Synthesis of gold nanoparticles from leaf Panax notoginseng and its anticancer activity in pancreatic cancer PANC-1 cell lines

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ABSTRACT
Development of novel methods is needed for the synthesis of nanoparticles. Attention on such particles has elevated disquiet about the eco-friendly manner of their fabrication methods. In the present study, we equipped and synthesized gold nanoparticles (AuNPs) from Panax notoginseng; investigated as an environmentally friendly and non-toxic substrate. Amalgamation of AuNPs was distinguished by numerous studies such as UV-absorbance and it shows peak values in the range of 520–550 nm. Nanoparticles sizes are confirmed by dynamic light scattering analysis and it shows 100 nm. Moreover, transmission electron microscopy (TEM) and energy dispersive X-ray analysis (EDX) confirmed the shape of the Au particles present in the synthesized materials. FTIR analysis studies showed the active molecules positioned in the plane of the synthesized particles. The anticancer potential of AuNPs was evaluated in PANC-1 cells. Additionally, AuNPs efficiently induced cytotoxicity, ROS and apoptosis by intonating intrinsic apoptotic gene expressions in PANC-1 cells. Finally, our study confirmed the synthesis of AuNPs from Panax notoginseng, which showed anticancer effects in an environmentally friendly manner.

Introduction
Nanotechnology has triggered the augmentation of resuscitation medicine, and with the progress in nanotechnology, the established design has resulted in improved resuscitative properties of tissues. The functions and interaction between the cells exist at a nanolevel; modern altered systems can amend the functions and behaviour, which results in the improved cellular level functions that can imitate indigenous organs and tissues [1]. Characteristics of nanomaterials exhibit different properties like morphology, distribution and size [2]. Nanodelivery systems are proficient of, in particular, targeting tumour tissues, which consent to exploit the improved dispersion and withholding effect [3]. Plentiful chemicals are used to extend as nanoparticles for the beneficial effect. Conversely, those resources are highly cost-effective and hazardous to the environment; consequently, uses of metal nanoparticles are measured as eco-friendly and economical [4,5].

Nanoparticles size ranges from 100 to 1000 times lighter than a human cell; as a result nanoscale devices can enter the cellular environment with no trouble and act together with, proteins, DNA, enzymes and receptors [6]. Numerous nanoparticle-based drug delivery systems have been intended to accomplish the constantly emergent needs of the basic and clinical research fields. Both in Indian and Chinese culture gold is used as health adjuvant in the form of “gold bhasma” for treatment of a variety of health issues and deficiencies like mounting imperative power and for the treatment of male impotence [7]. Gold has exclusive properties like limpness, harmless to the cellular environment and is a valuable and striking material for the biomedical researchers in addition to significance in healing and imaging systems [8–9].

Pancreatic cancer is the fourth foremost cause of cancer-associated transience with predictable 43,140 cases in line with 36,800 mortality in 2010 in America [10]. A recent study showed that the 5-year survival rate for patients is 33% who underwent surgery for stage-I pancreatic cancer [11]. Surgery remains the better way for the treatment of pancreatic cancer. Nonetheless, the majority of patients diagnosed are waiting for the tumour to attain a highly developed stage, and prognosis also deprived subsequent re-sectioning of the tumour. Radiation-oriented management is usually unsuccessful and fabricates only subsidiary survivals [12]. Furthermore, the uses of chemotherapy and radiotherapy result in prominent and considerable side effects on patients [13]. Numerous plants have been effectively used for the proficient and speedy extracellular synthesis of gold nanoparticles. Consequently, a plant-mediated amalgamation of nanoparticles have the utmost and ultimate loom for embattled delivery on cancer cells and avert the unwanted side effects.
A number of medicinal plants have been used at the side of the conservative treatment schedule [14].

Nanoparticles from plants could be more beneficial since it does not need complicated processes such as intracellular synthesis and numerous decontamination steps [15,16]. Panax notoginseng is an extremely cherished ginseng species in genus Panax, Araliaceae family [17]. Panax notoginseng is considered as a medication for averting blood loss and recovering from injury and also used broadly in the form of foods or drugs. This species is used in Chinese traditional medicine, which possesses the capacity to protect liver injury that was induced by D-galactosamine in mice [18]. Panax notoginseng and its active components seem to be efficient against colorectal cancer [19,20]. Ethanol extracts of Panax notoginseng also restrain spleen tumour and liver metastasis [21]. Previous reports have shown that Panax notoginseng extracts are effective against skin, lung, prostrate and liver cell lines [22–25].

In this study, we produced gold nanoparticles (AuNPs) from the ethanolic extract of Panax notoginseng and it was exemplified by numerous studies such as high resolution-transmission electron microscope (HR-TEM), UV-visible absorbance spectrum, dynamic light scattering analysis (DLS), FTIR, and energy-dispersive X-ray analysis (EDX). Furthermore, synthesized AuNPs were oppressed in the anticancer prospective studies by apoptotic regulation in pancreatic cancer cells (PANC-1).

Materials and methods

Chemicals, antibodies and solvents

Dulbecco's modified eagle's medium (DMEM), Foetal bovine serum (FBS), and trypsin-EDTA were purchased from Gibco (California, USA). Antibodies such as Bax, Bcl-xl, Bcl-2, β-actin, caspase-3 and goat anti-mouse IgG-HRP polyclonal antibody were purchased from Santa Cruz, USA. 3(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT), 2, 7-diacylt dichlorofluorescein (DCFH-DA), acridine orange (AO), propidium iodide (PI) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Other fine chemicals and solvents were used in the analytical grade.

Gold nanoparticles (AuNPs) synthesis and purification

Panax notoginseng leaves were worn out to make the aqueous extract. Cleaned Panax notoginseng leaves were weighing 25 g and rinsed with double-distilled water. Then, it was allowed to dry and firmly into 100 ml disinfected distilled water and it was filtered by Whatman No.1 filter paper (25 μm). For the synthesis of gold, the digestive growing method was used to generate gold nanoparticles from polyscattering nanoparticles by using digestive agents. Followed by heating colloidal suspension at elevated temperatures (~138 °C) for 2 min followed by reducing of temperature in the presence of alkanethiols. Temperature plays an imperative role in scheming the size allotment of the gold colloids [26].

Characterization of AuNPs synthesized from Panax notoginseng

Following synthesis and refinement of AuNPs, they were characterized by several experiments, which confirmed the size, shape and morphological characteristics of nanoparticles. The produced AuNPs were inveterated by ultraviolet-visible absorbance spectrum studies at wavelength ranges of 300–700 nm (Shimadzu UV spectrophotometer). Size and morphological distinctiveness of AuNPs were recognized and confirmed by transmission electron microscopy (TEM) images. Nanoparticles nature and stability were analyzed by energy-dispersive X-ray (EDX). Surface morphology and size of the nanoparticles were determined by dynamic light scattering (DLS). FTIR spectroscopy measurements were performed to examine the active compounds present on the synthesized AuNPs. The FTIR spectra of AuNPs were recorded in the range of 4000 and 1000 cm⁻¹ in KBr pellets using FTIR spectrophotometer. Atomic force microscopy (AFM) was used to determine the film with or without heat treatment using an Agilent 5500 AFM with silicon cantilever model and typical resonance frequency 2.7 N/m and 80 kHz, respectively, and tip radius <10 nm. Experiments were made in non-contact mode (topography and phase contrast). Gwyddion 2.10 software was used to analyze the AFM images.

Cell culture

Human pancreatic cancer cell lines (PANC-1) were used for this study. Cells were procured from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were preserved in DMEM medium enhanced with 10% foetal bovine serum (FBS) and 1% antibiotics at 37 °C in a humidified incubator at 5% CO₂. After 96 h, the culture was conquered with around 80% confluence and the cells were harvested with 1 ml of trypsin-EDTA solution. Finally, cells were ready to use for further studies.

Cell viability assay

After collecting PANC-1 cells, 5000–10,000 cells were added into 96-well plates and incubated for 24 h at 37 °C. Then, amalgamated AuNPs from Panax notoginseng were treated with various concentrations (5–100 μg/ml) and incubated for 24 h at 37 °C. After incubation, the cells were exposed with MTT reagent (1 mg/ml) in all the wells. Finally, the culture plate was incubated at 37 °C for 2–3 h to form purple formazan crystals. 200 μL of DMSO was added to each well to suspend formazan crystals, and then, the optical density was measured at 570 nm in a microplate reader.

Intracellular ROS measurement

AuNPs intervened ROS generation in PANC-1 cells was deliberated by fluorescent probe, 2,7-diacetyl dichlorofluorescein (DCFH-DA) staining [27]. Shortly, an aliquot of isolated PANC-1 cells (1 × 10⁶ cells/ml) was equipped and loaded in six-well plates. Then, the cells were indulged with 25 and 50 μg/
ml concentrations of AuNPs at 24-h incubation. Then, 1 μl of DCFH-DA (1 mg/mL) was added to all the wells and incubated at 37 °C in a CO₂ incubator under dark condition. The fluorescent intensity was calculated by spectrofluorimeter with excitation and emission correspondingly at 485 ± 10 and 530 ± 12.5 nm.

**Fluorescent terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-fluorescein nick end-labelling (TUNEL)**

AuNPs interceded nuclear fragmentation was examined by Sigma TUNEL assay kit. After treatment of AuNPs, the PANC-1 cells were cleansed with PBS twice, then fixed in paraformaldehyde (2%) for 30 min and permeabilized with 0.1% Triton X-100 for 30 min. Then, they incubated with TUNEL reaction buffer at 37 °C for 1 h in the dark, and again rinsed twice with phosphate buffer saline. Finally, cells were stained with DAPI, and it was visualized under a Nikon fluorescence microscope.

**Apoptotic studies for acridine orange/ethidium bromide**

AuNPs arbitrated apoptotic changes in PANC-1 cells were evaluated by acridine orange and ethidium bromide (AO/Et) staining. Shortly, 1 × 10⁵ cells were seeded in a 6-well plate, and then the cultured cells were mixed with 25 and 50 μg/ml concentrations of AuNPs incubation for 24 h. When incubation was over, the cells were cleaned with PBS (ice-cold) then discoloured with 20 μL of AO/PI staining (10 μL/mg AO and 10 μL/mg Et) solution at 37 °C for 15 min. The discoloured apoptotic and viable cells were observed using a fluorescence microscope.

**Caspase-8, 9 and 3 activity assay**

Caspase-8, 9 and 3 activities were deliberated by caspase assay kit, the methodology was followed as per manufacturer’s instruction. Shortly, the PANC-1 cells were loaded in 6-well plate and treated with AuNPs then allowed to incubate for 24 h at CO₂ incubator. Then, the cells were allowed to treat with suitable caspase-8, 9 and 3 reagents and then incubated for 2 h in dark room. Then, the optical density for caspase-8, 9 and 3 were measured using a microplate reader at 400 or 405 nm.

**Western blot analysis**

Apoptotic markers such as, Bcl-xl, Bax, Bcl-2 and caspase-3 were examined by western blotting. Shortly, 1 × 10⁶ cells were uncovered with AuNPs for 24-h incubation. Then, the proteins were extorted from cells using with RIPA buffer which contains cocktail protease. The protein concentration was measured using the Thermo scientific nanodrop instrument (USA). Then, the proteins were positioned to 10% SDS-PAGE and the gel was reassigned to PVDF membranes. The membrane was blocked with 5% bovine serum albumin for 3 h. The membranes were combined with a suitable primary antibody and kept for 24-h incubation at 4 °C. Additionally, it was incubated with suitable conjugated horseradish peroxidase secondary antibodies for 1 h. Bands were identified by using improved chemiluminescent substrates and band intensity was analyzed using Image J software.

**Statistical analysis**

All experiments were carried out in three self-determined experiments and the results were articulated as the mean ± standard deviation (Mean ± SD) by using one-way analysis of variance (ANOVA). Values of *p* < .05 were indicative of significant differences.

**Results and discussion**

Since splendid metal nanoparticles, especially gold nanoparticles, are extensively used in the human environment, there is a rising need to expand eco-friendly practices of nanoparticles synthesis using toxic free chemicals. Consequently, the organic process of nanoparticle production using plant extracts, by adding up with microorganisms, have been recommended as a promising eco-friendly substitute to both physical and chemical methods. By means of plants for nanoparticle, separation can be beneficial over other organic methods by eradicating the intricate process of preserving cell cultures [28]. Reasons for these kinds of nanosyntheses are their cost-effectiveness and eco-friendliness and their numerous biomedical functions [29,30]. In the present study, we have verified the preparing of gold nanoparticles from *Panax notoginseng* and it was illustrated by UV-visible absorbance spectrum, dynamic light scattering analysis (DLS), high-resolution transmission electron microscope (HR-TEM), FTIR, energy-dispersive X-ray analysis (EDX). Furthermore, production of AuNPs has exploited the anticancer potential studies by apoptotic regulation in pancreatic cancer cells (PANC-1).

**Characterization of gold nanoparticles (AuNPs) synthesized from Panax notoginseng**

The synthesis of AuNPs from *Panax notoginseng* was primarily indicated by the colour exchange and the formation of ruby-red colour indicated the formation of the AuNPs (Figure 1). The colour change was specified to excitation of surface Plasmon resonance synthesized AuNPs. UV-spectroscopy was used at dissimilar time indication while fixing the concentration of the *Panax notoginseng* and the aqueous solution of gold chloride. The ocular sign of the colour replacement and the formation of ruby-red colour indicated the formation of the AuNPs; that directs the formation of gold nanoparticles (Figure 1). This related data were previously proved by numerous researches on synthesis of gold nanoparticles [31].

Moreover, we established the *Panax notoginseng* arribated produced AuNPs by UV visible absorbance. A mounting concentration of *Panax notoginseng* extracts leads to increased intensity of absorption. The UV-visible spectra recorded after different time intervals such as 24, 48, 72 and 92 h from the initiation of reaction with different amounts of plant extract. This finding coincides with previous studies in...
which UV-Vis spectroscopy was used to examine the shape and size-controlled nanomaterials in the aqueous suspensions [32]. In the present study, the synthesized AuNPs displays the highest absorbance, which was found to be \( \sim 540 \) nm at 96 h prepare solution. The maximum absorbance bands were raised at a dissimilar concentration of AuNPs solution in different time points. When compared to other metal nanoparticles predominantly AuNPs have free electrons which contribute to SPR absorption bands due to the united vibration of electrons of metal nanoparticles in resonance with the lightwave [33]. Size distributions of synthesized gold nanoparticles from Panax notoginseng extract is done by dynamic light scattering (DLS) method. Herein histogram image indicated that the particle size of the Au nanoparticles from Panax notoginseng is 128 nm (Figure 1(B)). Furthermore, particle aggregation was not observed that results in the precision of particle size and allocation. Additionally, morphological uniqueness of AuNPs synthesized from Panax notoginseng was further considered and studied by HR-TEM analysis (Figure 2(A)).

HR-TEM image shows that the morphological structure of AuNPs which are observed in, hexagonal, spherical, oval and triangular with average sizes between 80 to 12 nm. This finding supports the statistically interrelated with DLS images for AuNPs produced from Panax notoginseng. EDX studies were examined to assess the shape of the particles synthesized from Panax notoginseng extract gold metal (Figure 2(B)). Moreover, the TEM figures afford high-density AuNPs synthesized by the Panax notoginseng extract in additional, which confirms the gold nanostructures development. In the
present study, sturdy signals were experienced in the gold region, which confirms the AuNPs formation. In our study, we obtained the strong signals of Au (1.4–2 and 2.5–3 keV). Due to surface plasmon resonance, the distinctive visual absorption for metallic crystal was generally found to be 3 KeV [34], which supports our finding. The topographical structure of biosynthesized AuNP’s was analyzed by atomic force microscopy (Figure 3).

The AFM analysis confirmed the nanosphere shapes of the gold nanoparticles which correlate with the TEM image. FTIR studies used to recognize the numerous functional groups present in the synthesis of AuNPs from Panax notoginseng extract by obtaining different spectrum values. In present study FTIR showed that 3713 cm\(^{-1}\), 3395 cm\(^{-1}\), 2811 cm\(^{-1}\), 1665 cm\(^{-1}\), and 1305 cm\(^{-1}\) which specify the hydroxyl (3550–3200 cm\(^{-1}\)), aromatic (1310–1200 cm\(^{-1}\)), carboxyl (1500–2000 cm\(^{-1}\)), amide and alkyl (2000–1650 cm\(^{-1}\)) groups that present on the surface of AgNPs (Figure 4(A)). Figure 4(B) shows the selected area electron diffraction (SAED) pattern of biosynthesized gold nanoparticles exhibits ring-like structure which indicates the crystalline nature of the nanoparticles. Our finding correlates with previous studies in which the synthesis of gold nanoparticles from different plants extracts has numerous functional groups [35].

Anticancer potential of AuNPs from Panax notoginseng pancreatic cancer cell lines (PANC-1)

AuNPs were tested for their anticancer activity against pancreatic cancer cells (PANC-1). Consequently, the anticancer potential of AuNPs was studied by MTT cytotoxicity assay. In this assay, the raised concentration of AuNPs considerably restrained the PANC-1 cells growth for 24 and 48 h incubation (Figure 5A). Hence, we found that 15 and 20 μg/ml concentrations of AuNPs revealed prominent cell death in PANC-1 cells. Consequently, we preferred 15 and 20 μg/ml concentrations of AuNPs for apoptotic studies. Synthesized AuNPs from Dendropanax morbifer leaf extracts shows significant anticancer activity HaCaT cells [36].

Intracellular reactive oxygen species (ROS) in a system induces oxidative stress which results in apoptosis [37]. Therefore, AuNPs-mediated ROS production was examined by DCFH-DA fluorescent assay. In the present study, we confirmed that AuNPs produced from Panax notoginseng induces ROS generation in pancreatic PANC-1 cells (Figure 5B). In addition, ROS overproduction leads to nuclear fragmentation and mitochondrial depolarization that leads to oxidative stress-mediated apoptosis [38–40] which supports our finding.

Tumour progress and development and resistance to the majority of oncologic treatment results mainly due to deficiency of the apoptotic stimuli. Apoptosis is a crucial controller of tissue homeostasis [41,42]. Consequently, apoptosis is a painstaking and important pathway for cancer treatment. AuNPs synthesized Panax notoginseng provoke ROS mediated apoptosis were evaluated by AO/PI dual staining. In the present study, control cells show acridine orange-stained green fluorescent which signifies viable cells. While treatment with AuNPs synthesized from Panax notoginseng showed
augmented apoptotic cells by observing EtBr stained red fluorescent cells (Figure 6).

AuNPs mediated apoptosis makers such as Bax, Bcl-2, Bcl-xl, Caspase-3, 8 and 9 were examined by caspase assay kit and western blotting. Figure 7 shows treatment with AuNPs synthesized from Panax notoginseng downregulated the expression of Bcl-2, Bcl-xl and upregulated the expression of Bax, caspase-8, caspase-9 and caspase-3 in PANC-1 cells. This data directly related to acridine orange/v staining. Finally, with these findings, AuNPs synthesized from Panax notoginseng successfully persuaded apoptosis by amending the intrinsic apoptotic pathway. Previous studies support our
present findings in which gold nanoparticles persuade apoptosis in MCF-7 human breast cancer cells.

Conclusion

In this study, we illustrate an effortful, rapid, and reproducible method for the eco-friendly synthesis of AuNPs without the need for costly reducing agents. The potential of AuNPs synthesized from *Panax notoginseng* were well demonstrated. The physio-chemical distinctiveness of synthesized AuNPs from *Panax notoginseng* is established by UV-absorbance, DLS, HR-TEM, EDX and FTIR. AuNPs successfully induces cytotoxicity, ROS and apoptosis by accenting intrinsic apoptotic gene expressions in PANC-1 cells. Finally, our findings confirm that the synthesis of AuNPs from *Panax notoginseng* demonstrates antimicrobial and anticancer effects.

Disclosure statement

No potential conflict of interest was reported by the authors.

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