Gold nanoparticles synthesized from Strychni semen and its anticancer activity in cholangiocarcinoma cell (KMCH-1)

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ABSTRACT
Nanotechnology has been materialized as a proficient technology for the development of anticancer nanoparticles all the way through an environment-friendly approach. Conventionally, nanoparticles have been assembled by dissimilar methods, but regrettably rely on the negative impact on the natural environment. Amalgamation of nanoparticles by means of plant extract is alternate conservative methods. In the present study, we equipped gold nanoparticles (AuNPs) from Strychni semen; displayed as a less toxic and environment-friendly. Integration of AuNPs was famed by UV-absorbance which displays peak values. Moreover, high-resolution transmission electron microscopy (HR-TEM), energy dispersive X-ray analysis (EDX) and atomic force microscopy (AFM) substantiate the shape of the AuNPs in the combined materials. FTIR results exhibit the active molecules positioned in the flat surface of the AuNPs. Similarly, the anticancer effectiveness of AuNPs is considered in KMCH-1 cells. Also, AuNPs successfully aggravate cytotoxicity and apoptosis by conjugating apoptotic gene expressions in KMCH-1 cells. Eventually, our results confirm the synthesis of AuNPs from Strychni semen shows anticancer effects with environment-friendly manner.

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Introduction
Challenges in anti-tumor tormented drug delivery system fabrication are to extend the appropriate transporter tools. In recent times, nanotechnology has happened to be a well-liked term, which represents the effectiveness of existing science and technology. When compared to other major contributions, nanomedicine field of research is an important area of nanotechnology which is also specific in many medical interventions at the molecular level for diagnosis and management of diseases [1]. The interdisciplinary occurrence of nano levels deals with partition, classification and purpose of resources [2]. Diseases such as cancer are the leading causes of transience worldwide and their occurrence is still mounting because of the increasing and aging worldwide population. Nanotechnology has transfigured conventional therapies by improving the precision of diagnosis and efficacy of drug delivery, therapeutics and regeneration of tissue due to the similar sizes of nanomaterials to subordinal arrangements of human tissues at the nanolevel. Various nanoparticles have been constructed to extend their total circulation half-life after general administration, scatter from blood into tumor region with improved blood vessel passage, choose and bind to tumor cells, envisage their position and tumor border and discharge antitumor drugs for enhanced cancer treatment [3].

Abundant chemicals are used to widen as nanoparticles for the hopeful effects. Evenly, those chemicals are destructive to the environment and extremely costly but conversely, innate metal nanoparticles are premeditated as low cost and conserved [4]. Numerous nanoparticles drug delivery systems have been predictable to attain the incessantly sprouting need of the essential and medical fields. During recent years, green composite metallic nanohybrids encumbered with bio-active complexes reveal numerous possible applications which include medical and pharmaceutical field [5,6]. Gold is an outstanding metal of option in the field of organic system, existing organism and medicinal field [7]. The powdered form of gold (bhasma) used as an immunological agent to treat many deficiencies like male impotency and numerous diseases especially in South Asian countries like China and India [8]. Primary reason for choosing gold as a candidate metal is when contrast to other inspected metallic particles, gold nanoparticles (AuNPs) are nontoxic in nature, possess many related medical applications and acquire biological properties [9]. Remarkable plane Plasmon resonance displayed by gold nanoparticles surface in conditional size which results in powerful obliteration of burning clarification. This unique property of gold is lost in other substantial materials [10].

Cholangiocarcinoma (CCA) is referred to as bile duct cancer instigated from the biliary tract epithelium. CCA is extremely hard to identify awaiting the disease development.
to the highly developed phase [11]. CCA is the second universal most important liver cancer worldwide, annually 1500 mortality cases due to this prime liver cancer in the United Kingdom. Conspicuously, the occurrence of CCA is mounting globally for unidentified reasons [12]. Too much of smoking, consumption of alcohol, exposure to fatty foods which results in obesity have not been time after time exposed to augmented menace, even though a small involvement cannot subsist to lined away [13]. Routine exploitation of persuaded phytochemicals, able to diminish the danger and improvement of specific cancers [14]. Strychni Semen, the seed of S. nux-vomica (Loganiaceae), generally known as “Ma qian zi” in China and used as folk medicine. Strychni Semen extracts are employed as a traditional medicine for the treatment of lung, stomach and esophageal cancers in Korea [15].

Numerous researches like pharmacological effects and chemical studies have been carried out in Strychni Semen [16–19]. Especially, Strychni Semen showed some actions against the growth of HepG2 (human hepatoma) cells [20]. In this present study, we shaped gold nanoparticles (AuNPs) from ethanolic extract of Strychni Semen and it was established by numerous studies such as UV-visible absorbance spectrum, resolution-transmission electron microscope (HR-TEM), energy dispersive X-ray analysis (EDX), FTIR with selected area diffraction, elevated size of AuNPs resoluted by atomic force microscopy image (AFM). Additionally, contrived AuNPs conquered the anticancer eventual studies by apoptotic induction and regulation by RT-PCR findings. Finally, intracellular ROS production and cytotoxic efficiency study is done in cholangiocarcinoma cells (KMCH-1).

Materials and methods

Chemicals and antibodies

Antibodies such as Caspase-3, Caspase-9, Bax, Bcl-2, Bid, and goat anti-mouse IgG-HRP polyclonal antibody were procured from Santacruz, USA. Dulbecco’s Modified Eagles Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA was acquired from Himedia, Mumbai, India. 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT), acridine orange (AO), propidium iodide (PI) and 2, 7-diacetyl dichlorofluorescein (DCFH-DA) were procured from Sigma chemical, MO, USA.

Gold nanoparticles (AuNPs) from Strychni semen

Strychni Semen dried seeds were decayed to make the aqueous extract. Dried seeds of Strychni Semen were weighing 15–20 g are hollowed with double distilled water. Then, it was allowed to dry and compressed into 100 ml disinfected distilled water, then it was filtered by Whatman No.1 filter paper. The gold particles have been equipped by Frens method [21] where the color of the exterior solution for AuNPs was pinkish red and with the UV-vis spectra, the observed maximum colloidal gold was in the range of 533 nm (Figure 1). Consequently, colloidal nanoparticles were equipped by adding 1 ml of 1% sodium citrate solution to 50 ml of a 0.01% chloroauric acid (boiling condition). The mixture was sustained at boiling point for 10 min and then stimulated for another 10 min after eliminating the heat source. Warmth plays a crucial role in devouring the size allocation of the gold colloids.

Categorization of AuNPs synthesized from Strychni semen

AuNPs were categorized by various experiments which recognize the size, shape and morphological individuality of nanoparticles. The formed AuNPs was embedded by ultraviolet-visible absorbance spectrum revised at wavelength ranges of 300–700 nm. Morphology and size of AuNPs were recognized and distinguished by high-resolution transmission electron microscopy (HR-TEM) and atomic force microscopy (AFM) images. Firmness of nanoparticles was observed by energy dispersive X-ray (EDX). Presence of active compounds in nanoparticles was studied by FTIR spectroscopy proportions. The FTIR spectra of synthesized nanoparticles were renowned in the arrangement of 1000 and 4000 cm$^{-1}$ in potassium bromide pellets by FTIR spectrophotometer.

Cell culture

In the present study, cholangiocarcinoma cells (KMCH-1) were used. Human cholangiocarcinoma cell lines (KMCH-1) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were sealed with DMEM medium enhanced with fetal bovine serum (10% FBS) and 1% antibiotics at 37 °C in a humidified incubator at 5% CO$_2$. After 96 h, the culture was vanquished approximately 80% confluent and the cells were harvested with trypsin-EDTA. Further studies are done with the prepared cells.

Cell viability assay

After assembling cholangiocarcinoma cells, nearly 10,000 cells were added in 96-well plates and it was reared for 24 h at 37 °C. Then, combined AuNPs particles from Strychni Semen was indulged with diverse concentrations (20–120 μg/ml) and kept in incubation for 24 and 48 h at 37 °C. After development, the cells were exposed with MTT reagent (1 mg/ml) in all the wells. Finally, culture plates were incubated for 4 h at
37 °C to form purple formazan crystals. About 200 μL of DMSO was supplemented to each well to hang up formazan crystals and then, the optical density was premeditated at 600 nm in microplate reader.

**Measurement of intracellular ROS**

AuNPs interceded ROS generation in KMCH-1 cells was deliberate by fluorescent probe, 2,7-diacetyl dichlorofluorescein (DCFH-DA) staining. Suddenly, an aliquot of isolated KMCH-1 cells (1 × 10^6 cells/ml) was equipped and overloaded in 6 well plates. Then, the cells were indulged with 15 and 20 μg/ml absorption of AuNPs at 24 h incubation. DCFH-DA (1 mg/mL) was added to 6 wells and kept in an incubator for 37 °C in a dark room. The fluorescent intensity was calculated by spectrofluorimeter with emission (490 ± 10 nm) and excitation (540 ± 12 nm).

**Apoptotic studies for acridine orange/propidium iodide**

AuNPs mediated apoptotic changes in KMCH-1 cells were assessed by acridine orange and propidium iodide (AO/PI) staining. In a while, 1 × 10^6 cells were seeded in 6-well plate and then cultured cells were indulged with 15 and 20 μg/ml concentrations of AuNPs incubation at 24 h. When incubation was over, the cells were sanitized with ice-cold phosphate buffer solution and then discolored with 20 μL of AO/PI staining solution at 37 °C for 20 min. The stained apoptotic and viable cells were scrutinized by a fluorescent microscope.

**RT-PCR studies**

Apoptotic markers such as Bax, Bcl-2, Bid, caspase-3& 9 were evaluated by RT-PCR studies. mRNA expression of Bax, Bcl-2, Bid, caspase-3& 9 genes concerned in apoptosis pathway was examined in KMCH-1 cells. RNA extraction of the cells was done according to RNX-Plus protocol. Eminence of RNA was qualified in KMCH-1 cells. RNA extraction of the cells was done at 72 °C. The cDNA amplification was carried out using SYBR green-based PCR Master Mix thermocycler (QIAGEN Corbett products, Hilden, Germany). Primers for cDNA amplification designed by Eacon Designer software, Boston, MA, USA. The total volume of amplification reactions was 25 μl and each well was included with SYBR Green PCR Master Mix (12 μl) 1 μl of cDNA, 900 nM of both forward and reverse primers. Thermal cycler steps were integrated with 95 °C for 10 min, 45 PCR cycles of 95 °C for denaturation step, annealing temperature 45 °C, and extension carried out at 72 °C, respectively. Lastly, the completion of amplifications done at 72 °C for 10 min.

**Statistical analysis**

All experiments were agreed out in three self-directed trials and the outcome results were expressed as the mean ± standard deviation (mean ± SD) by means of one-way examination of variance (ANOVA). The p values < .05 were considered significant.

**Results**

**Size, morphology and absorption of synthesized AuNPs**

In the current study, we have established that the preparation of gold nanoparticles from *Strychni Semen* and absorption pattern is illustrated by UV-visible absorbance spectrum analysis in Figure 1. UV-spectroscopy was used at dissimilar time proposition while the absorption of the *Strychni Semen* and the gold aqueous solution. The ocular sign of the colours with over and the formation of pinkish red colour precised the development of the AuNPs that express the pattern of gold nanoparticles (Figure 1). Size, the ultrastructure and morphological uniqueness of AuNPs fashioned from *Strychni Semen* were additionally precisely and premeditated by HR-TEM (high resolution-transmission electron microscope) in Figure 2(A). High resolution TEM image exemplifies the morphological array of AuNPs which are scrutinized with different size variations like spherical, hexagonal, oval and triangular with standard sizes between 80 and 10 nm.

**Characterization of AuNPs merged from Strychni semen**

The characterization of AuNPs from *Strychni Semen* is determined by energy dispersive studies to evaluate the shape of the particles formed from *Strychni Semen* extract (Figure 2(B)). Movement, size and force of the synthesized AuNPs from *Strychni Semen* determined by atomic force microscopy analysis (AFM) (Figure 3). In addition, FTIR is used to differentiate the numerous functional groups which are present in the synthesized AuNPs from *Strychni Semen* extract by obtaining different spectrum values. In the present study, FTIR showed values of 3907 cm⁻¹, 3745 cm⁻¹, 3500 cm⁻¹, 3378 cm⁻¹, 3210 cm⁻¹, 2341 cm⁻¹, 1725 cm⁻¹, 1645 cm⁻¹, 1459 cm⁻¹ and 1015 cm⁻¹ which recognizes the aromatic, hydroxyl, carbonyl alkyl and amide groups which are nearby to the surface of AuNPs in Figure 4.

**Anticancer potential of AuNPs from strychni semen in KMCH-1 cells**

AuNPs were knowledgeable for their anticancer proceedings against KMCH-1 cells. Consequently, the anticancer probability of AuNPs was intended by cytotoxicity assay (MTT), in which high AuNPs concentration considerably kept back the KMCH-1 cells expansion in both 24 and 48 h incubation in Figure 5. Therefore, we establish that 15 μg/ml concentrations of AuNPs exposed outstanding cell death in KMCH-1 cells. Therefore, we have chosen 15 μg/ml concentrations of AuNPs for further apoptotic studies.

**Apoptotic studies for acridine orange/propidium iodide**

Acridine orange/propidium iodide (AO/PI) double staining is done for cell morphological measurement. Morphological alterations in KMCH-1 cells pretreated with synthesized AuNPs from *Strychni Semen* for 24 h followed by succeeding revelation to 15 and 20 μg/ml concentrations of AuNPs. This was depicted in Figure 6 untreated cells (control); (B) 15 μg/ml,
Feasible cells are blemished green by the presence of acridine orange; necrotic and late apoptotic cells are blemished orange and red by the presence of propidium iodide.

**ROS, caspase-9 and 3 activity assay**

Consequently, AuNPs mediated ROS production that was analyzed by DCFH-DA fluorescent assay. In the present study, we recognized that AuNPs manufactured from *Strychni Semen* aggravate ROS production in KMCH-1 cells (Figure 7). Caspase-9 and 3 activities were premeditated by caspase assay kit, as per manufacturer’s direction, the methodology was chased. ELISA technique was used for evaluation of caspase-3 and 9 protein levels. Levels of caspase-3 and 9 were significantly increased in 15 and 20 µg/ml concentrations of AuNPs compared with control cells. There were significant differences in the levels of caspase-3 and caspase-9 is depicted in Figure 8.

**Real-time quantitative RT-PCR**

AuNPs intruded apoptosis markers such as Bax, Bcl-2, Bid, caspase-3&9 were analyzed by RT-PCR. Figure 9 illustrates...
treatment with AuNPs produced from *Strychni Semen* with the concentration of 15 and 20 μg/ml and diminished the expression of Bcl-2 and Bid at the same time elevated the expression of caspase -3 and 9 in KMCH-1 cells. Finally, with these findings, AuNPs produced *Strychni Semen* efficiently, persuaded apoptosis by regulating the apoptotic pathway.

**Discussion**

Even though there has been enormous innovation in indicative technique, surgical treatment and the development of new anti-cancer agent, the upshot of patients with bile duct cancer has improved discreetly in the past. In contrast to other metals, gold is environmentally friendly and low cost. Production of nanoparticles from plant extracts is an unsophisticated practice, in which metal salt is departed with plant extract and finishing point of reaction takes place within a short period. Specifically, gold nanoparticles (AuNPs) are non-toxic when compared to other metallic nanoparticles [22,23]. Nanoparticles separation in plants can be flattering over all other organic technique by eradicating the complicated process of preserving cell cultures [24]. In the present study, we recognized the *Strychni Semen* arbitrates the produced AuNPs by UV-visible absorbance. A mounting reflection of *Strychni Semen* seed extracts direct towards the improved intensity of absorption.

The UV-visible spectra distinguished with unrelated time intervals such as 24 h, 5th day, 15th day and 30th day from

![Figure 4: Atomic force microscopy analysis of gold nanoparticles synthesised from Strychni semen.](image)

![Figure 5: Cytotoxicity effect of gold nanoparticles synthesised from Strychni semen in cholangiocarcinoma cell (KMCH-1). The number of viable cells after treatment is expressed as a percentage of the vehicle-only control. This experiment was repeated thrice and the bars in the graph represent S.E. (*p* < .05).](image)

![Figure 6: Apoptotic effect of gold nanoparticles synthesised from Strychni semen in KMCH-1 Cells.](image)

![Figure 7: Intracellular production of ROS levels in KMCH-1 cells. This experiment was repeated thrice and the bars in the graph represent S.E. (*p* < .05).](image)

![Figure 8: Caspase-3, Caspase-9 activity in KMCH-1 cells. This experiment was repeated thrice and the bars in the graph represent S.E. (*p* < .05, *# p* < .01).](image)
the beginning of the reaction with various amounts of seed extracts. Present study exemplify the fashioned AuNPs reveal highest absorbance was found to be in the ranges of 533 nm at 60th day. This result matches with the prior studies in which UV-Vis spectroscopy tattered to examine the shape and size of the constrained nanomaterials in the aqueous suspensions [25]. This connected data were previously reputable by a number of investigations on a combination of gold nanoparticles [26]. When compared with the other metal nanoparticles, mainly AuNPs, enfold free electrons which are positioned in SPR (surface plasma resonance) absorption bands due to the united trembling of electrons of metal nanoparticles in consequence with lightwave [27]. In the current energy dispersive x-ray analysis study, tough and strong signal were recognized in the gold section which validates the AuNPs pattern. In the present study, we obtained the powerful signals of Au (i.e. 1.5–2.5 and 2.5–3.5 keV), in which previous surface resonance findings were very near to our present results with the unique optical absorption for metallic crystal typically found to be 3 KeV [28].

Gold nanoparticles normally display typically strapping ocular assimilation peak was experienced due to surface plasmon resonance [29]. The reinforcement of importance in a transmission electron microscope (TEM) pursue incessant modifications in examining latest diseases or supplementary features of aged diseases, new remedial drugs and their potential toxic effects, side effects upsetting the liver. In our findings, the development of gold nanostructures definite in TEM figures, in which morphological individuality provides high-density AuNPs shaped by the Strychni Semen extract. Previous studies confirm the size and shape of gold nanoparticles performed by TEM studies in Hep-2 cells, where the TEM examination of all gold nanoparticles was reliable with the ascending like 3, 11, 27, and 51 nm [30]. Many studies support our data like the TEM investigated the phase and contrivance of the cellular incorporations of protein-covered gold nanoparticles by means of the HeLa cell lines [31].

FTIR is used to distinguish the various functional groups which are embedded in the synthesized AuNPs from Strychni Semen extract. Present finding acquaintances with previous studies in which fabrication of AuNPs from various plants extracts have a number of functional groups [32]. Prior FTIR findingschains our data in which gold nanoparticles propose that the biological molecules like enzymes and proteins might perhaps implement the utility for the arrangement and consistency of the AuNPs using the Enterococcus species [33]. Atomic force microscopy images (AFM) corroborate the size of the formed nanoparticles. In (Figure 4), précised AFM images have enough pampered control through the progress of the AuNPs which matched with the previous findings [34]. A further AFM prior study shows a large number of AuNPs at the cell membrane after incubation of 1 h, in contrast where after 2 h, AuNPs are not noticeable [30]. Intracellular reactive oxygen species (ROS) in a system association influence oxidative stress which results in apoptosis [35]. In addition, prominent ROS directs to the disintegration of nucleus and mitochondria depolarization which effects in oxidative stress intervened apoptosis [36,37].

The propidium iodide/acridine orange stain (PI/AO stain) is a feasibility stain that notices the apoptotic cells. Apoptotic swots were executed with a staining method employing AO and PI according to the previous studies [38]. This was further correlated with prior studies in which vanillin, a flavonoid compound induces apoptosis and cell cycle detains in human colorectal cancer cell line HT-29 [39]. Caspases engage in recreation of crucial role in the extrinsic pathway of apoptosis via commencement of terminal caspases which were in effect [40,41]. RT-PCR investigation in cultured HA22 T, HepG2, and Huh7 human liver cancer cells in which the appearance of estrogen receptor and neurofibromin NF2 gene was assessed [42]. Development and sequence of tumor and alteration to numerous onologic remedial execution consequences are regularly scarce to apoptotic stimuli. Apoptosis is an essential watchdog in tissue preservation and maintenance [43]. Consequently, apoptosis is a scrupulous and important passegeway for cancer treatment. Previous studies support our current findings in which gold nanoparticles induce apoptosis in MCF-7 (human breast cancer cells) [44].

**Conclusion**

Finally, we demonstrate a simple and rapid technique with reproducibility for the environment-friendly fabrication of AuNPs, which lacks elegant reducing agents. The effectiveness of AuNPs produced from Strychni Semen was well established. The physiochemical distinctiveness of manufactured AuNPs from Strychni Semen is documented by UV-absorbance, EDX, FTIR, AFM and HR-TEM. AuNPs successfully convince cytotoxicity and apoptosis by inflicting intrinsic apoptotic gene expressions in KMCH-1 cells. So, finally, our study confirms the synthesized AuNPs from Strychni Semen, which displays anticancer effects.

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Disclosure statement
All the authors declared that there are no conflicts of interest.

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