International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 8 | Issue 2 | APR-JUN | 2018 | 169-177
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

GREEN SYNTHESIS OF BIOCOMPATIBLE GOLD NANOPARTICLES FROM GOLD MINE BACTERIA *BACILLUS OCEANISEDIMINIS* AND THEIR ANTILEUKEMIC ACTIVITY

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ABSTRACT

Green synthesis of gold nanoparticles (GNPs) has gained great interest as an eco-friendly, cost effective and a simple alternative to conventional physical and chemical methods. The aim of the present study is to determine the anticancer activity of biogenic gold nanoparticles against leukemic cell lines along their biocompatibility with red blood cells (RBC). The present work describes rapid eco-friendly approach for preparation of GNPs using novel isolate Bacillus oceanisediminis isolated first time from the Indian gold mines. GNPs were prepared using gold salt as the precursor and extracellular material from bacteria as a reducing and stabilizing agent. This method of GNPs synthesis is a cost effective, eco-friendly and non-toxic alternative to physical and chemical methods. The synthesis of GNPs was preliminary observed by a color change from yellow to red and confirmed by a peak at 535 nm using a UV– visible spectroscopy. X-ray diffraction studies used to study the crystalline nature of GNPs. The TEM image showed size of GNPs in the range of 5-10 nm. The functional groups on surface of the GNPs were studied by FTIR. The biocompatibility of biogenic GNPs were verified by incubating with RBCs and showed no significant hemolysis. In addition, these GNPs also proved to exhibit excellent anticancer activity against leukemic cell line with IC_{50} values 8.09 µg/ml. First time explored Indian gold mine bacteria for the synthesis of potent antiproliferative GNPs by Bacillus oceanisediminis can use as direct anticancer therapy and also as a vector in drug delivery, gene delivery.

KEY WORDS

Gold nanoparticles, Bacillus oceanisediminis, Haemolysis, X-ray diffraction, TEM.

INTRODUCTION

Metal nanoparticles have been synthesized using various methods including physical and chemical methods, photochemical reduction, electrochemical reduction and heat evaporation methodologies [1]. But these methods are expensive and moreover involve use of toxic ingredients and yield hazardous by-products which may be potentially harmful to either environment or human health. This greatly limits their use in biomedical fields, particularly in clinical applications [2]. Many microorganisms both unicellular and multicellular can produce nanoparticles either extracellular or intracellular routes. In the recent past, several studies have been reported the synthesis of GNPs by many species of bacteria such as *Streptomyces* sp. ERI-3 [3], *Thermos scotoductus* SA- 01 [4], *Enterobacter aerogenes* [5], *Staphylococcus epidermidis* [6] *bacillus marisflavi* [7] and *Bacillus cereus* [8]. Researchers, in most of these studies have used either bacterial cultures collected from culture collection centres or cultures isolated from different environmental soil samples. But there are no reports about the exploration of bacteria

from the Indian gold mines. Microbial species that reside in gold mines could possess high resistance against soluble gold toxicity and may produce gold nanoparticles efficiently.

Among nanoparticles, GNPs are inert, precious and non-oxidized on their expose to oxygen [9]. The *in vitro* and *in vivo* studies on the cytotoxic effect of GNPs have been reported in several studies. There are a few recent reports on biogenic GNPs used in cancer therapy are MCF – 7 breast cancer cells [10], mouse myoblast cancer cells [11], lung cancer cells, HepG2 cells [12] and colon cancer cells [13]. Leukemia is a group of cancer characterized by unregulated proliferation of the blood forming cells of bone marrow result in high number of abnormal white blood cells. Chemotherapy, radiation, surgery and photodynamic therapy are the common types of cancer treatment methods. But, these therapies kill normal cells as well as cancer cells and also cause side effects [14]. To overcome this problem, it is essential to develop an eco-friendly, cost effective and biocompatible method for treatment of cancer with fewer side effects. In the recent days nanoparticles are well accepted as therapeutic agents due to their high surface area and with strong binding affinity and they can easily enter cells by diffusion. Noble nanoparticles are having distinct properties compared to other nanoparticles due to their electronic, optical and molecular recognition properties are greatly utilized in imaging, sensors, cosmetics, drug delivery and cancer therapy [15].

As with any device or pharmaceutical, nanoparticles intended for biomedical applications must be subjected to biocompatibility testing before regulatory approval for an administration to patients. Determination of haemolytic property of synthesised GNPs is one of the most important tests in the studies of GNPs interaction with blood components [16]. Although many reports have been investigated the effect of GNPs on RBCs, the methods used for the synthesis of nanoparticles were different as they used chemical and physical methods for the GNPs synthesis. There are only a few reports on biologically synthesised GNPs used for hemolysis studies [17,18].

In the present study, we have isolated novel bacterium *Bacillus oceanisediminis* from the gold mines and used for GNPs synthesis. The synthesised GNPs were characterized by UV-vis spectroscopy, XRD, FTIR and TEM studies. The antiproliferative activity of biogenic GNPs was studied in leukemic cell lines and haemocopatability property was determined in chicken RBC cells.

MATERIALS AND METHODS

Isolation of bacteria from the gold mine

Soil samples from the Hutti gold mines, Karnataka state, India were used as inoculums, serially diluted and plated onto nutrient agar media. The plates were incubated at 37 °C for 24 h. The colonies obtained were further sub cultured on nutrient agar and stored at 4 °C for further experimental use. The bacterial cultures were inoculated in 250 ml flask containing 50 ml sterile nutrient broth and incubated for 24 h at 37 °C. After incubation, the bacterial cultures were centrifuged at 5000 rpm for 10 min at 4 °C to separate bacterial cells. The supernatant obtained after centrifugation was further used for the GNPs synthesis.

Biosynthesis of gold nanoparticles

The cell free extracts obtained from the above procedure were used for the synthesis of GNPs. In this process, bacterial supernatants were mixed with 1 mM Hydrogen tetrachloaurate ($HAuCl_4$) and the mixture was treated with microwave irradiation for 30 secs at 750 W power. The control which lacked biomass kept under the same experimental conditions. The tubes which showed color changes from yellow to red were selected for further study. Among different isolates based on the reduction efficiency, we have selected potential strain for the further GNPs synthesis. The synthesised GNPs were washed with distilled water, subjected to centrifugation at 14000 rpm for 10 min and used for further studies.

Identification of the active strain

For the identification of potential bacterial species from the gold mine soil, only the GNPs synthesizing bacteria were considered and was further characterised based on 16S rRNA gene sequence analysis. Genomic DNA was extracted according to the previously reported protocol [19] and DNA amplification of 16S rRNA gene was performed with a universal set of forward primer27F (5'AGAGTTTGATCCTGGCTCAG 3') and reverse primer 1492R (5' ACGGCTACCTTGTTACGCTT 3'). The DNA sequencing was performed, and the results were subjected to BLAST analysis. The nucleotide similarity was identified by the National Centre for Biotechnology Information databases (NCBI). The identified sequence

of bacterial isolate was deposited in GenBank database and accession number was assigned.

Characterization of biogenic GNPs

Bio reduction of GNPs was initially monitored by visual observation for color change from yellow to red and confirmed by recording the spectra of GNPs from 400-700 nm by UV-Visible spectroscopy (Thermo scientific, Multiskan Spectrum). The crystalline nature and mean size of the GNPs were analyzed by X-ray diffractometer (RigakuminiFlex 11) operating at 30 kV and a current of 15 mA with Cu Ka radiation (k = 1.5406 A °) and the 2θ scanning range was of 6–60 ° at 5 ° min^{-1}. The colloidal suspension of biogenic GNPs were dried and used for the powder XRD analysis. For the study of morphology, dispersity and size of biogenic GNPs, TEM (Tecnai G2 spirit BioTWIN, 20-120 kv, Netherland) was performed. Sample for TEM analysis was prepared by adding a drop of sonicated colloidal suspension of GNPs was drop cast on carbon coated copper grid and allowed to dry at room temperature. After drying it, the images were taken at different magnifications. Participation of the active functional groups in the reduction and stabilization process of GNPs was analysed by Fourier transform infrared spectrophotometer (FTIR, Jasco-460 plus) equipped with a horizontal attenuated total reflectance (ATR). For this the sample was dried and powder was used for the analysis of functional groups. The spectra were recorded with wavelength range from 4,000 to 400 cm^{-1} at resolution 4 cm^{-1}.

Chemical reagents and Cancer cell lines

All the chemicals used in the present study were purchased from Sigma Aldrich and Himedia, India. The cells used in the study were human myelogenous leukemia cell lines (K562) purchased from National Centre for Cell Science, Pune, India. Further the cells were grown in MEM/DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg of streptomycin/mL at 37 °C in 98% humidity with 5% CO_2.

Antileukemial activity

The selected leukemia cell lines were exposed to different concentrations of biogenic GNPs as described previously [20]. Cells were seeded at a density of 0.5×10^5 cells/ml in 96 well plates and the cells were treated with different concentrations (10, 50, 100, 250 and 500 µg/ml) of biogenic GNPs. After 48h of incubation, cells were processed for the proliferation assay and IC_{50} value was determined.

Haemolytic assay

Haemolysis assay was performed according to previous report [21]. Chicken blood was used to perform the haemolytic assay of the biogenic GNPs. RBCs were separated by centrifugation at 1500 rpm for 10 min and supernatant was removed. The RBCs were further washed for multiple times with sodium saline (0.85%) solutions. The RBCs pellet was made up to the volume of 20 ml with saline and 1 ml of the washed RBCs were mixed with two different concentrations (10 µg/ml and 20 µg/ml) of biogenic GNPs. Distilled water was used as positive control and 0.85% sodium saline was used as negative control. All the experimental and control samples were stored under static conditions for 4 h at 37 °C. After incubation the mixtures were centrifuged at 14,000 rpm for 10 min in order to remove the nanoparticles. The supernatants for haemolysis were measured at 540 nm in UV-visible spectrophotometer. The percentage of haemolysis was calculated by using the formula

$$\% \text{ Haemolysis} = \frac{\text{Absorption of test sample-Absorption of negative control}}{\text{Absorption of positive control-Absorption of negative control}} \times 100$$

RESULTS AND DISCUSSION

Screening of Bacteria for gold nanoparticles synthesis

Soil samples were collected from the Hutti gold mine, 4 morphologically different isolates were obtained, and one potential isolate was able to change the color from yellow to pink red. The change of color was preliminary observation for GNPs formation and confirmed by UV–Visible spectroscopy (Fig 1). Control tube, without adding bio molecules showed no color change, confirming the formation of GNPs in the presence of biomass only. In the present study the time period for nanoparticles formation was less than 30 sec. Previously, biosynthesis of GNPs by using bacteria has been reported by several researchers by incubating at 37 ºC for 24 h [7,8]. These studies used longer time duration for the synthesis of GNPs at 37 ºC. In our recent reports, temperature optimization studies revealed that the formation of GNPs was very high at boiling temperature, whereas at room temperature nanoparticles were not synthesised [6]. At high temperature, exposing of functional groups might be responsible for the rapid synthesis of nanoparticles [22]. In the present study, the time taken for formation of

GNPs was found to be less than 30 sec by microwave method. To best of our knowledge, this is the first report on rapid biosynthesis of GNPs within a very short time duration using bacteria.

Identification of GNPs producer

The potential strain for GNPs synthesis was further characterized by phylogenetic analysis. The phylogenetic relationship of the isolated strain was analysed based on the 16S rRNA gene sequence similarity with related species. After analysis of 16S rRNA gene sequence of the isolate, the highest similarity (99%) was observed to *Bacillus oceanisediminis*, which is deposited in Genbank with accession number KX007772. Supporting to our investigation, presence of heavy metal resistance genes, heavy metal efflux pumps and detoxifying enzymes in a bacterium *Bacillus oceanisediminis* have been previously reported [23].

UV–Visible spectroscopy

In the present investigation, a single SPR band was observed in the sample that is an evidence for spherical shape of GNPs. The spectra revealed a strong absorption peak at nearly 535 nm indicated the formation of gold nanoparticles (Fig 1).

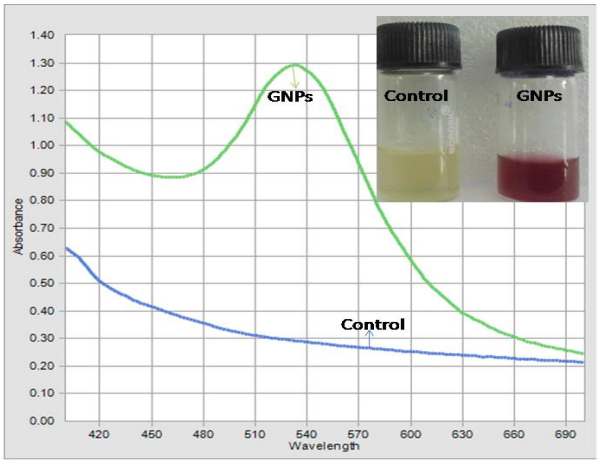

Figure 1: UV-visible spectra of biogenic gold nanoparticles shows peak at 535 nm indicated the formation of GNPs. The inset photo shows control and colloidal GNPs.

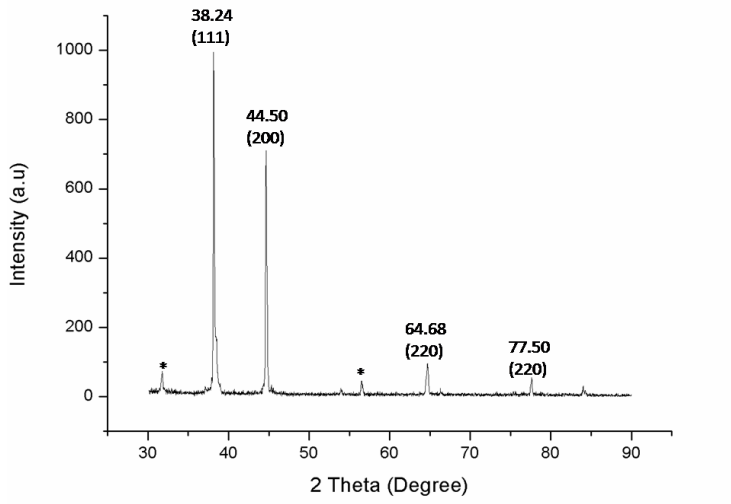

Figure 2: Powder XRD pattern of biogenic GNPs synthesised from *Bacillus oceanisediminis*

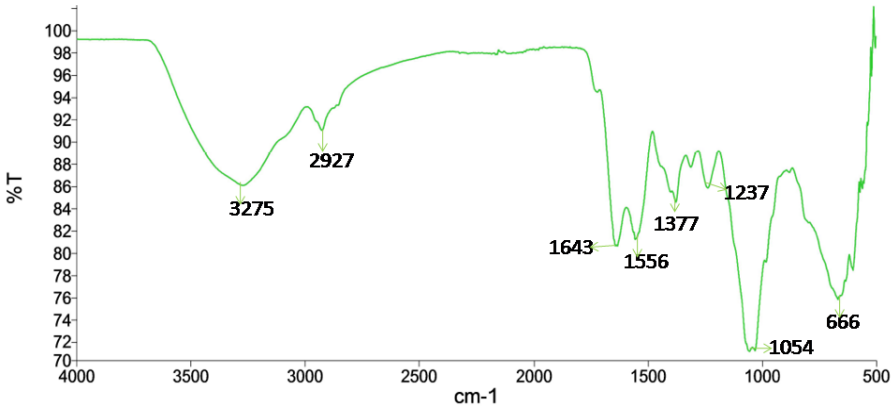

Figure 3: FTIR spectra of biogenic gold nanoparticles showed the presence different bands.

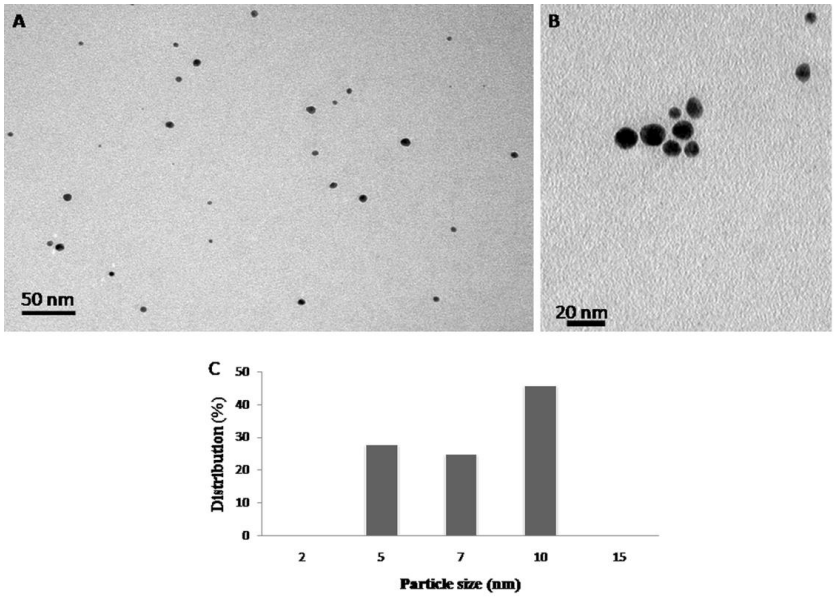

Figure 4: TEM images of biogenic gold nanoparticles size ranging from 5 to 10 nm (a and b). Size distribution histogram of GNPs (c).

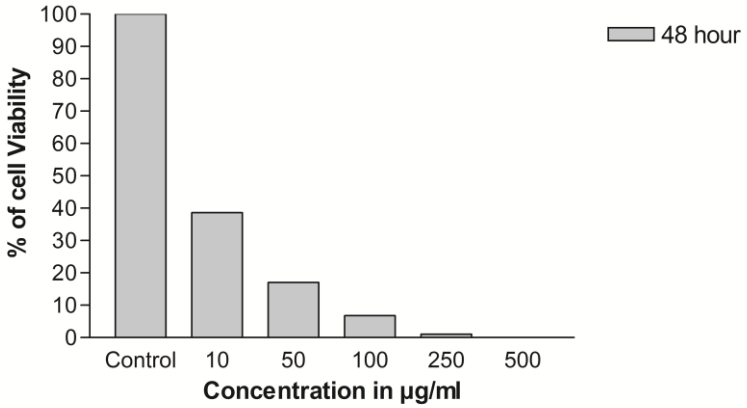

Figure 5: Anticancer activity of biogenic GNPs against leukemic (K562) cell lines.

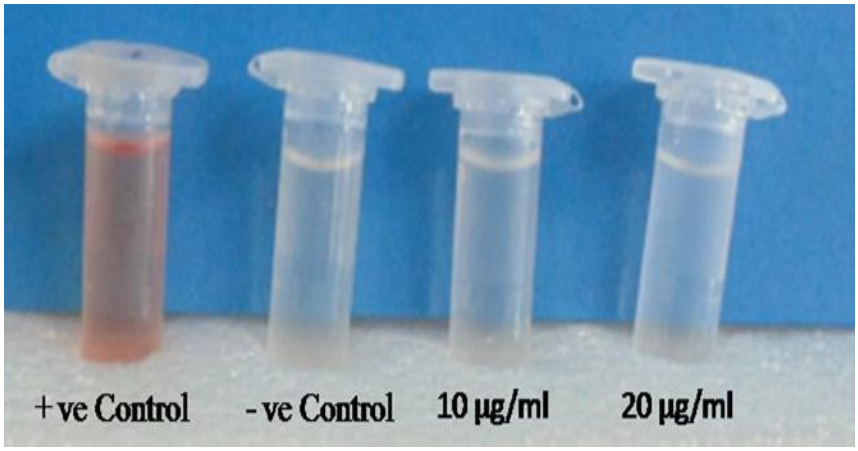

Figure 6: Hemolytic assay of RBCs incubated with biogenic GNPs.

The SPR of GNPs usually has a range of 520–560 nm in aqueous solutions depending on the shape and size of nanoparticles. With increase in size of the nanoparticles, the SPR shifts towards longer wavelength. The position of the absorption band mainly depends upon dielectric constant of the medium and surface-adsorbed species [24]. The sharp high intensity peak was observed in the present investigation evidenced for the formation of small spherical shaped GNPs.

XRD measurements

The XRD pattern of biogenic GNPs synthesised using *Bacillus oceanisediminis*is shown in (Fig 2). The strong narrow diffraction peaks observed indicates that the synthesised GNPs have well defined crystalline structure. The XRD pattern of GNPs with 2θ peaks at 38.24º, 44.50 º, 64.68 º, and 77.50 º corresponding to the Braggs reflections (111), (200), (220) and (311) respectively. These readings are matching with JCPDS data no. 04-0784 corresponding to the above-mentioned planes and face cantered cubic structure for GNPs. Furthermore, the spectra indicate a sharp and strong at 38.24 º suggesting the strong X-ray scattering centres in the crystalline phase due to the capping and stabilizing agents of the nanoparticles that is indexed to the (111) reflection of metallic gold with FCC structure [25]. The minor peaks (*) are due to the formation of amorphous or nanocrystals of biomolecules [26]. The average size of the biogenic GNPs was calculated using Debye-Scherrer equation was found to be 10.09 nm and it was further confirmed by TEM analysis.

FTIR studies

The identification of the functional groups responsible for reduction of $HAuCl_4$ to GNPs and active biomolecules in bacterial supernatant responsible for capping and stabilization of nanoparticles was analysed using FTIR studies.

Fig 3 presents the FTIR spectrum of biogenic GNPs showed the existence of bands at 3275, 2927, 1643, 1556, 1377, 1237, 1054 and 666 cm^{-1}. A broad band at 3275 cm^{-1} indicates the involvement of N-H stretch in reduction. A weak peak at 2927 cm^{-1} was observed due to asymmetric stretching of - C - H – groups in aromatic ring [26] and absorption peak at 1643 cm^{-1} was observed due to stretching frequency of carbonyl group (C=O) [27]. A band at 1556 cm^{-1} observed due the amide group II of proteins [28] and 1377 cm^{-1} corresponds to N=O group from nitro compounds present in the bacterial supernatant. The absorption peak at 1237 cm^{-1} was assigned as amide III band of proteins present in the bacterial extract. A strong intense peak at 1054 cm^{-1} was characteristic of C-OH stretching of secondary alcohols and while the intense band at 666 cm^{-1} due to alkynes (- C=C-H; C-H bend) [29]. The peaks observed in the FTIR spectra of the GNPs shows the presence of functional groups of proteins and enzymes on the surface of biogenic GNPs. This result proves that the biomolecules binds to metal nanoparticles and produced capped GNPs to prevent aggregation. Thus, it is found that the supernatant of *Bacillus oceanisediminis* perform dual functions of reduction and stabilization of GNPs.

TEM analysis

The size, shape and distribution of biogenic GNPs synthesised from supernatant of *Bacillus oceanisediminis* were studied by TEM analysis. Two TEM images were shown in the Fig 4 a,b was obtained from different magnifications of GNPs deposited on a carbon coated copper grid. The particles formed in this method

are mostly spherical in nature having almost uniform size distribution with size ranging from 5 to 10 nm. Particle histogram of TEM image is given in Fig.4 c. Most of the nanoparticles are spherical shaped and small in size. These kinds of spherical nanoparticles are formed only when the cell free extract has optimum stabilizing and capping property [30].

Antileukemial activity

Leukemic cells were exposed to biogenic GNPs at different concentrations of 0, 10, 50, 100,250 and 500 µg/ml for 48 h and anticancer activity of GNPs was determined using the MTT assay. The reduction in the viability of cancer cells treated with GNPs occurs in a dose dependent manner. As the concentration of GNPs increases up to 250 µg/ml a significant reduction in cell viability was observed (Fig 5). The GNPs exposed leukemic cells viability significantly decreased by 38 %, 17 %, 6 % and 1.05 % at concentrations of 10, 50, 100 and 250 µg/ml respectively. The result was found that the IC_{50} value of the biogenic GNPs was 8.09 µg/ml. The results obtained from our investigation, the anticancer activity was considerably more efficient than the previously reported biogenic GNPs against different cancer cell lines [31-33]**.** This may be due to the smaller sized GNPs as it is evident in the previous studies that the anticancer activity of GNPs is size dependent, i.e,in general, the smaller the particles, the greater the antiproliferative property and vice-versa. Different sizes and shapes of GNPs can promote antiproliferative activity via distinct mechanisms. For instance, 9 nm or smaller spherical shaped GNPs could cross the nuclear pore complex of the cancer cell [34]. Previously reported by Pan et al cytotoxicity of GNPs of size 0.8 – 15 nm on four different cell lines was determined that GNPs of smaller size resulted rapid cell death when compared to larger particles from which it is concluded that the cellular response to GNPs was size dependent [35]. In the present investigation the biogenic GNPs size was found to be less than 10 nm resulting efficient antiproliferative activity against leukemic cell lines. The IC_{50} value of the biogenic GNPs was found to be 8.09 µg/ml; therefore, the further haemolytic assay was carried out using the higher concentration than the IC_{50} value to check the hemocompatability effect of GNPs.

Haemolytic assay

Lysis of RBC *in vivo* can lead to a series of pathological conditions, so evaluation of the intravenously administered GNPs biocompatibility with blood is necessary by *in vitro* assays. Most of the previous reports on hemocompatibility of synthesised GNPs were studied by hemolysis of human red blood cells [19,36]. Among these, one of the studies showed the toxicity towards human RBC found to be less than 0.1 % hemolysis suggesting the safe nature of biosynthesised GNPs on human RBCs [19]. In previous report chicken embyo RBCs were exposed to platinum nanoparticles to check the biocompatibility results showed the percentage of hemolysis was 23 %. This experiment allows hemocopatability to be evaluated in a short time, as well as serving as a rapid, precise model for evaluating toxicity [37]. As shown in (Fig 6) the biogenic GNPs at the concentration of 10 and 20 µg/ml exhibited no significant hemolysis, which is not detected by UV-visible spectrometry. But, interestingly at the same concentrations of GNPs showed efficient antileukemic activity that evidenced the particles are toxic only to the cancer cells not to the normal cells. Similarly, recent studies reported that green synthesised silver nanoparticles show more toxic to cancer cells and less toxic to normal cells [38]. The exact reason for this property need to be investigated in future studies. This result shows that GNPs are biocompatible and non-toxic and can be utilised further in biological applications such as cancer drug development, drug delivery, labelling, photothermal therapy, tumor imaging and sensing etc.

CONCLUSION

This is a simple, rapid, eco-friendly and cost-effective protocol for the efficient synthesis of gold nanoparticles using *Bacillus oceanisediminis* as reducing and stabilizing agent. GNPs have been properly characterized by UV-visible spectroscopy, XRD, FTIR and TEM analysis. The XRD result confirms that biogenic GNPs are well crystalline nature and TEM analysis reveals that the size and morphology of biogenic GNPs were spherical in shape with an average size ranging from 5 to 10 nm. The synthesised GNPs were checked for their anticancer activity against leukemic cells and found to be potent anticancer agents with IC_{50} value 8.09 µg/ml. In addition, these biogenic GNPs elicited no haemolytic activity in red blood cells. The present study suggests that, the biogenic GNPs might be promising agents for anticancer drug development in future.

Acknowledgments

Authors gratefully acknowledge to Managing Director and General Manager, Hutti Gold Mines, Karnataka, India for permitted us to collect required samples to carry out our research work. The authors wish to acknowledge the University of Mysore for XRD and FTIR analysis of gold nanoparticles. The authors also acknowledge C-CAMP, National Centre for Biological Sciences (NCBS), Bangalore, Karnataka, India for TEM analysis of nanoparticles.

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