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Research Article

Antioxidant, cytotoxicity, and anticancer properties of biofabricated nanoparticles derived from animal source

Rathinam Latha*
Department of Zoology, N.M.S.S. Vellaichamy Nadar College (Autonomous), Madurai, (Tamil Nadu), India
Subramanian Sevarkodiyone
Postgraduate and Research Department of Zoology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi (Tamil Nadu), India
Jeyaraj Pandiarajan
Department of Biotechnology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, (Tamil Nadu), India

*Corresponding author. Email: vasikaranlatha@gmail.com

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Abstract
Green science has been witnessed in the advancement with nanobiotechnology enriched with nature-associated biogenesis of nanoparticles over the last three decades. Noble elements, including gold and silver, are the most promising developing trend in nanotechnology for designing bioengineering materials that might be used as modern diagnostic instruments and tools to combat major diseases. Silver and gold nanoparticles possess strong antimicrobial, antioxidant, cytotoxic and anticancer properties that enable the development of new processes with enhanced and target-specific actions. Siver and gold nanoparticles were synthesized using Prawn Head Extract (PHE) and characterized in a previous study. The objective of the present work was to investigate the antioxidant, cytotoxicity and anticancer activity of biosynthesized nanoparticles. The antioxidant properties were analysed by the DPPH(2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay, and the IC50 values were 1020 µg/mL for silver nanoparticles and 649 µg/mL for gold nanoparticles. The *in vitro* cytotoxicity of the vero cell line and anticancer activity in a human breast cancer cell line (MCF-7) were determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The cytotoxicity assay revealed IC50 values of approximately 118.75 and 93.75 µg/mL in silver and gold nanoparticles, respectively. The anticancer activity in the human breast cancer cell line MCF-7 showed IC50 values of approximately 93.75 and 46.8 µg/mL in silver and gold nanoparticles, respectively. The MTT assay, microscopic examination and DNA fragmentation assays confirmed morphological changes, membrane damage, cell shrinkage and mortality. Conclusively, the study revealed dose-dependently promising effects and can be further exploited in the field of biomedicine as a potential source with a standardized protocol for application.

Keywords: Antioxidant, Anticancer, Cytotoxicity DNA fragmentation, Green science

INTRODUCTION

Green nanotechnology offers tools for converting biological systems to environmentally friendly nanomaterial formulation methods by avoiding toxicity. Green approaches utilize biological sources due to the high number of harmful chemicals and severe settings used in the physicochemical creation of these nanoparticles. Green nanotechnology may bring out safe and environmentally friendly metal nanoparticles without using harmful chemicals in their synthesis by combining the principles of green chemistry and nanobiotechnology.Because of their small size can easily enter cells and migrate through cells, tissues, and organs (Foroozandeh and Aziz, 2018). Routes that can be used for diagnostic, imaging, and therapeutic purposes are available. These materials can be made from a variety of inorganic and organic materials, but because of their ease of modification, high drug loading capacity, and stability, inorganic sources are critical for simultaneous therapy and diagnosis. Nanoparticles can be utilized to deliver medications and to determine drug concentrations in pharmaceuticals. Due to its nanoscale size and high surface area, it has been available for further alteration with hydrophobic, hydrophilic, ionic or any neutral moieties compatible with the sur-

rounding environment, so they have wide application in biological sciences. They have been analysed and reported for various clinical applications, viz., drug carriers, gene delivery to tumorscontrast agents in bioimaging and biosensors (Krishnaraj *et al*., 2014).

Metal nanoparticles, such as gold, silver, platinum, aluminium, zinc, carbon, titanium, palladium, iron, and copper, have received massive attention in recent times because of their indispensable and technological importance (Khandel., 2018). Among the metallic nanoparticles, silver nanoparticles are most attractive due to their feasibility and biological medicinal properties, such as antimicrobial, adjuvanticity and antitumor properties (Zhang *et al*., 2020). Enhanced physicochemical and biological properties based on the type of extra element employed in the manufacturing of silver nanoparticles become a plus. Gold nanoparticles are used in a variety of applications, including biocatalysis, biosensing, and cancer treatments, as well as for immunoassays, targeted drug administration, bioimaging, and near-infrared photothermal ablation of microbes and cancer cells (Ou *et al*., 2019)and nanotechnology building blocks. Silver nanoparticles are employed in antibacterial coatings for medical devices, wound and burn dressings, several cosmetic goods, and food packaging because of their antibacterial, antiangiogenic, and anti-inflammatory qualities (Devi *et al*., 2019 and Aziz *et al*., 2017). Molecular imaging, targeted medication delivery, gene therapy, cancer treatment, and radiation use gold nanoparticles. According to researchers, the increased demand for gold and silver nanoparticles in cancer diagnostics and therapy is due to their unique optical features, simple surface chemistry, and optimal nanoscale dimension. Controlling the size and form of these nanoparticles, or conjugating them with certain ligands/ biomarkers, can help to speed tumour diagnosis and treatment (Jain *et al*., 2007). Because many prospective biomedical applications rely on direct contact between gold nanoparticles and the target biological structure, evaluating the potential health impacts of gold-created nanoparticles is particularly critical (Jahangirian *et al*., 2019).

Breast cancer is the most commonly diagnosed cancer in women and the leading cause of cancer-related deaths in women around the world. According to global reports in 2018, breast cancer caused 2.08 million new cancer cases (an 11.6 percent incidence rate) and 626,679 deaths out of 9.55 million cancer-related deaths (6.6 percent of all cancer-related fatalities) (Malvia *et al*.,2017). According to current reports from the International Agency for Research on Cancer (IARC), 13.1 million people will die from cancer by 2030. It is clearly recognized that the low survival rate is attributable to a lack of effective medication delivery systems rather than a lack of effective natural or synthetic antitumoral drugs (Yingchoncharoen., 2016). As a result, there is a genuine need to develop new carriers and delivery systems that can deliver chemotherapeutic drugs only to the precise target site, increasing treatment efficiency and reducing undesirable systemic side effects. Nanotechnology offers a novel cancer treatment platform. It promises to lower systemic toxicity by improving functionalized particles for targeted treatment. They also offer an alternative technique for dealing with multidrug resistance because they can bypass the drug efflux mechanism linked to this trait (Awasthi *et al*., 2018). The cytotoxicity assay is a type of biological test that measures the toxicity induced by chemotherapeutic agents acting on living cells. Cytotoxicity assays of biologically synthesized nanoparticles play a vital role in determining the proposed biomedical application (Li., 2015)

Metal nanoparticles that have been biologically produced also have antioxidant properties. Antioxidant agents primarily regulate the generation of free radicals through enzymatic and nonenzymatic compounds. These free radicals have been discovered to play a key role in developing cellular damage, such as cancer, atherosclerosis, and brain damage. Antioxidants protect the body from the damaging effects of free radicals. Gold nanoparticles have a noteworthy effect as antioxidant agents, terminating the synthesis of reactive oxygen species (ROS) and scavenging free radicals (Barathmanikanth *et al*.,2010). Gold nanoparticles have risen to prominence among existing nanomaterials because gold is an inert, oxidation-resistant substance with applications in nanoscale technologies. The size of gold nanoparticles reveals a wide range of uses, such as hollow particles in CT imaging cancer therapy (Wang *et al*., 2021). Nanoflowers have been employed as biosensors for disease detection and the removal of dyes and heavy metals (Shende *et al*., 2018 and Elahi *et al*., 2018)]. The antioxidant efficiency of silver nanoparticles has been observed to be higher than that of other widely available synthetic compounds, such as ascorbic acid. Antioxidant scavenging action is important in the treatment of various chronic diseases, including cancer, autoimmune disorders, ageing, cataracts, rheumatoid arthritis, and cardiovascular and neurodegenerative diseases (Pham –Hui., 2008). The effective transport of medications and tissue engineering capabilities of nanotechnology have made significant contributions to translational research on pharmaceutical goods and applications (Zhang *et al*. , 2016).

A previous study revealed the production and characterization of silver and gold nanoparticles from Prawn Head Extract (PHE), and their biological uses, such as antibacterial, antifungal, and antidiabetic activities, were also evaluated (Latha *et al*., 2022). To determine its role in extensive biological applications, the present work is framed (i) to screen antioxidant capabilities, (ii) to analyse cytotoxicity in Vero cells, and (iii) to investi-

gate anticancer activity in human breast cancer cells (MCF-7).

MATERIALS AND METHODS

Cell culture

Vero cells and human breast cancer cells (MCF-7) were procured from the National Center for Cell Science, Pune, India. The cell lines were grown as a monolayer in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin/ streptomycin(250 U/ml), gentamycin (100 mg/ml) and amphotericin B (1 mg/ml) and incubated at 37°C in a humidified atmosphere of 5%CO_2.Cells were grown to confluence for 24 h before use.

Synthesis and Characterization of nanoparticles

PHE was used to synthesize silver and gold nanoparticles, and characterization was performed using various techniques.UV–visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction(XRD), scanning electron microscopy (SEM) and energy dispersive X-ray(EDAX) techniques were used (Latha *et al*., 2022).

Agarose gel electrophoresis

DNA visualized by 1.5% agarose gel electrophoresis was stained with ethidium bromide according to size DNA Markers.

Antioxidant activity of nanoparticles

The antioxidant activity of the synthesized silver and gold was assessed using the DPPH assay (Tailor and Goyal, 2014)). The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry. The DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) free radical scavenging method is an antioxidant assay based on the transfer of electrons that produces a violet solution in ethanol. The stability of free radicals at room temperature is reduced in the presence of an antioxidant molecule and reduces the violet ethanol to colorless. Various concentrations of samples ranging from 100 to 500 µg were prepared and mixed with 0.1 % DPPH. The mixture was allowed to react well and maintained at room temperature for approximately 30 minutes. The antioxidants react with DPPH, and a reduction reaction occurs, and the intensity of the color decreases. Absorbance was recorded at 517 nm, and butylated hydroxytoluene (BHT) was used as a standard. The experiment was performed in triplicate, and the scavenging activity was calculated as the percent inhibition according to the following formula:

% Antioxidant activity = {(absorbance of blank-(absorbance of sample) / (absorbance of blank)} X 100 …Eq. 1

In vitro cytotoxicity of nanoparticles in Vero cell lines

The cytotoxicity of silver and gold nanoparticles synthesized from PHE against the Vero cell line was determined by MTT-reduction assay(Mosmann, 2016).Cells (1×10^5/well) were plated in 0.2 ml of medium well in 96-well plates and incubated ina 5% CO_2 incubator for 72 hours. Then,the cells were added to various concentrations of the samples ranging from 31.2 to 1000 µg and incubated for 48 hours. After removal of the sample solution, 20 µL of MTT reagent was added to each well, and 90 µL of serum-free medium was added. This mixture was incubated at 37°C for 4 hours. Next, the medium with MTT was aspirated and mixed with DMSO for solubilization for approximately 30 minutes. Then, viable cells were determined by the absorbance at 540 nm. Doxil was used as a standard. The50% inhibition of cell viability (IC50) value was determined. The cytotoxic effect of nanoparticles on Vero cells was calculated as the percent cell viability using the following formula:

% Cell viability = A540 of treated cells/A540 of control cells × 100% … Eq. 2

In vitro anticancer activity in human breast cancer cells (MCF-7)

The in vitro anticancer activity of silver and gold nanoparticles synthesized from PHE against a human breast cancer cell line was determined by an MTT-reduction assay (Mosmann,2016). Cell lines were maintained in MEM supplemented with 10% FBS. Cells were kept in monolayer culture at 37°C, which was maintained in a humidified incubator with 5% $CO_2$for 72 hours. Cells were subcultured by trypsinization with TPVG solution and maintained in a tissue culture unit. Cells (1×10^5/well) were plated in 0.2 ml of medium-well in 96-well plates and incubated in a 5% CO_2 incubator for 72 hours. Before the assay, the cells from the well were washed twice with serum-free medium and kept in a CO_2 incubator for 1 hr. Then, the cells were added to various concentrations of the samples ranging from 31.2 to 1000 µg and incubated for24 hours. Then, 0.1% DMSO was added, and the plates were agitated for 30 minutes in a plate shaker. After removal of the sample solution,20 µl/well MTT reagent was added. Viable cells were monitored by absorbance at 540 nm. Doxil cancer drug was used as a standard. The 50% inhibition of cell viability (IC50) value was determined. The effect of the samples on the proliferation of MCF7 cells was calculated as the percent cell viability using the following formula:

% Cell viability = A540 of treated cells/A540 of control cells × 100% …Eq. 3

DNA fragmentation assay

A DNA fragmentation assay was performed to investi-

gate the internucleosomal DNA fragmentation caused by synthesized silver and gold nanoparticles.This assay involves extraction of DNA from a lysed cell homogenate followed by agarose gel electrophoresis.MCF7 cells were plated in 1 ml of medium/well in 12-well plates and incubatedina 5% CO2 incubator for 72 hours. One milliliter of sample was added to the wells and incubated for 24 hours ina 5% CO_2 incubator. The sample solution was removed and trypsinized. After 3-5 minutes, 1 ml of 1X PBS solution was added and thoroughly mixed to collect the sample for DNA fragmentation. Furthermore, the DNA was isolated using a commercially available kit (Genei, Bangalore, India), following the manufacturer's instructions. DNA was resolved on a 1.2% agarose gel at 90 V for 1.5 hours, and the bands were visualized using a UV transilluminator.

RESULTS AND DISCUSSION

Antioxidant activity

The DPPH assay was used to determine the antioxidant activity of silver and gold nanoparticles, and the resulting percentage of antioxidant activity is shown in Fig. 1. The antioxidant activity of the nanoparticles was dose-dependent, meaning that as the nanoparticle concentration increased, so did the percent inhibition. The antioxidant property IC_{50} values of silver and gold nanoparticles are tabulated. (Table 1). Patra and Baek (2016) found that silver nanoparticle-mediated aqueous extract of silky hairs of maize has equally strong antioxidant activity in terms of DPPH radical scavenging with an IC_{50} value of 385.87 g/mL (Patra and Baek, 2016). The findings significantly support using AgNPs as natural antioxidants for health preservation against various forms of oxidative stress linked to degenerative illnesses. Earlier research employing mango seed extract (Mangifera indica) revealed antioxidant activity in biologically produced gold nanoparticles, with an IC_{50} value for DPPH activity of 256 g/ml (Dong *et al*., 2020). Gold nanoparticles in size range of 1 to 100 nanometers, manufactured using green technology, have antibacterial, anticancer, anti-inflammatory, and antioxidant activities (Usman *et al*.,2018).

Antioxidants are organic or inorganic substances that can prevent or delay oxidant-induced cell damage (free radicals, ROS, and other unstable molecules). An antioxidant is a substance that prevents, slows, or reverses oxidative damage to a target molecule. A substance must be active at low concentrations (phenolic antioxidants lose activity at high concentrations and act as prooxidants), have a sufficient amount to deactivate the target molecule, react with oxygen or nitrogen free radicals, and produce a less toxic product than the removed radical to be classified as an antioxidant. The activity can be measured using the IC_{50} values, which are the concentrations of samples capable of scavenging 50% of the free radicals present in the reaction mixture. When the IC_{50} is large, antioxidant activity suffers (Halliwell, 2006). Because of their role in inducing xenobiotic detoxification pathways, stimulating signal transduction pathways, and stimulating phagocytosis, free radicals might be considered biologically vital. Atherosclerosis, ageing, ischaemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer, and other disorders have been linked to the same radicals (Costa *et al*., 2009). Because they are able to operate as scavengers of singlet oxygen and free radicals in biological systems, several phenolic compounds including flavonoids, have antioxidant properties (Rice-Evans, 1997). At higher concentrations, the extract appears to be more effective in scavenging the artificial free radical (DPPH). The FTIR results revealed the presence of phenolic compounds in the PHE, which is the added supporting reasoning of possessing antioxidant properties. (Latha *et al*., 2022). Silver and gold nanoparticles derived from different biological resources have antioxidant effects comparable to those of phytosource nanoparticles (Vijayan *et al*., 2018). Alt-

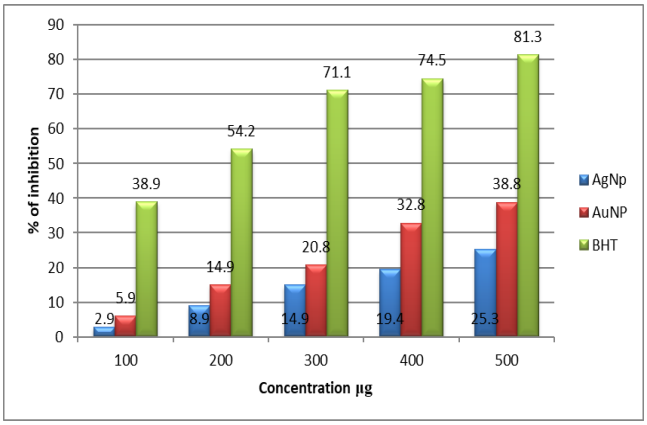

Fig. 1. *DPPH radical scavenging activity of silver and gold nanoparticles*

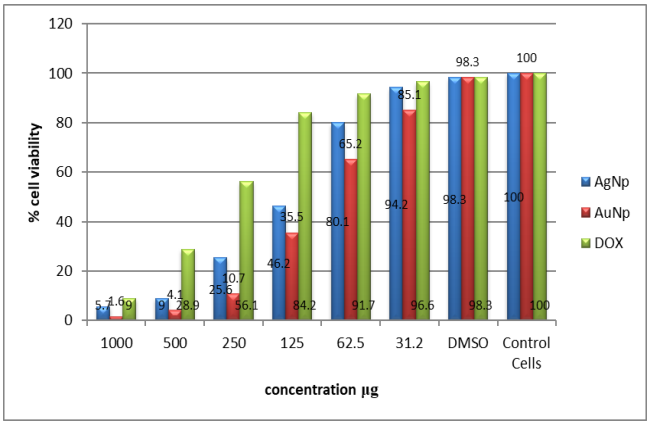

Fig. 2. *Percentage of cell viability against the in vitro cytotoxicity of silver and gold nanoparticles against the Vero cell line*

Table 1. IC50 values of antioxidant, cytotoxic and anticancer assays.

Assay	IC50 value µg/mL	
	Silver Nanoparticles	**Gold Nanoparticles**
DPPH	1020	649
MTT- Vero cell	118.75	93.75
MTT- Human Breast Cancer	93.75	46.8

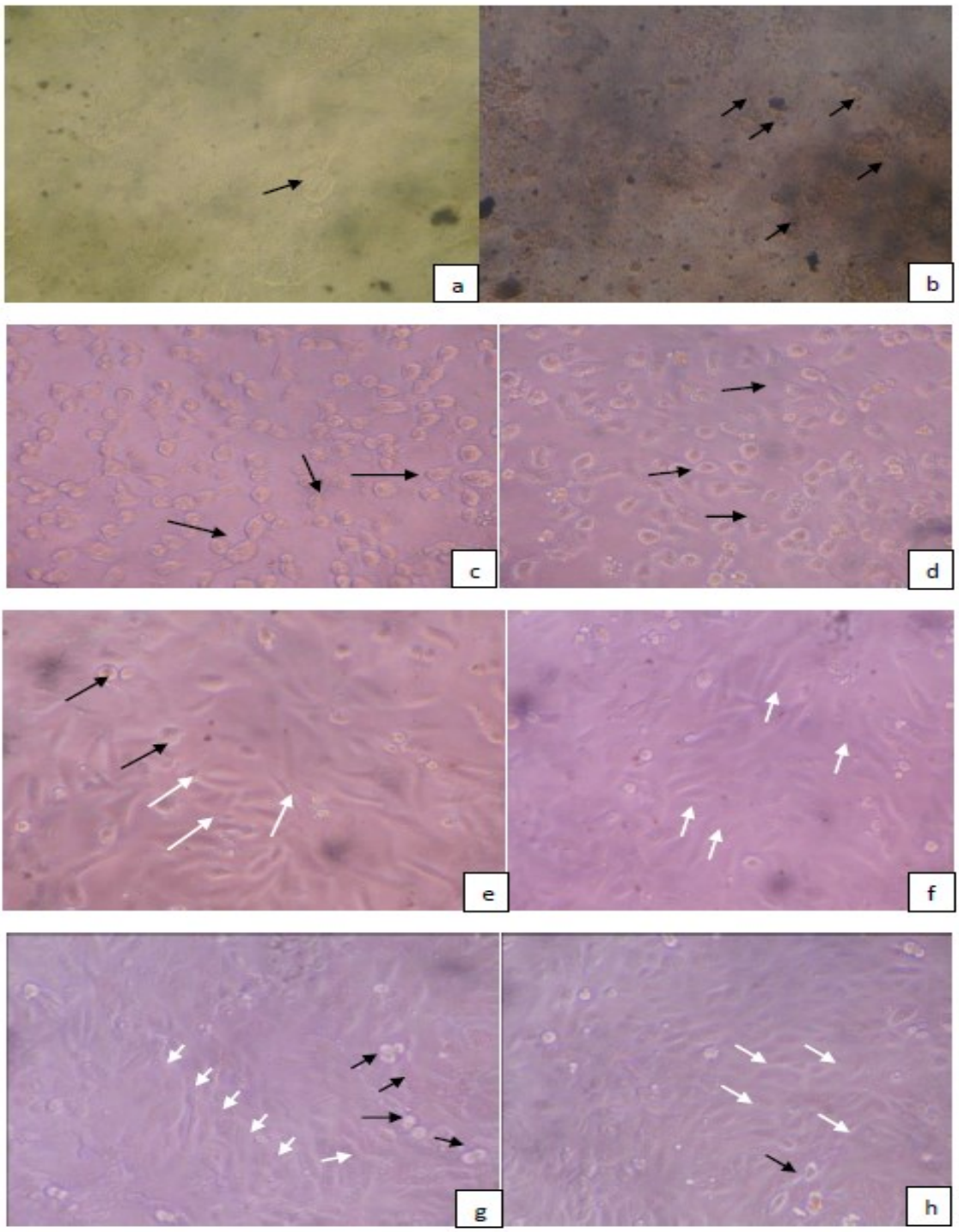

Fig. 3. *In vitro cytotoxicity of silver nanoparticles against Vero cells at various concentrations ranging from a-1000 µg; b- 500 µg; c-250 µg; d-125 µg;e-62.5 µg; f- 31.2 µg;g-DMSO;h -controlcells.Black Arrow- Dead cells: White Arrow – Live cells*

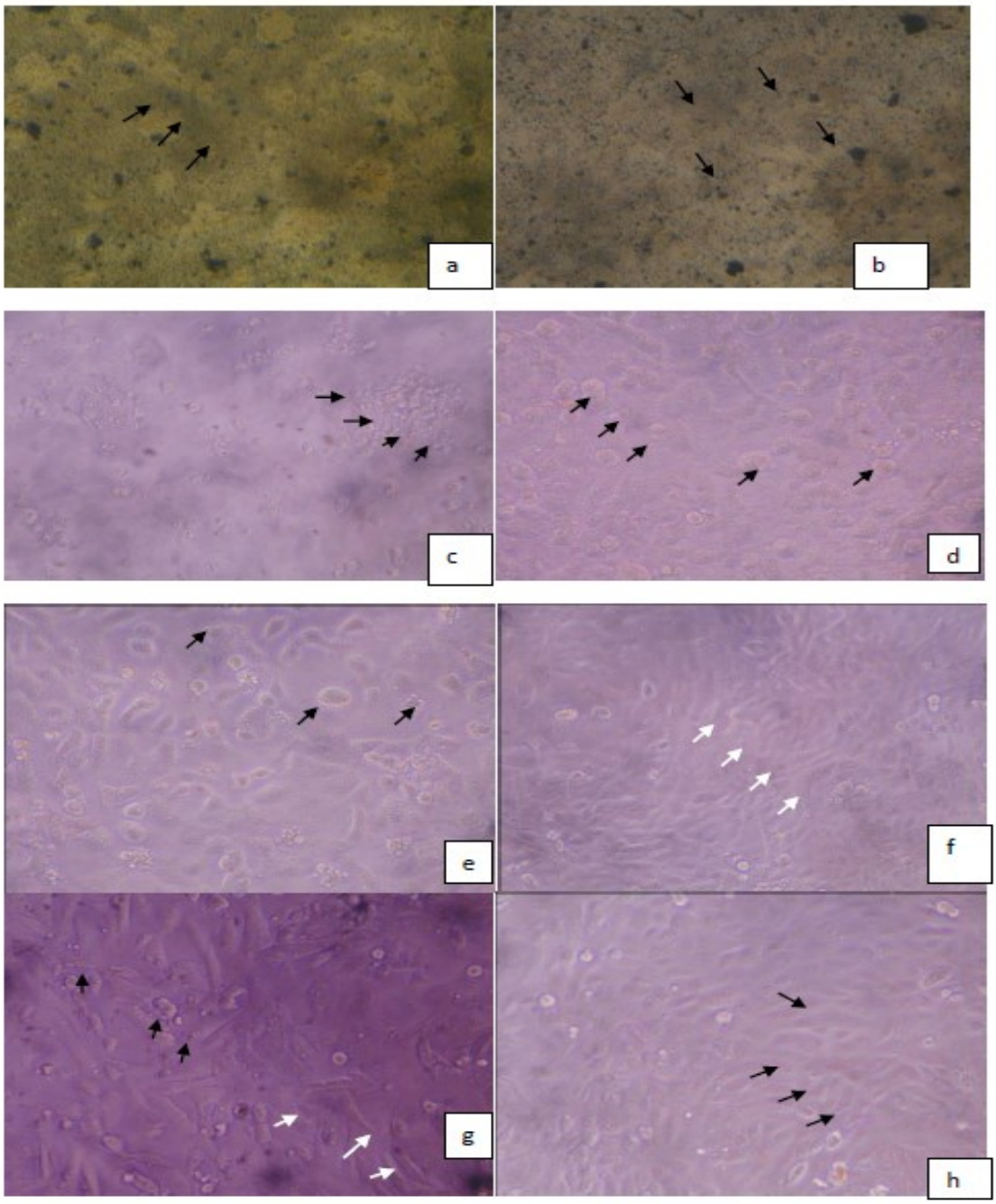

Fig. 4. *In vitro cytotoxicity of gold nanoparticles against Vero cells at various concentrations ranging from a-1000 µg; b- 500 µg; c-250 µg; d-125 µg;e-62.5 µg;f-31.2 µg;g-DMSO; h- Control cells (vero)Black Arrow- Dead cells: White Arrow –Live cells*

hough the $_{IC50}$ value is higher than that of various biological samples, such as phytosources and microorganisms, silver and gold nanoparticles generated from an animal source also have significant antioxidant effects, according to the present findings.

***In vitro* cytotoxicity of nanoparticles in Vero cell lines**

The cytotoxic effect of the produced nanoparticles was investigated using an *in vitro* MTT test on Vero cells. The percentages of cell viability against various concentrations of silver and gold nanoparticles are plotted in Fig. 2. Figs. 3 and 4 show the microscopic analysis of the cytotoxic effect of silver and gold nanoparticles at various concentrations ranging from 31.2 to 1000 µg/mL. This revealed the loss of the epithelial cell distinctive monolayer, which is indicated by the black arrow (dead cells) and white arrow (live cells) in silver (Fig.3c,dande) and gold (Fig.4d, e, and f) nanoparticles. The IC_{50} values for silver and gold nanoparticles were found to be 118.75 and 93.75 µg/mL, respectively

(Table 1). The cytotoxicity of silver and gold nanoparticles was dose-dependent, according to our understanding. Cytotoxicity is influenced by nanoparticle size and dose concentration (Liao *et al*., 2019). The size of the particles may have an impact on cytotoxicity in theory. For the same mass, smaller nanoparticles have a more accessible surface area to interact with biological components such as nucleic acids, proteins, fatty acids, and carbohydrates than larger nanoparticles. It has a higher chance of entering a cell and causing damage due to its tiny size (Huang *et al*., 2017).

***In vitro* anticancer activity in human breast cancer cells (MCF-7)**

The *in vitro* anticancer activity of silver and gold nanoparticles on the human breast cancer cell line MCF-7showed antiproliferative activity against the cancer cell

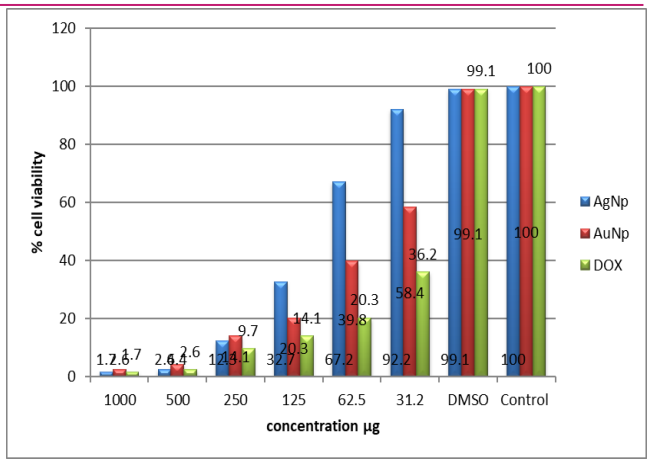

Fig. 5. *Percentage of cell viability against concentration in an in vitro anticancer study of silver and gold nanoparticles against the human breast cancer cell lineMCF-7.*

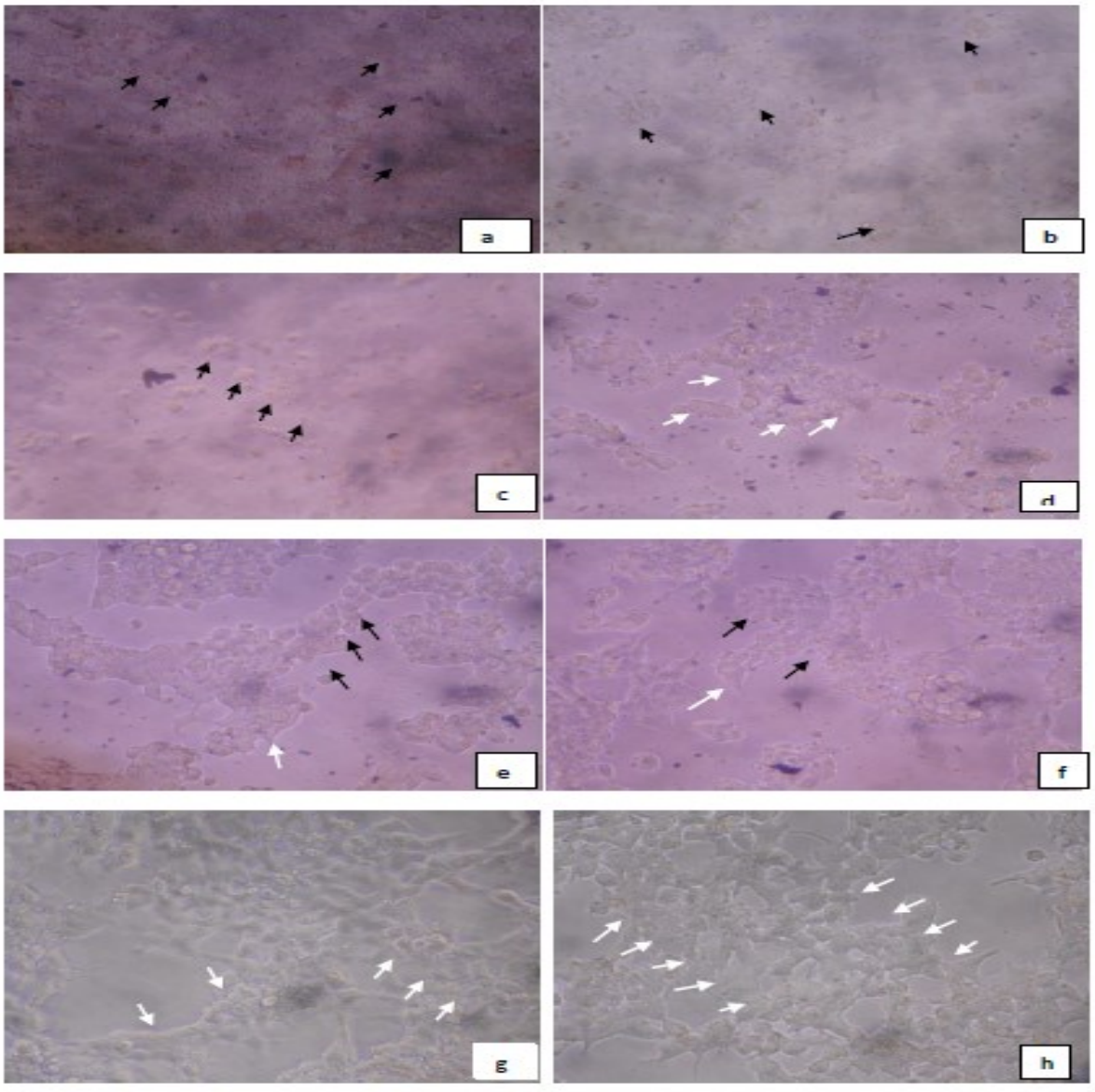

Fig. 6. *In vitro anticancer activity of silver nanoparticles against human breast cells (MCF-7) at various concentrations ranging from a-1000 µg; b- 500 µg; c-250 µg; d-125 µg;e-62.5 µg;f-31.2 µg.g-DMSO; h-Control cells. Black Arrow-Dead cells: White Arrow –Live cells*

line, which was visualized under a microscope (Figs. 6 and 7). Under a microscopic view, the control cells exhibited a greater number of well-adhered live cells than the cells treated with the synthesized nanoparticles. Considerable changes in the morphology and size are noted due to cell death and the scattering pattern. Cell mortality appeared more with the increase in the concentration, which is indicated by white and black arrows.The percentage of cell viability at various concentrations of silver and gold nanoparticles is shown in Fig. 5. The results proved to be efficient at the various concentrations tested. The IC50 values for silver and gold nanoparticles were found to be significant. (Table1). Based on these results, AuNPs were found to be most active against human breast cancer cells and showed strong cell growth inhibition at lower concentrations Hence, the results of these experiments showed that the inhibition rate is related to the dose, type of particle, concentration and type of cell line. The inhibition of cell growth by increasing the concentration of nanoparticles could be attributed to the apoptotic effect of nanoparticles (Zein *et al*., 2020).Earlier studies suggested that cell death was selectively induced by gold and silver nanoparticles depending on the type of cell line of breast cancer MCF-7 cells and colon cancer HT29 cells (He *et al*.,2016). Cell death was caused by apoptosis with no effect on normal cells. Silver and gold nanoparticles can cause cancer cell death by producing reactive oxygen species (ROS), DNA damage, and apoptosis. Similar *in vitro* anti-proliferation activities of silver and gold nanoparticles were studied on many cancerous cells (Yen *et al*., 2009). The inhibition of cell growth was reported to be due to the upregulated cytokine expression by silver and gold nanoparticles in cell lines. Silver nanoparticles can inhibit TNF-I, while gold nanoparticles can concentration-dependently inhibit IL-2 and IL-6 in leukemia cells, leading to anti-cell proliferation (Parnsamut and Brimson, 2015). This study revealed that the antiproliferative activity of gold nanoparticles is more effective

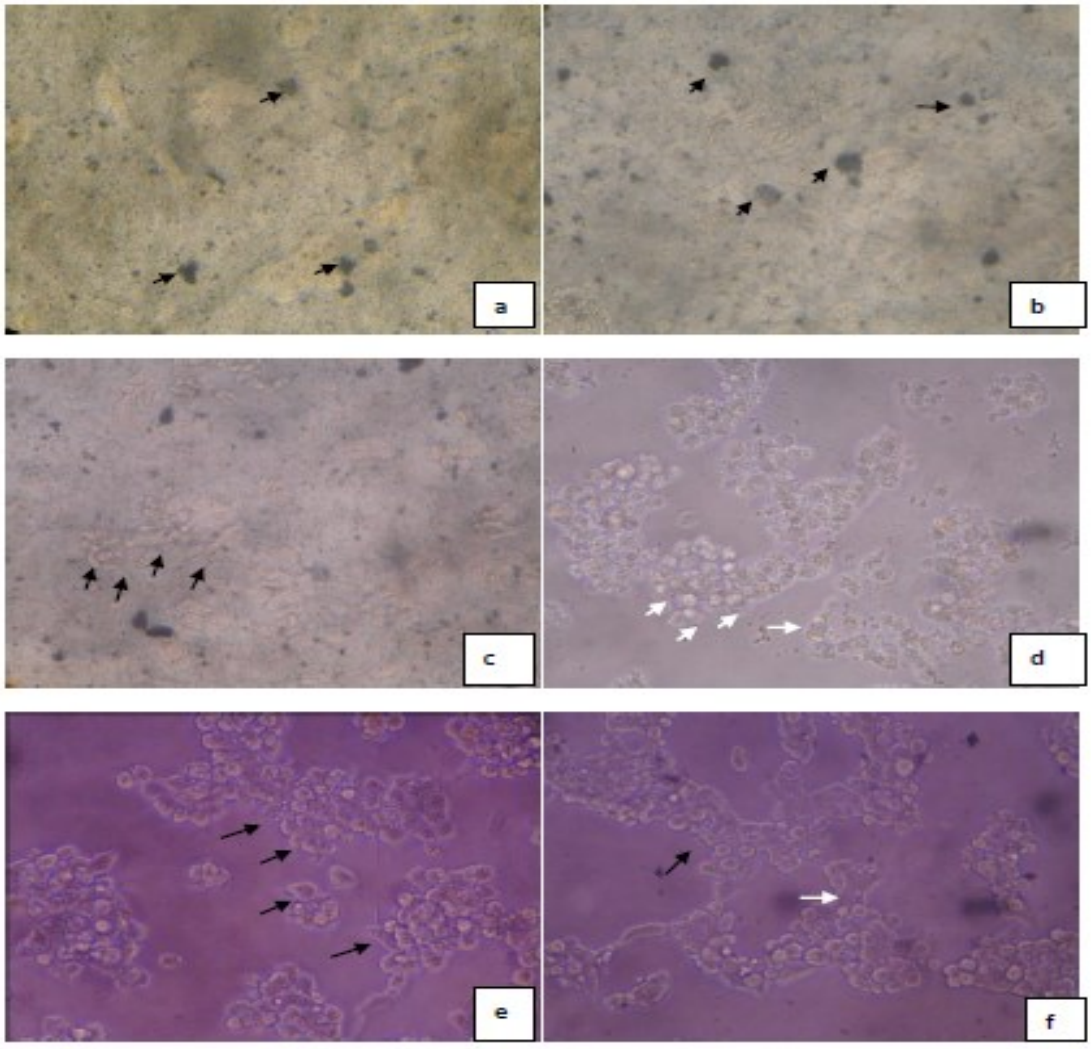

Fig.7. *In vitro anticancer activity of gold nanoparticles against human breast cells (MCF-7)at various concentrations ranging from a-1000 µg; b- 500 µg; c-250 µg; d-125 µg;e-62.5 µg;andf-31.2 µg. Black Arrow- Dead cells: White Arrow – Live cells*

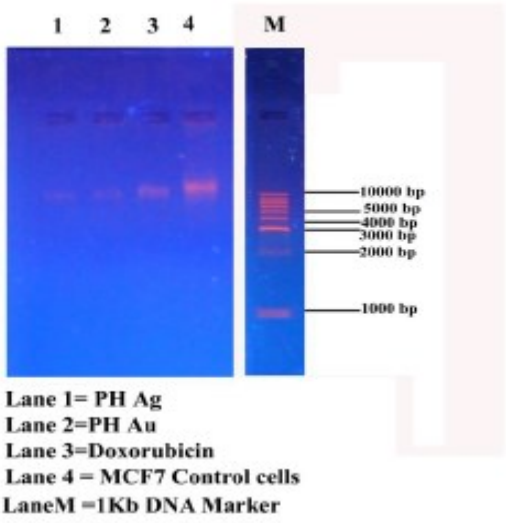

Fig. 8. *Effect of nanoparticles on the DNA fragmentation assay to study cell death (apoptosis).Lane 1- silver nanoparticles; Lane 2- gold nanoparticles; Lane 3- standard; Lane 4 – control cells.*

than that of silver nanoparticles. The reason supported the result that both nanoparticles can enter the cells, but only gold nanoparticles can upregulate the expression of proinflammatory genes. Positively charged, negatively charged, and neutral gold NPs (diameter 2–3 nm) produced DNA damage in human lung adenocarcinoma A549 cells following a 24-hour treatment (comet assay), with the positively charged gold NPs having the strongest genotoxic effect (May *et al*., 2018). In another study, 10-nm, 30-nm, and 60-nm gold NPs caused DNA damage in HepG2 cells, with the smallest NPs having the most effect (Lopez-chaves *et al*., 2018).

DNA fragmentation assay

Apoptosis is the late stage of morphological and biochemical events of programmed cell death, which is characterized by specific changes in the cell surface. A DNA fragmentation assay was conducted to study whether nanoparticles synthesized from PHE induced anti-proliferation/cell mortality through apoptosis. DNA laddering was performed by agarose gel electrophoresis,as shown in Fig.8. DNA fragmentation analysis revealed the apoptotic mode of cell death caused by synthesized silver and gold nanoparticles. Similar studies showed that the apoptosis mode of cell death in human breast and larynx cancer cell lines treated with Cassia auriculata leaf extract (Rajagopalan Prasanna et al., 2011) and Couroupitaguianensis flower extract-mediated gold nanoparticles induced apoptosis and enhanced DNA fragmentation in HL-60 cells (Geetha et al., 2013). Additionally, DNA fragmentation in an internucleosomal pattern is noticeable in mass cultures of apoptotic cells.The ability of AuNPs to induce apoptosis is also supported by measuring DNA damage in cells (Tao wu *et al*., 2019). The anticancer activity of green-synthesized gold nanoparticles through DNA damage and ROS-mediated apoptosis in HT-29 cells was confirmed (Mata *et al*., 2016).

Conclusion

Metallic nanoparticles such as silver and gold synthesized from animal sources (PHE) have been proven to possess significant antioxidant, cytotoxic and anticancer properties through various studies, such as DPPH radical scavenging effects, *in vitro* MTT assays in Vero cellsand human breast cancer cell lines and DNA fragmentation tests. The outcome of the study revealed thatgold nanoparticles have profound anticancer effects on the human breast cancer cell line MCF-7 compared to silver nanoparticles. Although the long-term toxicity of nanoparticles is a source of debate, the usage of gold and silver nanoparticles has a number of advantages over other available options (such as cytostatics). This work demonstrated that bioactive agents found in PHE can be discovered and used directly as medications or to separate bioactive compounds that can be employed as important ingredients in semisynthetic therapeutic formulations. Many innovative anticancer medications have been generated in the past from natural ingredients, and new ones are being developed all the time. Traditional chemotherapeutic drugs may improve the efficacy or lessen the toxicity of these naturally occurring anticancer chemicals, allowing them to be employed to treat certain tumours.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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