International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.8, No.5, pp 1011-1017, 2015

In vitro cytotoxicity of biosynthesized Ag/CS NP against MCF7, PC3 and A549 Cancer cell lines

Osman M.E.[1], Eid M.M.[2*], Khattab O.H.[1], El-Hallouty S.M.[4], El-Manawaty M.[4], Mahmoud D.A.[3]

[1]Botany and Microbiology Department, Faculty of Science, Helwan University, Cairo, Egypt.
[2]Spectroscopy Department, National Research Centre, 16226, Dokki, Giza, Egypt.
[3]Microbiology Department, National Organization for Drug Control and Research, Cairo, Egypt.
[4]Pharmacognosy Department, National Research Centre, 16226, Dokki, Giza, Egypt.

Abstract: We aim in this paper to verify the anti-cancer effect of biosynthesized silver/chitosan nanoparticles (Ag/CS NPs) on three different cell lines; Human cancer cell lines which used in this study were: MCF7 (Human Breast carcinoma cell line), PC3 (Human prostate carcinoma cell line) and A549 (Human Lung carcinoma cell line), to determine the sensitivity of these cell lines to the Ag/CS NPtreatment. In a previous part of this work, we identified the most efficient fungal isolates for the biosynthesis of Ag/CS NPs. Followed by the spectroscopic characterization of the physical properties and molecular structure of the nanoparticles, we reported Aspergillus and Pincilluim as the best two species for the biosynthesis of stable crystalline Ag/CS NPs with particle size ranging from 15-40nm. In this paper, after optimizing the pH and salt concentration, the Ag/CS NPs *from Aspergillus deflectus* under optimized condition *(OA), Aspergillus deflectus* under irradiated condition *(IOA), Penicillium pinophilum* under optimized condition(OP)and *Penicillium pinophilum* under irradiated condition (IOP) were tested for *In Vitro* cytotoxicity. Data show that the sensitivity of the lung cancer cell line was the highest for the four studied samples comparing to the breast and prostate cell lines.

Keywords: MCF7, PC3, A549, Ag/CS NPs.

Introduction

Nanomedicine is a burgeoning field of research with tremendous prospects for the improvement of the diagnosis and treatment of human diseases [1]. Dispersed nanoparticles are usually employed in nanobiomedicine as fluorescent biological labels [2], drug and gene delivery agents [3,4], and in applications such as biodetection of pathogens [5], tissue engineering [6,7], tumor destruction via heating (hyperthermia) [8], MRI contrast enhancement [9], and phagokinetic studies [10].

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. There is increasing demands for anticancer therapy [11]. *In vitro* cytotoxicity testing procedures reduces the use of laboratory animals [12] and hence use of cultured tissues and cells have increased [13]. The discovery and identification of new antitumor drug with low side effects on immune system has become an essential goal in many studies of immune-pharmacology [14]. 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 [15]. The Apoptotic effect of Nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells [16].

Silver Nanoparticles displays a synergistic effect [17] and also a cytotoxic effect on cell viability which have a chief role in antitumor and anticancer effect [18]. Silver nanoparticles aid in gathering and transporting drug into the cancer cells and they also obstruct with metabolism of cancer and tumor proliferation[19]. In previous work, synthesis[20], optimization of Ag/Cs NPs has been studied[21], inthis paper we evaluate the role of AG/CS NPs in enhancing the cytotoxic effect on breast, lung and hepatic cancer cell lines.

Material and Methods

Production of biomass

Aspergillusdeflectus and *Penicillium pinophilum* were isolated from soil samples collected from local areas in Egypt. Flasks containing potato dextrose broth were inoculated with tested fungal isolates at 25^{o}C, under shaking condition (180 rpm).

Synthesis of silver nanoparticles using silver nitrate

After incubation the culture was filtered (Whitman filter paper No.1) to separate the biomass from filtrate. Detection of silver nanoparticles was carried out in filtrate as well as the developed biomass. Then biomass was extensively washed with distilled water to remove any component. 1mM $AgNO_3$ was incubated with the filtrate of culture broth and the biomass of *Aspergillusdeflectus* and *Penicillium pinophilum* respectively in shaking incubator for 72 hs.

Cells and culture conditions:

Human cancer cell lines which used in this study were: MCF7 (Human Breast carcinoma cell line), PC3 (Human prostate carcinoma cell line) and A549 (Human Lung carcinoma cell line). These cell lines were grown as monolayer culture in (RPMI-1640 medium For MCF-7and DMEM for PC3 &A549) supplemented with 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B), 1% L-glutamine and supplemented with 10% heat inactivated fetal bovine serum. The cell lines were incubated at 37^{o}C in 5% CO_2-95% air in a high humidity atmosphere in the water-jacketed incubator (Revco, GS laboratory equipment, RCO 3000 TVBB, USA.).

It was regularly subcultured to maintain it in the exponential growth phase. Subcultures are done every 3 days by removal of culture medium, suspending the cell layer with 0.15% (w/v) Trypsin - 0.04% (w/v) EDTA solution. 2 ml of Trypsin solution were added to flask 1-5 min incubation during which cells are observed under an inverted microscope until cells are suspended. Complete growth medium was added, cells were aspirated by gently pipetting and appropriate aliquots of the cell suspension were added to new culture flasks. The sterile conditions were strictly attained by working under the equipped laminar flow (Micro flow Laminar flow cabinet, biosafety class II level).Cell Viability Assay: Chemicals:

MTT solution: 5.00 mg/ml of MTT in 0.9% NaCl for cytotoxicity assay.
Sodium chloride (NaCl): it was used in a concentration of 0.9 % as a buffering system and as solvent for silver nanoparticles.

Cancer cell lines (10X10^3 cells / well) were placed in a flat bottom 96-well microtiter plate, Cells were treated with silver nanoparticles to give a final concentration of 100 µg/ml for primary screening and incubated for 24 hr at 37ºC, in a humidified 5% CO_2 atmosphere.

Medium was aspirated,after 24 hr of incubation with silver nanoparticles, 40 µl MTT salt (2.5 µg/ml) were added to each well and incubated for further four hours at 37ºC under 5% CO_2.

Wells were then solubilized by added 200 µL of 10% Sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37 ºC. The color was developed and measured using a multi-well microplate ELISA reader at 595 nm and a reference wavelength of 620 nm.Control cells were treated

with vehicle alone. For each compound concentration, 3 wells were used (triplicate wells were prepared for each individual dose). The average was calculated.

The silver nanoparticles which gave 75% - 100% cytotoxicity in the primary bioassay were subjected to secondary screening for the determination of their IC_{50} and IC_{90} using different descending concentrations (50, 25 and 12.5 µg/ml).

The half maximal inhibitory concentration IC_{50} was then determined by statistical methods probit analysis (SPSS program for windows, statistical analysis software package / version 9 / 1989 SPSS Inc., Chicago, USA). Silver nanoparticles were arranged in a descending order and the most effective one having the least IC_{50}, it was selected for *in vitro* bioassay-guided fractionation.Percentage of survival fraction was calculated using the following equation:

[Absorbance of treated cells / Absorbance of control cells] X 100
The inhibition rate was calculated according to the formula below:
Inhibition rate (%) =1- ($Abs_{treated}/Abs_{control}$) × 100.

Results

***In vitro* cytotoxicity assay:**

Many medically relevant nanoparticles such as Ag/CS NPs were investigated for their cytotoxicity aspect. Ag/CS NPs showed different degrees of *in vitro* cytotoxicity and inhibited the growth of cancer cells significantly, in a dose- and duration dependent manner.

The cytotoxic effect of the silver nanoparticles was evaluated *in vitro* against three human carcinoma cell lines. Cytotoxicity of the tested Ag/CS NPsagainst three cancer cell lines is shown in tables (1, 2, 3 and 4). Cytotoxicity was measured and expressed as the survival fraction compared with untreated control cells, where the inhibition ratio was determined. The cytotoxic activity was determined according to the dose values of the exposure of the complex required to reduce survival to 50% (IC_{50}), compared to untreated cells, so IC_{50} was determined from a range of concentrations as shown in figures (1, 2, 3 and 4).

Table 1 Survival ratios and inhibition ratios of different cancer cell lines with silver nanoparticles of *A. deflectus* under optimized conditions at concentrations range from 12.5 to 100µg/ml.

Concentration (µg/ml)	MCF7		PC3		A549	
	Cell viability (control %)*	Inhibition ratio %	Cell viability (control %)*	Inhibition ratio %	Cell viability (control %)*	Inhibition ratio %
12.5	59.3[a]	20.7	76.0[a]	24.0	0.0	100
25	40.0[b]	60.0	74.0[b]	26.0	0.0	100
50	22.5[c]	78.5	22.0[c]	78.0	0.0	100
100	11.3[d]	88.7	2.0[d]	98.0	0.0	100
IC_{50} (µg/ml)	27.9		53			

*: Means in the same column with different letters have significant differences between each other (P<0.05).

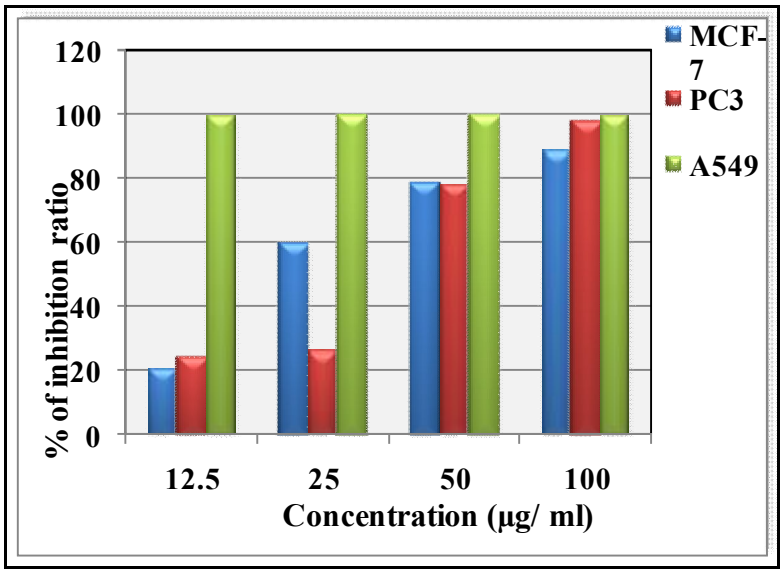

Figure 1 Inhibition ratios of different cancer cell lines with silver nanoparticles of *A. deflectus* under optimized conditions at concentration range from 12.5 to 100 µg/ml.

Table 2 Survival ratios and inhibition ratios of different cancer cell lines with silver nanoparticles of *A. deflectus* under irridated conditions at concentrations range from 12.5 to 100 µg/ml.

Concentration (µg/ml)	MCF7		PC3		A549	
	Cell viability (control %)*	Inhibition ratio %	Cell viability (control %)*	Inhibition ratio %	Cell viability (control %)*	Inhibition ratio %
12.5	95.9[a]	4.1	100[a]	0.0	83.5[a]	16.5
25	31.8[c]	68.2	85.0[b]	15.0	0.0	100
50	33.0[b]	77.0	39.0[c]	61.0	0.0	100
100	21.3[d]	88.7	8.0[d]	92.0	0.0	100
IC_{50} (µg/ml)	32.6		52.4			

*: Means in the same column with different letters have significant differences between each other (P<0.05).

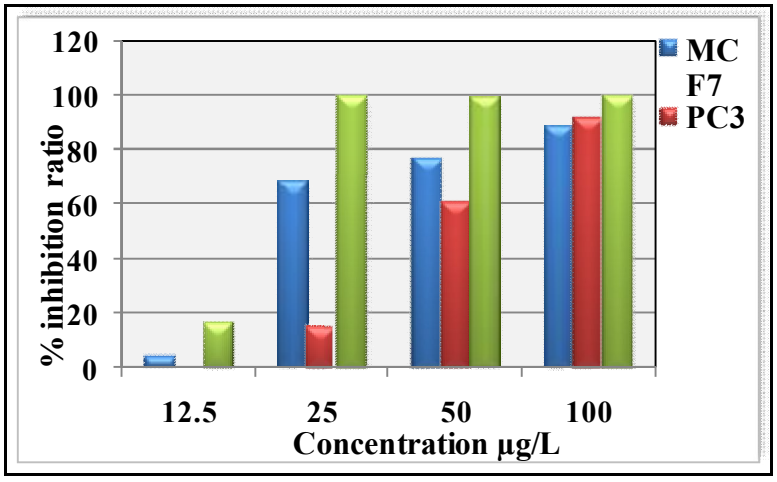

Figure 2 Inhibition ratios of different cancer cell lines with silver nanoparticles of *A. deflectus* under irradiated condition at concentration range from 12.5 to 100 µg/ml.

Table 3 Survival ratios and inhibition ratios of different cancer cell lines with silver nanoparticles of *P. pinophilum* under optimized conditions at concentrations range from 12.5 to 100µg/ml.

Concentration (µg/ml)	MCF7		PC3		A549	
	Cell viability (control %)*	Inhibition ratio %	Cell viability (control %)*	Inhibition ratio %	Cell viability (control %)*	Inhibition ratio %
12.5	100[a]	0.0	100[a]	0.0	100[a]	0.0
25	100[a]	0.0	100[a]	0.0	100[a]	0.0
50	100[a]	0.0	100[a]	0.0	100[a]	0.0
100	100[a]	0.0	100[a]	0.0	64.9[b]	35.1
IC_{50} (µg/ml)	No cytotoxic effect		No cytotoxic effect		Low cytotoxic effect	

*: Means in the same column with different letters have significant differences between each other (P<0.05).

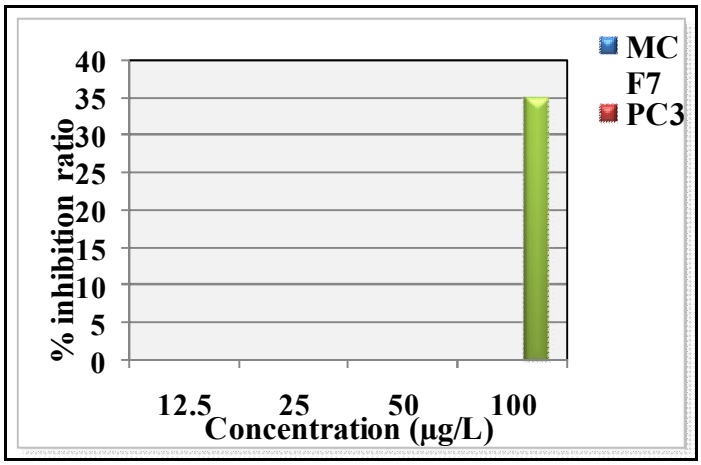

Figure 3 Inhibition ratios of different cancer cell lines with silver nanoparticles of *P. pinophilum* under optimized condition at concentration range from 12.5 to 100 µg/ml.

Table 4 Survival ratios and inhibition ratios of different cancer cell lines with silver nanoparticles of *P. pinophilum* under irridated conditions at concentrations range from 12.5 to 100µg/ml.

Concentration (µg/ml)	MCF7		PC3		A549	
	Cell viability (control %)	Inhibition ratio %	Cell viability (control %)	Inhibition ratio %	Cell viability (control %)	Inhibition ratio %
12.5	95.2[a]	4.8	100[a]	0.0	52.6[a]	47.4
25	77.3[b]	22.7	100[a]	0.0	4.2[b]	95.8
50	72.74[c]	27.6	100[a]	0.0	0.0	100
100	56.5[d]	43.5	100[a]	0.0	0.0	100
IC_{50} (µg/ml)	107.8		No cytotoxic effect		12.9	

*: Means in the same column with different letters have significant differences between each other (P<0.05).

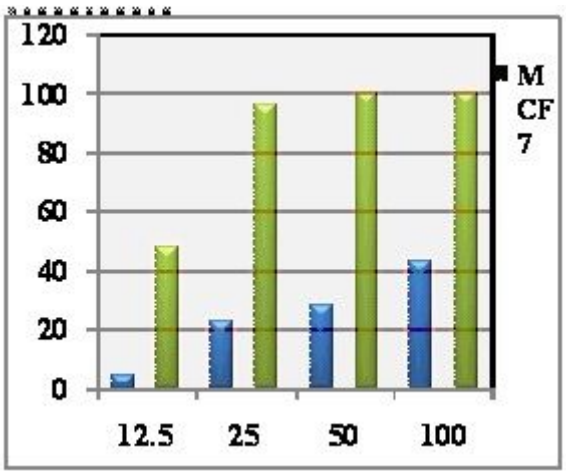

Figure 4 Inhibition ratios of different cancer cell lines with silver nanoparticles of *P. pinophilum* under irradiated condition at concentration range from 12.5 to 100µg/ml.

Discussion

Accordingly, *in vitro* inhibition ratio of MCF7 breast cell line by Ag/CS NPs of (*Aspergillus deflectus* under optimized condition *(OA), Aspergillus deflectus* under irradiated condition *(IOA), Penicillium pinophilum* under optimized condition(OP)and *Penicillium pinophilum* under irradiated condition (IOP)) at different concentrations (12.5, 25, 50 and 100 µg/ml) were studied. All Ag/CS NPs exhibited strong inhibition against cell growth at all concentrations except Ag/CS NPs of (OP). In addition, dose–response relationship between concentration of Ag/CS NPs and suppression of MCF7 cell proliferation was observed. The Ag/CS NPs of *Aspergillus deflectus* under optimized condition(OA)showed the highest significant cytotoxic effect (88.0 %) at concentration 100 µg/ml as compared with other AgNPs of the tested fungal strains.

Only the silver nanoparticles of *A. deflectus* under both condition (OA & IOA) revealed strong inhibitions against PC3 prostate cell line with inhibition ratios of 98.0 % and 92.0 % respectively at concentration of 100µg/ml, while silver nanoparticles of *P. pinophilum* (OP & IOP) not have any cytotoxic effect for all concentrations. The silver nanoparticles of *A. deflectus* under optimized and irradiated conditioninhibited the cell growth with IC_{50} values of 35µg/ml and 52.4µg/ml respectively

Also, the treatment of A549 lung cell line with the Ag/CS NPs at different concentrations revealed that all silver nanoparticles exhibited strong inhibitory effect against cell growth at all concentrations. Silver nanoparticles exhibited high inhibition ratios of 100 %, 100 %, 100 %, 35 % at the concentration of 100 µg/ml for (OA, IOA, IOP and OP)respectively.

The results suggested that silver nanoparticles of OA and IOA had the lowest IC50 and potential investigation for antitumor activities.This is an important observation, given that effectiveanticancer drug should demonstrate tumor specificity. Further studies are also in process to evaluate the efficacyof these two nanoparticles as an anticancer agent in animal models.

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