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Detection of Anti-cancer Activity of Silver Nanoparticles Synthesized using Aqueous Mushroom Extract of *Pleurotus ostreatus* on MCF-7 Human Breast Cancer Cell Line

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Abstract

In this research, silver nanoparticles (AgNPs) were manufactured using aqueous extract of mushroom *Pleurotus ostreatus.* Anticancer potential of AgNPs was investigated versus human breast cancer cell line (MCF-7). Cytotoxic response was assessed by MTT assay. AgNPs showed inhibition effect at the following concentrations 12.5, 25, 50, 100 and 200 µg/ml versus MCF-7 cell line, and all treatments had a positive result. The MCF-7 cells were inhibited up to 85.14 % at the concentration 200 µg/ml of AgNPs which reduced cells viability to 14.86%, while 12.5 µg/ml of AgNPs caused 24.23% cells inhibition with reduction of cells viability to 75.77%.

Keywords: Silver nanoparticles, Breast cancer, MCF-7cell line, Cytotoxicity, *In Vitro*.

الكشف عن النشاط المضاد للسرطان لجسيمات الفضة النانوية المُصنَّعة باستخدام مستخلص الفطر المائي من عيش الغراب *Pleurotus ostreatus* على خط خلايا سرطان الثدي البشري MCF-7

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الخلاصة

في هذا البحث تم تصنيع جزيئات الفضة النانوية (AgNPs) باستخدام المستخلص المائي من فطر عيش الغراب *Pleurotus ostreatus*. تم فحص إمكانية ضد السرطان لجزيئات الفضة النانوية لـ AgNPs ضد خط خلايا سرطان الثدي البشري (MCF-7). تم تقييم الاستجابة السامة للخلايا بواسطة فحص قياس السمية الخلوي (MTT) . أظهرت AgNPs تأثير التثبيط بالتركيزات التالية (12.5، 25، 50، 100 و200) ميكروغرام / مل ضد خط الخلايا MCF-7، وكان لجميع المعاملات تأثير إيجابي. تم تثبيط خلايا MCF-7 بنسبة تصل إلى 85.14٪ بتركيز 200 ميكروجرام / مل من AgNPs مما قلل من حيوية الخلايا إلى 14.86٪، بينما تسبب 12.5 ميكروجرام / مل من AgNPs في تثبيط الخلايا بنسبة 24.23٪ مع تقليل حيوية الخلايا إلى 75.77٪.

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Introduction

Recently, the technology of metal nanoparticles has focused on the growing curiosity of the scientific community, thanks to their glamorous applications in both biological and medical areas [1]. Silver nanoparticles (AgNPs), in particular, have gained attention due to their remarkable characteristics such as chemical stability, electrical conductivity, antibacterial, antifungal, anti-inflammatory, antiviral and anticancer activities [2-3]. Furthermore, nano-science can be used in many nano-based methods because of their various uses in biomedical applications such as human cancers treatment tracks [4-5]. Moreover, in order for nanoparticles to be used to treat diseases, some important information must be relied upon such as: particle size, form or organization and dimension in comparison with regular tiny size medicine particles [6-7].

Among many metal nanoparticles, AgNPs, turned out to be benefecial in industrial and health products, such as anticancer agents [8]. Moreover, the use of AgNPs in *In Vitro* studies against different types of cancer cells (because they have a strong anti-cancer effect) is one of the goals of recent scientific studies in this field where (AgNPs) were used along with manufactured drugs as anti-cancers for the purpose of increasing their efficiency against cancer in addition to benefiting from them when used synergistically with natural anti-cancer products, which use green chemistry approaches in their composition [9].

Cancer is the main cause of death worldwide. Therefore, scientists globally have directed their efforts to discover new drugs in order to treat multiple cancer types [10]. Different experiments are being done for using silver nanoparticles as anticancer or anti-malignancy agents, and surprisingly all of them have showed actual positive and promising findings. This makes the way much easier for other new directions in medical treatment area in order to substitute traditional chemo-therapies that imply the usage of cytotoxic elements (because of their significant side-effects, especially multidrug resistance microorganisms) to handle several kinds of cancer cells [11- 12]. One of the most common types of cancer is breast cancer which is an extremely manifold illness. It contains many histological and molecular subtypes which are associated with different clinical behaviors as well as therapeutic responding [13]. Furthermore, this type of cancer has remained at the forefront in terms of mortality rates around the world [14].

The breast cancer cell line (MCF-7) are cells that were first collected from a 69 years old woman of white origin. This cell line since then has had a fundamental impact upon breast cancer research and patient outcomes [15]. The most important characteristics of these cells in brief are: it is a type of primary tumor, epithelial-like cells which grow in monolayers presence of progesterone and estrogen receptors, have a proliferative response if they are exposed to estrogen, they originate from pleural effusion and luminal epithelial phenotype [16-17].

Therefore, the aim of this research was to study the anti-cancer effects of silver nanoparticles made from a natural substance,(the aqueous extract of the mushroom *Pleurotus ostreatus*), and the possibility of replacing them as an effective treatment at low concentrations against breast cancer, instead of using traditional treatments of chemical origin.

Materials and Methods

This study was performed at plant laboratories of Department of Biology, College of Science at the University of Baghdad, Baghdad Iraq, from September 2021 to March 2022.

Sample Collection

The samples of mushroom (*Pleurotus ostreatus*) were collected from fruiting bodies growing on peach trees in Salah Al-Din Governorate.

Preparation of Mushrooms Hot Aqueous Extracts

Mushroom fruit bodies were collected, washed, cut and dried in an oven at 45-50°C overnight, and the dehydrated mushroom was then grounded. Later, the gained powder was immersed in D.W at a ratio of 1:10 (w/v). The mixture was next placed on a heat source and brought to a boil continuously stirring at 60°C ± 2°C for 30 minutes. After this step, the mixture was covered and left to cool and settle down at room temperature for 30 minutes. Next, the formed sediment was removed by passing it through an autoclaved sterile gauze. The resulting fluid was then separated using a centrifuge at 10,000 rpm for 30 minutes at 4°C. Regarding the gained liquid, it was collected and filtered again using Whatman No. 1 filter paper. Finally, the collected aqueous extract was dried using a dryer freeze and the resulting powder was stored at 4 ± 2°C until used [18-19].

Biosynthesis of AgNPs from Mediate P. ostreatus Extract

The stock solution of silver nitrate ($AgNO_3$) was prepared by adding 1×10^{-3} mg of silver nitrate powder to sterile deionized water D.W, and then the succeeding concentrations of 12.5, 25, 50, 100 and 200 µg /mL were made from it [19]. The volume 10 mg/ml of aqueous extract solution was prepared using D.W and then it was passed through a syringe-filter (0.2 µm). Based on the data of an introductory trial, 2-7 ml out of 10 mg/ml aqueous extract of *P. ostreatus* (P2) was completed to a total volume of 10 ml with sterile D.W. *P. ostreatus* solution was then added to 5 ml of aqueous $AgNO_3$ solution and left at room temperature. The synthesis process occurred under dark conditions. Later, the solution was subjected to ultraviolet (UV) at 360- 550 nm wave lengths. After being incubated for 24 hours, the light-yellow solution mixture turned to dark yellow which indicated AgNPs existence [20].

The Strength of Particle Size Distribution Analysis

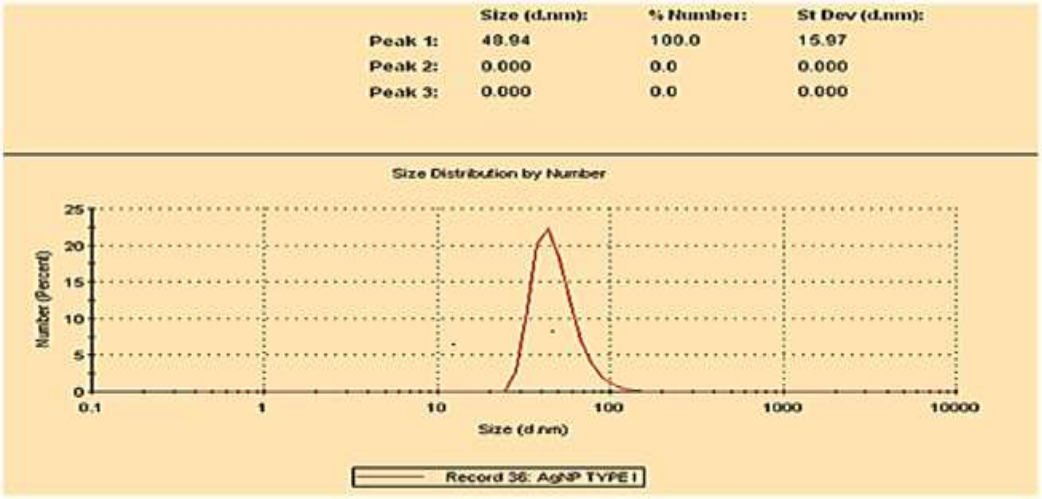

Figure 1: Size of silver nanoparticles synthesized from *P. ostreatus.*

For the purpose of knowing the exact size of the manufactured nanoparticles, distribution analysis for particles sizes was done using light scattering in aqueous solution. The obtained

data illustrated that nanoparticles' sizes ranged between 16-104 nm. On the other hand, mean size of AgNPs was detected to be 49 ± 16 nm as in (Figures 1, 2).

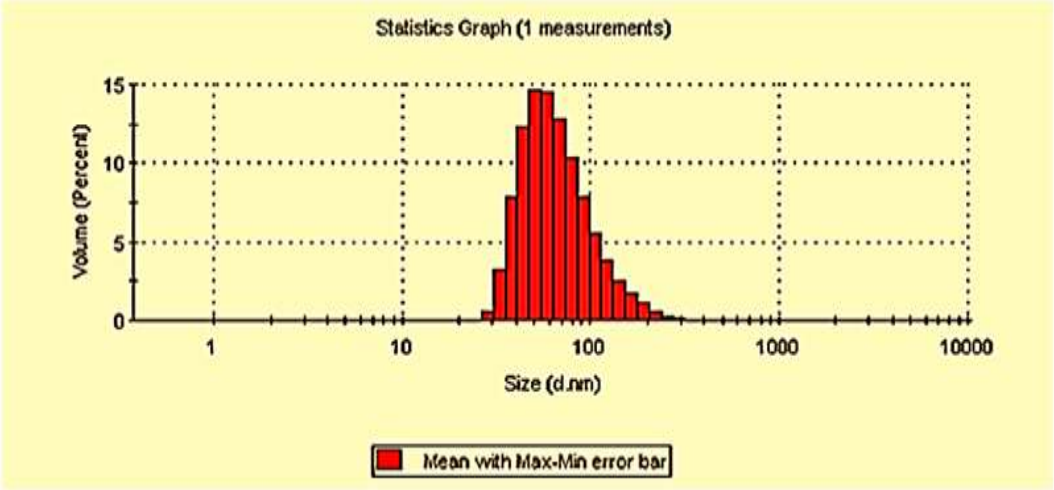

Figure 2: The distribution of *P. ostreatus* particles size in aqueous extract.

Detecting Anticancer Efficacy of AgNPs

The cytotoxic ability of AgNPs was examined on human breast cancer MCF-7 cell line using MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) -based cell proliferation assay. Human breast carcinoma cell line was acquired from Biotechnology Research Center. Department of Biomedical and Molecular Technology, AL-Nahrain University.

Maintenance of Cell Line Culture

RPMI 1640, (EuroClone) medium was used in culturing human breast cancer cell line MCF-7. Ten % of fetal bovine saline (FBS), 2 mM glutamine and 50 µg/ml of antibiotic (Penicillin- streptomycin) were added to the medium. The mixture was both incubated and maintained in a standard humidified incubator with 37°C, 5% carbon dioxide (CO2) conditions [21- 22].

MTT Assay

After culturing and maintaining MCF-7 cell line, $1x10^4$ cells/cm^2 cells were cultured in flat bottom 96 well plates for 24, hours and used experimentally thereafter. Cytotoxicity assays on MCF-7 cells treated with AgNPs was performed by MTT as follows:

Cancer cells were maintained with the fully prepared medium in both control (only medium added) and treatments with medium + different concentrations of AgNPs (12.5, 25, 50, 100 and 200 µg /mL) for 24 hours at 37ºC and 5% CO_2. Twenty-four hours later, MTT stain 5 mg/mL, 20 µl/well were added to the cells and incubated for 3 hours. Then, 125µl aliquot was removed and 50µl of dimethyl sulfoxide (DMSO) was added, and the plates were further incubated for 45 minutes at 37ºC. 150µl of medium alone containing 15µl of MTT stock solution was used as a negative control. The absorption was measured with a microplate reader at a 570 nm wavelength. This experiment was performed in five replications to strengthen the acquired results. Lastly, survival cells ratio was calculated using the following equations. (1) and (2): [23]

$$\text{Viability \%} = (\text{Test OD}/\text{Control OD}) \times 100 \quad (1)$$

$$\text{Cytotoxicity \%} = 100 - \text{Viability\%} \quad (2)$$

Where OD = Optical density

The fundamental assumption for determining the effectiveness of cells is that the NAD-dependent mitochondrial dehydrogenase of active cells is necessary to split the MTT for a quantifiable product of purple formazan formation [24, 25].

Statistical Analysis

Data was statistically analyzed by ANOVA using GenStat software [26]. As for mean values, they were compared using DUNCAN multiple range test which is considered to be statistically significant under probability value of $P<0.05$.

Results

The synthetic AgNPs were detected by UV-visible spectrophotometry. A strong peak was observed between the two wavelengths 420- 430 nm (Figure 3).

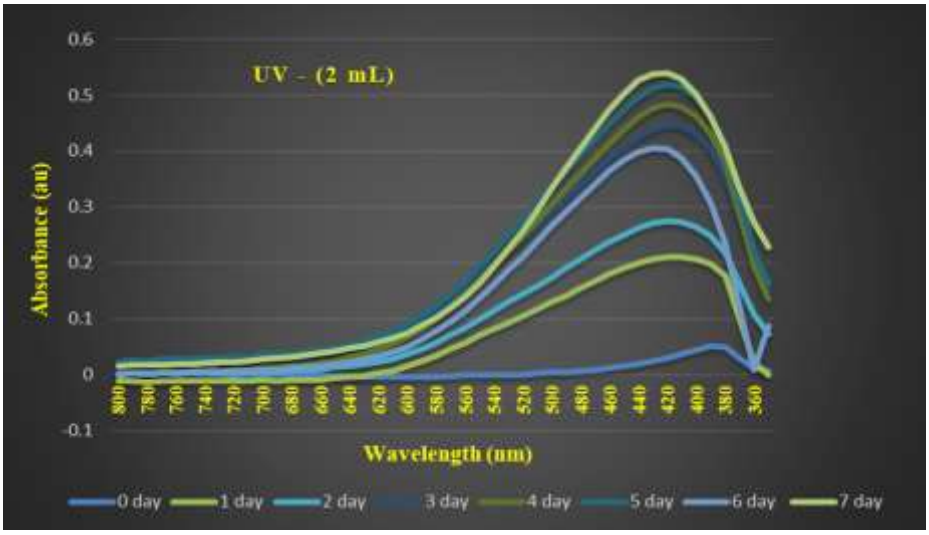

Figure 3: Detection the AgNPs using UV-visible spectrophotometry after bio- reduction by *P. ostreatus* aqueous extract.

The Cytotoxic Effect of Silver Nanoparticles (AgNPs) Prepared from P. ostreatus Using MTT Assay

The cytotoxicity of manufactured AgNPs was detected at various concentrations versus breast cancer cell line (MCF-7) using *in vitro* technique. This reaction continued for a 24-hours incubation period. It was observed that the manufactured particles had a strong cytotoxic effect when tested on MCF-7 cell line in comparison with control cells. The obtained data showed that the rate of cell death is directly proportional to the increase in nanoparticles' concentration. On the same time, results showed that cells were inhibited up to 85.14 % at the concentration 200 µg/ml of AgNPs which reduced cells viability to 14.86%, while 12.5 µg/ml of AgNPs caused 24.23% cells inhibition with reduction of cells viability to 75.77%. (Table 1).

Table 1: The effect of various concentrations of manufactured AgNPs from *P. oestratus* on MCF-7 cells viability after 24- hours incubation.

Concentrations (µg/ml)	Cells viability % ± SE	Cells inhibition %
control	100	0
12.5	75.77 ± 0.03	24.23
25	68.28 ± 0.04	31.72
50	52.74 ± 0.2	47.26
100	30.33 ± 0.03	69.67
200	14.86 ± 0.02	85.14

*The results illustrated above represent the mean of five replications ± standard error.
** Duncan multiple range test was used under probability value of P<0.05.

Discussion

MTT assay, a dose-dependent method, was used to estimate nanoparticles' toxicity against cancer cells. It's a colorimetric assay which is based on mitochondrial dehydrogenase enzyme of viable cells [27]. The results obtained by MTT assay in MCF-7 cells were exposed to several concentrations of AgNPs 12.5 µg/ml to 200 µg /ml for 24 hours are summarized in (Table 1). The results showed an important or significant connection between both cell viability % and cell inhibition % for MCF-7 cells with various treatments of silver nanoparticles. The observed lack of cell vitality (mortality data) confirms the anti-carcinogenic properties of these biosynthesized AgNPs which were attained from *P. ostreatus* [28-29, 23].

Current results were well supported by the presence of several evidences to confirm the toxic effect of the manufactured AgNPs at the cellular level, for example *R. Vivek et al.* [30] used *Annona squamosa* leaf extract versus breast cancer MCF-7 cell line; *Jacob et. al.* [31] used both *Piper longum*'s leaf extracts versus Hep-2 cancer cell line, and *Morinda citrifolia* versus different HeLa cell lines, all the mentioned experiments were performed using *in vitro* technique [32]. In a previous research, *Al-Sheddi et al.* [33] reported that the ultimate reduction in cells viability was measured as 9% each at 10, 25, 50, and100 μg/ml of ND-AgNPs respectively against human cervical cancer cells (HeLA) by MTT assay. Furthermore, *Farah et al.* [34] stated that the induction of toxic influence using biosynthesized silver nano-particles at low quantity / concentration could belong to certain mushroom's elements which adhere to silver-nanoparticles. Finding a new anticancer alternative treatment with relatively few side effects on the immune system has become an important goal for many studies in the field of pharmaceutical and immunological sciences. Hence, a lot of attention has been paid to natural compounds in plants [35].

Concerning the effectiveness of silver nano-particles, another research examined silver nano-particles technique on molecular level and discovered that the regulated death of cells was completely dependent on the gradation of concentration values [36]. In addition, *P. Gopinath et al.* [36] also stated that biosynthetic silver nano-particles provoked changes in cells structure, reduced both viability of cells and metabolic activity, raised oxidative stress which led to mitochondrial destruction. Besides, it raised the reactive oxygen species (ROS) production, and, finally, with all these influences destruction of cellular DNA occurred. It was also observed that the process of taking up nanoparticles by cells occurred mainly by the process of endocytosis [37].

Since getting the best cancer treatment is the primary goal of various researches, targeting cancer cells only is a very basic and important process. *Locatelli et al.* [38] developed nanoparticles with multiple functions characteristics and found that such particles contained both phosphate and silver PNPs and AgNPs polymeric nanocomposites. Such biologically manufactured AgNPs exhibited significant toxic effects on MCF-7 and T47D cell lines with increased endocytic activity when compared to normal breast cell line (MCF10-A) [39]. On another hand, regarding AgNPs mode of action, *Zhang et al.* [40] stated that biologically manufactured AgNPs have the ability to alter the cancer cell structure (a first indication of regulated cell death) which may be observed by alterations in cellular structure via transmitted light microscopy.

Conclusion

The results obtained through this research using manufactured silver nanoparticles from aqueous mushroom extract of *Pleurotus ostreatus* showed a clear efficacy against human breast cancer cell line (MCF7). The cytotoxic responses of the AgNPs suggested that the manufactured silver nano-particles may assist in the process of searching new alternative chemotherapeutic agents. Because of their low toxicity, silver NPs have been the focus of many scientists as they are interested in using these NPs as a splendid candidate for treating many diseases, cancer being the most important.

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