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ORIGINAL ARTICLE

Medicine Science International Medical Journal

Medicine Science 2020;9(3):760-5

Determination of Antioxidant and cytotoxic activities of king oyster mushroom mediated AgNPs synthesized with environmentally friendly methods

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Received 04 Februray 2020; Accepted 07 March 2020
Available online 26.08.2020 with doi: 10.5455/medscience.2020.02.014

Abstract

Synthesizing nanoparticles (NPs) using wild edible fungi with environmentally friendly synthesis methods is more preferred because of the advantages it provides. The fact that its synthesis is easy, economical, non-toxic and has a wide range of uses increases the interest in this subject. The aim of this study was to determine the antioxidant and cytotoxic activity of biomolecular synthesized *Pleurotus eryngii* silver nanoparticles (PE-AgNPs) against human prostate carcinoma (PC-3), human cervix (HeLa) and breast carcinoma (MCF-7) cells. PE-AgNPs showed significant cytotoxic activity against HeLa, PC-3, MCF-7 cell lines, and also dimethyl sulfoxide solvents of PE-AgNPs applied for their metal chelating activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity and antioxidant activity using the β-carotene linoleate model system. Biosynthetic PE-AgNPs were found to inhibit the proliferation of PC-3, HeLa and MCF-7 cells with IC_{50} values of 2.185, 46.594 and 6.169 µg / ml, respectively, during a 24-hour incubation period. With the parallel of increasing concentration (1, 2, 5, 10 mg/mL) the activities were also increased at all the tests studied. At 10 mg/ml antioxidant activities were 82%, 85% and 77% for chelation of ferrous ions reducing power, DPPH scavenging and β-carotene linoleate tests respectively. The results show that PE-AgNPs may contribute to the development of a suitable anticancer drug that can lead to a new development of nanoparticles for cancer treatment. It also appears to be advantageous to use nanotechnology and green chemistry to improve the existing therapeutic properties of *P.eryngii.*

Keywords: Metal nanoparticle, *Pleurotus eryngii*, HeLa, PC3, MCF-7, Antioxidant.

Introduction

The distinctive physical, chemical and biological properties of nanoparticles, which have the potential to be used in the fields of electronics, agriculture, medicine and health, have attracted worldwide attention to the nanoparticles [1].

It is stated that there are many Chemical methods for the synthesis of nanoparticles. In these methods, it is seen that organic solvents such as sodium borohydride, trisodium citrate, thiophenol, thiourea, macro capto acetate N-N dimethylformamide are used as chemical reducing agents [2,3].

One of the challenges faced by scientists is to create new nanoparticles that do not contain toxic chemicals to eliminate risks in the cosmetic, pharmaceutical and biomedical fields. The use of these reducing agents as toxic, flammable and environmentally malignant increases interest in the design of environmentally sensitive and biocompatible nanoparticles to find new and safe biomaterials [4].

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Plants, herbal products, bacteria, fungi, algae, yeasts and viruses have been used from various natural sources for the synthesis of NPs by biosynthetic methods [5-7]. Edible mushrooms, which have different application areas such as therapeutic, bioremediation, bioleaching and biocatalysis, are popular in NP synthesis because of their natural biomolecules as flavonoids, phenolic acids, tannins and oxidized polyphenols. [8,9].

It has also been reported that the high protein content in the mushroom extract and the carbonyl groups of amino acids can prevent the agglomeration of NPs and thereby stabilize them in the aqueous solution[10].

It is known that bacteria are widely used in the synthesis of metal nanoparticles. however, enzymes secreted by fungal micelles, biomolecules and the bio-potential of the fungal cell wall offer a large surface area for interaction. This contributes to the absorption and reduction of metal ions and the conversion of metal salts to metal nanoparticles. This makes the mushrooms advantageous for metal nanoparticle synthesis. [11]. The most prominent nanoproduct among several nanoproducts is silver nanoparticles. In the literature, AgNPs have been reported to have antimicrobial, antioxidant, anti-inflammatory and antifungal effects [7,12,13].

It is seen in the literature that fungal extracts such as *Volvariella volvacea, Pleurotus sajor Caju, Pleurotus Florida, Ganoderma lucidum, Agaricus bisporus, Inonotus Obliquius* are used in the production of AgNPs [14]. There are also many reports about nanoparticle synthesis using fungi [15].

Cancer is the most important cause of death in the world. The frequency of cancers in the world is of concern. The most common cancer in women is breast cancer while in men is prostate cancer. Another type of cancer that is common in women is cervical cancer [16]. A wide variety of cytotoxic agents such as doxorubicin, cisplatin and bleomycin are used in breast cancer and the others. However, there are drawbacks in their use and are not as efficient as expected [17]. It is stated that many cancers respond to chemotherapy in the first place, but later develop resistance [18,19]. Existing chemo preventives and chemotherapeutic agents appear to cause undesirable side effects and new methods need to be developed for biocompatible and cost-effective cancer treatment [20]. Therefore, finding new therapeutic agents against cancer is of great interest.

AgNPs have anti-cancer activity studies against different cancer cell lines performed by MTT method. [21-23]. AgNPs, which are generated by the continental methods, are less effective in the treatment of cancer than the AgNPs produced by biological methods. In the study, it was reported that the chemical synthesis of AgNPs was 2.8 times the IC_{50} of biosynthetic synthesis AgNPs (25 lg / ml) [24]. As a result, biologically synthesized AgNPs can be designed as an antitumor chemotherapeutic combination nano-drugs in the future. In literature, no data were found on HeLa, PC-3, MCF-7 cell lines and antioxidant activity by using PE-AgNPs. In the light of the above information, this study aimed to evaluate the antioxidant and cytotoxic activity by MTT analysis in HeLa, PC-3, MCF-7 cell lines using PE-AgNPs characterized according to Acay and Baran [25].

Materials and Methods

Mushroom

Pleurotus eryngii was used in the study. Mushroom, with fieldwork done in April-May 2018, was obtained from Hakkari/ Turkey. The samples obtained are stored in Mardin Artuklu University Microbiology-Biochemistry Research Laboratory.

Pleurotus eryngii silver nanoparticles (PE-AgNPs)

The synthesis and characterization of the AgNP using P. eryngii extract is reported in the previous study [25]. Briefly, 200 g of fresh mushrooms were cut into small pieces. After boiling at 90° C in 1000 ml pure water, it was left to cool at room temperature. Whatman No. 1 Hot water extract of the mushroom filtered with filter paper was then reacted with 1 mM $AgN0_3$ solution for use in AgNP synthesis. The extract and solution, mixed in a ratio of 1: 5, were left at room temperature for discoloration to occur. The conversion of the pale yellow solution to dark brown with the reduction of silver ions is considered as an indicator of the realization of nanoparticle synthesis [25]. The dark solution obtained was centrifuged at 10000 rpm for 5 minutes to remove the upper liquid phase. The remaining pellet was washed with distilled water until cleaned, then left to dry in the oven at 65 ° C for 48 hours. At the end of the procedure, AgNPs were provided and stored for characterization. Characterization of PE-AgNPs was performed according to the methods specified in the literature [26]. UV-Vis Spectrophotometer was used for primary characterization. FTIR analysis was analyzed with Perkin Elmer Spectrum One functional groups responsible for the reduction in synthesis. Evaluation of the particle element content was carried out with Bruker-125 eV (EDX). The appearance of AgNPs was checked by scanning electron microscope EVO 40 LEQ (SEM) data. Crystal structures were analyzed by RadB-DMAX II computer-controlled X-ray diffractometer (XRD) analysis, TGA-DTA decay temperature values were checked with Shimadzu TGA-50 device data.

Preparation of Cell Culture;

In the study, cell lines PC-3 *(CRL-1435, ATCC)*, HeLa *(CCL-2, ATCC)* and MCF7 *(HTB-22, ATCC)* supplied from the American Cell Culture Collection (ATCC) were used. Experiments İ.Ü. The Faculty of Pharmacy was carried out in the Cell Culture Laboratory of Pharmaceutical ToxicologyDepartment.

Serum media for PC-3 cell culture prepared 10 ml inactivated FBS (10%), 1 ml penicillin (100 U) / streptomycin (100 g / ml) solution (1%) with DMEM (Dulbecco's Modified Eagle Medium). Serum media for HeLa cell culture prepared 10 ml inactivated FBS (10%), 1 ml penicillin (100 U) / streptomycin (100 g / ml) solution (1%) with DMEM-F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12).Serum media for MCF7 cell culture prepared 10 ml inactivated FBS (10%), 1 ml penicillin (100 U) / streptomycin (100 g / ml) solution (1%) with EMEM (Eagle's Minimum EssentialMedium)

Determination of Cytotoxic effect of PE-AgNPs

Cytotoxic effect of fungus was determined according to the method of Alley et al [27] MTT test was used to evaluate in HeLa, PC-3, MCF-7 cell lines. MTT solution: 5 mg MTT was dissolved in phosphate buffer solution (CMFPBS) (pH = 7.0) without 1 ml divalent cations (Ca ++ and Mg ++). The solution was stored in the dark at 4° C. In the study, 24-h culture phases were applied in each well for HeLa, PC-3, MCF-7 cells in 200 µl medium with 105 cells. The 96-well microplate was incubated for 24 hours in a humidified incubator containing 5% CO_2 at 37° C. After 24 hours, the culture medium was removed from the wells using an 8-channel automatic pipette. 50 µl of PBS was added to the wells. 90 µl of fresh media was added to each well. Subsequently, 10 µl of the test substance was applied to the wells at a concentration of ½ percent dilution to the MTT test. Cytotoxicity tests were performed 7 times, 4 replicates for eachconcentration.

20 µl of MTT solution was added to each well of the 96-well microplate containing the cell line incubated for 24 hours at 37°C 5% CO_2 with fungus. After shaking at 150 rpm for 5 min, it was incubated at 37° C for 2-3 hours. The top liquid in the wells was discarded. 100 µl of DMSO was added to the wells. Shaken at 150 rpm for 5 min. The intensity of the resulting color was measured at 590 nm (versus 670 nm reference wavelength). By comparing the absorbance value of the tested compounds and the solvent control group, the number of cells that died in % (Inhibition concentration, IC) was calculated. The absorbance values of the wells (solvent controls) containing the solution of the test substance instead of

the test sample showed 100% viability. It was determined that DMSO used as 1% in the experiment had no cytotoxic effect on the cells. The absorbances at 590 nm for the MTT test were measured against the 690 nm reference wavelength. Corrected absorbance values were obtained by subtracting the blind absorbance from each solvent control and sample absorbance. Calculations were made by taking the average of absorbance values for repeats. in a micro plate. The relative inhibition activity (IC) was calculated according to the following formula (Eq.1) as a percentage of the solvent control;

% inhibition = 100 - (adjusted mean A sample x 100 / adjusted mean A solvent / positive control)(1)

% Viability was calculated using solvent control for MTT testing. The% inhibition curve against the exposure concentration was plotted. The concentration corresponding to 50% inhibition from the curve was determined as IC_{50}. Studies on cytotoxic activity were performed at the laboratories of Istanbul University Faculty of Pharmacy.

Antioxidant activities of DMSO-PE-AgNPs

DPPH activity, one of the antioxidant parameters, was measured according to the method determined by Hatano, Kagawa, Yasuhara and Okuda [28]. 1.0 ml 0.1 mM DPPH radical solution dissolved in methanol, the organic solvent, was treated with 0.5 ml DMSO-PE-AgNPs. In the experiment using various concentrations (1, 2, 5, 10 mg / ml), the reaction mixture was mixed and evaluated by measuring the absorbance at 520 nm. DPPH radical scavenging efficiency (%) was calculated by equation (Eq. 2):

I%=(A control − A sample)/A control × 100 (2)

where A control is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. The experiment was carried out in triplicate.

Total antioxidant activity was calculated by measuring with changes in the β-carotene linoleate model system. [29] In summary, the procedure was performed by adding 25 µL linoleic acid and 200 mg Tween 40 to 0.5 mg β-carotene solution dissolved in 1 ml of chloroform (HPLC grade). The chloroform was then completely evaporated and vigorously rinsed with 100 ml of oxygenated distilled water. Finally, 4.6 ml of the reaction mixture obtained was dispensed into test tubes.

The remaining 0.4 ml of various concentrations (1, 2, 5, 10 mg / ml) of DMSO-PEAgNP was added and the emulsion system was incubated (2h, 50◦C). At the end of this period, the absorbance of the mixes was measured spectrophotometrically at 490 nm. Absorbance measurement was repeated until color correction took place. Bleaching rate (R) of β-carotene, Eq. (3):

R = ln(a/b)/t (3)

where ln = natural log, a = absorbance at time 0, b = absorbance at time t (30, 60, 90, 120 min). The antioxidant activity (AA) was calculated in terms of percentage inhibition related to the control using Eq. (4):

AA = [(R control-R sample)/Rcontrol] × 100. (4)

The ability of DMSO-PE-AgNPs to chelate iron ions was achieved by modifying the method determined by Dinis et al. [30]. The sample (1-10 mg / L) in methanol (1 ml) was added to 3.7 ml methanol and mixed with 0.1 ml 2 mmol / L ferrous chloride.

The reaction started by adding 0.2 ml of 5 mmol / L ferrozine to the mixture was left to stand for 10 minutes at room temperature. At the end of the procedure, absorbances were measured against the blank at 562 nm. The data obtained were evaluated as the percent of inhibition of the formation of ferrozine-Fe^{2+} complex. The experiment was performed in three replicates and EDTA was used as a positive control. Percentage inhibition of the formation of the ferrozine-Fe^{2+} complex was calculated using the given formula (Eq. 5):

Chelating ability (%) = (A control − A sample)/A control × 100 (5)

Results

PE-AgNPs synthesized and characterized in the previous study [25] In the study, it is stated that PE-AgNPs are spherical and 18.45 nm in size. Here, the cytotoxic activity of the previously characterized nanoparticle in some cancer cells was evaluated. And also the potentialantioxidant capacity of PE-AgNPs was determined.

The cytotoxic activity of AgNPs was tested by MTT analysis against three human cancer cell lines (HeLa, PC-3, MCF-7) by measuring the number of live cells after 24 hours. Cell viability in all three of the cancer cells tested decreased with increasing concentration of AgNPs. Cytotoxic activity of PE-AgNPs in MCF-7 cell lines is shown in Table 1. In the study conducted on MCF-7 cell lines, the maximum inhibitory and minimum inhibitory effect was detected 93.89 % and 0.89 % at the concentration of 20 µg and 1.25 µg respectively.

The IC50 value was found to be 6,169 µg/ml (Table 1).

Table 1. Cytotoxic activity of PE-AgNPs in MCF-7 cell lines

Concentration (ug/ml)	% Inhibition
20	93.885
10	82.484
5	27.478
2.5	5.408
1.25	0.889
IC_{50}	**6.169**

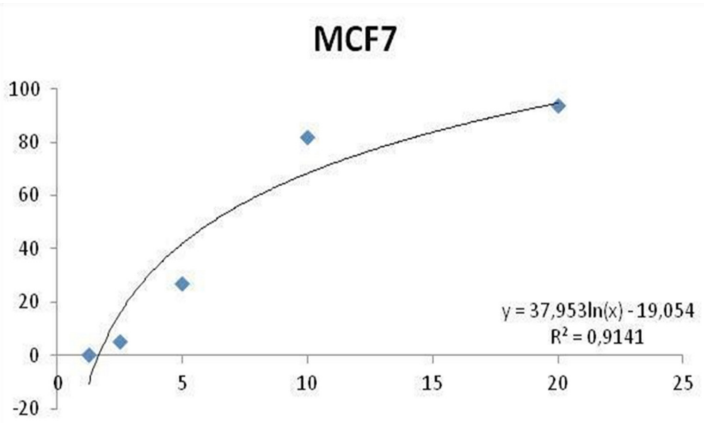

Figure 1. IC50 calculation curve for MCF-7 cell lines

Cytotoxic activity of PE-AgNPs in HeLa cell lines is shown in Table 2. As seen in Table 2, 73.46% inhibition was detected at 60 µg / ml concentration in HeLa cell lines. IC50 value was determined as 46,594 µg / ml.

Table 2. Cytotoxic activity of PE-AgNPs in HeLa cell lines

Concentration (ug/ml)	% Inhibition
60	73.468
50	52.129
40	33.637
30	29.139
20	18.318
15	4.498
10	5.715
IC	*46.5*

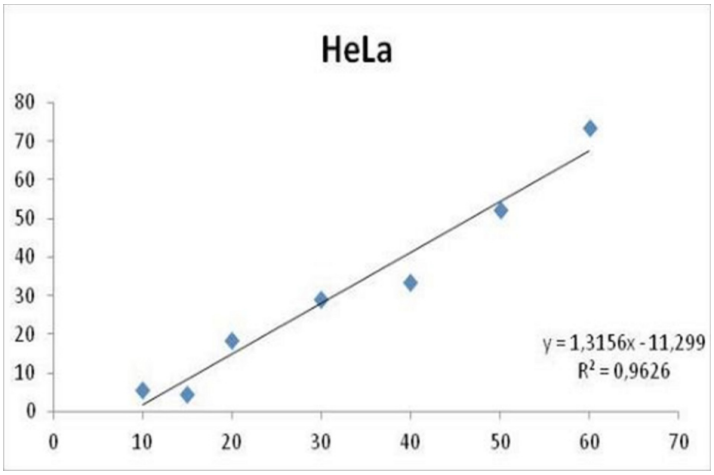

Figure 2. IC50 calculation curve for Hela cell lines

Cytotoxic activity of PE-AgNPs in PC-3 cell lines is shown in Table 3. As shown, the highest inhibition (99.02%) was detected at a concentration of 10 µg / ml and the lowest inhibition (2.59%) was obtained at a concentration of 0.31 µg / ml. The IC50 value of PE-AgNPs against PC-3 cells was 2.185 µg /ml.

In the study, the potential of cytotoxicity of PE-AgNPs was determined as PC-3> MCF7> HeLa.

Table 3. Cytotoxic activity of PE-AgNPs in PC-3 cell lines

Concentration (ug/ml)	% Inhibition
10	99.016
5	89.274
2,5	35.679
1,25	21.558
0,625	13.995
0,3125	2.597
IC_{50}	*2.185*

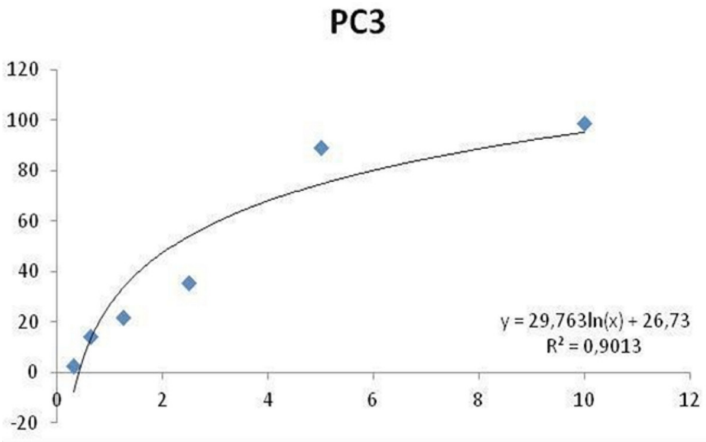

Figure 3. IC50 calculation curve for PC-3 cell lines

As seen in Table 4 the antioxidant activity of DMSO-PE-AgNPs samples was increased at all the tests when the concentration increased.

Table 4. Antioxidant activity of DMSO-PE-AgNPs.

	DPPHscavenging activity %	Metal activity %	Chelatingβ-caroten Method %
1mg/ml	45	38	26
2 mg/ml	57	43	38
5 mg/ml	76	71	65
10 mg/ml	85	82	77

Discussion

Mushrooms are considered to be extremely good candidates for the eco-friendly synthesis of metal nanoparticles. In the literature, we did not find any study that determined cytotoxic activity on MCF-7 cell lines of AgNPs synthesized using *P.eryngii*. Yehia and Al-Sheikh [31] reported that the AgNPs they synthesized using *Pleurotus ostreatus* caused a significant decrease in cell viability of the MCF-7 cell line. Gurunathan et al. [32] found the IC50 value as 6.0 µg/ml in the study of cytotoxic activity on MCF-7 cell lines. They also stated that this was significantly better than earlier findings. The data obtained in this study are in agreement with our study. Prabakaran et al. [33]. in their study using the *Beauveria bassiana* fungus found the IC_{50} value of the silver nanoparticles against a normal HeLa (cervical) cell as 28.8 ± 2.5 µg/ml. However, no other study has been found to determine the cytotoxic activity of nanoparticles synthesized using fungi against HeLa cells. Bio-synthesis nanoparticles based on bacteria were found to have high IC_{50} values against HeLa (cervical) cells [34,35]. Compared with bacteria, it is thought that fungal micelles give a large surface area for interaction and because of the enzymes they secrete, the effect of functional groups may increase. Based on published studies, metal NPs appear to exhibit dose-dependent cytotoxic activity in various cancer cell lines [36]. Prostate cancer is the most common cause of death in men after lung cancer. It is stated that approximately three-quarters of recorded cases occur in developed countries [37]. It is seen that the studies with fungal sources for the synthesis of bio-silver nanoparticles and anticancer activity are limited. Raman et al [38]. Reported that the biological synthesis of silver nanoparticles (AgNPs) using an aqueous

extract of *Pleurotus djamor* var. *roseus* was strong cytotoxic activity against human prostate carcinoma (PC-3) cells. But, no data on anticancer activity against PC-3 cells of PE- AgNPs was detected. Considering the mechanism of action of nanoparticles against cancer cells, as Sanpui et al [39]. we suggest that AgNPs do not only disrupt normal cell function but also induce various apoptotic signal genes of mammalian cells, affecting membrane integrity leading to programmed cell death or as indicated by Nakkala et al. [23]. The anticancer activity of AgNPs may be related to the biomaterial of fungal species adhering to the outer surface of AgNPs. The results were admirable when compared with former studies [40]. These activities obtained from the antioxidant studies may conclude from functional groups on the surface of DMSO-PE-AgNPs.

Conclusion

Pleurotus eryngii is one of the important edible and medicinal mushrooms because it contains various effective compounds. In recent years, *Pleurotus spp*. mushrooms have been in the first place in the synthesis and application fields of environmentally friendly nanoparticles by utilizing different organic materials of fungi. Recent advances in nanoscience and nanotechnology have contributed to methods of diagnosing, treating and preventing various diseases in all areas of human life. AgNPs are known to play an important role in nanoscience and nanotechnology, especially in nanomedicine. In this study, it was observed that *P. eryngii* AgNPs, which were first synthesized in an environmentally friendly, simple and efficient manner, had potential anticancer properties against some cancer cells such as HeLa, MCF-7 and PC-3. On the other hand, the strong antioxidant capacity of DMSO-PE-AgNPs identified in this study is encouraging for further studies. The relationship between cancer and antioxidant mechanism has shown that these nanoparticles can be used as supplements, preservatives or pharmaceutical agents in the biomedicine area. Also, further studies are needed to explain the mechanism of cytotoxic action. However, to generate data in clinical applications, animal studies must first be performed. Therefore, the data obtained in this study may form the basis for nanomaterials that can be used in the food and pharmaceutical industry.

Conflict of interests

The authors declare that they have no conflict of interest and any financial disclosures.

Financial Disclosure

All authors declare no financial support.

Ethical approval

All authors declare no ethical approval.

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