraction peaks at 2θ, values of 27.85°, 32.23°, 46.25°, 54.83°, 57.41°, 67.44°, and 76.67°, which were found to be (111), (200), (220), (311), (222), (400), and (420) reflections, respectively. The XRD analysis results proved the crystalline structure of the synthesized AgclNPs. The average crystalline size of NPs was calculated to be 22.8 nm, which was estimated by Debye–Scherrer's equation: $d = 0.89\lambda/\beta \cos\theta$, where d is the mean diameter of the nanoparticles, λ represents the wavelength of the X-ray radiation source, β denotes the full-width half maximum of the AgclNPs plane, and θ is the diffraction angle [23].

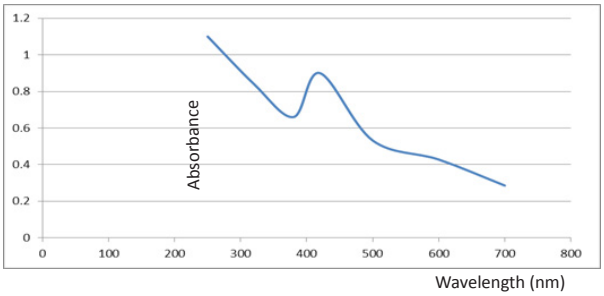

Figure 1) UV-Vis absorption spectrum of AgNPs synthesized using *P. chrysogenum*

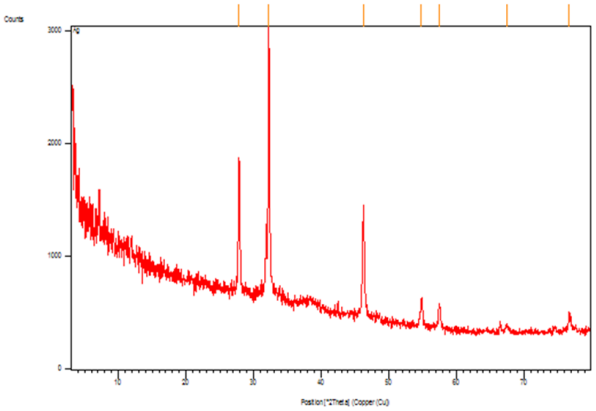

Figure 2) X-ray diffraction pattern of the green synthesized AgclNPs using *P. chrysogenum*

FTIR Analysis: FTIR analysis was performed to identify possible biomolecules which may be responsible for the reduction and stabilization of silver ions in the synthesis process of NPs. Figure 3 displays the FTIR spectra of *P. chrysogenum* extract and synthesized AgclNPs. The FTIR spectrum of the fungal extract alone showed distinct peaks in the range of 3412.29 (O-H bond in alcohols), 2930.08 (C-H bond in alkanes), 2346.71 (C≡C in alkenes), 1655.19 (C=O bond in amide), 1451.86 (C=C in aromatic ring), and 1106.55 (C–O bond in alcohols) cm^{-1}.

[DOI: 10.52547/iem.8.2.159]

Table 1) Molecular identification of fungal strain producing AgclNPs using ITS region*Accessions with the same similarity

PCR Product Size (bp)	Description	Accession	Coverage (%)	Identity (%)
597	*Penicillium chrysogenum*	*MH856357.1* AY373902.1* HQ026745.1* NR_077145.1**	99	99.81

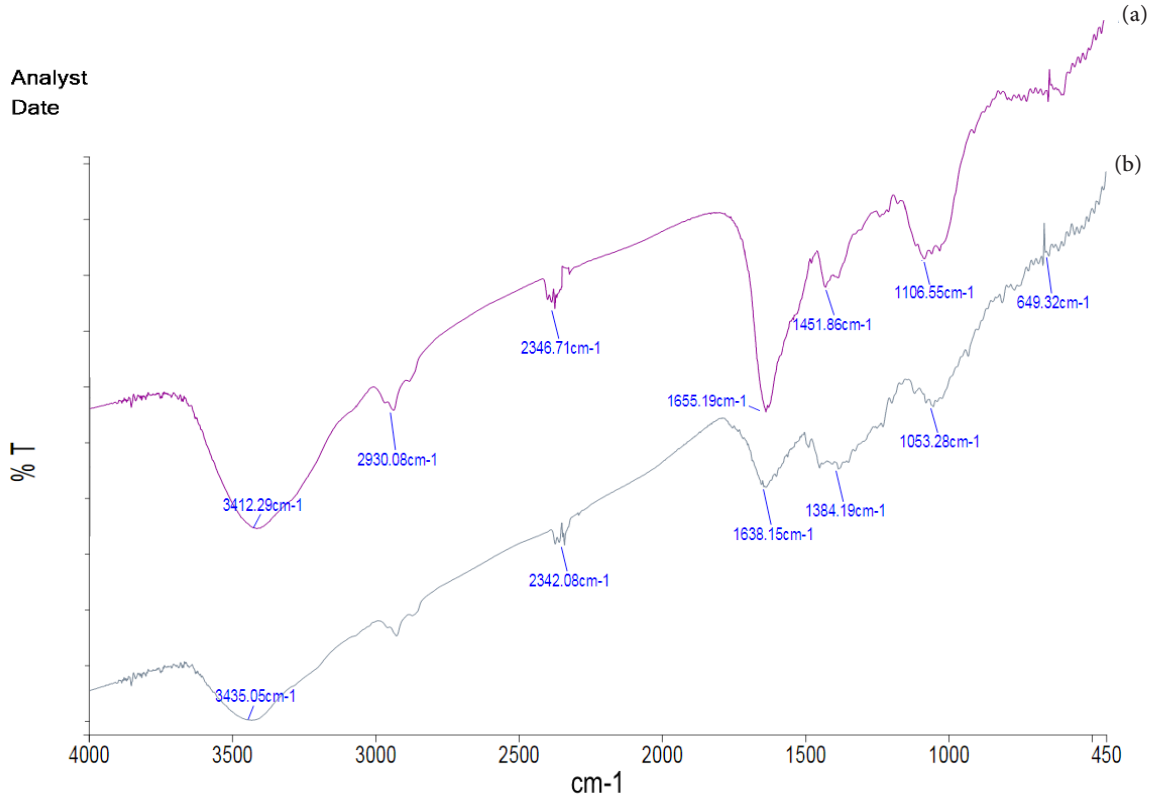

Figure 3) FTIR spectra of *P. chrysogenum* extract (a) and the green synthesized Agcl NPs (b)

TEM Analysis: TEM analysis of the green synthesized AgclNPs revealed that they were poly-dispersed with predominantly spherical and oval shapes. The diameter of AgclNPs ranged from 15 to 45 nm with an average diameter of 25 nm as illustrated in Figure 4.

MTT Assay: The cytotoxic activity of green synthesized AgclNPs against liver cancer cell line HepG2 was determined by MTT assay. The synthesized AgclNPs reduced HepG2 cells viability in a dose-dependent manner. The half maximal inhibitory concentration (IC_{50}) value of AgclNPs against HepG2 cells was 7.34 ± 0.71 µg/mL during 24 hours (Figure 5).

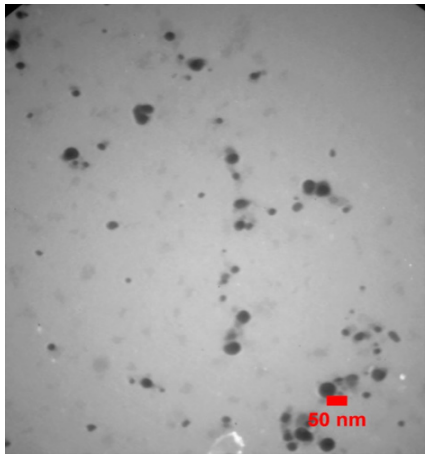

Figure 4) Transmission electron microscopy images of the green synthesized AgclNPs

[DOI: 10.52547/iem.8.2.159]

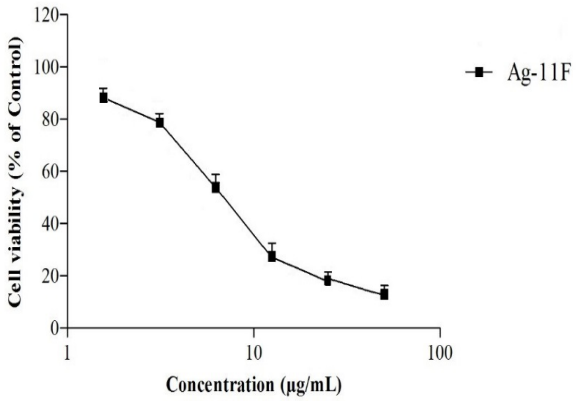

Figure 5) Effect of the green synthesized AgclNPs on HepG2 cell viability

Discussion

Biosynthesis of NPs using fungi has been widely used. They are considered as the best candidates for the biosynthesis of metal NPs due to their potential to produce a wide range of biomolecules that act as reducing agents. On the other hand, the growth of fungi is relatively fast and allows obtaining a high amount of biomass within a short time, making fungi a suitable option for large-scale green NPs synthesis. So far, many fungal species have been studied in terms of their potential for the synthesis of silver NPs, such as *Fusarium oxysporum* [24], *Aspergillus flavus* [25], *A. terreus* [26], *A. fumigatus* [27], *Cladosporium cladosporioides* [28], *P. fellutanum* [29], and *P. italicum* [30]. In this study, a fungal strain identified as *P. chrysogenum by* molecular method was used for green synthesis of AgNPs.

Biomolecules in fungal extracts reduce aqueous silver ions to silver NPs, which could be detected based on the color change of the reaction mixture to brown. Due to the surface plasmon resonance of AgNPs, UV-Vis spectroscopy is one of the main and important methods to detect the formation of silver NPs in colloidal solutions. Due to silver NPs size and shape, they exhibit a UV–Visible absorption in the range of 400–500 nm [31]. In this study, the maximum absorption was observed at 420 nm.

XRD is a non-destructive method that provides comprehensive information about the chemical composition and crystallographic structure of synthesized nanoparticles. In this study, X-ray diffraction analysis of lyophilized powder confirmed the formation of silver chloride nanoparticle crystals in the sample. Green synthesis of silver chloride NPs by bacteria [32], fungi [33], and plant extracts [34] has been reported by other researchers. The formation of AgclNPs could be due to the reaction between Ag^+ ions of $AgNO_3$ and Cl^- ions of biochemical compounds in the fungal extract.

In the FTIR spectrum of lyophilized *P. chrysogenum* extract, the major peaks were located at 3412.29, 1655.19, and 1106.55 cm^{-1}. The strong peak at 3412.29 cm^{-1} is attributed to the presence of the O-H group in alcohols and phenol functional groups. The absorption band at 1655.19 cm^{-1} region is mainly assigned to the stretching vibrations of C=O in the amide bonds in proteins. The peak at 1106.55 cm^{-1} is due to the stretching vibrations of the C–O bond in alcohols. The peak at 1451 cm^{-1} may be related to C=C stretching vibrations, while the peak at 2930.8 cm^{-1} represents C–H stretching vibrations. Overall, these results indicated the presence of alcohols, phenols, and proteins in *P. chrysogenum* extract and biosynthesized NPs. These biomolecules could act as reducing agents to reduce metal ions. Also, they could act as capping agents by binding to biosynthesized AgclNPs through free amino groups or electrostatic attraction of negatively charged carboxylate groups [35]. The obtained results were inconsistent with previous studies results. In a study by Taha et al. (2019), FTIR analysis of AgNPs synthesized by *P. italicum* revealed strong absorption peaks at 3404, 1658, and 1064 cm^{-1}, corresponding to stretching vibrations of O–H bond, binding vibrations

[DOI: 10.52547/iem.8.2.159]

of amide I, and OH deformation in alcohols and phenols, respectively [30]. In a similar report, FTIR analysis of NPs produced by *A. terreus* showed peaks corresponding to stretching vibrations of O-H bond in alcohols or phenols, rocking vibrations of C-H in alkanes, and stretching vibrations of C=O in carboxylic acid [26].

Nanoparticles show different physical and chemical properties depending on their size and shape. The study of the synthesized NPs by TEM provided useful information about the size and shape of NPs as well as their distribution. The TEM analysis results revealed that AgclNPs were not in direct contact with each other. The separation between these nanoparticles indicates the stabilization of these NPs by capping with proteins in the fungal extract [36].

In this study, the synthesized NPs were spherical and 15-45 nm in size. Estimation of NPs size using the Debye/Scherrer formula indicated that the average nanoparticle size was 22.8 nm, showing an acceptable agreement with the NPs size estimated by TEM analysis. Many factors such as the nature of the organism, temperature of the reaction, and pH of the solution affect the size, shape, and distribution of the green synthesized nanoparticles [37]. Synthesis of spherical silver chloride nanoparticles by green method has been reported in previous studies. Al Aboody et al. (2019) reported that Ag/AgclNPs synthesized by *Azadirachta indica* lalex were spherical, and their size ranged from 12.27 to 27.20 nm in diameter [34]. In a similar study, Patra and Baek (2016) reported that the synthesized AgclNPs using *Prunus persica* L. outer peel extract were spherical, and their size ranged from 15 to 50 nm [38].

Liver cancer is one of the most common cancers in the world and is considered as the second most common cause of cancer-related death worldwide [39]. In this study, silver chloride nanoparticles were screened for anticancer activity against HepG2 cell line. These nanoparticles showed a dose-dependent anticancer activity against HepG2. Their IC_{50} value against HepG2 was 7.34 ± 0.71 µg/mL during 24 hours. According to previous studies, the toxicity levels of AgNPs on HepG2 cells are variable. IC_{50} values of AgNPs against HepG2 cells have been reported to be 9.69 µg/mL by Al-Khedhairy et al. (2022) [40], 75 µg/mL by Ahmadian et al. (2018) [41], and 2.764 µg/mL by Faedmaleki et al. (2014) [42]. This variation in IC_{50} values could be related to the size, surface properties, distribution, and morphological shape of NPs. These factors affect the AgNPs conductivity, stability, as well as optical and catalytic properties. In addition, studies have shown that NPs of different sizes could enter the cell through different mechanisms. The findings of this study revealed that biosynthesized AgclNPs could be considered and used as potential chemotherapeutic agents in the treatment of liver carcinoma.

Conclusion

This study designed a rapid and eco-friendly method for the synthesis of NPs by *P. chrysogenum*. AgclNPs synthesis was confirmed by UV–Vis spectroscopy, XRD, and TEM analysis. TEM analysis revealed that AgclNPs were spherical and ranged from 15 to 45 nm. FTIR spectroscopy showed different functional groups of biomolecules responsible for capping of the synthesized NPs and enhancing their stability. Furthermore, MTT assay indicated that these NPs had a good anti-cancer activity against liver hepatocellular carcinoma. The IC_{50} value of AgclNPs against HepG2 cells was 7.340.71± µg/ml. Overall, the results suggest that biosynthesized AgclNPs could offer potential applications in cancer chemoprevention and chemotherapy.

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