

Cytotoxicity Effects of Green Synthesized Silver Nanoparticles by Using the Extract of Tuber Spp. on Breast cancer (MCF-7) cells

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ABSTRACT

BACKGROUND AND OBJECTIVE: Silver nanoparticles have antibacterial and anticancer activity. But the organic solvents used to produce these nanoparticles are toxic and can have devastating environmental effects. Therefore, there is a strong desire to use healthy methods for the synthesis of silver nanoparticles. Therefore, this study was performed to synthesize silver nanoparticles biologically from aqueous extract of Truffle and to study their biological effects on cancer cells.

METHODS: In this experimental study, to synthesize silver nanoparticles from aqueous extract of Truffle, 10 ml of fungus extract was added to 90 ml of 1 ml silver nitrate solution and incubated for 72 hours at room temperature. The physicochemical properties of the nanoparticles synthesized by XRD, FESEM and TEM were analyzed. Effects of Extract and Synthesized Nanoparticles were studied by MTT at concentrations of 0.025, 0.25, 0.25, 0.5 and 1 mg / ml on MCF-7 Cancer Cells at 72, 48, and 24 hours.

FINDINGS: The size of the nanoparticles was between 19 and 35 nm and their shape was mainly spherical. In cytotoxicity assay using MTT assay IC₅₀ calculated for silver nanoparticles and extract in MCF-7 cells showed that silver nanoparticles had more cytotoxic effects than extract. The calculated IC₅₀ for extracts and nanoparticles at 24, 48 and 72 hours were 0.73, 0.8, and 0.64 mg / ml, respectively, and for the nanoparticles were 0.6, 0.5, and 0.48, respectively (p < 0.05).

CONCLUSION: The silver nanoparticles produced by bio-synthesis method have a higher cytotoxicity than the truffle fungus (*Tuber spp.*) extract.

KEY WORDS: Silver Nanoparticles, Truffle Fungus (*Tuber spp.*), Bio-synthesis, MTT, MCF-7 Cell Line.

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Introduction

Cancer is a fatal disease with high mortality that results in many psychological and economic conflicts. Lifestyle and environmental factors are important and effective factors in cancer incidence (1). Despite various treatments such as chemotherapy, immunotherapy, and radiotherapy, cancer is still a major challenge. Therefore, the development of effective, biocompatible and low-cost approaches to cancer treatment is essential (2). It is now believed that silver nanoparticles can be used as a therapeutic agent in addition to the fight against bacteria and the effect of wound healing, to fight AIDS, viruses and especially cancer (3). But the organic solvents used to produce these nanoparticles are toxic and can have adverse environmental effects. Therefore, there is a strong desire to use healthy methods for the synthesis of silver nanoparticles (4).

One of the methods of nanoparticle production is bio-production and attention to the production of nanoparticles is rising. Substances such as plants, algae, fungi, yeast, bacteria, and viruses are used in the biological production of nanoparticles (5). Most fungi that produce underground organ are from the category of Ascomycete and are identified and called Truffle. More than 15 types of protein, Gallic acid, catechin, flavonoid, tannin and some other compounds, including beta-carotene and linoleic acid, have been identified. Medicinal properties of this valuable fungus include the treatment of gastric cancer, hepatitis A, B, C, nephritis, osteoarthritis, nerve pain, insomnia, bronchitis, asthma, gastric ulcer, hypertension, and high cholesterol (6,7). Al-Laith after studying the antioxidant/antioxidant components of desert Truffle from Bahrain, Iran, Morocco and Saudi Arabia found that Iranian mushrooms have the highest antioxidant activity (8). Zabihi et al. Studied the effect of hydro alcoholic extract of Truffle on estrogen and progesterone levels in experimental model of multiple sclerosis in female rats and the results showed that Truffle hydro alcoholic extract increased the amount of estrogen and progesterone in experimental model of multiple sclerosis rats (9).

Nanoparticles have received increasing attention due to the therapeutic efficacy and reduced unwanted toxicity of anticancer drugs, and Since chemical and physical methods are generally expensive, time consuming and dangerous to the environment in nanoparticle synthesis, use of fungi, Bacteria, plants and algae (as an intermediate in the synthesis of nanoparticles from inorganic compounds) can be

another method alongside chemical and physical methods for the production of nanoparticles. Biopreparation of nanoparticles lowers risk for humans, air and overall ecosystem (10, 11).

This study was performed to synthesize aqueous silver nanoparticles using Truffle fungus extract for 72 hours and to investigate its anticancer activity on MCF-7 breast cancer cells by MTT assay.

Methods

Preparation of aqueous extract of fungus (Tuber spp.): This applied experimental study was conducted from April to September 2016. For this purpose, Truffle (Tuber spp.) was prepared from Meshgin city of Ardebil province (from Agricultural Jihad province). Then rinse thoroughly with distilled water and shake into small pieces in a dark environment to dry completely. 100ml of distilled water was added to 100 grams of fungus, to reduce aqueous extract of Truffle. It was then boiled at 90 ° C for 10 minutes. After cooling, the aqueous extract was purified by Whitman No. 1 filter paper and stored in the refrigerator.

Synthesis of silver nanoparticles from fungal extract (Tuber spp.): To synthesize silver nanoparticles from aqueous extract of Truffle, 10 ml of aqueous extract of Truffle was added to 90 ml of 1 mM silver nitrate solution then incubated for 72 h at room temperature on a shaker (200 rpm). The amount of solution absorbance was investigated using a UV2550 spectrophotometer made in Japan in the range of 300-700 nm. The resulting nanoparticles were isolated by centrifugation (12000 rpm for 15 min) and then dried at room temperature in a closed glass container and kept in the dark until using time.

Determination of physicochemical properties of silver nanoparticles: To determine the size, structural properties, optical properties, morphology of silver nanoparticles synthesized from Tuber spp., respectively, by X-ray diffraction (XRD) microscopy Emission field scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) were used.

Also, the crystalline constants of silver nanoparticles were analyzed by Philips X'pert Pro X-ray powder diffraction (XRD) machine made in the Netherlands and investigated for morphology, size and dispersion distribution of silver nanoparticles synthesized from Truffle extract, respectively, the transmission electron microscope (LEO-912AB) made in England and the

scanning field scanning electron microscope were used (Mira-Xmu made in Czech).

Evaluation of the effect of aqueous extract and nanoparticles obtained from Truffle fungi on killing of MCF-7: MCF-7 cell line was prepared from Pasteur Institute of Iran cell bank and in RPMI 1640 medium containing 10% fetal bovine serum (FBS). 1% of antibiotics (Pen/Strep) were cultured and incubated at 37 °C, 95% humidity and 5% CO₂. After MCF-7 cells were grown, the cells were isolated from the bottom of the flask by EDTA-Trypsin and suspended at 7×10^3 / ml, and then 200 µl of the suspension was added on each of the 96 well plates. Plates were incubated for 24 h in the above-mentioned conditions to adhere the cells to the bottom of the plate.

Then, each of the 96-well plate wells were treated separately with different amounts of aqueous extract and silver nanoparticles obtained from Truffle (0.0625, 0.25, 0.25, 0.5 and 1 mg/ml) and incubated at 37 °C, 95% humidity and 5% CO₂ for different periods (24, 48, 72 h). After the elapsed time, the supernatant was removed and 180 ml of culture medium without FBS was added to each well with 20 µL of MTT solution. Plates were then incubated in the incubator with the above conditions.

After four hours of incubation, the wells were evacuated and 200 µl of DMSO was added to each well. In order to calculate cell viability, 10 min after cell treatment by DMSO, the absorbance of each 96-well plate was measured by Elisarader at a wavelength of 570 nm. In this test, DMSO solution was used as blank and untreated cells with aqueous extract and nanoparticles obtained from Truffle was used as control group. The percentage of living cells was calculated using the following equation for each row:

$$\text{Viability percentage of cells} = 100 \times \frac{\frac{\text{Mean of optical absorbance of treated cells}}{\text{Mean of optical absorbance of control cells}}}{\text{cells}}$$

The data were analyzed by SPSS 22 software and one-way ANOVA and Tukey test. All tests were performed in at least three replications and $p < 0.05$ was considered significant.

Results

Synthesis and Ultraviolet spectroscopy of nanoparticles: After biosynthesis, the pale yellow solution changed to light brown. As one of the indications for the reduction of silver ions in the process of silver nanoparticles production is its color change to

light brown, in addition to spectroscopy by UV-Vis spectrophotometer of aqueous extract and nanoparticles formed from Truffle fungus, a relatively strong peak formed in the range of 400 to 450 nm (Fig 1).

Investigation of silver nanoparticles by XRD spectra: X-ray diffraction spectra of the synthesized nanoparticles at 72 h showed that the peaks observed at angles of 38.22°, 44.19°, 64.60°, and 77.51°, respectively, were consistent with the crystalline plates (111), (200), (220) and (310) of which these results were consistent with the standard spectrum of the silver bulk (Fig. 2).

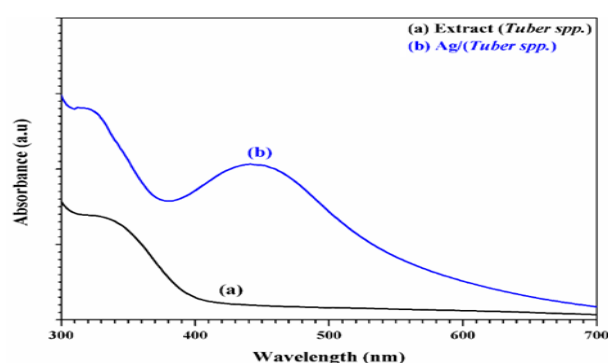


Figure1. UV-Vis spectrophotometry (a) extract, (b) silver nanoparticles

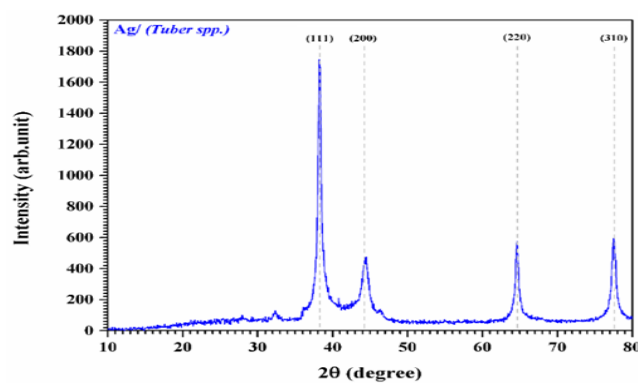


Figure2. X-ray diffraction pattern of synthesized silver nanoparticles within 72 h of Tuber spp

Investigation of morphological properties of silver nanoparticles by FESEM and TEM electron microscopy: Images of silver nanoparticles by field emission scanning electron microscopy (FESEM) obtained from aqueous extract of Truffle had a relatively high spherical structure (Fig 3). In addition to the spherical structure, silver nanoparticles were also observed by TEM microscopy in triangular, elliptical, and rod shapes. The results of TEM electron microscopy examination of silver nanoparticles showed that most of the nanoparticles had size in the range of 26 to 35 nm (Fig 4).

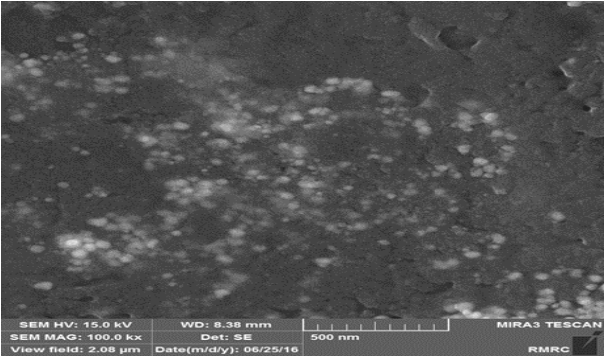


Figure 3. FESEM images of synthesized nanoparticles from aqueous extract of *Tuber spp*

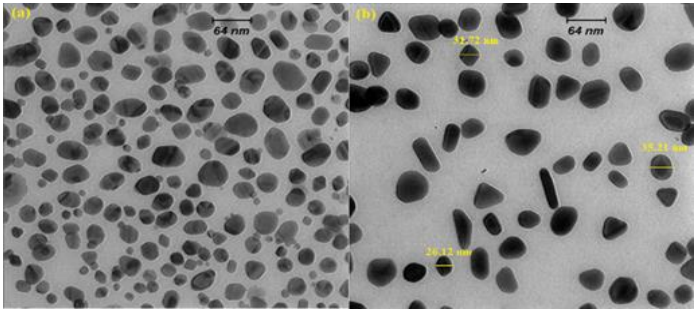


Figure 4 (a,b). TEM images of synthesized silver nanoparticles from aqueous extract of *Tuber spp*

Evaluation of the effect of silver nanoparticles and aqueous extract of Truffle on MCF-7 cell killing: Results showed that there was a significant linear relationship between increasing concentrations of each

treatment with different concentrations of silver nanoparticles and aqueous extract of Truffle and the killing percentage of MCF-7 cells, as the concentration of each of the above treatments increased, the killing rate of MCF-7 cells increased too (Fig 5).

Cell viability was decreased by 65 and 48 percent, respectively by increasing the concentration of nanoparticles and aqueous extract of Danbolan from 0.065 mg/ml to 1 mg/ml after 48 hours of treatment with MCF-7 cells. The results also showed that silver nanoparticles obtained from aqueous extract of Truffle had a higher lethal effect on MCF-7 cancer cells compared to aqueous extract of Truffle so that at the weight ratio of 0.125 and at 48 hours of treatment, the survival rate in MCF-7 cells after treatment with aqueous extract and silver nanoparticles obtained from Truffle was 63 and 95%, respectively. The results also showed a similar trend for the minimum concentration of aqueous extract and silver nanoparticles obtained from Truffle for 50% killing of MCF-7 (IC₅₀) cells. The IC₅₀ content of silver nanoparticles obtained from aqueous extract of Truffle was significantly lower than that of aqueous extract of Truffle.

For example, the IC₅₀ for aqueous extract of Truffle for 24, 48, and 72 hours was 0.73, 0.8, and 0.64 mg/ml, respectively. However, for silver nanoparticles obtained from the aqueous extract of Truffle were 0.6, 0.56 and 0.48 mg / ml, respectively (Table 1).

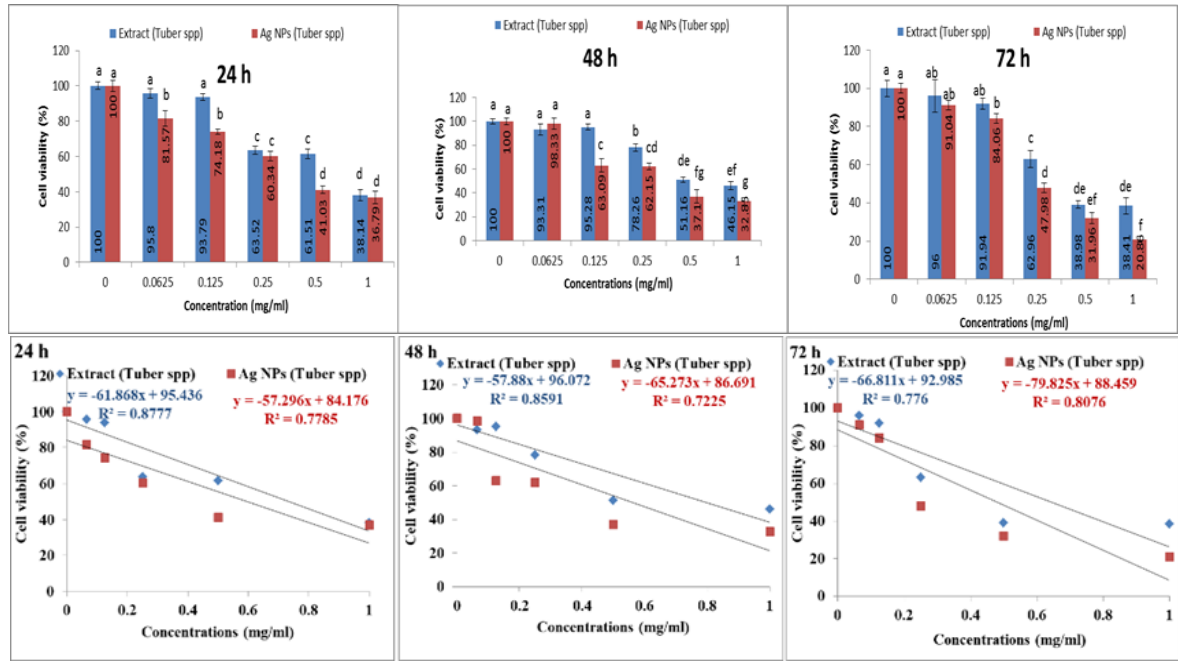


Figure 5. Comparison of the average effect of different concentrations of aqueous extract and nanoparticles obtained from Truffle on the survival of MCF-7 cells over 24, 48 and 72 hours and the mean survival of MCF-7 cells (all treatments were performed in 3 replications, UN-common small letter indicates significant at 5% probability level)

Table1. IC50 value calculated on MCF-7 cells for 24, 48 and 72 hours

Variable	Time	24 h (mg/ml)	48 h (mg/ml)	72 h (mg/ml)
Extract (Tuber spp)		0.73	0.8	0.64
Ag NPs (Tuber spp)		0.6	0.56	0.48

Discussion

In this study, it was found that Truffle fungus extract was able to reduce Ag⁺ ions to silver nanoparticles. Also, biologically produced silver nanoparticles are more cytotoxic to MCF-7 cancer cells. Shankar et al. have produced silver nanoparticles from *Azadirachta indica*. This group reported the size of nanoparticles between 5 and 35 nm and their shape was reported spherical (12).

Sankar and his colleagues synthesized silver nanoparticles in a biological way from an extract of *Origanum vulgare*. After extraction from the plant, the silver nitrate solution combined with 1: 9 molar ratio of extract and after 15 minutes discoloration was observed and UV-vis analysis showed an absorbance peak at 440 nm. The size and shape of the nanoparticles were determined using the SEM apparatus about 63-85 nm and spherical (13). The observed color change from yellow to dark brown indicates the reduction of silver ions and the formation of nanoparticles, that is the first indication of the production of silver nanoparticles due to surface plasmon vibrations in the nanoparticles. The results showed that max λ is about 450 nm, which is consistent with the results of other researchers (14,15). The crystalline size of silver nanoparticles is obtained from the Scherrer relation $k\lambda/(\text{B} \cos \theta) = D$, which is 25 nm. This finding is consistent with the findings of other researchers (15).

Field scanning electron microscopy images of the synthesized silver nanoparticles showed that the shape of the nanoparticles was completely spherical and the distribution of the nanoparticles was uniform. The approximate size of the 72 h nanoparticles is about 26.8 nm. The TEM image showed the nanoparticles to be predominantly spherical, but other shapes such as triangles can be seen in the image, ranging from 26 to 35 nm. However, variations in temperature, pH, and duration and concentration of the interactions of the salt solution and the desired extract can affect the magnitude of the nanoparticle size changes (13). By comparing the

calculated IC50 of the extract and the nanoparticles, it was found that the IC50 content of the nanoparticles lower than the extract and the nanoparticles at lower concentrations of the extract have a high toxic effect on the cancer cell, meaning that the nanoparticles are more cytotoxic than the extract. The results of MTT assay showed that by increasing treatment time to 72 hours the effects of cytotoxicity were increased. In general, it can be said that nanoparticles can decrease cell viability in a concentration- and time-dependent manner.

The shape of the nanoparticles will affect the level of contact and release of the silver ion. Since proteins tend to adhere to sharp edges, the tendency to adhere to cubic or triangular particles is therefore greater (16). In a study by Devi et al., they synthesized silver nanoparticles through the reduction of *Ulva lactuca* in the range of 20 to 56 nm and showed its anticancer effects on MCF-7, HT29, HepG2 and normal cell lines. In their study, more toxic effects of IC50 of 12.5 $\mu\text{g} / \text{ml}$ were observed in the 24-hour treatment of the liver cancer cell line than the other and normal cell line (17). In the synthesized silver nanoparticles in this study, a triangular shape is also observed, which could be one of the causes of the lethality of these nanoparticles. The pathway of cell death may involve activation of proapoptotic events of the mitochondrial organelle in the cell, which begins with the release of cytochrome C. In addition, the effects of silver nanoparticles on increased caspase-3 protease gene expression in cancer cells and induction of programmed cell death have been demonstrated in various studies (18, 19).

Therefore, due to the high activity of mitochondria in the process of respiration in cancer cells compared to normal cells, a suitable substrate for silver is provided for the destruction of cancer cells. The results showed that Truffle fungus extract was able to reduce Ag⁺ ions to silver nanoparticles.

The silver nanoparticles produced by the green method also have more cytotoxicity than the fungal

extract, an effect that proceeds from a mechanism that inhibits the cell cycle progression from G1 to S and increases cell death and apoptosis in MCF7 cells, in addition, biosynthesized silver nanoparticles can be used as a viable treatment for cancer, but much research is needed.

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