



Green synthesized silver nanoparticles: Catalytic dye degradation, *in vitro* anticancer activity and *in vivo* toxicity in rats

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ABSTRACT

In this study, silver nanoparticles were synthesized (AgNPs) using aqueous rhizome extract of *Acorus calamus* (ACRE) and evaluated their *in vitro* anticancer activity and *in vivo* toxicity in a Wistar rat model. The synthesized AgNPs showed good catalytic activity against different organic pollutant dyes. *In vitro* cytotoxic effects of AgNPs were assessed in Hep2, COLO 205 and SH-SY5Y cells using MTT assay. Further, the apoptotic changes induced by AgNPs in more susceptible Hep2 cells were observed through AO/EB, DCFH-DA, Rhodamine 123, PI/DAPI staining, oxidative stress markers and Western blotting. *In vivo* toxicity study revealed substantial alterations in the levels of serum biochemical markers including AST, ALT, LDH and inflammatory markers such as TNF- α and IL-6 on day 29 when rats treated with AgNPs as compared to control, however, these levels were restored to normal at the end of washout period on day 89. No remarkable changes were observed in liver oxidative stress enzymes. ICP-OES analysis indicated bio-distribution of silver in spleen (5.67 $\mu\text{g/g}$) and liver (4.98 $\mu\text{g/g}$) in rats treated with 10 mg/kg b.w of AgNPs on day 29 and elimination of silver from all organs was observed at the end of washout period on day 89. Histopathological analysis revealed no significant changes in kidney, spleen, lungs, heart, testis and brain with 5 and 10 mg/kg b.w of AgNP. However, 10 mg/kg b.w of AgNPs showed moderate degree of cell swelling and vacuolar degeneration in liver and these alterations were reverted back to normal at the end of washout period. Findings from this study signify green synthesized AgNPs at low concentrations might be useful in many ways with ecofriendly nature.

1. Introduction

In recent years, nanotechnology has fabulous growth due to their various applications in several fields such as material science, chemistry, medicine, bionanotechnology, etc. Nanoparticles (NPs) have high attention because of their great surface to volume ratio, and tremendously small size, which leads to difference of physical and chemical properties as compared to same composition of bulk material [1]. Among the NPs, the researchers have shown more interest in silver NPs (AgNPs) because of their wide range of applications in antimicrobial, catalysis, medical, optics and energy. These NPs have been commonly synthesized using electrochemical [2], photochemical [3], microwave [4], thermal decomposition [5], chemical reduction [6], and radiant assisted process [7] for a long time.

The AgNPs synthesized from various chemical approaches showed toxicity in *in vivo* studies. The behavior of AgNPs is influenced by the size, concentration, coating, and distribution level of the particles [8,9].

Alternately, the microbial and biological system (green chemistry) approaches are developed to synthesize NPs without the use of harsh and expensive toxic chemicals [10,11]. The microorganism based NPs synthesis is rapidly developed due to their eco-friendly formation of NPs. However, microbial method has disadvantages due to the consumption of more time for maintaining cultures [12]. Nowadays, the NPs are synthesized using different plant sources due to their natural availability, rapid formation, and the ecofriendly nature [13]. The AgNPs were synthesized using *Acorus calamus* rhizome. *Acorus calamus* is also called as sweet flag. In Indian Ayurvedic system this herb is used to treat brain disorders, chronic diarrhea, liver troubles, rejuvenator for nervous system, etc. [14]. The rhizome is also used in the preparation of modern herbal medicine as it has diuretic, laxative, sedative and carminative properties [15].

The present study aims to evaluate the catalytic activity of green synthesized AgNPs on different organic pollutant dyes, *in vitro* anticancer activity in cancer cells and *in vivo* toxicity in a Wistar rat model.

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2. Materials and methods

2.1. Collection and preparation of extracts

The *Acorus calamus* rhizome powder was collected from the Puducherry local market, Puducherry, India. The powder was authenticated by Prof. Parthasarathy, Department of Ecology and Environmental Science, Pondicherry University, India. The dried powder was mixed with deionized water and the mixture was filtered through Whatman No. 1 filter paper. The water from the filtered solution was evaporated using a rotary evaporator followed by lyophilizer in order to obtain the residue. The phytochemical screening was performed using the residue.

2.2. GC–MS analysis

The phyto-constituents of ACRE were tested using GC–MS (GC and MS JEOL GC mate supplied with the secondary electron multiplier). The interpretation of GC–MS spectra was performed by comparing the data of National Institute Standard and Technology (NIST) database.

2.3. Synthesis of AgNPs

The extract was prepared by mixing the 1 g of the dry powder of ACRE in 100 mL of double distilled water and then kept in boiling water bath for 15 min. The Whatman No. 1 filter paper was used for filtering the extract which was then stored at 4 °C for further use. The NPs were synthesized by mixing different ratios of 1 mM silver nitrate and prepared extract at room temperature. The color change observed in the mixture is an indicative of formation of NPs. The formation of AgNPs was further confirmed through periodical observation of absorption of the solution using UV-visible spectroscopy in the range of 300–700 nm. The synthesized *Acorus calamus* silver NPs (ACAgNPs) were collected by using repeated centrifugation at 18000 rpm for 25 min. The obtained pellet was further repeatedly washed three times using double distilled water in order to remove the unbounded biomolecules which may be the secondary metabolites or the proteins. The purified AgNPs pellet was dried at room temperature and their characterization was performed by using different techniques.

2.4. Characterization of AgNPs

The formation of ACAgNPs was determined using UV-visible spectrophotometer (UV-1700 Shimadzu), with optical density measurements executed at various time intervals between the wavelength ranges of 300–700 nm. The baseline was corrected using double distilled water. The micrograph image and elemental composition of the ACAgNPs were determined by TEM along with EDAX (JEOL 3010). The sample was prepared by placing dry powdered AgNPs on the carbon coated copper grid, which have been dried under a mercury lamp for 5 min. The excess powder was removed by using tissue paper. The functional groups present in the ACRE and ACAgNPs were identified by FTIR spectroscopy (Thermo Nicolet Nexus 670 equipped with KBr optics and a DTGS detector). The instrument was operated with a resolution of 4 cm⁻¹ and scanned between the frequency ranges of 500–4000 cm⁻¹. The average hydrodynamic diameter and polydispersity nature of ACAgNPs were determined by the DLS particle size analyzer (ZETA Sizers Nanoseries). The zeta potential of the ACAgNPs in pure water was further analyzed by using electrophoretic light scattering at 25 °C in 150 V (Malvern Instruments Nano ZS).

2.5. Catalytic activity

The catalytic role of ACAgNPs was assessed using 4-nitrophenol (4-NP), 3-nitrophenol (3-NP), 2, 4, 6-trinitrophenol (2, 4, 6-TNT) or picric acid (PA), coomassie brilliant blue (CBB), congo red (CR), eosin Y (EY),

rhodamine B (RB), methylene blue (MB), methyl red (MR), methyl orange (MO), cresol red (CRR), acridine orange (AO), eriochrome black T (EBT), and phenol red (PR). Briefly, the experiment was performed by mixing 10 mM of 0.3 mL of 4-NP, 1.7 mL of deionized water, 1 mL of 150 mM NaBH₄ and 15 µg/mL of ACAgNPs. The 10 mM of 30 µL picric acid (PA) or 2, 4, 6-TNT was mixed with 1.97 mL of deionized water, 1 mL of 150 mM NaBH₄ and 15 µg/mL of ACAgNPs. Similarly the 10 mM of 100 µL 3-NP, CBB, CR, EY, RB, MB, MR, MO, CRR, AO, EBT, and PR were individually mixed with 1.9 mL of deionized water, 1 mL of 150 mM NaBH₄ and 15 µg/mL of ACAgNPs. The absorption of the mixture was monitored periodically with different time intervals between the ranges of 200 to 800 nm using UV-visible spectrophotometer.

2.6. Cell culture

The Hep2 (human epidermoid carcinoma), COLO 205 (human colon adenocarcinoma) and SH-SY5Y (neuroblastoma) cancer cells were cultured in Dulbecco's modified Eagle medium with L-glutamine and 1000 mg/L glucose (DMEM) which was supplemented with 10% fetal bovine serum, streptomycin sulfate (0.1 mg/mL) and penicillin G (100 units/mL) in the humidified environment consisting of 5% CO₂ at 37 °C.

2.6.1. MTT [(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay

The anti-proliferative ability of ACAgNPs on Hep2, COLO 205 and SH-SY5Y cancer cells was assessed based on the previously reported by MTT assay [16]. The cell viability was determined by the following equation and then IC₅₀ values were calculated.

$$\text{Percentage of viability} = \frac{\text{Absorption (test)} \times 100}{\text{Absorption (control)}}$$

2.6.2. Cytomorphological analysis

The cultured more susceptible Hep2 cells were seeded (1 × 10⁵ cells/well) in a 12 well chamber plate and maintained for 12 h in a humidified environment consisting of 5% CO₂ at 37 °C. The seeded cells were then treated with ACAgNPs with their respective IC₅₀ values and then maintained for 24 h. The cytomorphology of the treated and untreated cells was examined in an OPTIKA (Italia) inverted phase-contrast microscope.

2.6.3. Acridine orange/ethidium bromide (AO-EB) staining

The induction of apoptosis by ACAgNPs in Hep2 cells was examined using AO/EB double staining technique. The cultured Hep2 cells were seeded (1 × 10⁵ cells/well) in a 12 well chamber plate and maintained for 12 h in a humidified environment consisting of 5% CO₂ at 37 °C. Then the seeded cells were treated with the respective IC₅₀ values of ACAgNPs and incubated at 37 °C for 24 h. Finally, 50 µL of dye mixture (AO-EB) was added to the cells and observed under fluorescence microscope (Nikon Eclipse Ti Japan) to detect any apoptotic cell death.

2.6.4. Detection and quantification of intracellular ROS

In this assay, Hep2 cells (1 × 10⁵ cells/well) were plated in a 6 well plate and treated with respective IC₅₀ value of ACAgNPs for 24 h. Following treatment, 10 µM of DCFH-DA was added and then kept for 30 min incubation at 37 °C. The treated and untreated cells were observed under the fluorescence microscopy (Nikon Eclipse Ti Japan) to detect any ROS production. Similarly, ACAgNPs treated cells were trypsinized and separately collected in aluminum foil wrapped Eppendorf tubes for ROS quantification. The DCFH-DA (25 µM) solution was added to cells followed by incubation for 45 min at 37 °C. The intensity of fluorescence was noted by using the Fluorolog-FL3-11 spectrofluorometer (HORIBA JobinYvon, NJ, USA) with respective excitation and emission wavelengths.

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