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Biosynthesis of Silver Nanoparticles Using Mushroom Extracts: Induction of Apoptosis in HepG2 and MCF-7 Cells via Caspases Stimulation and Regulation of BAX and Bcl-2 Gene Expressions

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ABSTRACT

Green synthesis of metal nanoparticles is a rapidly growing field due to their potential applications in the medical and search fields. This study was conducted for the silver nanoparticles biosynthesis; using *Pleurotus ostreatus* edible mushroom extracts and investigating the anticancer activity against human liver (HepG2) and breast (MCF-7) adenocarcinoma cancer cell lines. The formation of silver nanoparticles (Ag-NPs) was confirmed by Fourier transform infrared (FTIR), Ultraviolet-visible spectroscopy and Transmission Electron Microscopy (TEM). The data showed that spherical Ag-NPs with a mean diameter size of 17.5 nm and absorption maxima at 450 nm were produced. The anticancer activity of Ag-NPs on HepG2 and MCF-7 cancer cell lines was assessed using the MTT method. The results demonstrated that Ag-NPs showed cytotoxicity against HepG2 and MCF-7 cancer cells, with the median growth inhibitory concentrations (IC_{50}) 20.0±1.15 and 37.0±1.15 µg/ml, as compared to the anticancer drug doxorubicin (IC50: 18.67±1.20 and 30.33±0.88 µg/ml, respectively. Further, the apoptotic effect of Ag-NPs was investigated by DNA damage. Furthermore, the transcriptional expression of Bcl-2 (B-cell lymphoma 2), BAX (Bcl2-Associated X Protein), caspase-3, -8,-9, cytochrome c and P53 were also evaluated by RT-PCR. The Ag-NPs induced DNA fragmentation and apoptosis in HepG2 and MCF-7 via suppressing of bcl-2 gene expression, up-regulation of BAX, down-regulation of Bcl2, simulation of caspases, P53 and cytochrome c gene expression. In conclusion: Ag-NPs prepared by *Pleurotus ostreatus* edible mushroom extracts showed antitumor cytotoxicity against HepG2 and MCF-7 via caspase-dependent apoptosis, associated with the activation of p53 and the down-regulation of Bcl-2.

KEYWORDS: Silver nanoparticles, *Pleurotus ostreatus* extract, apoptosis, human liver HepG2 adenocarcinoma cell line and Breast cancer MCF-7 adenocarcinoma cell lines.

INTRODUCTION

The discovery and identification of new antitumor drugs with low side effects on the immune system has become an essential target in many studies [1]. Even though there is an extensive range of cytotoxic agents used in the cancer therapy, such as doxorubicin, cisplatin and bleomycin, their use have shown drawbacks and are not as efficient as expected [2]. Nowadays, great efforts have been enforced to develop bio-active antitumor nanomaterials. Nanoparticles are used for solid tumor treatment via their selective accumulation in the tumor tissues due to the passive targeting effects [3]. Numerous microorganisms from bacteria to fungi have been documented to synthesize inorganic materials either intra- or extracellularly and thus to be potentially exploited as eco-friendly nano-factories [4-6]. Some naturally occurring edible mushroom extracts were efficiently used for the biosynthesis of gold (Au-NPs), Silver (Ag-NPs) and Au-Ag nanoparticles in water [6]. In particular, several researchers used *Pleurotus ostreatus* mushroom extract for Ag-NPs biosynthesis [7,8].

Silver nanoparticles had a broad spectrum of biological activities as anti-bacterial, anti-fungal, and anti-viral activities [9]. Kovács et al [10] demonstrate that Ag-NPs have anti-proliferative effect and induce apoptosis mediated cell death, both in drug sensitive and in multidrug resistant cancer cells. A recent study showed that, silver nanoparticles prepared by Mora leaf had *in vitro* cytotoxicity on hepatic cancer cell line (Hep-G2) and in vitro antioxidant efficiency [11].

Mushrooms have many different bioactive compounds with various biological activities. The content and bioactivity of these compounds depend on the preparation and consumption of mushroom [8]. It is well known that mushrooms are rich in proteins, vitamins and amino acids. Edible mushrooms have antitumor, anti-inflammatory, cardiovascular, antiviral and antibacterial [12,13]. In addition, the progress of green synthesis of Ag-NPs and their applications in the medical field are of important [14]. The antibacterial, antioxidant and antitumor activities of Ag-NPs have been reported previously that depend on the size, shape and stability of the nanoparticles [15-19].

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Accordingly, this study aimed to biosynthesis of Ag-NPs, using mushroom (*Pleurotus ostreatus*) extract. In addition, evaluation of the antitumor activity and the possible mechanism of action of Ag-NPs on human liver HepG2 and breast cancer MCF-7 adenocarcinoma cell lines.

MATERIALS AND METHODS

Chemicals and Reagents

Pleurotus ostreatu mushroom was obtained from the Agriculture Research Center, Giza, Egypt.The human liver HepG2 and MCF-7 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was purchased from Carbosynth Limited (Berkshire, UK). Dulbecco's modified Eagle's medium (DMEM) was purchased from Lonza (Verviers, Belgium). Fetal Bovine Serum (FBS) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Silver nitrate ($AgNO_3$) was purchased from Fischer Scientific (USA). All chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Biosynthesis of Ag-NPs

Silver nanoparticles (Ag-NPs) were prepared using *Pleurotus ostreatus* extract according to the method of **Philip** [6]. Briefly, edible mushroom, *Pleurotus ostreatus*, was washed several times with deionized water. Then, 68 g of finely cut mushroom was boiled for 2 min in 300 ml deionized water and filtered. The filtrate was cooled at room temperature and used as reducing and stabilizer agent. To a 30 ml aqueous solution of $AgNO_3$ (50 mg dissolved in 240 ml deionized water), 6 ml mushroom extract was mixed and agitated in an orbital shaker at 25 °C in the dark for 48 h. The formation of silver nanoparticles is indicated by yellowish brown color formation.

Characterization of Silver nanoparticles (Ag-NPs)

Fourier transform infrared (FTIR) spectra (KBr) of Ag-NPs were determined using JASCO FTIR 6300.The nanoparticles and all spectrophotometric measurements were also characterized by ultraviolet-visible spectrum using Unicam UV/VIS spectrophotometer (Helious alpha, England) (200 to 800 nm), by taking 2 ml of reaction mixture at different time intervals of 1, 2, 12, 24, 48 and 72 h. The size and morphology characterization of Ag-NPs Samples were deposited onto carbon-coated copper TEM grids using Transmission electron microscopy (TEM) JEOL model 100CX.

Cell culture

The human liver HepG2 and breast cancer MCF-7 adenocarcinoma cell lines (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 µg/ml) at the 37°C in a humidified atmosphere containing 5% CO_2. HepG2 and MCF-7 cancer cells at a concentration of 5 x 10^4(cells/ml) were grown in a 25 cm^2 flasks in 5 ml of complete culture media.

MTT Cytotoxicity assay

MTT was used as a colorimetric assay to assess cell viability [20]. It was utilized in examined to evaluate Ag-NPs or the standard reference drug, doxorubicin (DOX) activity on the cell proliferation.HepG2 and MCF-7 cancer cells were plated in 96-well plates, at an initial density of 5×10^5 cells, with 200 µl media per well. The cells (5×10^4) were allowed to attach overnight and were then treated with different concentrations of the Ag-NPs for 48 h. MTT was then mixed with HepG2 or MCF-7 cancer cells at 37°C for 2 h in a humidified CO_2 incubator at 5% CO_2. MTT formazan product was dissolved in DMSO and absorbance was then measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek Instruments, USA).Cell viability was expressed as a percentage of the control (untreated) culture value. Experiments for each extract were carried out in triplicate; each concentration was repeated in three wells under the same experimental conditions. The results were compared with the cytotoxic activity of DOX, the reference anticancer drug.

Apoptosis assay:

Detection of DNA damage

DNA fragmentation in HepG2 and MCF-7 cancer cells was performed the alkaline Comet assay proposed by **Singh et al.** [21]. Tail length, tail DNA % and extent tail moment were measured in the cells using a Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera.

Quantitative Real Time PCR (qRT-PCR)

Real Time PCR was performed to determine relative gene expression ratio of P53, Bcl-2, BAX, caspase-3, caspase-7, caspase-8 and caspase-9, Cytochrome C in HepG2 and MCF-7 cancer cells.

-RNA Isolation and Reverse Transcription

Briefly, HepG2 or MCF-7 cancer cells ($5x10^5$ cells/ml)were homogenized in 1 ml ice-cold TRIzol reagent following the manufacturer's instruction. The RNA quality was verified using spectrophotometric and agarose gel electrophoresis. cDNA was synthesized from 2 µg total RNA using Revert Aid^{TM} first strand cDNA synthesis kit (Ferments life science,

Thermo Scientific, USA) by incubating at 37ºC for 1 h with reverse transcriptase with random hexa-nucleotides according to the manufacturer's instructions.

-Quantitative Real Time PCR

QRT-PCR was performed using the Real-Time PCR Systems (Step One instrument, Applied Biosystems, Foster City, CA, USA). Each 10 µl reaction contained 5 µl SYBR Green Master Mix (Applied Biosystems), 0.3 µl gene-specific forward and reverse primers (10 µM), 2.5 µl cDNA and 1.9 µl nuclease-free water. The relative expression of the studied genes was calculated using the comparative threshold cycle method. Table (1) showed the forward and reverse primers used in this study [22].

Assay of oxidative stress parameter

The oxidative stress parameters malondialdehyde (MDA), nitric oxide (NO) and glutathione reduced (GSH) are determined in the HepG2 cell lysates ($5x10^5$ cells/ml) treated with 20 µl of 1/10 of IC_{50} values of Ag-NPs [23]. Lipid peroxidation (LPO) level was estimated as MDA by thiobarbituric acid (TBA), according to the method of **Buege and Aust** [24]. NO level was measured using the method of **Granger et al.** [25]**.** GSH content was determined according to **Ellman** [26]**.** The hydrogen peroxide levels (H_2O_2) were estimated by ferric-xylenol orange (FOX) methods [27]. The superoxide dismutase (SOD) activity was determined as described by **Marklund and Marklund** [28]**.**

Statistical analysis

Data are expressed as mean ± SD for three different determinations. Statistical significance was analyzed by one-way analysis of variance ANOVA, using SPSS (Statistical Package for the Social Sciences), Version 15.0 for Windows (SPSS, Inc., Chicago, IL, USA). Mean separations were performed using the least significant difference method. Values with $P< 0.05$ considered as statistically significant.

Table 1: Forward and reverse primers sequence

	Forward primer sequence	Reverse primer sequence
Bcl2	5′-ATGGACGGGTCCGGGGAG-3′	5′-TCAGCCCATCTTCTTCCA-3′
BAX	5′-CAGCTGCACCTGACG-3′	5′-ATGCACCTACCCAGC-3′
P53	5′-GGGACAGCCAAGTCTGTTATG-3′	5′-GGAGTCTTCCAGTGTGATGAT-3′
Cyto C	5′-TTTGGATCCAATGGGTGATGTTGAG-3′	5′-TTTGAATTCCTCATTAGTAGCTTTTTTGAG-3′
Caspase-3	5′-CAGTGGAGGCCGACTTCTTG-3′	5′-TGGCACAAAGCGACTGGAT-3′
Caspase-7	5′-AGTGACAGGTATGGGCGTTC-3′	5′-CGGCATTTGTATGGTCCTCT-3′
Caspase-8	5′-GGGAAGTGTTTTCACAGGTT-3′	5′-TTCTTGCTTCCTTTGCGGAAT-3′
Caspase-9	5′-CTGCGAACTAACAGGCAAGC-3′	5′-CTAGATATGGCGTCCAGCTG-3′
β-actin	5′-ATCCGTAAAGACCTCTATGC-3′	5′-AACGCAGCTCAGTAACAGTC-3′

RESULTS

Biosynthesis and characterization of Ag-NPs

In the present study, *Pleurotus ostreatus* mushroom extract was used to prepare Ag-NPs. The FTIR, UV-Vis spectra and the typical TEM image obtained for Ag-NPs were represented in **Fig. 1.** In the present work, FTIR spectra showed intense absorption at 2073, 1639, 1446, 1399, 1315, 1249, 1108, 603 cm^{-1}. The IR bands at 1639, 1315 cm^{-1} are characteristic of the C-O and C=O stretching modes of the carboxylic acid group.UV-visible spectroscopy of prepared Ag-NPs was carried out after 12, 24, 48 and 72 h exposure at different wavelengths. $AgNO_3$ treated mushroom extract showed maximum absorption corresponding to the absorption maxima of Ag-NPs at 450 nm. In this work, at the higher magnification of TEM image, the morphology of the Ag-NPs are predominantly spherical with the size distribution ranged from 13.1 to 24.1 nm (mean diameter is 17.5 nm) **(Fig. 1).**

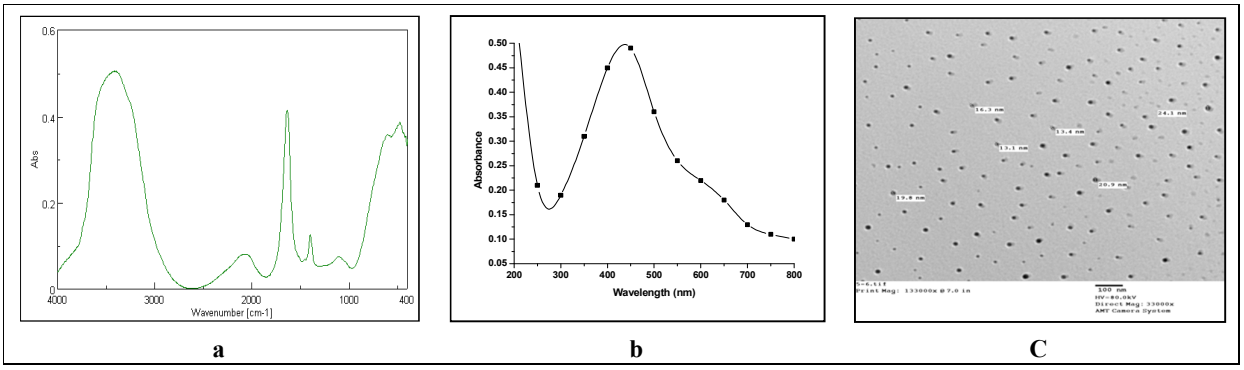

Figure 1. Characterization of Ag-NPs. Fourier transform infrared (FTIR) (a), ultraviolet-visible (UV/Vis) spectrum (b) and Transmission electron microscopy (TEM) image (c).

***In vitro* anti-proliferation activity of Ag-NPs**

The anti-proliferative activity of Ag-NPs on HepG2 and MCF-7 cell lines was evaluated using MTT assay in comparison with doxorubicin as a reference drug. The present results showed that Ag-NPs caused significant cytotoxicity in HepG2 and MCF-7 cells, with the median growth inhibitory concentrations (IC_{50}) 20.0±1.15 and 37.0±1.15µg/ml, as compared to the standard anticancer drug; DOX, IC_{50}: 18.67±1.20 and 30.33±0.88 µg/ml, respectively (Table 2).

An important feature of cell apoptosis is the fragmentation of genomic DNA. DNA fragmentation was demonstrated by incubating HepG2 and MCF-7 cells with Ag-NPs for 48 h. Treatment of Ag-NPs led to a significant increase ($P<0.05$) of DNA damage (tail length, tail DNA% and extent tail moment) in a dose dependent manner in comparison to DNA from control cells (Fig. 2 a, b, c).

Table 2. *In vitro* anti-proliferation activity of Ag-NPs against HepG2 and MCF-7 cancer cell lines.

Compound	IC_{50} (µg/ml)	
	HepG2	MCF-7
Ag-NPs	20.0±1.15	37.0±1.15
DOX	18.67±1.20	30.33±0.88

Data are expressed as means±S.E. of three separate experiments. IC_{50}; (concentration at which 50% of the cells were dead), DOX; doxorubicin, HepG2 and MCF-7; Human liver and breast cancer adenocarcinoma cell lines.

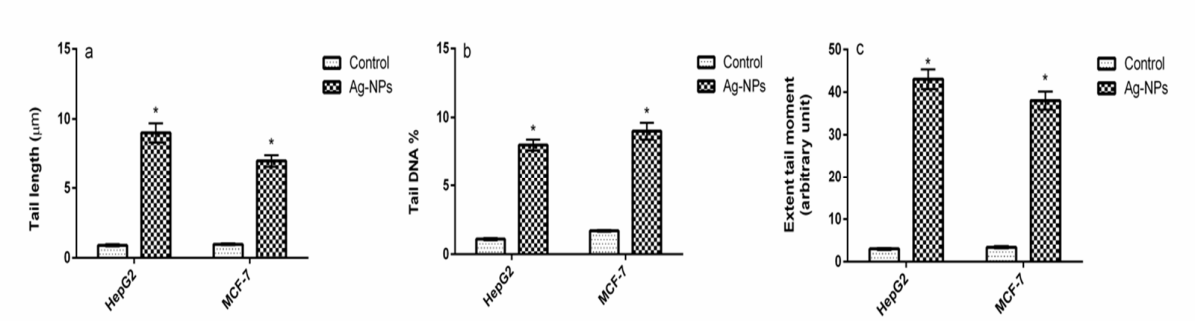

Figure 2. Effect of Ag-NPs on DNA damage. *HepG2 and MCF-7 cancer cells were treated with Ag-NPs for 48 h. The apoptotic DNA fragmentation, tail length (a), tail DNA% (b) and extent tail moment (c), was measured in HepG2 and MCF-7 cells using the comet assay. Data represent as mean±S.E. of three experiments. *P< 0.05 compared to control.*

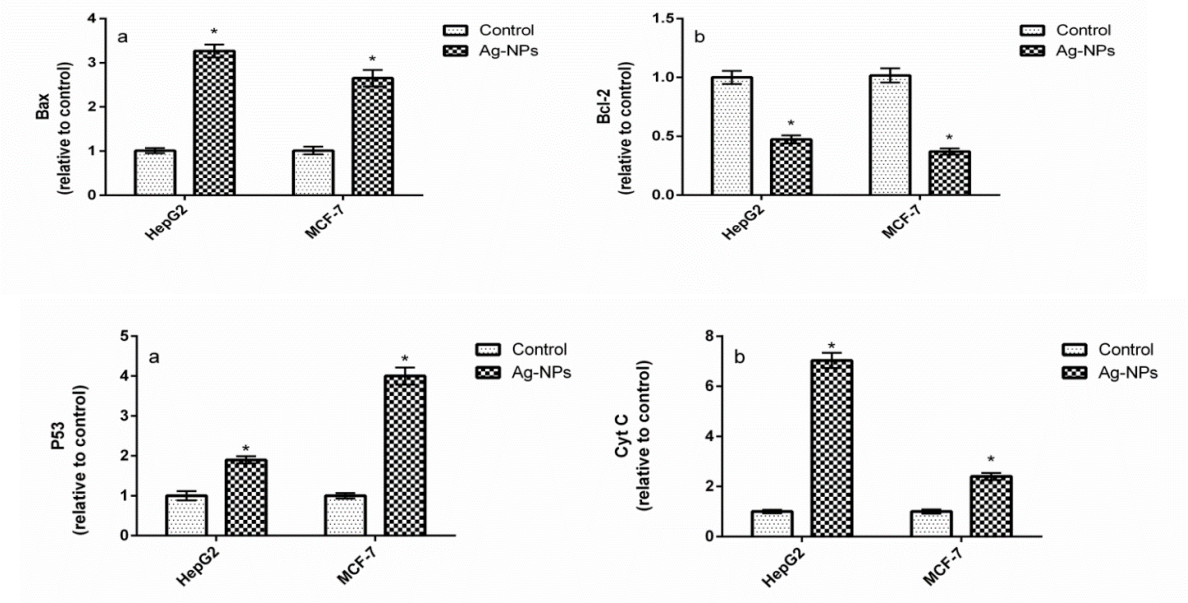

Figure 3. Effect of Ag-NPs on Bcl-2, BAX, P53 and Cytochrome c gene expression. *HepG2 and MCF-7 cancer cells were treated with Ag-NPs for 48 h. Gene expressions of BAX (a) and Bcl-2 (b) were measured in cell supernatants using the RT-PCR. Data represent as mean±S.E. of three experiments. *P≤ 0.05 compared to control.*

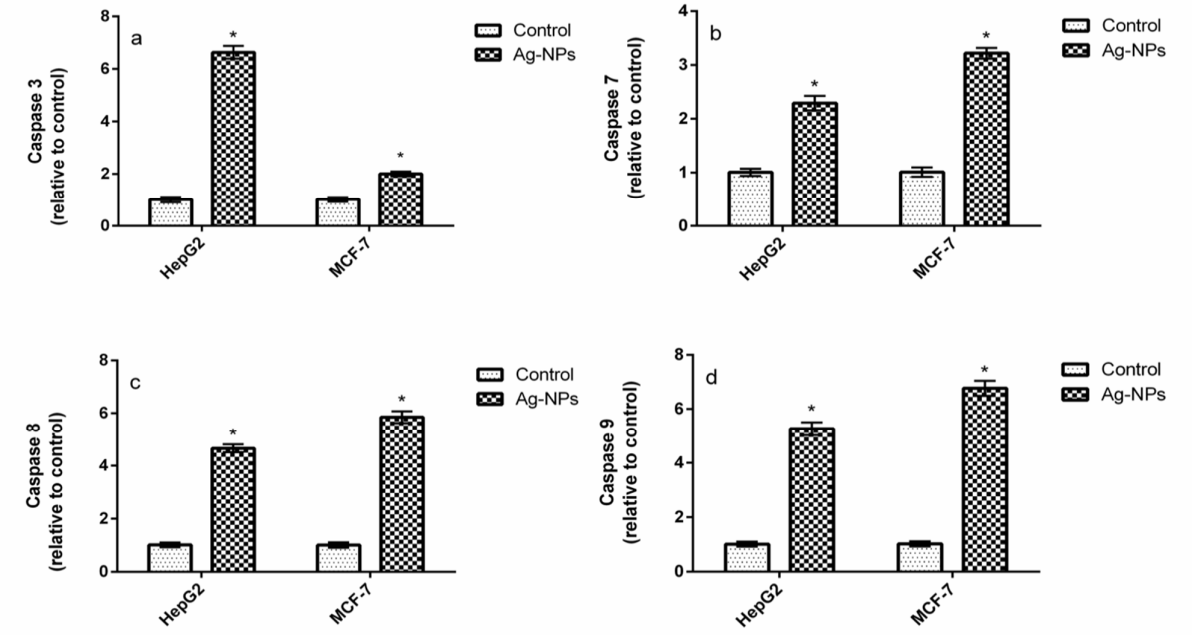

Figure 4. Effect of Ag-NPs on caspase-3, caspase-7, caspase-8 and caspase-9 gene expression. HepG2 and MCF-7 cells were treated with Ag-NPs for 48 h. Gene expression of caspase-3 (a) caspase-7 (b) caspase-8 (c) and caspase-9 (d) were measured in cell supernatants using the RT-PCR. Data represented as mean±S.E. of three experiments. $^{*}P<0.05$ compared to control.

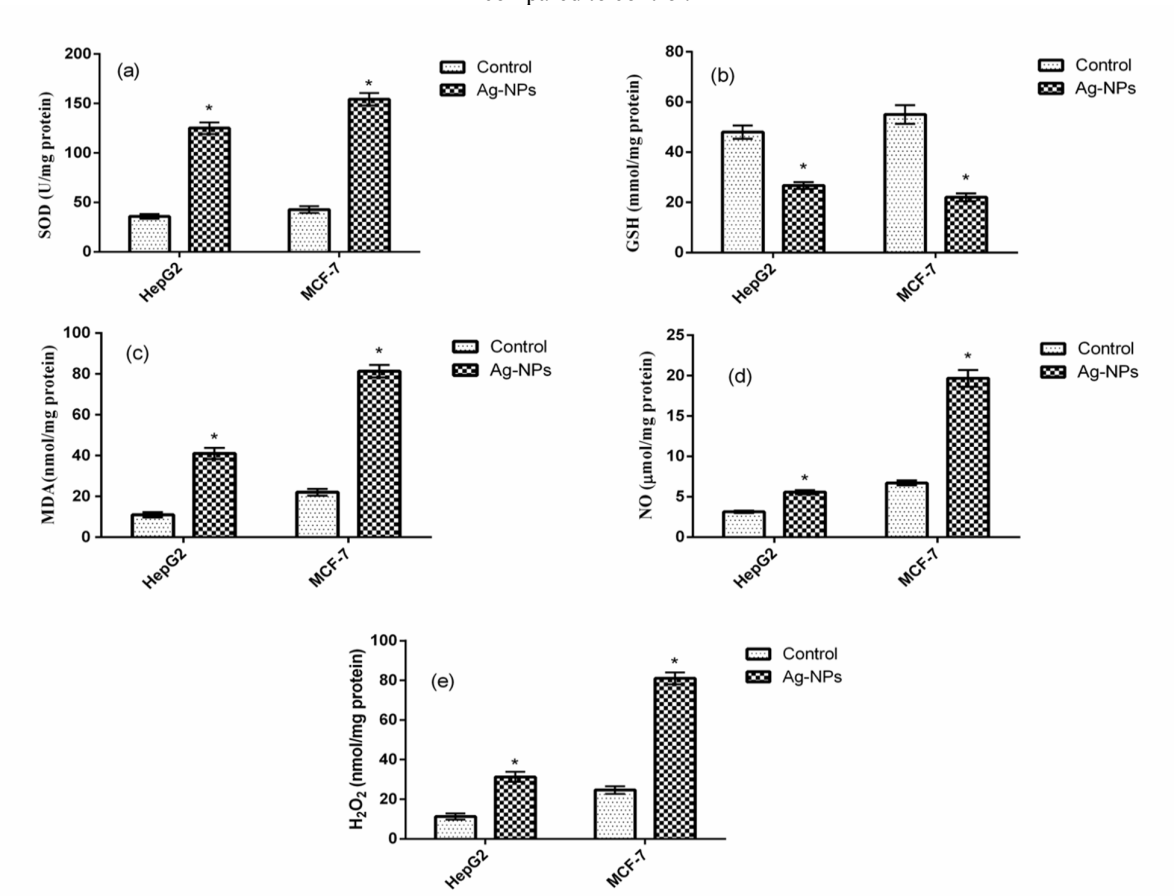

Figure 6. Effect of Ag-NPs on oxidative stress parameters (SOD, GSH, MDA, NO and H_2O_2). HepG2 and MCF-7 cells were treated with Ag-NPs for 48 h. Oxidative stress of SOD (a), GSH (b), MDA (c), NO (d) and H_2O_2 were measured in cell supernatants. Data represent as mean±S.E. of three experiments. $^{*}P< 0.05$ compared to control.

To elucidate the apoptotic mechanism of Ag-NPs on HepG2 and MCF-7 cells, RT-PCR analyses were used to measure the mRNA expressions of Bcl-2, BAX, p53, cytochrome-c caspase-3, -7, -8, and -9 as well as the expression of the β-actin (an internal standard). The result of the present study demonstrated that, a significant decrease in the level of Bcl-2 expression in parallel with significant increases in BAX, P53 and cytochrome c expression ($P<0.05$) was observed due to the treatment of HepG2 and MCF-7 cells with Ag-NPs for 48 h, as compared with the level of the corresponding control values (Fig. 3 a and b). In addition, as shown in Fig 4 a, b, c and d, significant increases ($P<0.05$) in expressions of caspase-3, -7, -8 and -9 were observed in HepG2 and MCF-7 cells treated with Ag-NPs for 48 h when compared to control cells.

The SOD activity and the level of some oxidative stress parameters MDA, GSH, NO as well H_2O_2 was measured in HepG2 and MCF-7 cell lines treated with Ag-NPs (Fig. 5). The present study showed that the SOD activity, the levels of MDA, NO and H_2O_2were significantly ($P<0.05$) increased, but, GSH content was decreased in Ag-NPs treated HepG2 and MCF-7 cells, as compared to the corresponding control values, which reflected the induction of oxidative stress in the HepG2 and MCF-7 cancer cells

DISCUSSION

The use of plants as the production assembly of silver nanoparticles has drawn attention, because of its rapid, eco-friendly, non-pathogenic, economical protocol and providing a single step technique for the biosynthetic processes. Silver nanoparticles synthesized through green method have been reported to have biomedical applications. The current study focused on the biosynthesis of silver nanoparticles using *Pleurotus ostreatus* mushrooms extract and investigation of its anticancer activity. Currently, biosynthesis of Ag-NPs has been receiving great interest in the scientific community due to their therapeutic applications, such as their promising role as anticancer agents [29-31]. The carbonyl groups of amino acid residues and peptides have a strong ability to bind to silver ions [32]. It was accounted that proteins could bind to nanoparticles either through free amine or cysteine groups in proteins and thereby stabilizing the Ag-NPs [33]. The proteins present over the Ag-NPs surface acts as capping agents. Since mushrooms are highly enriched with proteins, it is suspected that they act as reducing as well as capping agent. In the current study, spherical Ag-NPs were successfully synthesized, using *Pleurotus ostreatus* mushroom extract, with λ_{max} at 450 nm and the size distribution ranged from 13.1 to 24.1 nm. Further, the FTIR spectrum of Ag-NPs showed that, Ag is bound to proteins through the carboxylate group of the amino acid residues. The polysaccharide/oligosaccharide fractions of the mushroom extract could be reliable for the reduction of metal ions to the corresponding metal nanoparticles [5]. Furthermore, when the mushroom extract was subjected to an aqueous solution of silver nitrate, a gradual change to yellowish brown color was observed after 48-h. This change of color could be attributed to the formation of silver nanoparticles of varying shape and size. Generally, the cytotoxicity of nanomaterials depends on the nanoparticle size and concentration, which is associated with their reactivity [34,35]. Ag-NPs size affects the cell viability, induction of oxidative stress, and release of cytokines [36]. Overall, the small size as well the surface coatings of Ag-NPs could induce stronger cytotoxic effects, depending on the cell type [37]. Moreover, the anticancer properties of Ag-NPs could be attributed to their functionalization with the organic moieties in the *Pleurotus ostreatus* mushroom extract.

In the present study, the antitumor activity, induction of apoptosis and the mechanism of cell death of Ag-NPswere investigated in Hep-2 and MCF-7*in vitro.* The results obtained in this study revealed the potent anticancer activity of the Ag-NPs that compared with doxorubicin as a reference anticancer drug onHepG2 and MCF-7cancer cell lines. Our results are in accordance with that of **Sahu et al.** [38] **and Devi et al.** [39] who observed *in vitro* cytotoxic activity of Ag-NPs against several human cancer cell lines, including HepG-2, and MCF-7 cell lines. The previous *in vitro* cytotoxic studies proved that Ag-NPs have dose-dependent cytotoxic effects in human liver HepG2 cells due to trigger of oxidative stress and induction of apoptosis, mediated by mitochondrial injury [36,38,40]. Previous studies have indicated that nanoparticles motivate cytotoxicity via the generation of reactive oxygen species or increases in intracellular oxidative stress and trigger cell death process including apoptosis and necrosis [41-43]. Also, it was reported that Ag-NPs have dose-dependent cytotoxic effects in MCF-7 breast cancer cells through induction of apoptosis, ROS generation, activation of caspase 3 and DNA fragmentation [36,44-46].

Cell apoptosis is the mechanism provoked by the biosynthesized Ag-NPs [47,48].Apoptosis or programmed cell death is a gene-regulated phenomenon, which is important in both physiological and pathological conditions. The death receptors, activation of caspases, mitochondrial responses and the regulation of Bcl-2 and BAX gene expression are significant guiding mechanisms of apoptosis [49,50]. The apoptotic effect of the synthesized Ag-NPs was explored via assessment of the relative ratio of caspase-3, caspase-7, caspase-8 and caspase-9 gene expression and DNA damage in Hep-G2 and MCF-7 cells treated with Ag-NPs (1/10 of the IC_{50}) for 48 h. Our results showed that caspase activity increased in treated cells. Caspase-3 and caspase-9 are members of the cysteine protease family, which have been identified as major regulators of programmed cell death. Caspase-9 activates caspase-3 by proteolytic cleavage and caspase-3 then cleaves vital cellular proteins or other caspases, which are dependent on the release of mitochondrial cytochrome C. The cytochrome C gene expression was also induced in the HepG2 and MCF-7 cells treated with Ag-NPs, which could indicate the involvement of the mitochondrial pathway [51].

On the other hand, ROS are highly reactive and result in oxidative damage to proteins and DNA, which induce mitochondrial dysfunction. To determine whether Ag-NPs could significantly increase the intracellular ROS level, we assessed the SOD activity, MDA, NO, H_2O_2 levels and GSH contents in HepG2 and MCF-7 cancer cells treated with Ag-NPs. The results revealed a remarkable increase of the SOD activity, MDA, H_2O_2 and NO levels, in parallel with depletion of GSH content in the HepG2 and MCF-7 cancer cells that were treated with Ag-NPs, indicating that Ag-NPs induced oxidative stress within the cancer cells. Overproduction of ROS can induce oxidative damage to vital cellular molecules and structures, including lipids, proteins and DNA, activate the intrinsic apoptotic pathway due to mitochondrial dysfunction and finally lead to apoptosis [52]. In addition, it has been reported that Ag-NPs could readily diffuse into, and translocate to, the nucleus through nuclear pore complexes, thereby leading to the formation of ROS, which directly trigger DNA damage and chromosomal abnormalities [53]. **Rahman et al.** [54] indicated that the up-regulation of metabolism and oxidative stress genes by Ag-NPs might increase the production of ROS as a byproduct of oxidation. Hence, Ag-NPs might cause cytotoxicity in HepG2 and MCF-7 cancer cells via elevating the ROS levels and increasing lipid peroxidation as well as GSH depletion in cancer cells; leading to genotoxicity via inducing DNA damage.

Conclusion

Biosynthesis of silver nanoparticles using *Pleurotus ostreatus* mushroom extract were developed in a very simple and eco-friendly method. Generally, mushrooms containing proteins have played a major role in acting as a reductant as well as a capping material in order to synthesize Ag-NPs that functioned effectively as an anticancer agent against HepG2 and MCF-7 cancer cells. The antitumor activity of the synthesized Ag-NPs mediated by fragmentation in nucleic acid and induction of apoptosis in HepG2 and MCF-7 via suppressing of bcl-2gene, up-regulation of BAX, down-regulation of Bcl2, simulation of caspases, P53 and cytochrome c gene expression. Therefore, the present work also explored the potential anticancer activity of biologically synthesized Ag-NPs in tumor cell lines, HepG2 and MCF-7, *in vitro* and indicated they might be an effective alternative for the treatment of tumors.

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