Cancer Nanotechnology

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Investigation of mitochondria-dependent apoptosis pathway and lipid peroxidation level induced by biosynthesized silver nanoparticles: caspase-3 activation, BAK1/BCLx regulation and malondialdehyde production

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Abstract

Nowadays, silver nanoparticles (AgNPs) have attracted the attention of many researchers due to their special physical, chemical, and biological properties. There is strong evidence that biogenic AgNPs can act as potent anticancer agents through the production of reactive oxygen species (ROS) and initiate the mitochondrial pathway of apoptosis. That is why we decided to use *Nepeta bracteata Benth* flower extract for the first time to bio-synthesize AgNPs and study their cytotoxic and apoptotic effects on SK-BR-3 cells. AgNPs were biosynthesized at 70 °C after mixing silver nitrate and flower extract with a specific ratio and concentration, then were characterized using various analytical techniques, such as FESEM, FTIR, EDS, and zeta potential. Studies have shown that AgNPs have an irregular and circular shape, with about 99% by weight of silver, carbon, and oxygen. On the other hand, the appropriate size (below 57 nm) and surface charge (− 11.52 mV) make them stable in biological fluids. The better cytotoxic effect of AgNPs compared to flower extract on SK-BR-3 cells was investigated using the MTT method. The positive effect of AgNPs on inhibiting the growth of SK-BR-3 breast cancer cells was again confirmed by the sulforhodamine B staining method, so that AgNPs were able to decrease the density of cancer cells in a concentration-dependent manner. In addition, the flow cytometry test proved that biosynthesized AgNPs using *Nepeta bracteata Benth* flower extract can induce apoptosis in SK-BR-3 cancer cells. Real-time PCR then proved that the ratio of Bak1/Bclx, as well as caspase-3 expression, was increased due to active ROS-producing biomolecules present in the plant extract, and therefore, AgNPs can activate the mitochondria-dependent apoptosis pathway in breast cancer cells. Finally, their negligible oxidative stress on erythrocytes was confirmed by the lipid peroxidation method and showed that biosynthesized AgNPs can be used for breast cancer treatment without showing adverse effects on erythrocytes.

Keywords: Real-time PCR, Flow cytometry, Lipid peroxidation, Sulforhodamine B staining, *Nepeta bracteata Benth*, Caspase-3, Bak1/Bclx ratio

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Introduction

Nanoparticles (NPs) are a broad class of materials that have at least one dimension less than 100 nm. In addition, because of this particular size, they can have different physiochemical properties and applications, specifically in the field of medicine. For example, they can be used for drug or gene delivery, fluorescent biological labels, bio-detection of pathogens or proteins, magnetic resonance imaging (MRI) contrast, probing of deoxyribonucleic acid (DNA) structure, and tissue engineering (Kumar et al. 2023).

Among these, metallic NPs are one of the most attractive areas of research that has attracted significant attention, because they deal with all the fields of sciences, such as physics, chemistry, engineering, pharmacy, computational, and clinical application (Sharma et al. 2023; Khashan et al. 2018). Unlike traditional methods that use toxic chemicals as reducing and capping agents during the production of nanoparticles, the synthesis of metal nanoparticles employing biological materials (e.g., fungi, algae, bacteria, and plant extracts) with an environmentally friendly approach can save time and cost, and produce non-toxic products (Bhattacherjee et al. 2018; Cruz-Hernández et al. 2023). Among the available sources for synthesis of biogenic nanoparticles, using plant extracts is a rather simple approach for producing nanoparticles at a large scale relative to fungi or bacteria, due to active biomolecules of plants may bind on the surface of the nanoparticles and act as good stabilizing agents (Kandemir et al. 2023; Yan et al. 2021).

In addition to electronic, physical, bandaging, surgical, and other environmental applications, silver nanoparticles (AgNPs) due to their suitable size, can show a wide range of medical applications, such as antiviral, antifungal, antibacterial, anti-inflammatory, anticancer, and antioxidant (Burange et al. 2021; Kumar Bachheti et al. 2020). Although the anti-pathogenic mechanism of AgNPs is not well-understood, there is evidence that they act through the release of free silver ions (Ag^{+}) and the generation of reactive oxygen species (ROS) in macrophage cells (Akter et al. 2018). After that, activated macrophage cells elevate Tumor necrosis factor alpha (TNF-α) secretion which leads to cell membrane damage and apoptosis (Park et al. 2010). In addition, another study showed that AgNPs could increase the expression of the Bak1 (BCL2 Antagonist/Killer 1) gene via the mitochondrial pathway which is associated with the generation of ROS (Sargazi et al. 2020). Therefore, biosynthesized silver nanoparticles can be expected to be useful in the programmed death of cancer cells and the induction of apoptosis (Zhang et al. 2022). However, the anti-cancer activity of AgNPs is strongly influenced by various factors, such as size, morphology, surface charge, and especially surface coating (Alexandra-Cristina et al. 2018). On the other hand, the different studies improved that the morphology, size, and surface coating are dependent on biological materials that are used to produce AgNPs (Alsmadi et al. 2022).

In this study for the first time, we used *Nepeta bracteata Benth* for the bio-reduction of AgNPs, which contain significant amounts of terpenoids, diterpenes, flavanones, flavonoids, and phenolic acid derivatives (Hajiheydari et al. 2017). It has been proven that active compounds such as flavonoids and terpenoids in flower extract can act as effective stabilizing and capping agents, leading to the formation of AgNPs with an average size below 50 nm that have potential medical applications (Hasanien et al. 2023; Kannan et al. 2013). Based on this, Zhang et al. (2021) confirmed that the new diterpenoids in *Nepeta bracteata Benth* can show moderate cytotoxic effects in human intestine

ileocecal adenocarcinoma (HCT-8) cell line. In addition, these bioactive molecules such as flavonoids, vitamins, polyphenols, and carotenoids cause the reduction of metal precursors and act as biological factories to produce pure metal particles and metal oxides on a nanometer scale (Kim et al. 2012). Therefore, we speculate that they both stimulate the biosynthesis of nanoparticles and play a role in inducing apoptosis and inhibiting the growth of cancer cells.

Therefore, considering the importance of AgNPs in the treatment of breast cancer and the need for newer drugs, our main goal is the biosynthesis of AgNPs by plant compounds and the investigation of their cytotoxic effects on SK-BR-3 cells (human breast cancer) using MTT and sulforhodamine B staining methods to make sure that this biocompounds can prevent the growth of cancer cells. In addition, due to the presence of active compounds in biogenic nanoparticles and the non-toxic nature of covering agents, for the first time we evaluate their ability to induce apoptosis in SK-BR-3 cancer cells by flow cytometry. In addition, we investigate the changes in the expression of genes involved in apoptosis (Bak1, Bclx, and Caspase-3) in SK-BR-3 cells treated with biosynthesized AgNPs using *Nepeta bracteata Benth* flower extract to find a new way to treat breast cancer. Finally, erythrocytes are prone to oxidative stress, and therefore, it is necessary to investigate the safety of AgNPs for erythrocytes, so that we can recommend them for the treatment of breast cancer.

Experimental

Materials

Solvents such as distilled water and ethanol were of high purity and were all purchased from Merck. SK-BR-3 (human breast cancer cells) and L-929 (normal mouse fibroblast cells) were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China). RPMI-1640 medium was obtained from Caisson Labs (North Logan, UT, USA). Fetal bovine serum (FBS) and penicillin G-streptomycin were purchased from Gibco (Carlsbad, CA, USA). Acetic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), sulforhodamine B (SRB), and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) were obtained from Sigma Aldrich (St. Louis, MO, USA). Silver nitrate ($AgNO_3$) and Tris base (Trizma® base) were purchased from Merck (Darmstadt, Germany). High Pure RNA Isolation Kit was related to Roche (Rotkreuz, Switzerland). Fluorescein isothiocyanate–(FITC)-conjugated annexin V was provided from Beyotime (Shanghai, China). PrimeScript™ RT reagent kit was related to Takara Bio Inc (Mountain View, CA, USA). *Nepeta bracteata Benth* (code: KF1251) whose native range is Iran to Central Asia, Afghanistan, and Pakistan, was obtained from the Medicinal Plants Center in the Hospital of Nanchang University in China. The primers were purchased from Bioneer (Daejeon, South Korea) with the reference code T950213.

Isolation of hydro-alcoholic extract of *Nepeta bracteata Benth*

The flowers of *Nepeta bracteata Benth* were dried by lyophilization to preserve the bioactive compounds of the plant. For this purpose, the flowers were cut into small pieces and then freeze-dried at −60 °C under a vacuum condition (Sargazi et al. 2020). Then, 20 g of flowers were added to 100 mL of 50% v/v water/ethanol, and the extraction of bioactive materials was done at room temperature by an orbital shaker (speed = 160 rpm

(revolutions per minute), orbit 5 cm) for 72 h. A closed conical flask (250 mL) was used for this purpose. After this, a cold Buchner funnel with vacuum suction was used to separate the supernatant, and excess ethanol was removed under vacuum in a rotary evaporator (BIOBASE, RE 100-Pro, Jinan, Shandong, China). To prevent the degradation of bioactive compounds and to maintain antioxidant properties, the aqueous extract was stored at − 20 °C (Pham et al. 2017).

Biosynthesis of AgNPs using green approach

Biosynthesis of AgNPs was done based on previously reported methods with some modifications (Yan et al. 2021). First, 90 mL of $AgNO_3$ solution (2M) was prepared in an Erlenmeyer flask and then 10 mL of *Nepeta bracteata Benth* aqueous extract was added slowly to the solution at 70 °C, and stirring was continued until the color of the solution changed to brown. To precipitate AgNPs, the solution was incubated overnight at 4 °C, and AgNPs were then harvested by centrifugation at 3000 rpm for 5 min. Removing the remaining water and creating a dry powder was performed utilizing lyophilization (TOPTION, Xi'an, China), and the weight of dry AgNPs was calculated to be 4.6 g.

Characterization of AgNPs

The morphology and size of biosynthesized AgNPs were studied utilizing the field emission scanning electron microscopy (FESEM) (Brno, Czech Republic) device. To capture FESEM images of the biosynthesized nanoparticles, a drop of AgNPs suspension was placed on a conventional carbon-coated copper grid and allowed to air dry at room temperature (RT). Images were collected at an operation voltage of 15 kV with 500 nm magnification (Benakashani et al. 2016). In addition, the elemental composition of AgNPs was evaluated by an energy-dispersive X-ray spectroscope (EDS) combined with FESEM (Sargazi et al. 2018a; Ali et al. 2019). The Fourier transform infrared spectroscopy (FTIR) (LABOAO, Zhengzhou, Henan, China) spectra were obtained in the whole region of 4000 to 500 cm^{-1} to investigate the bio-reduction of Ag^{+} ions using *Nepeta bracteata Benth* flower extract and compare the parameters and functional groups of AgNPs with flower extract. It was gained at a resolution of 16 cm^{-1} using potassium bromide (KBr) pellets (Baygar et al. 2017; Jabir et al. 2019). According to different papers, measuring the zeta potential of colloidal solution of nanoparticles in the range of − 200 mV to + 200 mV is useful for determining the surface charge.

In vitro cell viability assay using MTT

SK-BR-3 (a human breast cancer cell line) and L-929 (a normal mouse fibroblast cells) were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin G–streptomycin according to available instructions (Shahraki et al. 2019; Sorinezami et al. 2019). After transferring 1.2×10^4 cells to each well of a 96-well culture plate, SK-BR-3 human breast cancer cells were treated with five different concentrations of AgNPs (1.56 mg mL^{-1} to 25 mg mL^{-1}) as well as five concentrations of aqueous extract (3.125 to 50 μL mL^{-1}) for 48 and 72 h. In addition, L-929 cells were exposed to five concentrations of AgNPs and flower extract only for 72 h. Cell viability was measured by MTT according to previous works (Oveisi Keikha et al. 2023).

Cytotoxicity screening by sulforhodamine B colorimetric method

After transferring SK-BR-3 to a 96-well plate and treated with five different concentrations of AgNPs (1.56 mg mL^{-1} to 25 mg mL^{-1}), the cells were incubated for 72 h at 37 °C. After carefully removing the culture medium, 100 µL of 10% TCA was added to each well and incubated for 1 h in the dark at 4 °C. The excess TCA solution was washed with sterile distilled water 4 times. Adding and removing water was done very carefully. Then, 80 µL of 0.5% SRB solution was added to each well and left in a dark cabinet at normal room temperature for 30 min. The excess dye was immediately removed by 0.5% acetic acid solution and left for 1 h to dry the wells. Finally, 150 µL of 10 mM Trizma base solution was added to each well and shaken for 20 min to dissolve the protein-bound dye, and the absorbance was read at 510 nm.

Apoptosis assay by flow cytometry

SK-BR-3 cells were cultured with a density of 1.0×10^6 cells/flask before treatment with 2 mg mL^{-1} of AgNPs for 24 h. The concentration was selected according to the value of IC_{50}. One flask was considered as a control without any treatment. All preparation and testing steps were performed in accordance with previous work (Sargazi et al. 2018b; Shahraki et al. 2017). Briefly, cell culture media were carefully discarded and cells were washed three times with phosphate-buffered saline (PBS), then adherent tumor cells were detached using 0.25% trypsin. Afterward, the cells were collected by centrifuge, washed with PBS, and labeled with FITC-conjugated annexin V according to the manufacturer's instructions. After incubation at 37 °C in the dark, the samples were labeled with propidium iodide (PI), and dot plots for each reagent were recorded using a flow cytometer (Beckman Coulter, Epics XL, Holliston, MA, USA).

Real-time polymerase chain reaction (PCR) for apoptosis evaluation

SK-BR-3 cells were maintained in T25 cm^2 flasks under standard conditions to reach a density of 1.0×10^6. One flask without treatment was considered as control and the other was treated with 4 mg mL^{-1} of biosynthesized AgNPs for 24 h (He et al. 2021). After that, treated cells were rinsed twice with sterile PBS, dissociated using trypsin, and collected by centrifuge at 4000 rpm for 5 min. The supernatants were removed well and the cells were re-suspended in 200 µL PBS. The next step, which is the isolation of ribonucleic acid (RNA), was followed according to the manufacturer's protocol of the High Pure RNA Isolation Kit (Sargazi et al. 2020; He et al. 2021). To determine the concentration and purity of the eluted RNA, a WPA Biowave II spectrophotometer (Biochrom Ltd, Cambridge, England) was used, and then it was stored at − 80 °C for the next step (Barar et al. 2015).

Also, the details of complementary deoxyribonucleic acid (cDNA) synthesis using the PrimeScriptTM RT reagent kit were given by Dou et al. (2022). The expression of the Bak1, Bclx, and Caspase-3 genes (Table 1) was measured using the Real-time PCR method and according to the appropriate thermal cycle by the Applied Biosystems StepOne™ device (Thermo Fisher Scientific, Waltham, MA, USA) (Sargazi et al. 2020).

Table 1 Primers used for real-time polymerase chain reaction (RT-PCR). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) is considered as housekeeping gene

Gene	Accession number	Primer sequence
GAPDH	U02886	Forward: TTGCCATCAATGACCCCTTCA Reverse: CGCCCCACTTGATTTTGGA
Bclx	U51277	Forward: TGGAAAGCGTAGACAAGGAGA Reverse: TGCTGCATTGTTCCCATAGA
Caspase-3	MN612052	Forward: TGCAGTCATTATGAGAGGCAAT Reverse: AAGGTTTGAGCCTTTGACCA
Bak1	AT4G33430.2	Forward: TCTGGGACCTCCTTAGCCCT Reverse: AATGGGCTCTCACAAGGGTATT

Lipid peroxidation assay on red blood cells

Two mL of ready erythrocytes were prepared from the blood bank of the hospital of Nanchang University and mixed with 8 mL of PBS. Then, two samples with concentrations of 0 and 2 mg mL^{-1} of AgNPs were prepared in erythrocyte suspension. At 0, 12, and 24 h, 1 mL of the sample was withdrawn and mixed with 1 mL of 20% TCA and 0.5 mL of 0.028 M TBA and incubated at 100 °C for 15 min. Then, centrifugation was performed and the supernatant was separated and its absorbance was measured at a wavelength of 532 nm (the test was repeated three times).

Statistical evaluation

Cytotoxic data were analyzed using a one-way analysis of variance (ANOVA). In addition, the comparison of data was done utilizing Tukey's range test (Jabir et al. 2022). Data are shown as mean values ± SD of four independent experiments and p values of <0.05 are accepted as the minimum level of significance.

Results and discussion

Characterization of biosynthesized AgNPs

In the current research, the flower extract of *Nepeta bracteata Benth* was used as a capping, reducing, and stabilizing agent for the biosynthesis of silver nanoparticles. According to our research, this is the first case in which AgNPs have been synthesized by mediating *Nepeta bracteata Benth*. Active biomolecules of flowers such as terpenoids, diterpenes, flavanones, flavonoids, and phenolic acid derivatives act as templating agents for the green biosynthesis of AgNPs thereby determining their morphology and size. The use of these environment-friendly capping agents at 70 °C resulted in a dark brown suspension of AgNPs (Fig. 1) which shows an irregular and circular shape (Fig. 2a). The average size of AgNPs was estimated to be between 36 to 57 nm according to FESEM measurements. Likewise, various comparisons showed that the use of different reducing agents for the green synthesis of AgNPs can lead to a flake-like appearance (Ashique et al. 2022).

The main items required for the synthesis of green nanoparticles include a suitable non-toxic reducing and stabilizing agent plus a suitable and environmentally friendly solvent, such as water, ethanol, or a mixture thereof (Malik et al. 2022; Huang et al. 2023). Plant extracts that have recently been used to synthesize green nanoparticles are rich in bioactive compounds that can act as natural stabilizing and/or reducing agents.

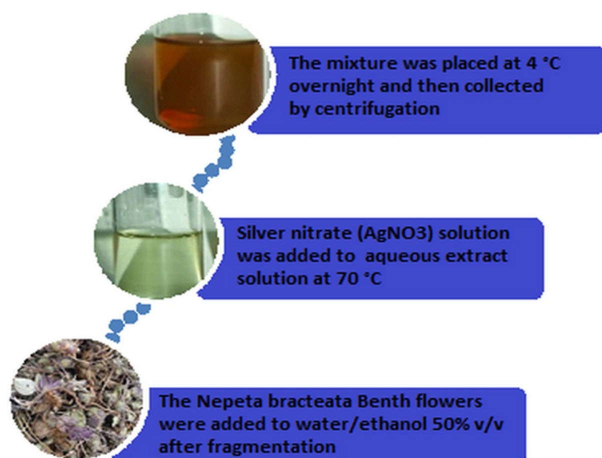

Fig. 1 Pictorial presentation of AgNPs biosynthesis

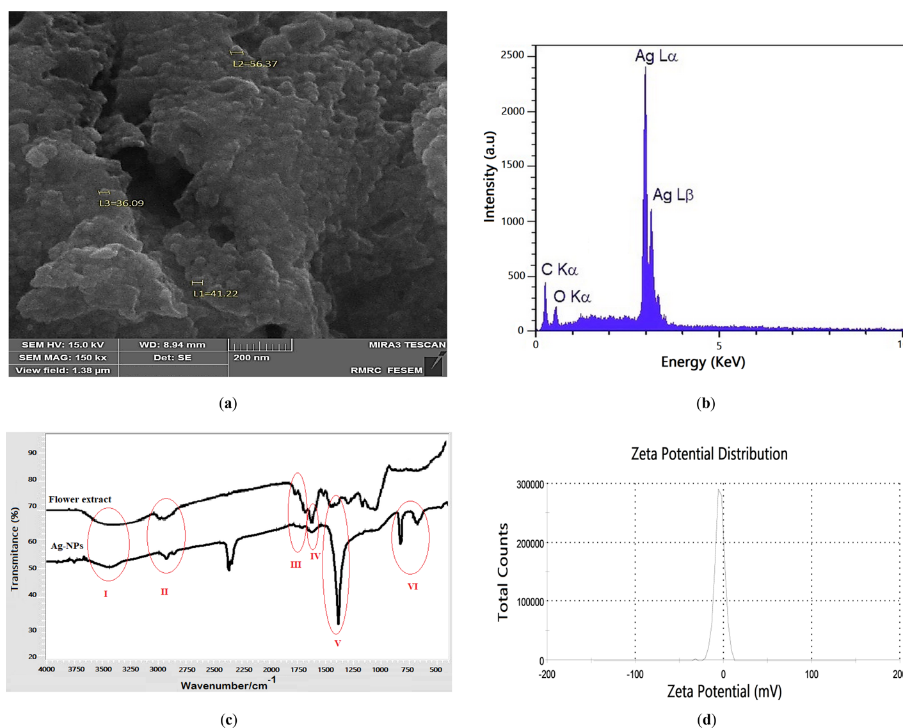

Fig. 2 **a** Morphological studies were performed using FESEM (Mira 3 TESCAN instrument) at good magnification (200 nm). **b** EDS analysis of the biosynthesized AgNPs between 0 and 10 keV. **c** FTIR spectrum of biosynthesized AgNPs and *Nepeta bracteata Benth* flower extracts using Shimadzu IR PRESTIGE 21 spectrophotometer (Tokyo, Japan). **d** Zeta potential of AgNPs obtained using Malvern Zetasizer_nanoZS (England)

In fact, they are very important for reducing metal ions to NPs in a single-step green synthesis process and can affect the size, shape, and composition of nanoparticles (Le et al. 2023). Thus, the obvious signals of C and O besides the high signals of Ag certified the presence of the plant residues on the surface of the biosynthesized AgNPs. The EDS analysis (Fig. 2b) revealed the high signal and homogenous distribution of the chemical

composition of the biosynthesized AgNPs between 0.5 and 5 keV. In addition, the high ratio of weight percentage (Table 2) of silver, carbon, and oxygen was a good reason for the high purity of AgNPs.

Extraction of *Nepeta bracteata Benth* flower extracts was performed at room temperature to preserve the volatile compounds of the flower. Based on the EDS analysis, it was found that plant residues and the chemical composition of the flower are present in the structure of AgNPs and can act as stabilizing and bio-reducing agents of silver ions. To further confirm this, the main groups of flower extracts were determined using the FTIR spectrum and compared with the FTIR spectrum of AgNPs.

As shown in Fig. 2c, the marked region **I** using a red line belonged to the hydroxyl groups (–OH), the marked region **II** using a red line corresponded to asymmetric stretching vibrations of methylene groups (CH_2), the marked region **III** using red line has related to the stretching vibration of the C=O bonds, the marked region **IV** using red line has belonged to the carbon–carbon (C=C) double bonds, and the marked region **V** using red line has characterized with the CH_2 bending and CH_2 wagging modes. In addition to them, a sharp peak can be detected in the fingerprint region, **VI**, which clearly proves the presence and synthesis of AgNPs.

AgNPs with high amounts of surface loads provide higher repulsive forces between the particles, which lead to high physical colloidal stability and less aggregation (Nguyen et al. 2022). Therefore, zeta potential is a key indicator that can be useful for distinguishing and comparing the relative stability of various forms of nanoparticles in a colloidal suspension (Iso et al. 2001). By measuring the zeta potential, it was found that there is a negative charge of − 11.52 mV on the surface of AgNPs, which belongs to the biological components of flower extract (Fig. 2d). Similar negative values of zeta potential for many silver nanoparticles synthesized with biomolecules have been reported in various papers (Erdogan et al. 2019; Gemishev et al. 2022). A noteworthy point in Fig. 2a is the partial aggregation of AgNPs when the solvent dries for FESEM imaging and its relationship to the surface charge (− 11.52 mV). It has been proved that when the particle size of nanosilver becomes larger than 50 nm, agglomeration occurs due to their high surface tension and energy (Wang et al. 2020).

Estimation of cell proliferation using MTT

The cytotoxicity of the AgNPs was evaluated using a cell viability assay which is based on the evaluation of mitochondrial dehydrogenase activity.(Yan et al. 2022; Kaba et al. 2015) Since the AgNPs can aggregate and enter cells using phagocytosis, they can weaken the antioxidant defense function of the cells, disrupt the mitochondrial role, damage DNA,

Table 2 Weight and atomic percentage of the constituent elements of silver nanoparticles obtained from energy-dispersive X-ray spectroscope (EDS) analysis

Element	Weight (%)	Atom (%)
C	25.57	44.05
O	19.53	42.11
Ag	54.71	13.58
	99.81	99.74

and finally, lead to cell death and apoptosis (Akter et al. 2018). To investigate the influence of the biomolecules and stabilizing agents of AgNPs on cell proliferation of SK-BR-3 cells, in parallel with the AgNPs the cells were incubated with an aqueous extract of *Nepeta bracteata Benth*. The percentages of viable cells as a function of AgNPs concentration are shown in Fig. 3a. The effect of AgNPs on SK-BR-3 cells is dose-dependent so at high concentrations, it reduces cell viability by about 21% compared to the control. The IC_{50} values of 1.95 and 2.079 mg mL^{-1} for AgNPs were obtained after 48 and 72 h of treatment, respectively (Fig. 3d). *Nepeta bracteata Benth* extract, unlike AgNPs, has weak cytotoxic effects on breast cancer cells. Chart results related to flower extracts (Fig. 3b) showed that 45% of SK-BR-3 cells were still viable after 72 h of treatment and at the highest concentration. These results are in line with the previous work in which Zhang et al. (2021) confirmed that the new diterpenoids in *Nepeta bracteata Benth* flower extract have moderate activity with an IC_{50} value of about 40 µM on HCT-8 cells.

The better effect of AgNPs than flower extract can be related to several factors. For example, small AgNPs are more likely to cross the cell membrane and accumulate in the cell and, therefore, can induce more cytotoxicity (Zhang et al. 2018). Moreover, smaller AgNPs have a larger specific surface area which can lead to more stability in bio-fluids and also more ROS production in the host cell (Orlowski et al. 2018). Therefore, it can be concluded that the synthesis of AgNPs by the reducing agents of *Nepeta bracteata Benth*

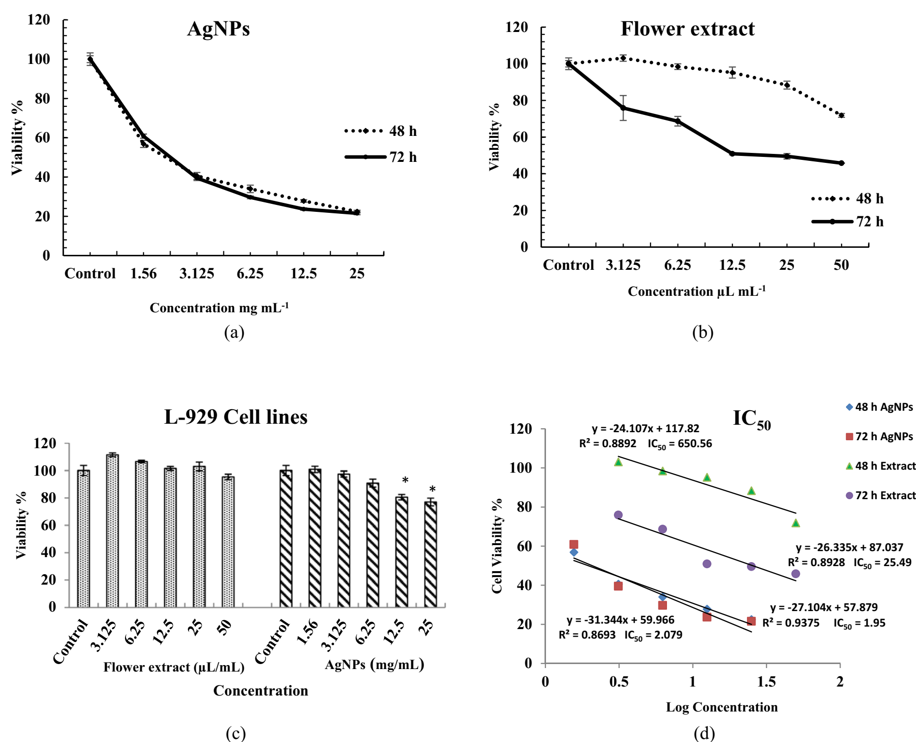

Fig. 3 Anticancer activity of AgNPs against SK-BR-3 cancer cells after 48 and 72 h of treatment as indicated **a** AgNPs and **b** *Nepeta bracteata Benth* extract. Panel **c** demonstrates the effect of flower extracts and AgNPs on noncancerous L-929 cell lines. **d** IC_{50} value was obtained by plotting the cell viability % (*Y*-axis) against the logarithm of the concentration (*X*-axis) when Y equals 50 in the equation of the line. Mean ± standard deviation (SD), $p < 0.05$

can prevent the proliferation of cancer cells and be a step forward in producing a new anti-cancer drug.

On the other hand, the results of in vitro cytotoxicity tests on L-929 as noncancerous cell lines showed that AgNPs do not have adverse effects on these cell lines and, therefore, can be used as a safe compound for the treatment of patients (Fig. 3c). AgNPs showed a slight negative effect on normal cells only at two high concentrations and inhibited only about 10% of normal cells. Studies by Khorrami et al. (2018) showed that synthesized AgNPs by walnut green husk exhibited 70% cytotoxicity against breast cancer cells (MCF-7), while they showed much less cytotoxic effect on noncancerous cell line (L-929).

Estimation of cell viability using SRB

The SRB staining was used to determine cell viability based on the protein content of cancer cells. The process of MTT reduction by the cell is a metabolic activity and some compounds or environmental factors may interfere with this process and increase or decrease it (Plumb et al. 1989). On the other hand, the optimal average size for the passive uptake of AgNPs by biological cells is about 50 nm (Chen et al. 2015) so we expect that AgNPs can cross the membrane of cancer cells and show a positive effect on inhibiting the growth of cancer cells. For this purpose, the SK-BR-3 cells were treated with the AgNPs, and their viability was checked by SRB staining to make sure that AgNPs have a good ability to reduce the protein content of cancer cells. As Fig. 4a shows, AgNPs have been able to reduce the density of SK-BR-3 cells, so that at the highest concentration, only 32% of the cells are alive. The amount of IC_{50} was calculated to be 4.13 after 72 h exposure to the AgNPs (Fig. 4b) which is almost close to the IC_{50} value calculated by the MTT method.

The role of AgNPs in apoptosis regulation of SK-BR-3 cells

The positive effect of AgNPs on the induction of apoptosis as well as autophagy in various types of cancer cells including ovarian, breast, cervix, and lung has been identified (Yuan et al. 2017). This fact is well evident in flow cytometry results, because AgNPs were able to induce apoptosis in the SK-BR-3 cell line after 24 h. Represented results in

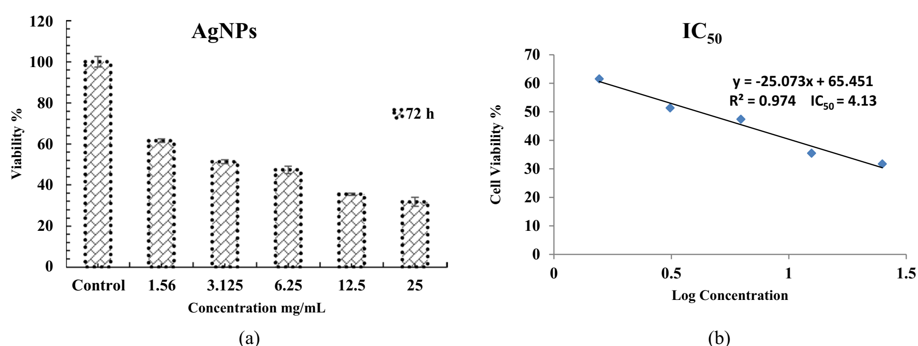

Fig. 4 **a** Investigating the inhibition rate of SK-BR-3 cells by SRB staining method. $\%\ \textit{cell viability for each concentration} = \frac{\textit{the mean OD of each concentration}}{\textit{the mean OD of the control}} \times 100$. **b** Calculation of IC_{50} for cells treated with AgNPs after 72 h

Fig. 5 confirm the success of AgNPs (18.8% apoptosis) in the induction of apoptosis in SK-BR-3 breast cancer cells compared to the control.

Also, it is proved that treatment with AgNPs can cause mitochondrial damage by producing excessive amounts of ROS and unbalancing at oxidation/anti-oxidation levels. These dysfunctions can lead to Parkin-mediated mitochondrial fragmentation and mitochondrial-mediated apoptosis (Li et al. 2021). Since the active compounds in flower extracts of *Nepeta bracteata Benth*, especially terpenoids, induce the cell cycle arrest and apoptosis in cancer cells by producing ROS (Yang et al. 2021), we expect the bio-synthesized AgNPs to have a good ability to induce apoptosis.

Mitochondrial damage by ROS and mitochondrial outer membrane permeabilization (MOMP) can activate executioner caspase and kill cancer cells. On the other hand, the interaction between members of the Bcl-2 family can control MOMP. Therefore, research on Bcl-2 family proteins can determine the pathway of apoptosis by AgNPs (Lebeaupin et al. 2018). Bcl-2 family proteins are classified into two groups; one group such as Bcl-2 and Bcl-x can inhibit pro-apoptotic proteins and show their anti-apoptotic functions by preventing caspase activation in the cytoplasm, while the other group such as Bak and Bax are the main cause of apoptosis and create MOMP (Chen et al. 2018). Therefore, we decided to investigate for the first time the effect of AgNPs bio-synthesized by the flower extract of *Nepeta bracteata Benth* on the expression of Bclx and Bak1 genes by the real-time PCR method. The results of Table 3 show that biosynthesized AgNPs were able to increase the expression of the Bak1 pro-apoptotic gene by 34 times compared to the control group. In addition, the Bak1/Bclx ratio has increased significantly (p value < 0.05) in comparison with the control (151 against 1) and indicates a positive effect of AgNPs on the induction of apoptosis.

The increase in the expression of the apoptosis-coordinating enzyme, caspase-3 (14.5 times) was due to the increase in the Bak1/Bclx ratio and confirmed that caspase-3 activation is related to the intrinsic (mitochondria-mediated) pathway (Brentnall et al. 2013). According to all the results, it can be expected that bio-synthesized AgNPs as a therapeutic agent can be effective on breast cancer cells and induce apoptosis in them.

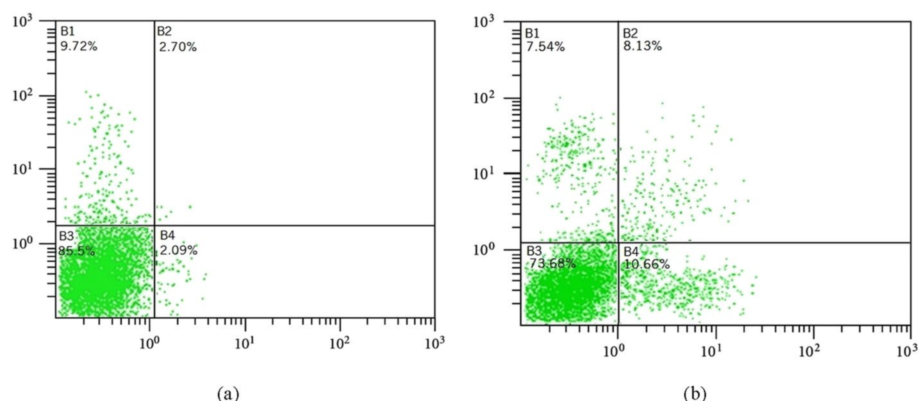

Fig. 5 AgNPs induces apoptosis in SK-BR-3. **a** Untreated cells and **b** treatment with AgNPs following 24 h exposure. The rate of apoptosis is obtained from the sum of panels B4 and B2, which are related to early and late phase of apoptosis, respectively. Panel B1 shows the amount of necrosis in cancer cells, and panel B3 present the amount of viable cells

Table 3 Expression of genes involved in apoptosis was performed by real-time polymerase chain reaction (PCR)

Genes	Sample	CT (mean)	ΔCT	ΔΔCT	RQ
BCLx	Control	15.5125	− 3.5735	0	1
	AgNPs	32.3232	− 1.4181	2.1554	0.2245
BAK1	Control	26.1379	7.0518	0	1
	AgNPs	35.7057	1.9643	− 5.0875	34.001
Caspase-3	Control	19.9565	0.8705	0	1
	AgNPs	30.7537	− 2.9876	− 3.8581	14.501
GAPDH†	Control	19.086			
	AgNPs	33.7413			

For this purpose, the Applied Biosystems StepOne™ instrument (USA) was used. RQ (relative quantification) was considered equal to $2^{-\Delta\Delta CT}$. ΔΔCT is comparative cycle threshold. †GAPDH is an endogenous control and used to normalize data from other genes

Similar to our work, biosynthesized AgNPs using Artemisia turcomanica leaf extract were able to up-regulate the BAX (Bcl-2-associated X) genes and down-regulate the Bcl2 genes in gastric cancer cell lines (AGS) (Mousavi et al. 2018). On the other hand, Fard et al. (2018) examined the ability of induction of apoptosis by AgNPs synthesized using Centella Asiatica leaf extract on MCF-7 cells. The results showed that AgNPs have the ability to increase the expression of genes encoding caspases 3 and 9 in MCF-7 cancer cells. Since the expression of caspase-3 indicates mitochondrial disturbance, they did not investigate the effect of green AgNPs on the expression of BAk and Bcl genes and, therefore, differ from our work.

Investigating the possibility of oxidative stress in red blood cells

Since the *Nepeta bracteata Benth* flower extract used for the biosynthesis of AgNPs contains significant amounts of terpenoids, diterpenes, flavonoids, and phenolic acid derivatives (Hajiheydari et al. 2017), it may have a dual function and induce or even inhibit oxidative stress. Of course, many researchers have proven that biological molecules such as phenols, sugars, and flavonoids in plant extracts have antioxidant properties and prevent the creation of free radicals, which is the final result of eliminating oxidative stress in living cells (Lobo et al. 2010; Zhang et al. 2023). However, when these biomolecules are used to synthesize green AgNPs, they may cause oxidative stress after entering the cell due to the release of Ag^{+} ions (Ferdous et al. 2020).

On the other hand, red blood cells are susceptible to oxidative stress damage due to their high oxygen content, having unsaturated fatty acids in the cell membrane, and lacking the endoplasmic reticulum and nucleus to repair damaged proteins (Maurya et al. 2015). Therefore, it is necessary to place red blood cells in the vicinity of AgNPs and check the possibility of oxidative stress in them. The best method to investigate oxidative stress is to evaluate the amount of malondialdehyde (MDA) produced by erythrocytes after exposure to NPs, which is known as an index of lipid peroxidation (Cherian et al. 2019).

As shown in Fig. 6, after exposure to 2 mg mL^{-1} of AgNPs (based on IC_{50} value) with erythrocyte, only after 24 h the MDA value increased slightly and confirmed that the presence of active biomolecules can significantly reduce the toxic effects of AgNPs on

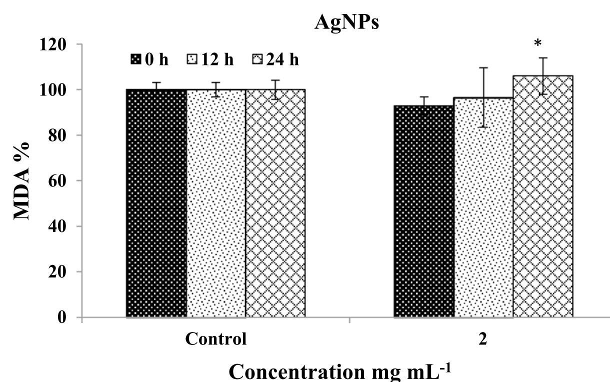

Fig. 6 Investigating the level of lipid peroxidation based on the percentage of malondialdehyde ($n = 3 \pm$ SD)

red blood cells. In the same study conducted by Mondal et al. (2020) they biosynthesized AgNPs using culture supernatant of strain Shewanella sp ARY1 and evaluated the biocompatibility of AgNPs against rat red blood cells (RBC). The results of their hemolytic assay showed that biosynthesized AgNPs are biocompatible at low doses.

Conclusion

Based on the available research, we decided for the first time to synthesize a silver nanoparticle using *Nepeta bracteata Benth* flower extract and to study its cytotoxic and apoptotic properties on SK-BR-3 breast cancer cells. In vitro results using MTT assay and SRB staining proved that AgNPs were able to inhibit the proliferation of cancer cells in a concentration-dependent manner, while flower extract alone was not able to inhibit cancer cells. Flow cytometry test proved that AgNPs can induce apoptosis in SK-BR-3 cancer cells. In addition, due to their ability to produce ROS, AgNPs were able to increase the expression of apoptosis-dependent genes, including Bak1 and Caspase-3, as well as reduce the level of anti-apoptotic Bclx. In addition, the percentage of MDA confirmed that the presence of active biomolecules can significantly reduce the toxic effects of AgNPs on erythrocytes. Therefore, it can be concluded that AgNPs due to their active biomolecules can induce apoptosis in breast cancer cell lines and be useful in treating patients.Considering that the entry of AgNPs into cancer cells is passive, their function may be reduced. Therefore, we suggest using targeting agents for active drug delivery to reduce side effects and increase the performance of AgNPs. Also, we suggest investigating the effect of AgNPs on animal models (in vivo study), so that they can be used as a new drug for breast cancer treatment in the future.

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Author contributions
HX and XN conceived the study and wrote the manuscript, WC conducted the experiments (such as PCR and MTT), JZ, and JC analyzed the data, RL wrote the manuscript. MHM and YL Guide experiment and writing. All authors read and approved the final version.

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Availability of data and materials
The data of this article will be made available by the corresponding author upon request.

Declarations

Ethics approval and consent to participate
This work did not have any animal or human studies; however, it has been reviewed by the Ethics Committee of the first affiliated hospital of Nanchang University.

Competing interests
The authors declare no competing interests.

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