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RESEARCH ARTICLE

ANTIHELMINTHIC, ANTICANCER, ANTIOXIDANT ACTIVITY OF SILVER NANOPARTICLES ISOLATED FROM *F.oxysporum*

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Abstract

Nanoparticles have shown exceptional electronic, catalytic, optical, magnetic and other physical and chemical properties that are quite different from the bulk one. Silver nanoparticles have proven useful in antibacterial clothing, burn ointments and as coating for medical devices because of their mutation resistant antimicrobial activity. To fulfill the growing need of environmental friendly nanoparticles, researchers are using microorganisms for the synthesis of various silver nanoparticles of *F. oxysporum* has been reported to possess potent anthelmintic, anticancer and antioxidant activity .

Keywords: Silver Nanoparticles, anthelmintic, anticancer and antioxidant activity .

Introduction

Human beings are often infected by microorganisms such as bacteria, molds, yeasts, and viruses present in their living environments. Because of the emergence and increase in the number of multiple antibiotic-resistant microorganisms and the continuing emphasis on health-care costs, many scientists have researched methods to develop new effective antimicrobial agents that overcome the resistances of these microorganisms and are also cost-effective. Such problems and needs have led to resurgence in the use of silver-based antiseptics that may be linked to a broad-spectrum activity and considerably lower propensity to induce microbial resistance compared with those of antibiotics. In particular, silver ions have long been known to exert strong inhibitory and bactericidal effects as well as to possess a broad spectrum of antimicrobial activities. Silver ions cause the release of K+ ions from bacteria; thus, the bacterial plasma or cytoplasmic membrane, which is associated with many important enzymes and DNA, is an important target site of silver (Abhilash *et al.,* 2011) .

Biological methods have emerged as an alternative to the conventional methods for synthesis of NPs. Synthesis of inorganic nanoparticles by biological systems makes nanoparticles more biocompatible and environmentally benign. Moreover, the process is cost effective too. Many bacterial as well as fungal species have been used for silver nanoparticles synthesis (Ahmad A *et al*., 2002). But most of them have been reported to accumulate AgNPs intracellularly. Intracellular synthesis always takes longer reaction times and also demands subsequent extraction and

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recovery steps. On the contrary, plant extract mediated synthesis always takes place extracellularly, and the reaction times have also been reported to be very short compared to that of microbial synthesis. Most importantly, the process can be suitably scaled up for large scale synthesis of NPs (Arora *et al.,* 2008).

Cancer is an abnormal type of tissue growth in which the cells exhibit an uncontrolled division, relatively in an autonomous fashion, leading to a progressive increase in the number of dividing cell. There is increasing demands for anticancer therapy. *In-vitro* cytotoxicity testing procedures reduces the use of laboratory animals and hence use of cultured tissues and cells have increased (Hasan *et al.,*(2008). The discovery and identification of new antitumor drug with low side effects on immune system has become an essential goal in many studies of immuno-pharmacology. With this aim, many attentions have been paid to natural compounds in plants, marine organism and microorganisms.

Many medically relevant nanoparticles such as AgNPs were investigated for their cytotoxicity aspect. AgNPs showed different degrees of *in vitro* cytotoxicity . The Apoptotic Effect of Nanosilver is mediated by a ROS- and JNK-Dependent Mechanism Involving the Mitochondrial Pathway in NIH3T3 Cells . The present study was carried out to verify the possible cytotoxic action of AgNPs synthesized using *Gelidiella sp.* on HEp2 cells, evaluating morphology and the number of viable cells after incubation with the plant extract in different concentrations and duration of exposure.

Materials and Methods

Preparation of Fungal Extract

20 gm of fresh fungal mycelium were washed thoroughly with double-distilled water and were then cut into small pieces. These were then mixed with 100 mL doubled-distilled water, and this mixture was kept for boiling for a period of 5 minutes. After cooling, it was filtered through Whatman Filter paper no. 1.

Synthesis of AgNPs and Evaluation of Reducing Potential of the Extract

10 mL of aqueous extract of fungal mycelium was added to 90 mL of silver nitrate solution so as to make its final concentration to 10^{-3} M. The solution was allowed to react at room temperature. Periodic sampling after 30 minutes was carried out to monitor the formation of AgNPs. The qualitative evaluation of reducing potential of aqueous of *F.oxysporum* silver nanopartilces was carried out as per the method reported by Saifuddin et al.

Antihelminthic activity

Earthworms

Indian adult earthworms (*Pheretima posthuma*) collected from moist soil, washed with normal saline to remove the external matter and were used for anthelmintic activity. Adult Indian earthworms *Pheretima posthuma* of 3-4 cm in length and 0.1-0.2 cm in width were used for all the experimental protocol due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings .

Antihelmintic activity

Prepared *F.oxysporum* extract and silver nanoparticles were used for antihelmintic study. Solution of standard antihemintic drug (piperazine citrate, 10 mg/ml) was also prepared in distilled water. Normal saline is used as a control. Eight groups of approximately equal size of earthworms, consisting of six in number in each group, were released into each petridish.

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Observations were made for the time taken for paralysis and death of individual worms. Time for death of worms were recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water at 500C. The anthelmintic activity was evaluated by adopting the standard method.

Anticancer activity

In vitro assay for Cytotoxicity activity (MTT assay).

Hep-2 (Human Epidermoid larynx carcinoma) cell lines were were maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO2 at 37 °C. The Cytotoxicity of synthesized AgNPs on Human Epidermoid Larynx cancer cells was determined by the MTT assay (*Mosmann et al*., 1983). Cells (1 × 105/well) were plated in 100 µl of medium/well in 96-well plates. After 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48h at 37°C. After removal of the sample solution and washing with phosphatebuffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide cells (MTT) phosphate-buffered saline solution was added. After 4h incubation, 0.04M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570nm with reference at 655nm.

Measurements were performed in 3 times, and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The absorbance at 570 nm was measured with a UV spectrophotometer, using wells without sample containing cells as blanks. All experiments were performed in triplicate. The effect of the AgNPs on the proliferation of Human Epitheloid larynx cancer cells was expressed as the % cell viability, using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100%.

Evaluation of *in vitro* cytotoxic activity of the silver nanoparticles on cell lines

MTT assay was performed to determine the cytotoxic property of synthesized AgNPs against Hep-2, MF7, HT29 and Vero cell lines. Cell lines were seeded in 96-well tissue culture plates. Stock solutions of AgNPs (5 mg/ml) were prepared in sterile distilled water and diluted to the required concentrations (10, 5, 2.5, 1.25, 0. 625, 0.312, 0.156 mg/ml) using the cell culture medium. Appropriate concentrations of Ag-NP stock solution were added to the cultures to obtain respective concentration of Ag-NP and incubated for 48 hrs at 37°C. Non-treated cells were used as control. Incubated cultured cell was then subjected to MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) colorimetric assay [19]. The tetrazolium salt 3- [4, 5- dimethylthiazol - 2-yl]- 2,5-diphenyltetrazolium bromide (MTT) is used to determine cell viability in assays of cell proliferation and cytotoxicity. MTT is reduced in metabolically active cells to yield an insoluble purple formazon product. Cells were harvested from maintenance cultures in the exponential phase and counted by a hemocytometer using trypan blue solution. The cell suspensions were dispensed (100µl) in triplicate into 96-well culture plates at optimized concentrations of 1 × 105/well for each cell lines, after a 24- hr recovery period.

Assay plates were read using a spectrophotometer at 520 nm. The spectrophotometrical absorbance of the samples was measured using a microplate (ELISA) reader. The cytotoxicity data was standardized by determining absorbance and calculating the correspondent AgNP

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concentrations. Data generated were used to plot a dose-response curve of which the concentration of extract required to kill 50% of cell population (IC50) was determined.

Cell viability (%) = Mean OD/ control OD × 100

Following Ag-NP treatment, the plates were observed under an inverted microscope to detect morphological changes and photographed.

Antioxidant activity

Free radical scavenging activity of *silver nanoparticles*

Determination of total antioxidant capacity

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the procedure of Prieto et al., . The assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95○C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. For standard, L-Ascorbic acid was used as a control and prepared by dissolving 2mg of L-ascorbic acid in 10ml. The antioxidant activity of sample was expressed as %.

Iron reducing power assay

The Fe3+ reducing power of the of *F.oxysporum* silver nanopartilces was determined by the method of Oyaizu with slight modifications. The of *F.oxysporum* silver nanopartilces (0.75ml) at various concentrations was mixed with 0.75ml of phosphate buffer (0.2mole, pH 6.6) and 0.75ml of potassium hexacyanoferrate [K3Fe (CN) 6] (1%, w/v), followed by incubating at 50oC in a water bath for 20min. The reaction was stopped by adding 0.75ml of trichloro acetic acid (TCA) solution (10%) and then centrifuged at 3000r/min for 10min. 1.5ml of the supernatant was mixed with 1.5ml of distilled water and 0.1ml of ferric chloride (FeCl3) solution (0.1%, w/v) for 10min. The absorbance at 700nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Hydrogen peroxide scavenging activity assay

Hydrogen peroxide scavenging activity of the of *F.oxysporum* silver nanopartilces was estimated by replacement titration (Khan et al., 2011). Aliquot of 1.0ml of 0.1mmole of H2O2 and 1.0ml of various concentrations of *F.oxysporum* silver nanopartilces were mixed, followed by 2drops of 3% ammonium molybdate, 10ml of 2mole of H2SO4 and 7.0ml of 1.8mole KI. The mixed solution was titrated with 5.09mmole of NaS2O3 until yellow color disappeared. Percentage of scavenging of hydrogen peroxide was calculated as: % Inhibition = (V0 - V1) / V0 100 Where V0 was volume of NaS2O3 solution used to titrate the control sample in the presence of hydrogen peroxide (without of *F.oxysporum* silver nanopartilces), V1 was the volume of NaS2O3 solution used in the presence of the of *F.oxysporum* silver nanopartilces *rum* **.**

Results and Discussion

Antihelmintic activity analysis

Silver nanoparticles using *Saraca indica* leaves showed better anthelmintic activity when compared with the extract of *F.oxysporum* silver nanopartilces.

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Nanoparticles show least time to cause paralysis and death of worms followed by aqueous extract.Overall the anthelmintic activity revealed the concentration dependent nature of the extracts and silver nanoparticles. It was found that colloidal solution of silver nanoparticles using of *F.oxysporum* silver nanopartilces leaves possessed more anthelmintic From this study it may be concluded that, in addition to products of plants, silver nanoparticles using of *F.oxysporum* silver nanopartilces have more anthelmintic activity.

Anticancer activity

Cytotoxicity of silver nanoparticles

The *invitro* cytotoxicity of the AgNPs was evaluated Hep-2 cell lines at different concentrations. Our cytotoxicity analysis of the sample shows a direct dose-response relationship; cytotoxicity increased at higher concentrations. The IC50 value was plotted by taking the concentration of AgNPs on X-axis versus percentage of cell viability on Y-axis (Fig.7). The samples demonstrated a considerable cytotoxicity against the Hep-2 cell lines. The result showed that Hep2 cells proliferation were significantly inhibited by AgNPs with an IC50 value of 31.25 *µg/ml* of the concentration. In contrast the presence of 15.62*µg/ml of* AgNPs significantly inhibited the cell line's growth (> 60%). Previous study shows that phytochemicals depletes intracellular antioxidants thereby induced cancer cell death (Kleemann, 1978).

In vitro cytotoxic activity against Hep2, MCF7, HT29 and Vero cell line at different concentrations was evaluated and compared with the standard drug 5-fluorouracil. The *in vitro* screening of the AgNPs showed potential cytotoxic activity against the human laryngeal cancer (Hep-2) cell line, human breast cancer (MCF 7) cell line and human colon cancer (HT 29) cell line.

The plates were observed under an inverted microscope to detect morphological changes. The result showed that Hep2 cells proliferation were significantly inhibited by AgNPs with an IC50 value of 12.5µg/ml of the concentration, MCF7 cells with an IC50 value of 37µg/ml of the concentration and HT29 cells with an Iwith an IC50 value of 49µg/ml of the concentration. Thus the synthesized nanoparticles were found to be potently cytotoxic agent against Hep 2 cell lines and mildly cytotoxic against MCF 7 and HT 29 cell lines. These results indicate that the sensitivity of human cancer cell line for cytotoxic drugs is higher than that of Vero cell line for the same cytotoxic agents. There are reports that marine macroalgae belonging to Phaeophyta group possess antitumor activity, and sterols from of *F.oxysporum* silver nanopartilces exhibited cytotoxic activity against several cultured cell lines (Mohamed , 2007). Several cytotoxic compounds such as fucoidans, laminarians, and terpenoids stated to posses anticancer, antitumor, antibacterial and anti-proliferative properties are reported to be abundant in seaweeds (Kandile et al., 2009). These compounds could be further explored as novel leads to cancer chemoprevention and chemotherapy and necessitates further investigation (Mohamed et al., 2005).

Cytotoxic activity

Colony formation assay

This assay identifies the cell populations that are destined to die or survive following a cytotoxic drug treatment. Cells were seeded at a density of 2.5×10^4 cells in T-25 flasks and treated with 0, 25, 100, 200 and 400 µg/mL of Ag-np and incubated for 48 hours. At the end of the incubation period, medium containing Ag-np was replaced with fresh medium and formation of colony and recovery period were recorded. Two experiments were conducted for fibroblasts

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to ensure complete recovery from Ag-np induced stress. In the first batch of experiments fibroblasts recovered were subjected to cell cycle analysis (Duran et al., 2005). Second set of recovered cells were assayed for the concentration of silver inside the cells.

Antioxidant activity

Total antioxidant capacity of *F.oxysporum* is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract is in the increasing trend with the increasing concentration of the silver nanopartilces . The observed scavenging effect AgNPs and standard on the total antioxidant activity decreases in the following order: AgNPs >L ascorbic acid > plant extract. Among this AgNPs possess potential antioxidant activity as compared with ascorbic acid. Positive Hydrogen peroxide tests demonstrated that AgNPs and ascorbic acid are free radical scavengers. The Hydrogen peroxide scavenging assay exhibited effective inhibition activity of AgNPs and ascorbic acid. The Hydrogen peroxide activity of the nanoparticles was found to increase in a dose-dependent manner. The identification of antioxidant is beneficial to biological system against ROS ravage. Recently importance has been given for *in vitro* antioxidant study to understand the pharmacological role of medicinal plant and its isolate. *In vitro* techniques have been used for detection of antioxidants, which are based on the ability of compounds to scavenge peroxy radicals (Duran,et al., 2005).

Total antioxidant activity

Total antioxidant capacity of *F.oxysporum* silver nanopartilces is expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to *F.oxysporum* silver nanopartilces (Vigneshwaran, et al., 2001). Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract is in the increasing trend with the increasing concentration of the plant extract, ascorbic acid and AgNPs. Among this AgNPs possess potential antioxidant activity as compared with *F.oxysporum* silver nanopartilces.

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H2O2 can probably react with Fe2+, and possibly Cu2+ ions to form hydroxyl radical and this may be the origin of many of its toxic effects (Cho et al., 2005). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The study reveals that hydrogen peroxide scavenging activity of the *F.oxysporum* silver nanopartilces is in the increasing trend with the increasing concentration of the plant extract, ascorbic acid and AgNPs. Among this AgNPs possess hydrogen peroxide scavenging activity as compared with *F.oxysporum* silver nanopartilces.

Iron reducing power activity

For the measurements of the reducing ability, the Fe3+–Fe2+ transformation was investigated in the presence of *F.oxysporum* silver nanopartilces. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, reductive capacity and radical scavenging (Wright, 2000, 2005). The study reveals that reducing power activity of the extract is in the increasing trend with the increasing concentration of the plant extract, ascorbic acid and AgNPs. Among this AgNPs possess potential reducing activity as compared with plant extract. The present study concluded that the bio-reduction of silver ions through *F.oxysporum* silver nanopartilces flower extract and testing for their *in vitro* antioxidant activity.

On the basis of the results of this study, it clearly indicates that *F.oxysporum* silver nanopartilces possessing antioxidant activity against various *in vitro* antioxidant systems. The free radical scavenging activity of AgNPs was found to be higher than that standard confirmed in the present investigation. From the above assays, the possible mechanism of antioxidant activity of AgNPs includes reductive ability, electron donating ability and scavengers of radicals. These obtained silver nanoparticles are advantageous in medical and pharmaceutical purposes. It also has potential applications in the biomedical field and can be produced commercially at large scale.

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