



Evaluation of the Cytotoxicity and Molecular Docking Properties of the Green Synthesis of Ag₂S Nanoparticles

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Abstract

Pancreatic cancer is a type of cancer that is aggressive and has a high mortality rate with a five-year survival rate. The primary treatment option commonly used today is chemotherapy, which has very severe side effects. Considering all these side effects, the search for a new treatment for pancreatic cancer is extremely important. In this study, the cytotoxicity effect of silver sulfide nanoparticles (Ag₂S NPs) synthesized with sweet cherry (*Prunus avium* L.), gemcitabine (GEM), a chemotherapy drug, and their combination (Ag₂S NPs—GEM) on PANC-1 cancer cell lines was evaluated by MTT method. Additionally, the interaction of Ag₂S NPs, GEM, and their combination with DNA was examined by conducting agarose gel electrophoresis. Also, interactions of Ag₂S NPs, GEM, and their combination (Ag₂S NPs—GEM) with key enzymes involved in nucleotide synthesis and DNA replication were investigated using molecular docking. The cytotoxicity results in PANC-1 cancer cell lines indicated that the most effective incubation period for Ag₂S NPs was 24 hours (IC₅₀: 75.77 µg/mL), while the most effective results for gemcitabine were obtained at 72 hours (IC₅₀: 32.77 µg/mL). The combined application of Ag₂S NPs and GEM produced significantly more effective results compared to monotherapy (IC₅₀: 18.97 µg/mL in 72 h). DNA cleavage study showed that both Ag₂S NPs and GEM bind to DNA, especially Ag₂S NPs has affinity for G-G bases. The Ag₂S NPs—GEM combination showed highly favorable binding affinities compared to Ag₂S and metabolites of GEM, particularly against thymidylate synthase ($\Delta G = -6.52$ kcal/mol) and DNA polymerase alpha catalytic core ($\Delta G = -6.65$ kcal/mol), correlating with its potent cytotoxicity in PANC-1 cells. Additionally, docking simulations with B-DNA revealed that Ag₂S NPs—GEM exhibits the strongest DNA-binding affinity ($\Delta G = -7.93$ kcal/mol), acting as an effective DNA binder that may enhance cytotoxicity in PANC-1 cells.

Keywords Green synthesis · Ag₂S · Cytotoxicity · DNA cleavage · Molecular docking

Introduction

The demand for environmentally sustainable methods in nanomaterial synthesis has risen significantly in recent years, driven by both ecological concerns and the potential

for biomedical applications. The metal nanoparticles (NPs) can be synthesized with different physical and chemical methods. However, traditional methods of synthesizing Ag₂S often rely on hazardous chemicals (i.e., sodium borohydride and hydrazine) and high-energy inputs, which can lead to adverse environmental impacts and limit their practical application in biological systems [1, 2]. The best alternative to this situation is green synthesis. Green synthesis offers an eco-friendly alternative, utilizing natural reducing agents such as plant extracts, microorganisms, or biomolecules to produce nanoparticles under mild conditions [3, 4]. This approach not only minimizes the generation of toxic byproducts but also can enhance biocompatibility, making green-synthesized Ag₂S particularly suitable for biomedical applications [5, 6]. The use of natural capping agents can further stabilize the nanoparticles, improving their interaction with cellular targets and enhancing their cytotoxic

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efficacy against cancer cells [7–9]. Sweet cherry (*Prunus avium* L.) is a member of the Rosaceae family known as wild cherry; it is worth studying due to its antioxidant, antitumor, and anti-inflammatory properties such as flavonoids, anthocyanins and polyphenols and its benefits to human health [10–13]. Silver sulfide nanoparticles (Ag₂S NPs), known for their unique optical, electrical, and antimicrobial properties, have emerged as promising candidates in various fields, including drug delivery, sensors, biolabeling, deep tissue imaging, and photodynamic therapy applications [9, 14].

Cancer is recognized as a major global health concern, consistently ranking among the leading causes of mortality worldwide. It also diminishes the quality of life for patients due to the impairment of organ functions [15–17]. According to data from the World Health Organization, cancer has consistently ranked among the top ten causes of death over several years [18]. As a gastrointestinal malignancy, pancreatic cancer ranks as the sixth leading cause of cancer-related mortality. In 2022, this aggressive malignancy accounted for 510,992 cases and 467,409 fatalities, as reported by GLOBOCAN data [19]. Today, it has become the third leading cause of cancer-related deaths in the United States, surpassing breast cancer, and it is expected to surpass colorectal cancer soon, becoming the second leading cause of cancer-related deaths after lung cancer [20–23]. Despite being compatible with surgical resection, it often presents at an advanced stage upon diagnosis. It is characterized by aggressive behavior and a low 5-year survival rate, leading to unfavorable outcomes as patients frequently present with regional or distant metastasis [24, 25]. Patients with early-stage disease have treatment options that include surgery, radiotherapy, and chemotherapy [24]. These treatment options, while effective against cancer, also present certain adverse side effects. Side effects involve sudden or late chronic toxicity, treatment-induced secondary cancers, infertility, damage to various systems and organs, and drug resistance, all of which seriously compromise patient quality of life and health [26]. Besides side effects, cancer cells may initially respond favorably to treatments but often develop resistance in later stages. Consequently, patients require alternative options, as both longevity and quality of life decline with the development of resistance to existing therapies [27, 28]. Alternative options include natural products and nanoparticles, which play a crucial role in enhancing the bioavailability of these natural products [29–31].

In recent years, particularly over the past two decades, the molecular docking method has emerged as a pivotal technique for determining the interactions, binding modes, and binding affinities of synthetic nanomaterials with protein and DNA molecules [32, 33]. Furthermore, it has proven to be an invaluable tool in elucidating the molecular mechanisms underlying the cytotoxic effects

observed in cancer cell lines [34, 35]. This molecular-level understanding of cytotoxic responses in cancer cells offers a robust advantage in optimizing and further improving the design of synthesized nanomaterials.

In this study, we explore the physicochemical properties and cytotoxic effects on cancer cell lines by using Ag₂S nanoparticles prepared with green synthesis from sweet cherry fruit. The sweet cherry extract was preferred as a reducing agent instead of harmful reducing agents in this work. The sweet cherry is a beneficial fruit with antioxidant, antitumor, and anti-inflammatory properties [12]. By leveraging an environmentally sustainable synthesis pathway, we aim to highlight the potential of green-synthesized Ag₂S in cancer treatment applications, while emphasizing its compatibility with biological systems and reduced ecological footprint. Furthermore, molecular docking studies were conducted to elucidate the potency and molecular mechanism underlying the cytotoxic effect observed in the PANC-1 cancer cell line and to gain insights into the interactions between intracellular target enzymes, DNA and Ag₂S, gemcitabine (GEM), and the Ag₂S—GEM mixture (Scheme 1).

Materials and Methods

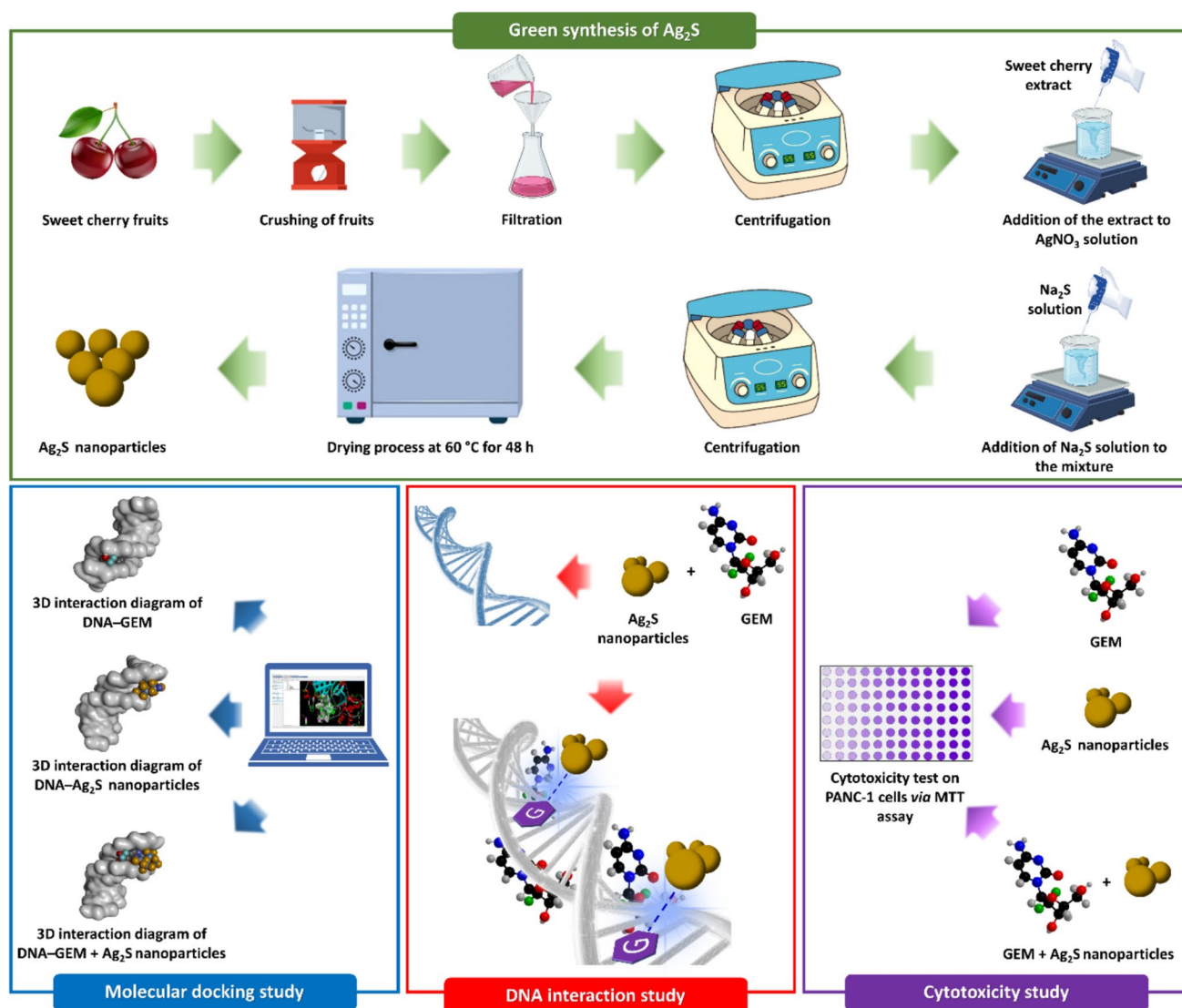
The materials, apparatus, and synthesis of nanomaterial used in this study are presented in the Supplementary Material.

Cell Culture

Pancreatic ductal epithelioid carcinoma cell line PANC-1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), which contains 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin/streptomycin). The culture medium's ingredients were purchased from Gibco in Grand Island, New York, USA. Every three days, the medium was changed and cells were passaged using a trypsin-EDTA solution when 80–90% of the culture flask was covered. Cells are incubated at 37 °C in an appropriate incubator with 5% CO₂. Cell growth rates and bacterial/fungal contamination were tracked using an inverted microscope.

Dose Preparation

A stock concentration of 1 mg/mL of Ag₂S nanoparticles (Ag₂S NPs) was prepared in the culture medium. After that, the concentrations of the dilutions were adjusted to 250, 125, 62.5, and 31.25 µg/mL. Gemcitabine, a chemotherapy drug used to treat cancer was dissolved in physiological saline to prepare a stock solution at a concentration of 40 mg/mL, which was then diluted to concentrations of 250, 125, 62.5,



Scheme 1 Schematic representation of Ag₂S nanoparticles synthesis and their application in cytotoxicity, DNA interaction, and molecular docking studies.

and 31.25 µg/mL. The study used 5-fluorouracil as a positive control, diluting it to the IC₅₀ dose [36].

MTT Assay

The cytotoxic effects of Ag₂S nanoparticles, gemcitabine alone, and a 1:1 mixture of both on PANC-1 cells were evaluated using the MTT assay. The method was used previously described [37], with a few minor adjustments. In a 96-well plate, 104 cells were seeded per well in 100 µL of DMEM culture medium. The cell culture medium was removed from the incubation plate after 24 hours. Ag₂S NPs, gemcitabine, and the combination mixture were added to the wells in 100 µL of DMEM at the indicated concentrations. Following 24, 48, and 72 hours of incubation, 200 µL of

MTT (Sigma) solution (0.5 mg/mL) was added to each well and left in the incubator for 2 hours. The formed formazan crystals were dissolved in 200 µL of DMSO and measured at a 570 nm wavelength. Cell viability was determined using eq. (1) [37].

$$\% \text{Viability} = 100 \times \left[\frac{\text{OD}(\text{Treated Cells})}{\text{OD}(\text{Untreated Cells})} \right] \quad (1)$$

DNA Interaction Assay

A DNA cleavage assay was performed to determine the interaction of Ag₂S NPs, gemcitabine alone, and a 1:1 combination of the two with DNA *in vitro*. For this

purpose, supercoiled pBR322 plasmid DNA was incubated in the dark at 37 °C for 24 h with specific doses of Ag₂S NPs, gemcitabine alone, and a 1:1 combination of the two. Following incubation, treated plasmid DNAs were electrophoresed on a 1% agarose gel in the presence of 1×TAE at 100 V for 1 h. After electrophoresis, the gel was stained in ethidium bromide and then imaged in a UV transilluminator.

DNA Cleavage Assay

The Ag₂S, Gemcitabine, or their combined-DNA mixtures were initially incubated for 24 hours and subsequently digested with the restriction enzymes BamH I and Hind III for 1 hour at 37 °C. BamH I and Hind III are known for their ability to recognize the sequences G/GATCC and A/AGCTT, respectively. The pBR322 plasmid DNA contains a unique restriction site for each enzyme, which transforms supercoiled Form I DNA and open circular Form II DNA into linear Form III DNA. Following a one-hour incubation, the restricted DNA was subjected to electrophoresis in a 1% agarose gel for one hour at 100 V in TAE buffer. The gel was stained with ethidium bromide and subsequently examined using a UV transilluminator.

Molecular Docking Studies

Rationale of Ligand Selection

When gemcitabine (GEM) enters the cell through a human nucleoside transporter (hNT), it is first converted to gemcitabine monophosphate (GEM-MP) by deoxycytidine kinase (dCK), then to gemcitabine diphosphate (GEM-DP) by pyrimidine nucleoside monophosphate kinase (UMP-CMP kinase), and finally to gemcitabine triphosphate (GEM-TP) by the catalytic activity of nucleoside diphosphate kinase A [38, 39]. Of these sequentially produced metabolites, pharmacologically the most active ones are GEM-DP (a ribonucleotide reductase inhibitor) and GEM-TP (a DNA chain terminator). Therefore, the major metabolites produced after GEM enters the cell and persist intracellularly are GEM-DP and GEM-TP. In this study, 5-fluorouracil (5-FU) was used as a positive control (reference compound). 5-FU is an antimetabolite frequently used in cancer treatment, especially in colorectal cancer [40]. After entering the cell through facilitated transport similar to uracil, 5-FU is converted to fluorodeoxyuridine monophosphate (FdUMP, 5-FU-MP), one of the most important metabolites of 5-FU, by thymidine kinase [40]. In terms of receptor-ligand relationship, GEM-DP is an inhibitor of ribonucleotide reductase (RNR), GEM-TP is an inhibitor of DNA synthesis via ‘*masked DNA chain termination*’ mechanism [38], and FdUMP (5-FU-MP) is

an inhibitor of thymidylate synthase (TS). Therefore, the triphosphate structure of GEM (GEM-TP), the diphosphate structure of GEM (GEM-DP), as well as 5-FU-MP (positive control) were used as ligands in the docking simulations against protein receptors.

Selection of Target Receptors and Protein-Ligand Preparation

As stated in the previous section, ribonucleotide reductase (RNR), catalytic DNA polymerase, and thymidylate synthase (TS), which are intracellular targets of GEM-DP, GEM-TP, and 5-FU-MP, respectively, were selected as receptors in the docking experiments. In this context, crystal structures of human RNR, the catalytic core of human DNA polymerase alpha (α), and human TS were retrieved from the RCSB Protein Data Bank (PDB) with PDB IDs 5 TUS (resolution: 2.66 Å), 4Q5 V (resolution: 2.52 Å), and 1HVV (resolution: 1.90 Å), respectively. On the other hand, in the DNA docking study, a high-resolution B-DNA dodecamer (PDB ID: 7RQT, resolution: 1.26 Å) was used as the target receptor.

Before conducting the docking simulations, the 3D structures of the receptors, namely the human RNR, the catalytic core of human DNA polymerase alpha, and the human TS, were subjected to a refinement procedure using the Discovery Studio Visualizer v16 software. This refinement entailed the removal of water molecules, inhibitors, and non-interacting ions. Afterward, the energy minimization of human RNR, the catalytic core of human DNA polymerase alpha, and human TS was performed using the NAMD (NANoscale Molecular Dynamics) module implemented in the Vega ZZ program (version 3.2.2.21), via the selection of the CHARMM22_PROT force field [41]. After completion of the final step of energy minimization, the resulting 3D structures of the three enzymes were saved in *pdb* format as the lowest energy conformers.

On the other hand, the 3D coordinates of GEM-TP and GEM-DP were downloaded from the NIH PubChem database as a structure data file (*sdf*), and energetically minimized using MMFF94 force field [42–44] with conjugate gradients algorithm in the Avogadro software [45]. In a similar way, the 3D structure of 5-FU's active metabolite, FdUMP (5-FU-MP), which was used as a positive control in the MTT test performed on PANC-1 cells, was downloaded from the NIH PubChem database in *sdf* format and minimized using the MMFF94 force field (conjugate gradient algorithm) [46]. The nanoparticle, silver sulfide (Ag₂S), used in the docking study was drawn manually in ChemBioDraw Ultra 15.0 program, saved in *mol* format, and energetically optimized using the Universal Force Field (UFF) [47] with conjugate gradients algorithm also implemented in the Avogadro software [45].

In drawing the 3D structure of Ag₂S, the experimentally determined monoclinic crystal system was used as the original structure [48]. Subsequently, optimized structures of GEM and Ag₂S structures were combined to create the Ag₂S—GEM, where the 4-NH₂ functional group of GEM was chosen as the processing site (4-NH₂ modification) as reported in the literature [49]. Energy minimization of ligands is an important step before molecular docking, as it increases the accuracy of the docking process by helping to eliminate steric clashes, strained bonds and angles, or other unfavorable contacts that may occur between the ligand and the receptor [50]. Finally, prior to docking, all ligand and receptor structures were converted to *pdbqt* format using AutoDock Tools 1.5.6 [51].

Molecular Docking

In this study, the latest version AutoDock 4.2.6 was utilized to dock 5-FU-MP, GEM-DP, GEM-TP, Ag₂S (nanoparticle), and Ag₂S—GEM ligands against the three protein target receptors [51].

During the docking experiments, the polar hydrogen atoms in the structures of the ligands (5-FU-MP, GEM-DP, GEM-TP, Ag₂S, and Ag₂S—GEM) and receptors (RNR, DNA polymerase alpha catalytic core, and TS) were retained, while non-polar hydrogens were merged using AutoDock Tools 1.5.6. The Kollmann charges were assigned to the receptors and the Gasteiger charges to the ligands. In this applied semi-flexible docking protocol, the ligands' rotatable bonds were allowed to rotate freely. Grid box sizes of 15 Å × 15 Å × 15 Å points (center x: 13.63 Å; center y: -19.48 Å; center z: -25.47 Å) for RNR, 14 Å × 14 Å × 14 Å points (center x: 81.68 Å; center y: 98.99 Å; center z: 81.43 Å) for DNA polymerase alpha catalytic core, and 20 Å × 20 Å × 20 Å points (center x: 0.40 Å; center y: 12.39 Å; center z: 17.76 Å) for TS were adjusted. For B-DNA, a grid box size of 60 × 70 × 110 Å points (center x: 15.10; center y: 20.95; center z: 10.85) with 0.375 Å grid spacing was set, a default value in AutoDock 4.2.6 that maintains an optimal balance between adequately elucidating ligand–target interactions while managing computational efficiency. The coordinates and dimensions of these grid boxes were determined by carefully examining and extracting the coordinates of the bound co-crystallized inhibitors on the proteins. Moreover, these grid box sizes allowed the investigated five ligands to interact easily with the active site and adjacent residues of the three protein receptors as well as the entire surface of B-DNA to capture both minor and major groove interactions. The binding energy values (ΔG : kcal/mol) obtained by re-docking of the co-crystallized inhibitors (naphthyl salicyl acyl hydrazone [NSAH] and aphidicolin) of human RNR and DNA polymerase alpha catalytic core enzymes were used as positive (reference) control values for these two

enzymes. On the other hand, 5-FU (as 5-FU-MP), which was used as the positive control in the MTT test, was used as a reference inhibitor of the human TS enzyme in the docking experiments.

The docking protocol was performed for 5-FU-MP, GEM-DP, GEM-TP, Ag₂S (nanoparticle), and Ag₂S—GEM along with the mentioned three reference compounds against each of the three target proteins. In the DNA docking studies only the 5-FU, Ag₂S, GEM, and Ag₂S—GEM were used as the ligands against the DNA since the formation of 5-FU and GEM metabolites is not observed in cell-free plasmid assays.

The number of *genetic algorithm runs* was set to '100', and the *maximum number of energy evaluations* was set to '5,000,000'. Following docking, the resulting binding conformations (poses) of the ligands, along with the reference compounds, were clustered by AutoDock 4.2.6 and ranked based on their binding affinities, starting from the most negative binding free energy (ΔG ; kcal/mol) against each target receptor. The top-ranked binding poses of 5-FU, 5-FU-MP, GEM, GEM-DP, GEM-TP, Ag₂S, and Ag₂S—GEM, as well as the positive controls, were visualized and analyzed using Discovery Studio Visualizer v16 program.

CompuSyn-Based Computerized Analysis of Ag₂S—GEM Interaction in PANC-1 Cells

Dose–effect data, including the fractional inhibition (Fa) values—considered reliable only when they were within the acceptable assay range (extremes such as Fa=0 or Fa=1 were avoided)—were analyzed using CompuSyn software, which employs the Chou–Talalay method [52, 53]. In this approach, the combination index (CI) was calculated to quantitatively assess the nature of the drug interaction. Notably, the CI analysis was conducted on 72-hour treatment data from Ag₂S nanoparticles (NPs) and GEM in PANC-1 cells, where synergistic cytotoxicity was markedly observable. CI values were interpreted as follows: a CI value less than 1 (CI < 1) indicates synergism, a CI value equal to 1 (CI = 1) signifies an additive effect, and a CI value greater than 1 (CI > 1) denotes antagonism. These parameters and their respective ranges provided a rigorous framework for evaluating the fidelity of the dose–response data and the interaction between Ag₂S and GEM.

Statistical Analysis

Cytotoxicity graphs and statistical analysis were performed using GraphPad Prism version 8.0.2 software (GraphPad Software, Inc., San Diego, CA). For MTT assays, each concentration was repeated in triplicate, the results were expressed as mean ± standard deviation (SD). IC₅₀ was defined as the minimum concentration required to inhibit 50% of PANC-1 cells and was determined using nonlinear

regression analysis in GraphPad Prism software. Statistical significance was considered at $p < 0.05$. Data were analyzed using one-way ANOVA, followed by Tukey's post hoc test to identify differences between groups.

Results and Discussion

Structural and Morphological Characterization of Ag₂S NPs

Fig. 1a shows the X-ray diffraction (XRD) pattern of Ag₂S NPs synthesized from sweet cherry by green synthesis. The main three peaks correspond to (111), (−121), and (121) assigned to the 2θ values at 28.51° , 34.51° , and 36.99° , respectively (PDF # 00–014–0072). The XRD pattern of Ag₂S NPs fabricated by green synthesis was similar to many studies in the literature [1, 8, 9, 54].

The FT-IR spectrum of Ag₂S NPs was recorded in the range of $4000\text{--}400\text{ cm}^{-1}$ (Fig. 1b). The absorption peaks are observed at around 2340, 1660, 1530, 1390, 760, 675, and 540 cm^{-1} . These peaks suggest the formation of Ag₂S NPs with Ag–S and Ag–N bonds. The broad IR peak at around 3400 cm^{-1} is indexed to the presence of adsorbed water molecules on Ag₂S NPs. The values of stretching frequencies obtained from the FT-IR analysis are in good agreement with the previous reports [54, 55].

Fig. 2a illustrates the scanning electron microscope (SEM) photograph of Ag₂S nanoparticles (NPs) at a scale of 1 micrometer, distinctly revealing their cubical and cylindrical morphologies [1, 9]. Figure 2b depicts the energy dispersive X-ray spectroscopy (EDS) spectrum of Ag₂S NPs, containing Ag (84.66 wt%) and S (15.34 wt%) elements (inset Fig. 2b). Fig. 2c displays the EDS mapping images of Ag₂S NPs. The transmission electron microscope (TEM) photos of Ag₂S NPs are illustrated in Fig. 2d and e. Fig. 2d presents the morphology of Ag₂S, indicating nanosphere structure. In addition, Ag₂S NPs have agglomerated structures. In Fig. 2e,

a 0.31 nm lattice fringe ascribed to the (111) crystal plane of Ag₂S was matched [8, 56]. In light of these results, TEM images of Ag₂S nanoparticles match with XRD results.

Cytotoxicity Studies

This study assessed the cytotoxic effects of Ag₂S nanoparticles, synthesized *via* green synthesis with sweet cherry fruits, on the pancreatic cancer cell line PANC-1. Additionally, the consequences of Ag₂S nanoparticle combined application with gemcitabine on PANC-1 cells were also evaluated. For this purpose, the MTT assay was used to determine the cytotoxicity of Ag₂S nanoparticles and gemcitabine monotherapy and their combination. In order to evaluate the reliability of the experimental design, 5-FU (5-Fluorouracil), a chemotherapy agent used in the treatment of pancreatic cancer, was included as a positive control. In cells treated with Ag₂S nanoparticles (NPs), both a decrease in cell number and distinct morphological changes were observed during all incubation periods, especially at high concentrations such as $250\text{ }\mu\text{g/mL}$ and $125\text{ }\mu\text{g/mL}$ (Fig. 3). As a result of detailed examination of cell morphology, swelling in the cytoplasm and remarkable rounding compared to normal cell morphology were detected. While cell swelling and rounding were predominantly observed in cells exposed to Ag₂S NP application, intense apoptotic cell morphologies were observed in Gemcitabine application as monotherapy. In the case of combined application of Ag₂S and Gemcitabine, both swelling and rounding and apoptotic cell morphologies as well as abnormal vesicle formation in the cytoplasm were noted, especially in the high dose groups. These abnormal morphological changes are indicated in Fig. S1.

The IC₅₀ values for Ag₂S NPs in monotherapy treatments after 24, 48, and 72 hours were $75.77\text{ }\mu\text{g/mL}$, $87.51\text{ }\mu\text{g/mL}$, and $91.36\text{ }\mu\text{g/mL}$, respectively. For Gemcitabine, the values were $>250\text{ }\mu\text{g/mL}$, $>250\text{ }\mu\text{g/mL}$, and $32.77\text{ }\mu\text{g/mL}$, respectively. These results indicated that the most effective incubation period for Ag₂S NPs was 24 hours, whereas the most

Fig. 1 XRD pattern (a) and FT-IR spectrum (b) of Ag₂S NPs

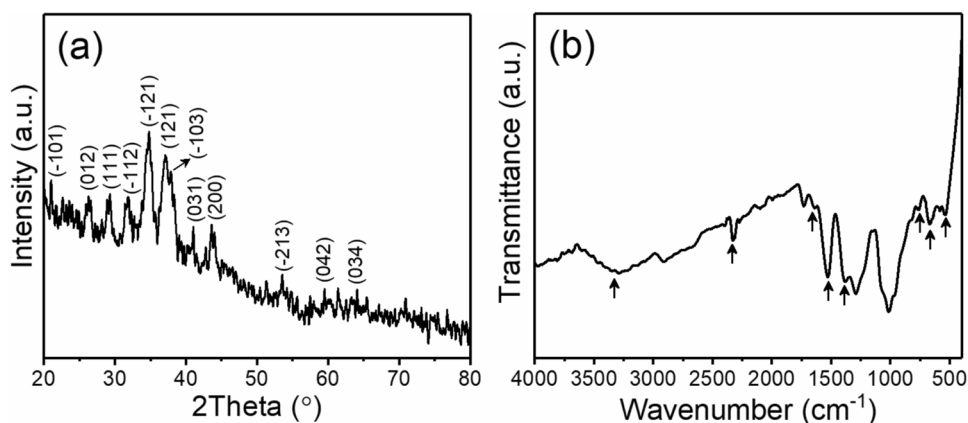


Fig. 2 SEM photo (a), SEM-EDS spectrum (b), EDS mapping (c), TEM (d), and HR-TEM (e) of Ag₂S NPs

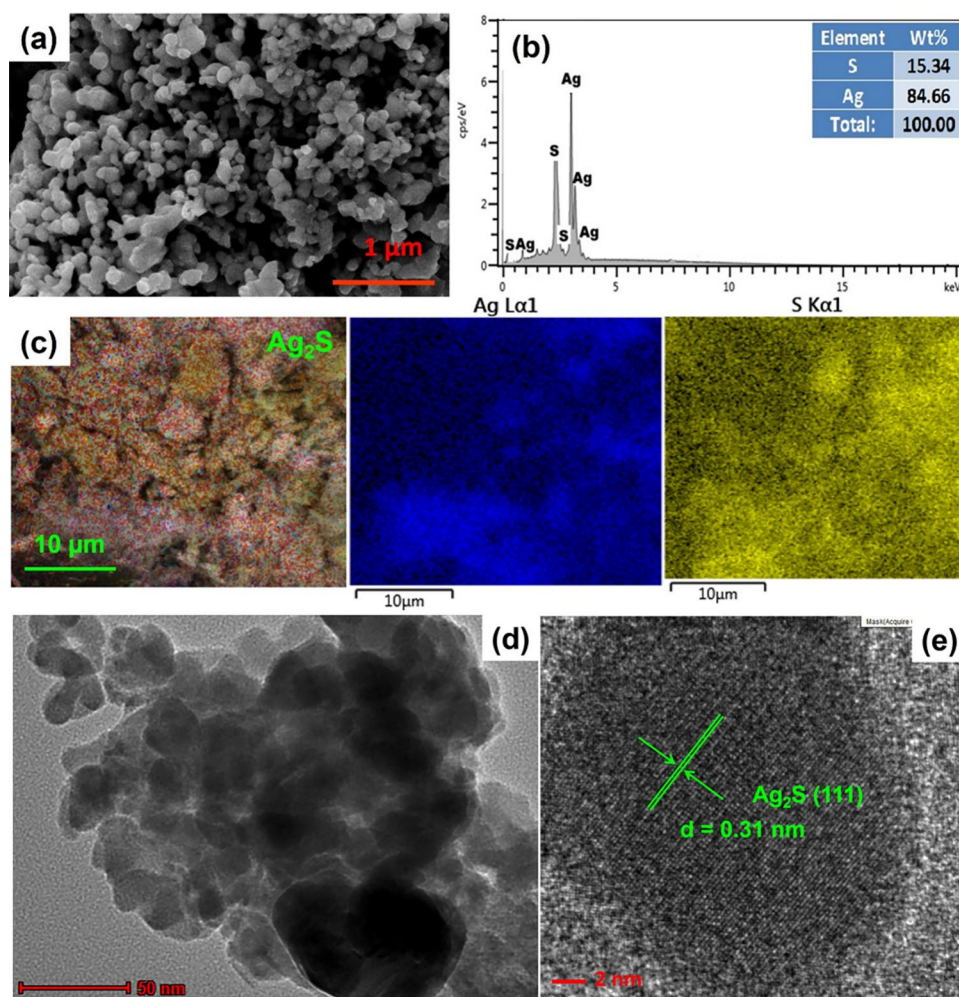
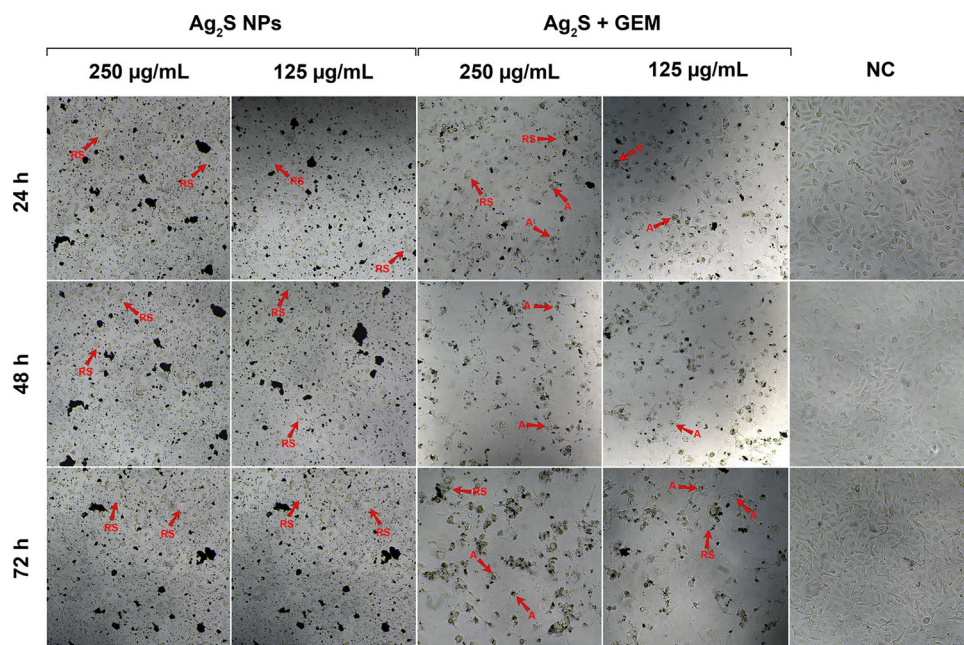


Fig. 3 The morphological changes of Ag₂S and Ag₂S + Gemcitabine on the PANC-1 cells in vitro. Arrows represent only a few of the morphological changes in the cells. *Gem* Gemcitabine, *NC* Negative control (untreated cancer cells), *RS* Cell swelling and rounding, *A* Apoptotic cell morphology.



effective results for gemcitabine were achieved after 72 hours. Therefore, the data indicate that the nanoparticles synthesized during this study exhibit higher effectiveness against PANC-1 cells compared to gemcitabine. The combined application of Ag₂S NPs and gemcitabine showed a dose- and time-dependent reduction in the viability of cancer cells, with IC₅₀ values recorded at 82.88 µg/mL, 46.31 µg/mL, and 18.97 µg/mL for the incubation periods of 24, 48, and 72 hours, respectively (Figs. 4 and 5). The combination approach produced significantly more effective results compared to monotherapy when evaluating the 48- and 72-hour incubation periods. To

validate these observations, a one-way ANOVA followed by Tukey's post hoc test was conducted to evaluate the differences among the treatment groups. The analysis identified statistically significant differences between the groups: The mean difference between Ag₂S NPs and GEM was 75.77 (95% CI [72.19, 79.36]), with a p-value < 0.0001; the mean difference between Ag₂S NPs and Ag₂S NPs + GEM was -7.11 (95% CI [-11.12, -3.10]), with a p-value of 0.0051; and the mean difference between GEM and Ag₂S NPs + GEM was -82.88 (95% CI [-86.89, -78.87]), with a p-value < 0.0001. These results demonstrate that all pairwise comparisons between

Fig. 4 The cytotoxic effects of Ag₂S and Gemcitabine on the growth of PANC-1 cells in vitro. PANC-1 cancer cells were treated with Ag₂S, Gemcitabine, and a combination of both at different concentrations (0, 31.25, 62.5, 125, 250 µg/mL) for **A** 24 h, **B** 48 h, and **C** 72 h, and the cell viability was assessed by an MTT assay (*n*=3). 5-FU positive control cancer drug. NC negative control (untreated cancer cells)

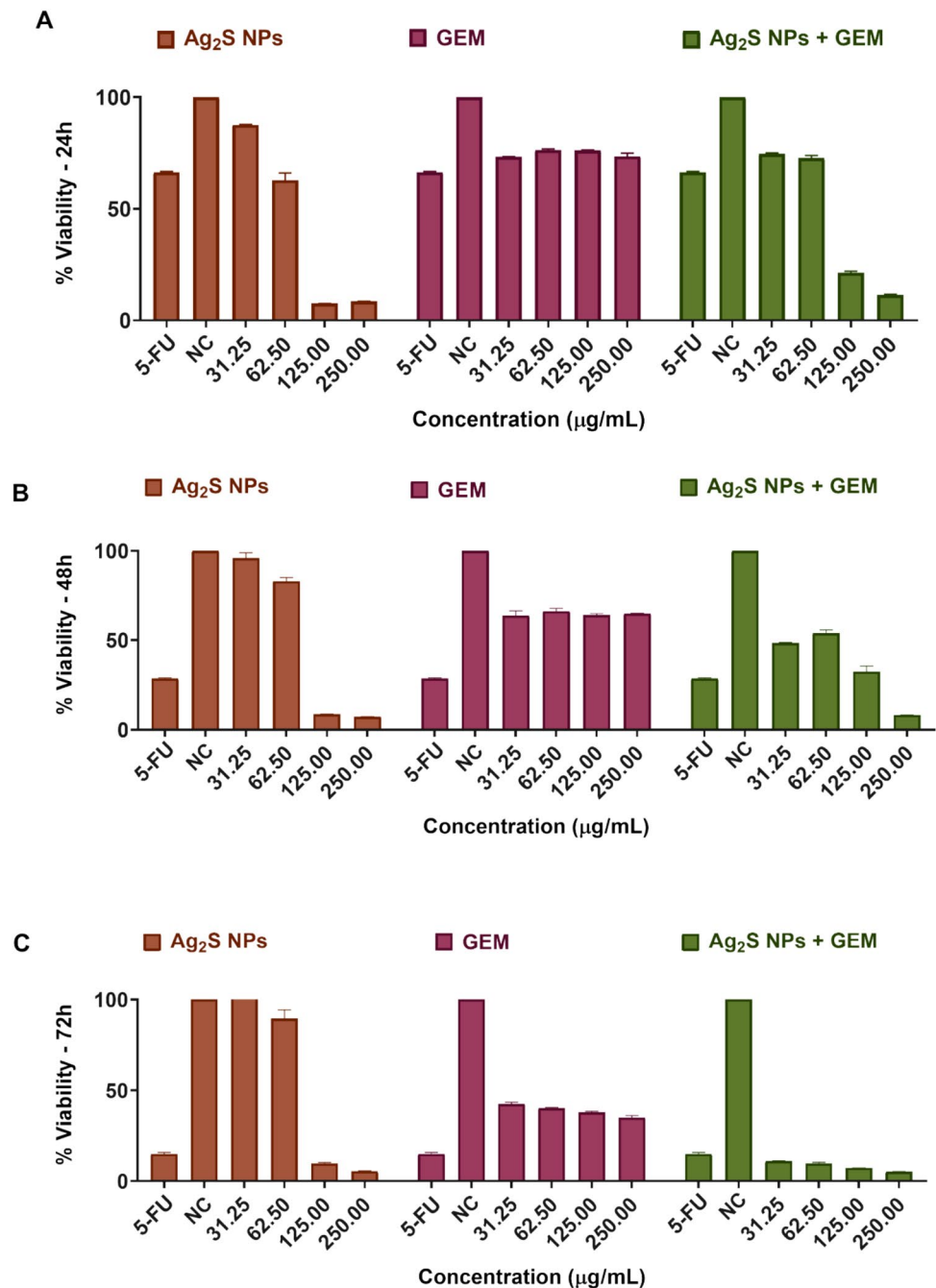
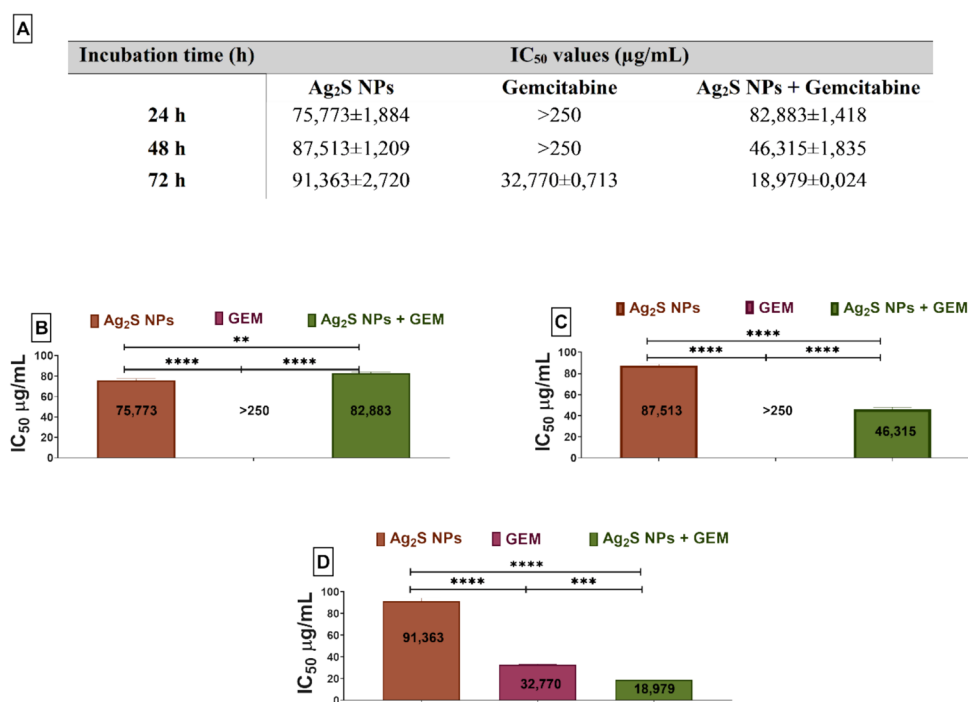


Fig. 5 IC₅₀ values **A** after treated with Ag₂S, Gemcitabine, and a combination of both for **B** 24 h, **C** 48 h, and **D** 72 h. Statistical comparison of IC₅₀ values for Ag₂S, Gem, and Ag₂S+Gem was performed using one-way ANOVA followed by Tukey's post hoc test, ns: $P > 0.05$ (non-significant), *: $p < 0.05$ (significant), **: $p < 0.01$ (more significant), ***: $p < 0.001$ (highly significant), ****: $p < 0.0001$ (extremely significant).



treatment groups yielded statistically significant differences, further supporting the robustness and reliability of the findings. Additionally, the one-way ANOVA revealed a significant effect of the treatment groups on the outcome ($F(2, 5) = 3196$, $p < 0.0001$), with an exceptionally large effect size ($\eta^2 = 0.9992$), highlighting the strength of the treatment effects. The findings also indicate that the synergy between nanoparticles and gemcitabine might reduce the negative side effects related to gemcitabine treatment. Due to its extensive efficacy against various cancer types, gemcitabine is considered the gold standard treatment for pancreatic cancer. Nonetheless, the main challenges associated with the use of gemcitabine include significant systemic side effects and the development of drug resistance in cancer cells [57]. In corroboration of this literature, the application of Gemcitabine to PANC-1 cells over 24 and 48 hours resulted in IC₅₀ values over 250 mg/mL (Fig. 5) in this investigation, suggesting that these cells exhibit resistance to Gemcitabine. On the other hand, evaluating the results of the combined application suggests that applying equal amounts of nanoparticle-gemcitabine could offer an alternative strategy for overcoming potential drug resistance.

Computerized Analysis of Synergism Between Ag₂S and Gemcitabine (GEM)

In vitro treatment of PANC-1 cells with Ag₂S and gemcitabine (GEM) at increasing concentrations (31.25, 62.5, 125, and 250 μg/mL) over a 72-hr period resulted in high inhibitory (cytotoxic) responses, with fractional inhibition (Fa) values ranging from 0.8909 at 31.25 μg/

mL to 0.9488 at 250 μg/mL indicating a robust inhibitory response that is reliably measurable without approaching the extreme limit ($Fa=0$ or $Fa=1$). Notably, the corresponding combination index (CI) values, which ranged from 0.09464 to 0.65822 (approximately 0.09–0.66), were markedly below 1 ($CI < 1$)—demonstrating a strong synergistic interaction between Ag₂S and GEM in inhibiting PANC-1 cells proliferation. Thus, our results showed that the combination treatment of Ag₂S + GEM at 72 h robustly enhances cytotoxicity compared to the effects of the individual agents (see Supplementary Information Fig. S2 for the isobologram calculated by CompuSyn).

DNA Interaction

Plasmid DNA may appear in three distinct forms on an electropherogram: Form I (supercoiled or closed circular), Form II (open circular), and Form III (linear). The migration rates of these plasmid DNA forms differ; Form I migrates faster, whereas Form II migrates at a slower rate. A linear DNA fragment cleaved by a restriction enzyme is positioned between the two other configurations [58].

The results demonstrated that applying Ag₂S nanoparticles to the plasmid DNA restricted the migration of pBR322 plasmid DNA in both form I and form II structures on agarose gel compared to the negative control. The highest concentration of Ag₂S nanoparticles applied on plasmid DNA significantly decreased the mobility of plasmid DNA on the agarose gel in comparison to other concentrations and the negative control (Fig. 6). Furthermore, the two lowest

concentrations of Ag₂S nanoparticles used decreased the density of the form II structure of plasmid DNA. This conclusion indicates that the Ag₂S nanoparticle interacts with both configurations of DNA and may have also partially degraded the open circular form of DNA. The analysis of the interaction between gemcitabine and plasmid DNA indicated that the Form II structure of the plasmid exhibited less interaction with NP applications and demonstrated a migration rate comparable to the control in agarose gel across all doses. Nevertheless, the Form I configuration of the plasmid exhibited a significant delay in the agarose gel relative to both the control and NP treatment. Only the open circular DNA form (Form II) of the plasmid was cleaved at the two lowest concentrations, resulting in a reduction in its density. The examination of plasmid DNA interaction with Ag₂S NPs and gemcitabine revealed a reduction in the mobility of both DNA forms following treatment. In the combined application, when the concentration decreased, particularly at the lowest level, it was noted that Form I and II showed characteristics similar to the control, however, the intensity of Form II reduced. The interaction of the chemotherapeutic agent 5-FU with DNA significantly decreased the mobility of Form I, suggesting the treatment affects the supercoiled DNA configuration.

DNA Cleavage

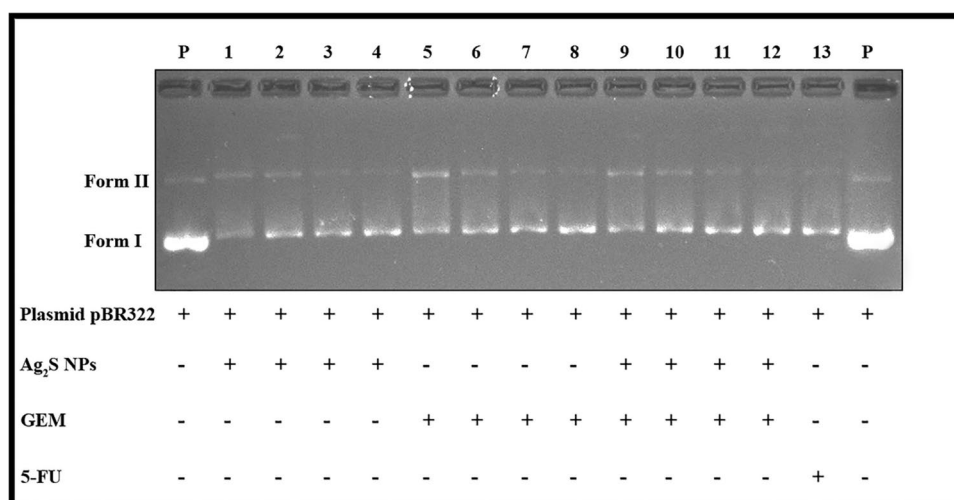
After the interaction of Ag₂S NPs and plasmid DNA, it was observed that the BamHI restriction enzyme does not cut the plasmid DNA at any of the four treated concentrations. This finding indicates that Ag₂S nanoparticles are associated with the guanine-guanine nucleotides of DNA. The HindIII enzyme partially cleaved forms I and II during the cleavage procedure but did not completely cleave them. Consequently, the electropherogram revealed the presence of all three forms, including form III, which is linear DNA

(Fig. 7). Applying gemcitabine monotherapy concentrations caused the BamHI enzyme to cleave the plasmid at the first three highest concentrations, targeting the supercoiled form I structure. However, it did not cleave the open circular form II structure, except for the lowest concentration. In the lowest concentration of gemcitabine monotherapy, it did not completely cut form I, causing partial cuts and allowing the observation of all three forms. Gemcitabine monotherapy doses caused the HindIII enzyme to partially cut the plasmid DNA. It specifically targeted supercoiled DNA. At the highest concentration, it completely cut form I, but at the lowest concentration, it only partially cut both forms. No BamHI cuts were detected in the plasmid DNA interacting with the Ag₂S NP and gemcitabine combination at any of the four concentrations. However, the HindIII enzyme performed partial cleavage, and all three forms were observed. The interaction between plasmid DNA and the chemotherapy drug 5-FU caused the BamHI enzyme to completely cut the plasmid, while HindIII only partially cut it, targeting form I (Fig. 8). Consequently, it was observed that Ag₂S nanoparticles, through their affinity for G-G base pairs and binding to them, inhibited the BamHI cutting. Additionally, Ag₂S nanoparticles show a partial affinity for A-A base pairs, resulting in partial cutting by the HindIII enzyme.

Molecular Docking Studies with Protein Receptors

In this study, molecular docking was performed to determine the binding affinities of synthetic Ag₂S (nanoparticle), gemcitabine (GEM), and Ag₂S—GEM towards potential target enzymes (thymidylate synthase [TS], DNA polymerase alpha [Pol α], and ribonucleotide reductase [RNR]) that play role in nucleotide precursor synthesis and DNA replication. Moreover, the intermolecular interactions between these agents and target proteins were visually elucidated. While 5-fluorouracil (5-FU), the

Fig. 6 Electropherograms were applied to the interaction of pBR322 plasmid DNA with decreasing concentrations (250, 125, 62.5, and 31.25 µg/mL) of Ag₂S (1–4), Gemcitabine (5–8), Ag₂S-Gemcitabine combination (9–12), and 5-FU (13). Line P represented untreated plasmid DNA to use as a control.



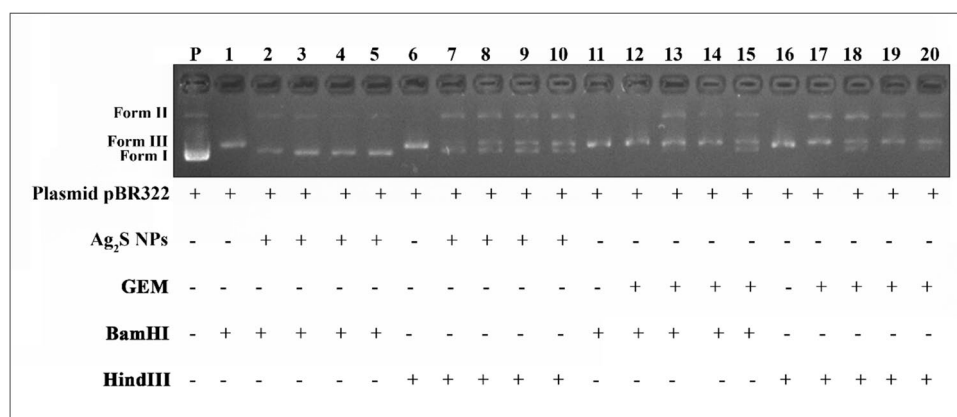


Fig. 7 An electrophoretogram was applied to incubated mixtures of plasmid DNA and Ag₂S or Gemcitabine, followed by digestion with BamHI or HindIII. Lane P: untreated and undigested plasmid DNA. Line 1: untreated plasmid DNA digested with BamHI. Line 2–5: BamHI digestions of plasmid DNA interacted with decreasing concentrations of Ag₂S (250, 125, 62.5, and 31.25 µg/mL). Line 6: untreated plasmid DNA digested with HindIII. Line 7–10: HindIII digestions of plasmid DNA interacted with decreasing concentrations

of Ag₂S (250, 125, 62.5, and 31.25 µg/mL). Line 11: untreated plasmid DNA digested with BamHI. Line 12–15: BamHI digestions of plasmid DNA interacted with decreasing concentrations of Gemcitabine (250, 125, 62.5, and 31.25 µg/mL). Line 16: untreated plasmid DNA digested with HindIII. Line 17–20: HindIII digestions of plasmid DNA interacted with decreasing concentrations of Gemcitabine (250, 125, 62.5, and 31.25 µg/mL).

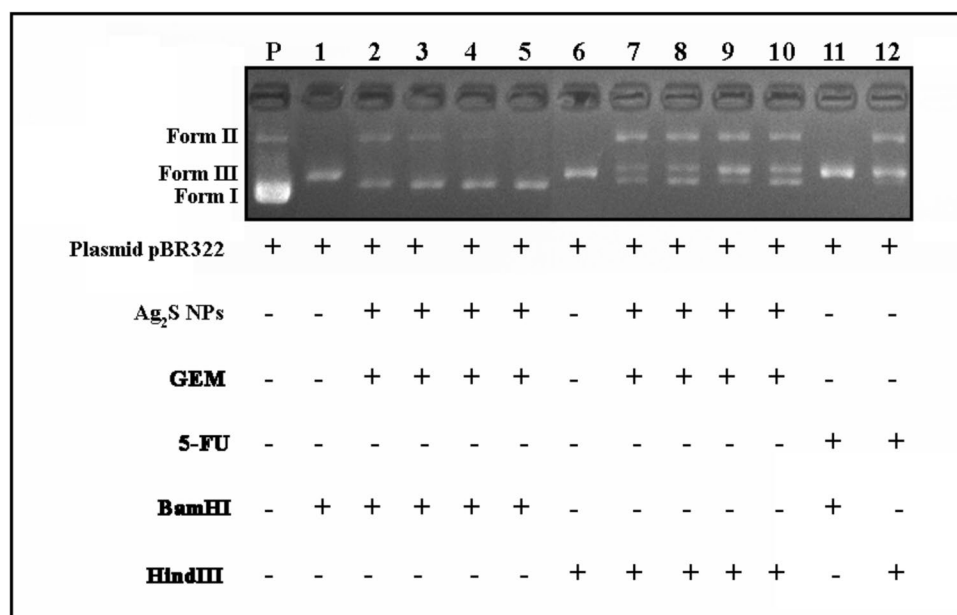


Fig. 8 An electrophoretogram was applied to incubated mixtures of plasmid DNA and the Ag₂S-Gemcitabine combination, followed by digestion with BamHI or HindIII. Lane P: untreated and undigested plasmid DNA. Line 1: untreated plasmid DNA digested with BamHI. Line 2–5: BamHI digestions of plasmid DNA interacted with decreasing concentrations of the Ag₂S-Gemcitabine combination

(250, 125, 62.5, and 31.25 µg/mL). Line 6: untreated plasmid DNA digested with HindIII. Line 7–10: HindIII digestions of plasmid DNA interacted with decreasing concentrations of the Ag₂S-Gemcitabine combination (250, 125, 62.5, and 31.25 µg/mL). Line 11: BamHI digestions of plasmid DNA interacted with 5-FU. Line 12: HindIII digestions of plasmid DNA interacted with 5-FU.

positive control in the MTT test, inhibits the TS enzyme in its monophosphate form (5-FU-MP), cytotoxicity happens through coordinated interactions of many proteins within the cell. Therefore, a series of docking calculations were also performed against RNR and Pol α enzymes, which

are the intracellular targets of the diphosphate (GEM-DP) and triphosphate (GEM-TP) forms of GEM. Thus, *a.* the binding strengths of ligands (Ag₂S, GEM, and Ag₂S—GEM) to the TS enzyme were compared with the reference compound 5-FU, *b.* their inhibitory potentials on the other

two enzymes that play a direct (Pol α) or indirect (RNR) role in DNA synthesis were also determined. Using this holistic approach, it was determined which of the ligands had the higher inhibitory potential against these target enzymes, thus, providing a rational and mechanistic ground to the MTT test results.

Intermolecular Interactions with Thymidylate Synthase

The molecular interactions and binding free energy (ΔG =kcal/mol) values between thymidylate synthase (TS) and ligands (5-FU-MP, Ag₂S, GEM-DP, GEM-TP, and Ag₂S—GEM) are presented in Table 1 and Fig. 9. The positive control compound, 5-FU-MP, interacts with the active site of TS through six hydrogen bonds with Glu87, Ile108, Cys195, Asp218, and Asn226. It also forms a hydrophobic contact with Cys195, an electrostatic attraction with Glu87, a halogen interaction with Asn226, and several Van der Waals interactions (Table 1). The molecular affinity of 5-FU-MP against TS was found to be -5.97 kcal/mol.

The interactions of Ag₂S nanoparticles towards TS consist of 5 H-bonds with Lys77, Lys107, Leu221, and Ile307, two pi-sulfur interactions with Phe80 and Phe225, and a certain number of Van der Waals interactions with several residues (Table 1, Fig. 9A). Compared to 5-FU-MP, Ag₂S showed a weak binding affinity against TS (ΔG = -4.36 kcal/mol). As the interactions of GEM-DP with TS examined, it is seen that GEM-DP formed 8 H-bonds with Lys77, Phe80, Glu87, Ile108, Leu221 and Gly222, 3 hydrophobic contacts with Ile108 and Trp109, one halogen interaction with Ile108, and a moderate number of Van der Waals contacts with several residues (Table 1).

Compared with 5-FU-MP, the binding strength of GEM-DP against TS was moderately strong (ΔG = -5.09 kcal/mol), but not as potent as 5-FU-MP (Table 1, Fig. 9B). The interactions of GEM-TP with TS include nine hydrogen bonds with Lys77, Phe80, Asn112, Leu221, Asn226, Gly222, Lys308, and Met309. It also has five hydrophobic contacts with Ile108, Trp109, and Phe225, a halogen interaction with Glu87, a pi-lone pair interaction with Ile108, and several Van der Waals contacts with various residues (Table 1, Fig. 9C). When compared to 5-FU-MP, the molecular affinity of GEM-TP towards the TS enzyme is weak, as evidenced by ΔG = -4.25 kcal/mol. Upon examining the interactions of the Ag₂S—GEM with TS, it can be observed that it forms 12 hydrogen bonds with Lys77, Phe80, Glu87, Ile108, Leu221, Gly222, Asn226, and Met309, two hydrophobic contacts with Ile108 and Phe225, as well as halogen and pi-sulfur interactions with Glu87 and Phe225, respectively. Furthermore, Ag₂S—GEM is surrounded by a considerable number of Van der Waals contacts with various residues localized in the enzyme active

site (Table 1, Fig. 9D). The binding energy of the Ag₂S—GEM against TS is remarkably strong (ΔG = -6.52 kcal/mol) when compared to 5-FU-MP, and it exceeds that of the positive control.

Intermolecular Interactions with DNA Polymerase Alpha Catalytic Core

The molecular interactions of the co-crystallized ligand (reference compound), aphidicolin (APH), in the catalytic region of DNA pol α involve 5 H-bonds with Leu864, Ser955, Gly958, and Asp1004. Additionally, it encompasses 6 hydrophobic contacts with Tyr865, Pro866, and Tyr957, as well as a few Van der Waals interactions with certain residues. APH also shows an energetically highly favorable binding strength (ΔG = -7.17 kcal/mol) in the active site of the enzyme (Table 1). GEM-TP, the intracellularly active metabolite of GEM, forms 9 H-bonds with Leu864, Asn954, Asp1004, and Pro866, one hydrophobic contact with Tyr957, 3 electrostatic interactions with Tyr865, Asp1002, and Asp1004, as well as two halogen interactions with Ser863 and Asn954. Also, a certain number of Van der Waals interactions can be observed surrounding the drug in the active site (Table 1, Fig. S3 A). However, the calculated binding strength of GEM-TP against the DNA pol α is considerably weaker (ΔG = -3.77 kcal/mol) when compared with the co-crystallized inhibitor APH. In the DNA pol α active site, Ag₂S nanoparticles formed three hydrogen bonds with Tyr839 and Gly958, as well as three pi-sulfur interactions with Tyr839, Tyr865, and Tyr957. Additionally, there were many Van der Waals contacts with other residues during the binding of the nanoparticles (Table 1, Fig. S3B). However, when compared to the native ligand APH, the binding affinity of Ag₂S against the DNA pol α is found weak (ΔG = -4.27 kcal/mol). The Ag₂S—GEM exhibits interactions within the active site of DNA pol α , involving a total of 9 hydrogen bonds with Tyr839, Tyr865, Pro866, Asn954, Tyr957, and Asp1004. Additionally, it forms a hydrophobic contact with Tyr865, an electrostatic interaction with Asp1004, and a total of 5 halogen and pi-sulfur contacts with Ser863, Tyr865, Asn954, and Tyr957. Moreover, numerous Van der Waals contacts occurred with certain residues also contributed to the stabilization of the Ag₂S—GEM conformation in the enzyme's active site (Table 1, Fig. S3 C). When compared to the co-crystallized inhibitor APH, the binding strength of Ag₂S—GEM in the active site of DNA pol α is highly favorable (ΔG = -6.65 kcal/mol).

Table 1 Predicted docking binding free energies and intermolecular interactions of gemcitabine (GEM-DP, GEM-TP), silver sulfide (Ag₂S; nanoparticle), Ag₂S—GEM, and positive control compounds (5-FU-MP, aphidicolin, and NSAH) against target receptors (thymidylate synthase, DNA polymerase alpha catalytic core, and ribonucleotide reductase) and the resulting molecular interactions.

Compound	Molecular weight (g/mol)	Target	ΔG_{best} (kcal/mol)	H-bond	Hydrophobic interaction			Electrostatic interaction	Other (Halogen*, Pi-lone pair, Pi-Sulfur ^o)
					Van der Waals	π - π / π -alkyl interaction			
5-FU-MP (pos. control)	228.07	TS	-5.97	Glu87 (1), Ile108 (1), Cys195 (1), Asp218 (2), Asn226 (1)	Phe91, Trp109, Asn112, Gln214, Gly217, Leu221, Gly222, Val223, Phe225, Met311	Cys195 (1)	Glu87 (1)	Asn226 (1)*	
Ag ₂ S	1341.88	TS	-4.36	Lys77 (2), Lys107 (1), Leu221 (1), Ile307 (1)	Val79, Ile108, Pro224, Lys308, Met309, Glu310, Met311	-	-	Phe80 (1) ^o , Phe225 (1) ^o	
GEM-DP	423.16	TS	-5.09	Lys77 (2), Phe80 (1), Glu87 (1), Ile108 (1), Leu221 (2), Gly222 (1)	Val79, Phe91, Gly220, Pro224, Phe225, Asn226, Met311	Ile108 (1), Trp109 (2)	-	Ile108 (1)*	
GEM-TP	503.14	TS	-4.25	Lys77 (1), Phe80 (1), Asn112 (2), Leu221 (1), Asn226 (1), Gly222 (1), Lys308 (1), Met309 (1)	Val79, Leu192, Gly220, Pro224, Met311	Ile108 (2), Trp109 (2), Phe225 (1)	-	Glu87 (1)*, Ile108 α	
Ag ₂ S—GEM	1570.96	TS	-6.52	Lys77 (2), Phe80 (1), Glu87 (1), Ile108 (2), Leu221 (2), Gly222 (1), Asn226 (2), Met309 (1)	Arg78, Phe91, Lys107, Tyr135, Cys195, His196, Gly220, Lys308, Met311	Ile108 (1), Phe225 (1)	-	Glu87 (2)*, Phe225 (1) ^o	
APH (co-crystallized inhibitor)	373.4	Pol α	-7.17	Leu864 (1), Ser955 (1), Gly958 (1), Asp1004 (2)	Phe861, Ser863, Asn954	Tyr865 (3), Pro866 (1), Tyr957 (2)	-	-	

Table 1 (continued)

Compound	Molecular weight (g/mol)	Target	ΔG_{best} (kcal/mol)	H-bond	Hydrophobic interaction			Electrostatic interaction	Other (Halogen*, Pi-lone pairing, Pi-Sulfur ^o)
					Van der Waals	π - π / π -alkyl interaction			
GEM-TP	295.8	Pol α	-3.77	Leu864 (2), Asn954 (4), Asp1004 (2), Pro866 (1)	Gly842, Ser955, Gly958, Thr1003, Lys1053	Tyr957 (1)	Tyr865 (1), Asp 1002 (1), Asp1004 (1)	Ser863 (1)*, Asn954 (1)*	
Ag ₂ S	1341.88	Pol α	-4.27	Tyr839 (1), Gly958 (2)	Gly841, Gly842, Pro866, Asn954, Ser955, Gly961, Thr1003	-	-	Tyr839 (1) ^o , Tyr865 (1) ^o , Tyr957 (3) ^o	
Ag ₂ S—GEM	1570.96	Pol α	-6.65	Tyr839 (1), Tyr865 (1), Pro866 (1), Asn954 (4), Tyr957 (1), Asp1004 (1)	Gly841, Gly842, Leu864, Ser955, Gly958, Thr977, Thr1003	Tyr865 (1)	Asp1004 (1)	Ser863 (1)*, Tyr865 (1) ^o , Asn954 (1)*, Tyr957 (2) ^o	
NSAH (co-crystallized inhibitor)	306.1	RNR	-9.14	Ser202 (1), Thr604 (2), Ala605 (1), Ser606 (1)	Thr199, His200, Pro203, Ser217, Ala447, Ser448	Ala201 (1), Cys429 (1), Pro603 (1)	-	Cys218 (2) ^o , Met602 (1) ^o	
GEM-DP	423.16	RNR	-6.34	Ser202 (1), Ser217 (2), Ala245 (1), Cys429 (1), Thr607 (2)	Ala201, Pro203, Gly246, Gly247, Leu428, Leu446, Met602, Pro603, Thr604, Ala605, Ser606	Cys218 (1)	-	Cys429 (1) ^o	

Table 1 (continued)

Compound	Molecular weight (g/mol)	Target	ΔG_{best} (kcal/mol)	H-bond	Hydrophobic interaction		Electrostatic interaction	Other (Halogen*, Pi-lone pairs, Pi-Sulfur ^b)
					Van der Waals	π - π / π -alkyl interaction		
Ag ₂ S	1341.88	RNR	-4.24	Ser202 (1), Ser217 (1), Ala245 (1), Ala605 (1), Ser606 (2)	Ala201, Pro203, Gly247, Gln288, Ala296, Leu428, Leu446, Met602, Thr604, Thr607, Tyr737	Cys429 (1), Pro603 (1), Ala605 (1)	-	Phe206 (1) ⁰ , Ser202 (1) ⁰
Ag ₂ S—GEM	1570.96	RNR	-5.41	Ala201 (1), Gly247 (1), Leu446 (1), Thr604 (1), Ala605 (1)	Thr199, His200, Ser202, Pro203, Ser217, Ala245, Gln288, Leu428, Cys429, Ala447, Ser448, Ser606, Thr607, Tyr737	Met602 (1), Met603 (1)	-	Cys218 (1) ⁰ , Phe206 (1) ⁰ , Thr604 (2)*

TS Thymidylate synthase (PDB ID: 1HVY), Pol α DNA polymerase alpha catalytic core (PDB ID: 4Q5 V), RNR Ribonucleotide reductase, (PDB ID: 5TUS), ΔG_{best} Most favorable binding free energy (kcal/mol)

The numbers within parentheses immediately adjacent to the amino acid residues depict how many times the corresponding chemical interaction has occurred

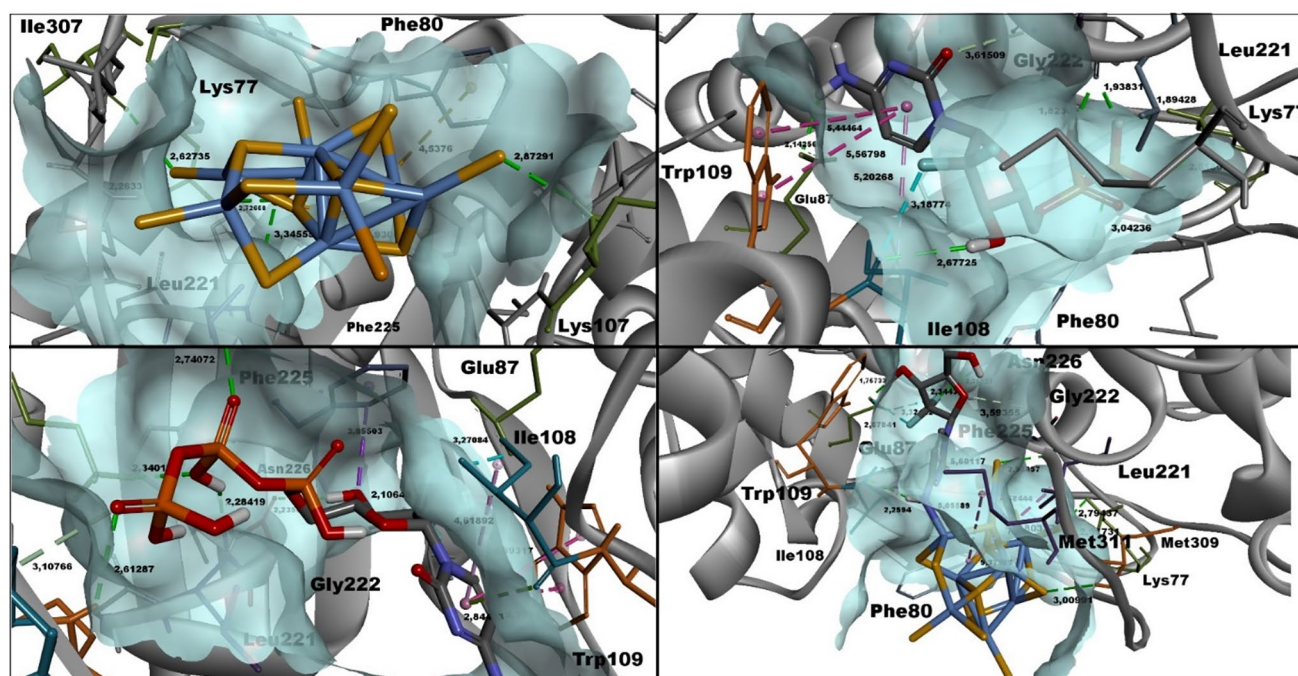


Fig. 9 Top-ranked conformations of the molecular complexes formed by Ag_2S (nanoparticle), GEM-DP, GEM-TP, and Ag_2S —GEM ligands with thymidylate synthase (TS) enzyme: **A** Ag_2S —TS complex, **B** GEM-DP—TS complex, **C** GEM-TP—TS complex, **D** Ag_2S —GEM—TS complex. The TS protein is represented in solid ribbon mode, while the ligands are depicted in stick mode. Green and light grey dashed lines represent hydrogen bonds, dark and light pur-

ple dashed lines indicate hydrophobic contacts, cyan dashed lines represent halogen (fluorine) contacts, light yellow dashed lines represent pi-sulfur interactions, and light green dashed lines represent pi-lone pair interactions. To accentuate the surface topology of the ligand-binding sites, the receptor's active sites were rendered in the 'receptor surface' mode. 3D figure was prepared using the Discovery Studio Visualizer program (Color figure online).

Intermolecular Interactions with Ribonucleotide Reductase

The interactions of the co-crystallized inhibitor, naphthyl salicyl acyl hydrazone (NSAH), with RNR show that it forms five hydrogen bonds with Ser202, Thr604, Ala605, and Ser606. It also has three hydrophobic contacts with Ala201, Cys429, and Pro603, and two pi-sulfur interactions with Cys218 and Met602 (Table 1). Additionally, the orientation of NSAH in the enzyme active site is further stabilized by a moderate number of Van der Waals contacts with several residues. The calculated docking binding energy of NSAH towards RNR ($\Delta G = -9.14$ kcal/mol) is highly favorable, indicating a strong inhibition. The intermolecular interactions of GEM-DP with RNR consist of a total of 7 H-bonds with Ser202, Ser217, Ala245, Cys429, and Thr607, one hydrophobic contact with Cys218, one pi-sulfur interaction with Cys429, and Van der Waals contacts involving numerous residues (Table 1, Fig. S4 A). Although the binding energy of GEM-DP towards RNR ($\Delta G = -6.34$ kcal/mol) is not highly comparable to that of the native inhibitor NSAH, it still provides the expected level of inhibition from a hit molecule and is considered strong. The molecular interactions of Ag_2S nanoparticle

with the RNR active site consist of 6 H-bonds with Ser202, Ser217, Ala245, Ala605, and Ser606, three hydrophobic contacts with Cys429, Pro603, and Ala605, two pi-sulfur interactions with Phe206 and Ser202, and numerous Van der Waals contacts with several residues (Table 1, Fig. S4B). The calculated binding energy value of Ag_2S towards RNR ($\Delta G = -4.24$ kcal/mol) is weak. Upon examination of the molecular interactions between the final ligand, Ag_2S —GEM, with RNR, it is observed that Ag_2S —GEM forms 5 H-bonds with Ala201, Gly427, Leu446, Thr604, and Ala605, two hydrophobic contacts with Met602 and Met603, two pi-sulfur and one halogen contact with Phe206, Cys218, and Thr604, respectively. Furthermore, the Ag_2S —GEM has formed numerous Van der Waals contacts with multiple residues in the active site of RNR (Table 1, Fig. S4 C). When compared to NSAH, the binding energy of Ag_2S —GEM within the active site of RNR indicates a moderately strong interaction ($\Delta G = -5.4$ kcal/mol).

Interactions of 5-FU, Ag_2S , GEM and Ag_2S —GEM with B-DNA

In this study, the DNA affinities, binding modes, and the nucleotides interacting with 5-fluorouracil (5-FU), Ag_2S ,

gemcitabine (GEM), and the Ag₂S-GEM combination were examined through docking simulations performed using a 12-nucleotide B-DNA dodecamer (PDB ID: 7rq1). These findings (Table 2) were subsequently used to rationalize the results obtained from plasmid DNA interaction and cleavage assays. Furthermore, to rule out non-specific ligand binding, DNA docking was also performed with aflatoxin (AFL)—a known genotoxic agent and DNA binder—and the binding free energy of this group was used as a positive control for comparison (see for results, Supplementary Information, Fig. S5, Table S1).

In its interaction with B-DNA, 5-FU exhibited minor groove recognition (Fig. S6a) and demonstrated binding affinity to a GC-rich region of the oligonucleotide structure (Table 2). The binding affinity of 5-FU to B-DNA was strong ($\Delta G = -6.34$ kcal/mol). Ag₂S nanoparticles (NPs) recognized B-DNA through the minor groove (Fig. S6b), interacting with nucleotides present in equal proportions of guanine (G), cytosine (C), and adenine (A) (G=C=A) (Table 2). The binding affinity of Ag₂S to B-DNA was moderate ($\Delta G = -4.81$ kcal/mol). The docking interaction results revealed that Ag₂S binds equally to G, A, and C nucleotides, which is consistent with the findings of the DNA cleavage assay (Fig. 6) that demonstrated Ag₂S preferentially target guanine–guanine nucleotide pairs in DNA. These results suggest that Ag₂S NPs could act as physical blockers, preventing enzymatic access to specific DNA sequences.

In docking experiments with GEM, the compound recognized B-DNA from the minor groove (Fig. S6c) and displayed a higher affinity for sequences containing adenine (A) and cytosine (C) nucleotides, with relatively lower affinity for thymine (T) and guanine (G) (Table 2). GEM exhibited strong binding affinity to B-DNA, ($\Delta G = -6.67$ kcal/mol). The combination of Ag₂S and GEM demonstrated binding to B-DNA from the minor groove (Fig. S6 d), with a pronounced preference for regions enriched in guanine (G) and, to a lesser extent, adenine (A) and cytosine (C) nucleotides (Table 2). This combination exhibited a significantly

strong binding affinity to B-DNA ($\Delta G = -7.93$ kcal/mol). Moreover, when compared with the binding free energy of the positive control AFL ($\Delta G = -7.79$ kcal/mol) (see Supplementary Information, Fig. S4, Table S1 for details), 5-FU, GEM, and Ag₂S displayed slightly weaker binding affinities of -6.34 , -6.67 , and -4.81 kcal/mol, respectively. Although these ligands exhibit weaker interaction energies toward DNA compared to AFL, these values still suggest relatively strong affinities (with the exception of Ag₂S). Notably, the Ag₂S–GEM combination achieved a more favorable binding free energy ($\Delta G = -7.93$ kcal/mol) than AFL, supporting the observed significant cytotoxic effect in PANC-1 cells treated with Ag₂S–GEM combination. These results were also consistent with the observations in the DNA cleavage assay (Fig. 8), which showed complete inhibition of BamHI cleavage at the preferred binding site of the Ag₂S–GEM complex, providing a plausible explanation for the observed cytotoxic effect in PANC-1 cells (Figs. 3, 4 and 5).

Our docking data showed that 5-FU, GEM, and the Ag₂S–GEM combination exhibited considerable binding affinities (Table 2) for DNA *via* interactions within the minor groove. In this context, while such binding with DNA can enhance therapeutic efficacy in cancer cells (e.g., by impeding DNA replication and increasing DNA damage in tumor cells), it could also raise concern about genotoxicity and off-target effects on healthy cells such as genome damage, and non-specific binding due to concentration-dependent effects, since our data suggest that toxicity of ligands, specially the Ag₂S–GEM combination, is tightly correlated with compound concentration and treatment duration (Fig. 4C). Thus, to reduce non-specific toxicity *in vivo*, mitigation strategies—such as targeted delivery (directly into tumor tissue) [59, 60] or antioxidants to lessen DNA damage [61, 62] and side effects in healthy cells—can be proposed. Moreover, further experimental validation (i.e. *in vivo* comet assay and micronucleus tests) in healthy cells may prove useful to fully characterize and manage off-target effects.

Table 2 Binding free energies (kcal/mol) predicted from molecular docking for 5-fluorouracil (5-FU), Ag₂S, gemcitabine (GEM), and the Ag₂S–GEM with the 12-nucleotide double-stranded B-DNA dodecamer.

Compound	Molecular weight (g/mol)	Receptor	ΔG_{best} (kcal/mol)	Binding mode	Binding sequence
5-FU	228.07	B-DNA	−6.34	Minor groove	5'-GCGCTTAAGCGC-3' 3'-CGCGAATTCGCG-5'
Ag ₂ S	1341.88	B-DNA	−4.81	Minor groove	5'-GCGCTTAAGCGC-3' 3'-CGCGAATTCGCG-5'
GEM	247.20	B-DNA	−6.67	Minor groove	5'-GCGCTTAAGCGC-3' 3'-CGCGAATTCGCG-5'
Ag ₂ S–GEM	1570.96	B-DNA	−7.93	Minor groove	5'-GCGCTTAAGCGC-3' 3'-CGCGAATTCGCG-5'

Ag₂S Silver sulfide, DNA B-DNA dodecamer (PDB ID: 7rq1), Ag₂S–GEM Silver sulfide-gemcitabine combination, ΔG_{best} Most favorable binding free energy (kcal/mol)

The specific TS inhibitor, 5-FU-MP, exhibited a higher cytotoxic effect compared to GEM (GEM-DP: RNR inhibitor; GEM-TP: DNA pol α substrate) at treatment periods of 24, 48, and 72 hours (Figs. 4 and 5). Therefore, TS inhibition possesses a more potent cytotoxic signaling ability in the pancreatic ductal epithelioid carcinoma cell line (PANC-1) compared to RNR or DNA pol α inhibition. Our docking results also support this hypothesis, as 5-FU-MP exhibits a higher ability to form an energetically more favorable complex with TS compared to GEM's diphosphate (GEM-MP) and triphosphate (GEM-DP) forms (Table 1). On the other hand, the Ag₂S—GEM has demonstrated a more potent cytotoxic effect than the reference compound 5-FU-MP at all treatment durations in PANC-1 cells, particularly at high (125 and 250 $\mu\text{g/mL}$) concentrations (Figs. 4 and 5). The calculated binding energy of Ag₂S—GEM ($\Delta G = -6.52$ kcal/mol) in the docking experiment against the TS enzyme, as shown in Table 1, supports this finding. In terms of IC₅₀ values, the Ag₂S—GEM exhibits a more effective cell-killing potency in PANC-1 cells compared to Ag₂S NPs, particularly at treatment periods of 48 and 72 hours (Figs. 3, 4, and 5). The physical combination of Ag₂S and GEM into a mixture has increased the docking binding energy to the enzymes TS and DNA pol α , compared to the individual forms of the ligands (Table 1).

This observation shows that Ag₂S and GEM work together synergistically, meaning the Ag₂S—GEM complex can interact more favorably with the active sites of the TS and DNA pol α enzymes than Ag₂S and GEM do on their own (specifically GEM-DP and GEM-TP) (Table 1). Furthermore, considering its favorable docking interaction with DNA pol α , the Ag₂S—GEM holds potential as an effective DNA replication inhibition strategy in cancer cells.

Although resistance to Gemcitabine in cancer treatment is a common problem, the mechanisms responsible for this resistance have not yet been completely explained [63]. This uncertainty has increased the importance of combined treatment approaches using Gemcitabine along with therapeutic agents, different chemicals, extracts, or nanoparticles, leading to numerous studies in this field. The main objective of these combination therapy approaches is to use synergistic interactions to overcome the resistance that occurs against Gemcitabine [36, 63–67]. The synergistic interaction between gemcitabine and synthetic silver nanoparticles has been thoroughly assessed in several of recent studies, which have found that using these nanostructures in conjunction with gemcitabine significantly increases its therapeutic efficacy. In a study conducted by Yuan et al. (2017), the application of non-conjugated AgNPs and Gemcitabine in combination demonstrated higher cytotoxic activity in human ovarian cancer cell lines than either treatment independently [68]. Also, it was found that when Gemcitabine conjugated with silver nanoparticles was

applied together, the cytotoxic effects on MDA-MB-453 cells were significantly higher than when the treatments were administered as monotherapy [69]. In the study by El-Sheikh et al. (2021), the cytotoxic potential of gemcitabine-loaded silver nanoparticles (GEM-AgNPs) was evaluated in the human hepatocellular carcinoma cell line (HepG2) in the study, showing an increase of both apoptotic and necrotic cell death pathways in addition to G1 phase cell cycle arrest. The authors further showed that the combinatorial treatment regimen produced superior antitumor effects compared to gemcitabine monotherapy, and these findings were substantiated by complementary *in vivo* experiments [70]. Similarly, Yang et al. (2020), investigated the combinatory effects of silver nanotriangles (AgNTs) with various chemotherapeutic agents—including cyclophosphamide (CTX), 5-fluorouracil (5-FU), oxaliplatin (OXA), doxorubicin (DOX), and gemcitabine (GEM)—across different glioma cell lines (U87, U251, and C6), with an aim to explain the underlying mechanisms of synergy. Among the agents tested, gemcitabine exhibited the most determined synergistic interaction with AgNTs, which was associated with a significant reduction in cell viability, disruption of mitochondrial membrane potential (MMP), and an elevation in apoptotic cell death in glioma cells [71].

Consistent with these previous findings our results similarly indicate that the combined application of Ag₂S nanoparticles and gemcitabine exhibits a synergistic cytotoxic effect, a conclusion that is further reinforced by comparable findings reported in the existing literature. Moreover, previous studies have provided various evidence into the cytotoxic mechanisms of gemcitabine and silver Ag NPs on cells. Especially, Ag NPs generate reactive oxygen species (ROS) that can damage proteins, cell membranes and interfere with DNA synthesis, finally leading to DNA damage. Meanwhile, gemcitabine promotes DNA damage and apoptosis by integrating into DNA by intracellular phosphorylation. Exposure to these substances leads to cell cycle arrest and subsequently apoptosis [69, 72–75]. However, for the effects of drugs or substances to occur, they must pass through various biological barriers and reach the target molecules they will interact with [76]. For this purpose, it is recognized that the main pathway for nanoparticles to enter cells is endocytosis [77]. A previous study by AshaRani et al. (2009) evaluated the application of silver nanoparticles (Ag-NPs) to normal human lung fibroblasts (IMR-90) and human glioblastoma cells (U251). Their findings provided that Ag-NPs were internalized through clathrin-mediated endocytosis and macropinocytosis, with Ag-NPs detected both in the cytoplasm and the nucleus [78]. In the context of this literature, our findings suggest that Ag₂S NPs may induce apoptosis in PANC-1 cells through DNA damage, which

occurs as a result of cellular internalization *via* endocytosis. Moreover, the intracellular uptake of gemcitabine (GEM) could be enhanced through non-covalent electrostatic interactions between Ag₂S NPs and GEM. Given these observations, it is probable that the combination treatment of GEM and Ag₂S NPs induces apoptosis by causing DNA damage in the cells. This hypothesis is also strongly supported by the results of the molecular docking and in-vitro DNA cleavage analyses conducted in this study.

Reactive oxygen species (ROS), which are commonly triggered by nanoparticles, have been reported to induce cellular degeneration by increasing intracellular oxidative stress. This increasing intracellular oxidative stress disrupting cell membrane integrity and permeability. As a result of this causing cell swelling and rounding [79]. Supporting these findings, in this study, morphological alterations characterized by cellular swelling and rounding as well as apoptotic cell morphologies were observed in cells treated with 250 µg/mL and 125 µg/mL concentrations of Ag₂S nanoparticles. These morphological changes may also indicate that AgNP exposure can trigger apoptotic or necrotic pathways in the affected cells [79, 80].

Conclusion

In this study, we successfully synthesized silver sulfide nanoparticles (Ag₂S NPs) using an environmentally friendly approach with sweet cherry extract, demonstrating their potential as effective agents against pancreatic cancer. Our findings reveal that Ag₂S NPs exhibit significant cytotoxic effects on PANC-1 cancer cell lines, particularly when combined with gemcitabine (GEM). The most effective cytotoxicity was observed with the Ag₂S NPs-GEM combination, achieving an IC₅₀ value of 18.97 µg/mL at 72 hours, which underscores the synergistic potential of this therapeutic strategy. Molecular docking studies indicated that the Ag₂S NPs—GEM complex shows favorable binding affinities with key enzymes involved in DNA replication, such as thymidylate synthase and DNA polymerase alpha. This suggests that Ag₂S NPs not only enhance the efficacy of GEM but may also act as effective DNA binders, contributing to increased cytotoxicity through DNA damage mechanisms. The implications of our findings are significant for future cancer treatment strategies. The combination of Ag₂S NPs and GEM could provide a dual mechanism of action, potentially overcoming drug resistance commonly associated with chemotherapy. However, in order to identify the signals that trigger death in PANC-1 cancer cells and to develop targeted treatment strategies, apoptosis gene-pathway relationships (gene expression etc.) and apoptotic effects in cells need to be clarified by examining them with some methods (TMRE and AnnexinV/7 AAD staining

etc.). As such, further research is warranted to explore the in vivo efficacy and safety of Ag₂S NPs in combination therapies. Additionally, the green synthesis approach presents a scalable and ecologically sustainable method for nanoparticle production, making it a promising avenue for developing new cancer treatments. Future studies should focus on optimizing the formulation and delivery systems of Ag₂S NPs to maximize their therapeutic potential in clinical applications.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare that they have no conflict of interest. No potential conflict of interest was reported by the authors.

Ethical Approval Not applicable.

Consent to Participate The authors declare our consent to participate.

Consent for Publication The authors declare our consent for publication upon acceptance.

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