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PII: S0141-8130(20)34654-7

DOI: https://doi.org/10.1016/j.ijbiomac.2020.10.032

Reference: BIOMAC 16922

To appear in: *International Journal of Biological Macromolecules*

Received date: 3 September 2020

Revised date: 28 September 2020

Accepted date: 5 October 2020

Please cite this article as: M. Vijayakumar, K. Priya, S. Ilavenil, et al., Shrimp shells extracted chitin in silver nanoparticle synthesis: Expanding its prophecy towards anticancer activity in human hepatocellular carcinoma HepG2 cells, *International Journal of Biological Macromolecules* (2018), https://doi.org/10.1016/j.ijbiomac.2020.10.032

Shrimp shells extracted chitin in silver nanoparticle synthesis: Expanding its prophecy towards anticancer activity in human hepatocellular carcinoma HepG2 cells

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ABSTRACT

In this study, a well-organized, simplistic, and biological route of AgNPs (AgNPs) was synthesized using shrimp shell extracted chitin as reducing, capping and stabilizing factor under the optimized conditions. Also, the anticancer potential of synthesized biogenic AgNPs was evaluated against human hepatocarcinoma (HepG2) cells. Ultraviolet visible spectroscopy (UV-Vis spec) study indicated that the development of AgNPs present in the colloidal solution was single peak at 446 nm. FTIR results showed a strong chemical interaction between the chitin and biogenic AgNPs; whereas, XRD studies confirmed AgNPs presence in the composites. The SEM TEM analytical studies confirmed the synthesized AgNPs had a spherical shape crystalline structure with size ranges from 17 to 49 nm; EDX study also confirmed the percentage of weight and atomic elements available in the colloidal mixture. Furthermore, the synthesized AgNPs showed significant cytotoxic effect on the HepG2 cells with an IC_{50} value shown at 57 ± 1.5 µg/ml. The apoptotic and necrotic cell death effects of AgNPs were also confirmed by flow cytometry. The upregulated apoptotic related proteins Bax, cytochrome-c, caspase-3, caspase-9, PARP and downregulated anti-apoptotic related proteins Bcl-2 and Bcl-xl in cancer cells, confirmed the anticancer potential of AgNPs. These findings suggest that the AgNPs possess significant anticancer activity against HepG2 cells which could play major role in the therapeutic

drug development to treat cancer in future.

Keywords: chitin, AgNPs, TEM, HepG2, HCC, cytotoxicity, apoptosis

1. Introduction

Chitin is the largest biopolymer present in the natural sources next to cellulose, which found majorly in insects cuticles, cell walls of fungi, yeast, and green algae [1]. Interestingly, the shrimp and crab shells waste from the aquatic processing plants have been used as starter culture for the production of chitin in many industries, which has an approximately 20% of chitin [2]. The concepts of "shell bio-refinery", referring to the fractionation and value-added applications of shells (such as shrimp shells) have been proposed and practiced [3, 4]. This chitin is composed of N-acetyl D-glucosamine residues, whose side chains are linked by β-1,4 linkage which is linear [5]. Chitin and their derivatives can be reformed into various forms like hydrogels, nanofibers, beads, membranes, scaffold sponges and nanoparticles [6]. It is biodegradable, biocompatible , non-antigenicity, less or non-toxic effects, chitin and its derivative could be used in various biomedical application [7] and they also possess antioxidant, anti-inflammatory, immune-stimulatory, wound healing, antimicrobial and anticancer activity [8].

Polymers or nanocomposites are hybrid materials that contain at least one phase whose dimension in the nanoscale level [9]. The polymer matrix containing metallic nanoparticles is a group of composite materials known for its wide applications in cancer treatment, drug delivery, bio-sensing, molecular imaging, cell labeling, diagnosis, pharmaceutical applications and material chemistry [10-13]. There are unique physiochemical properties in metal nanoparticles (Pd, Pt, Ag, Au, Cu, Fe and Ni) when compared to individual molecules or bulk metals or core shells [14-17]. Due to the beneficial utilization in biomedical, drug delivery, food industries,

agriculture, textile industry, and water treatment, among the noble metals, silver have drawn the interest of researchers [10, 18]. Chitin along a metallic nanoparticle synthesis has got great attraction due to the unique optical, electronic and catalytic activity of the composites. Chitin exhibits anticancer activity through a mechanism like inhibition of anti-apoptotic genes [19]. AgNPs have been showed a higher efficiency to treat cancer [20, 21]. Though silver has the potential to treat cancer it also exhibits a certain level of toxicity to normal cells. In order to overcome these disadvantage AgNPs is complexed with a natural polymer. This nanocomposite is highly specific to cancer cells while silver nano-particles alone does not show high specificity and at the same time, the concentration of AgNPs required to show beneficial effect is relatively high. Chitin a biopolymer, when complexed with AgNPs shows anti-cancer effect even at low concentration of AgNPs. Therefore, in this study we focused on shrimp shells extracted biopolymer of chitin in synthesis of AgNPs and expanding its potential towards anticancer activity in human hepatocellular carcinoma HepG2 cells.

2. Materials and Methods

2.1. Chemicals

Silver nitrates ($AgNO_3$), and Sodium hydroxide (NaOH), were obtained from Himedia, Mumbai, India. Hydrochloric acid (HCl) and glacial acetic acid used in this study are of SRL grade (Sigma-Aldrich SRL, India).

2.2. Processing of shrimp shells

The shrimp (*Metapenaeus dobsoni*) shells waste was collected from the fish market during autumn season, Coimbatore, India. Shrimp shells were removed and washed with water thoroughly to remove the flesh parts. The Cleaned shells were dried under shadow condition and

then samples were kept at -20°C in an airtight container for further use.

2.3. Chitin preparation from shells

Demineralization process, the estimated amounts of dried shrimp shells were transferred into a beaker and added four volumes of 1.25N HCl and incubated for 3 h, and then shells were removed and washed thoroughly. Again shells were placed in a beaker with 1.25N HCl and repeated this process 3 to 4 times. Afterwards, the shells were subjected to deproteinization by addition of 5 % NaOH (fivefold W/W))and heated h at 70-75 °C for one hour in the water bath, this step is repeated again and again until the protein portion is removed completely and shells were washed and dried at 65 °C for 8 h and then made powder to get a chitin and stored in air-tight container until further study [22].

2.4. AgNPs synthesis and characterization

Chitin mediated AgNPs was synthesized [23] and characterized by different analytical methods such as UV-visible spectrophotometer, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscope (SEM) and Transmission electron microscopy (TEM) and Zetasizer Nano ZS [23].

2.6. Antiproliferative activity of AgNPs

2.6.1. Cell culture

The HepG2 cells (HB-8065) (National Center for cell science (NCCS), Pune, India) were cultured and maintained in DMEM (ATCC-30-2003) medium with 10% FBS and antibiotics and incubated at 37 °C with 5% CO_2. After confluent, the cells were tripsinized and re-seeded in flask/ plate and incubated for 24 hr.

2.6.2. Cell cytotoxicity and *Flow cytometry study*

IC_{50} (Inhibitory concentration) for AgNPs was determined by the method of Castro-Aceituno et al [24]. FACS flow cytometer (Becton Dickenson Biosciences, CA, USA) equipped with an excitation laser line at 488 nm was used detect the apoptosis levels [25].

2.6.4. Western blot analysis

After exposure of HepG2 cells ($3x10^6$/well) with 40 and 50 µg/ml of AgNPs for 24 h, Cells lysate were prepared with 0.1mL of lysis buffer (1x lysis buffer, 200 mM PMSF, 100 mM sodium orthovanadate) including protease inhibitor cocktail (Santa Cruz Biotechnology, CA, USA) / well and incubated for 5 min at 4 °C. The cells were rapidly scraped and collected, then centrifuged at 10000xg for 5 min at 4 °C. The concentration of protein was quantified by protein assay kit (Thermo Scientific, IL, USA). The same concentration of protein (µg/well) was loaded onto SDS-polyacrylamide gel electrophoresis. Protein was then transferred into a nitrocellulose membrane (Millipore, Bangalore, India). Membrane was blocked with 10% skimmed milk for one hour. Membrane was then washed thrice with PBST (PBS, 0.1% Tween 20) and incubated with respective target antibodies Bax (sc-20067), cytochrome-c (sc-13560), caspase-3 (cs-56046), caspase-9 (cs-56073), PARP (sc-8007), Bcl-2 (sc-7382), Bcl-xL (56021) and β-actin (sc-47778) (1:1000) (Santa Cruz Biotechnology, CA, USA) at 4 °C for overnight. Target proteins were detected with secondary antibody (sc-2004) for 1h at room temperature. Protein band was detected with chemiluminescence substrate (Santa Cruz Biotechnology, CA, USA). Intensity of protein was quantified by densitometric analysis via ImageJ software.

2.7. Statistical analysis

Statistical analyses were performed with SPSS-16 (Chicago, IL, USA). The data are

represented as the mean ± SEM; significance level was considered at least with $p < 0.05$.

3. Results and discussion

Biological/ green synthesis of AgNPs is one of the most emerging technologies which overcome the limitations of the conventional physical and chemical methods [26]. Biological extracts or its chemical constituents of plant, fungal cell walls, yeast or green algae act as potent reducing agents and capping molecules for the AgNPs preparations [27]. Thus, first we synthesized AgNPs by a reported biological method using chitin as reducing agent. Chitin belongs to de-*N*-acetylated chitin family with various points of deacetylation, this is a cationic biopolymer, possess an excellent biodegradability, bio compatibility, and nontoxicity. The interactions of chitin with polyanions by electrostatic interaction due to protonated amino groups in $AgNO_3$ were stabilized as well. Hence, in this study we presented the green synthesis AgNPs using chitin extracted from shrimp shells and extended their impacts on the HepG2 carcinoma cells.

3.1. Characterization of AgNPs

3.1.1. UV-Vis spectroscopy analysis

UV-Vis spectroscopy is the most widely used analytical tool to determine the optical property of the biologically synthesized AgNPs. It is well known factor that the absorption pattern of the solution containing biogenic AgNPs was kinetically monitored in the ranges between 200 - 600 nm due to surface plasmon resonance (SPR). The AgNPs development was confirmed visually by changing its color creamy white into yellowish brown. This colour intensity of the absorption and scattering in much higher than that of nonplasmonic nanomaterials and supports to SPR. However, the characteristic absorbance peak of synthesized

AgNPs was observed at 446 nm (Fig.1a). The appearance of single SPR peak revealed that the AgNPs embedded in chitin matrix was spherical in shape [5]. The study results agreed with Saber et al. [28] who previously reported that the absorption peak of AgNPs are mainly organized by SPR and the peak associated with size of the particle.

3.1.2. FTIR analysis

The FTIR spectrum of biologically synthesized AgNPs was used to detect the organic molecules present on the surface of the nanoparticles. In this investigation, the FTIR spectrum analysis was used to recognize the biomolecules that are responsible for the conversion of silver ion to AgNPs. The FTIR spectrum showed the bands were present in the AgNPs with range of 4000 to 500 cm^{-1} (Fig. 1b). The results of the current study were in accordance with the FTIR spectra of standard chitin [29]. The spectrum of Ag NPs absorption band of N-H and O-H stretching vibrations are 3248 and 3456 cm^{-1}, and C-H vibration at 2953 $cm^{-1,}$ the C-HN stretch vibration at 1416 cm^{-1} [30]. The absorption at 3456 indicative of bonded hydroxyl (-OH) group and the absorption peak at 571 from Ag NPs, this implies that the amide group and nitro compounds plays major role in the interaction and stabilization of silver particles to the chitin matrix.

3.1.3. EDX analysis

EDX Spectrum showed the presence of composition in synthesized AgNPs (Fig. 1c). The mass weight percentage of the elements present in the AgNPs presented in Table 2. From this data the mass weight percentage of Ag was found to be 48.5 %, while the mass weight percentage was attributed to the elements like C (23.71%) and O (27.79%). In the EDX spectrum there is presence of three peaks between the energy levels 2.9 KeV-3.5KeV, these three peaks

corresponds to the characteristic L lines of silver element. Absorbance peak around 3KeV is due to the surface plasmon resonance of metallic AgNPs [31]. These elements act as stabilization and capping agent on the surface of the AgNPs. Similar results were stated on synthesis of AgNPs using biological method [31-33].

3.1.4. X-ray diffraction analysis

The compositions pattern of the AgNPs was determined using XRD. The XRD pattern of the AgNPs (Fig. 1d) had Bragg's reflections with 2θ values of 29°, 59° and 74° corresponding to (111), (222) and (331) planes indicated crystallinity nature and spherical shape of the AgNPs as validated by results from other researchers in literature [34-36]. A broad peak around 29° was due to the presence of chitin in the nanocomposites [5]. From the XRD pattern it was confirmed that the synthesized AgNPs is amorphous in nature. The cross links produced by the intercalating polymer chains was responsible for the amorphous nature of AgNPs [37].

3.1.5. SEM analysis

The SEM analysis was used to determine the morphological character and size details of the synthesized AgNPs. The SEM image (Fig. 2a) depicted the morphology of as prepared AgNPs were in the form of small spherical balls. Similar results reported by Gomathi et al. [38]. These particles were dispersed on the closely packed chitin matrix, because of the strongly crosslinked chitin matrix AgNPs are partially visible, the visibility of the silver particles was increased when the composite was viewed under nanometer range, this is further evident in the TEM image. The chitin matrix present in the AgNPs prevented the agglomeration and precipitation of silver particles.

3.1.6. TEM analysis

The structural organization and size distribution of the AgNPs dispersed in the composites were acquired using Transmission Electron Microscopy. The TEM image (Fig. 2b) showed that the AgNPs were distributed all over the chitin matrix and the size of the silver particles was found to be at the range of 17–49 nm, the composite itself was found to be at the range of nanometer scale with the help of the micrograph this result was in accordance to [37] they reported chitin nanofibre with evenly distributed AgNPs.

3.1.7. Zeta potential and particle size distribution

The surface charge of the nanomaterial plays a key role in stability, functionalization, and applications as well. The acceptable range of synthesized particle zeta is greater than +30 mV and less than -30 mV for colloidal dispersions in the absence of steric stabilization [39]. As synthesized Ag NPs by chitin reduction, the zeta potential was observed -18.92 mV (Fig. 2c). Size distribution, shape, and surface charge are the major factors (optical, anticancer effects) influence nanoparticle activity applied to biosystem. Therefore, in this study, the size distribution of AgNPs was measured by dynamic light scattering property. According to the previous literature, PDI value is more than 0.7 indicated that the sample has broad size distribution [40]. But, we observed the unusual structure of Ag NPs size of 109 ± 63 nm with the polydispersity index (PDI) of 0.352 ± 0.71 (Fig. 2d). The study results confirmed that the size distribution of the synthesized AgNPs in nanosize with average particle diameter range. Our current study results agreed with Khorrami et al. [41] who reported on selective cytotoxicity of green synthesized AgNPs against the MCF-7 tumor.

3.2. Antiproliferative activity

3.2.1. MTT assay

In recent years, researchers have growing interest on biologically introduced AgNPs for the treatment of various cancers. In this content, biologically synthesized AgNPs are being tested to discover the potential anticancer agents. Therefore, the cytotoxicity effect of AgNPs on HepG2 cells was measured using the MTT assay. The cells were treated with various concentrations of chitin, $AgNO_3$, doxorubicin (Dox) and synthesized AgNPs for 48 h against inhibitory percentage of cells growth. Results showed that the percentage of growth inhibition obtained with constant exposure for 48 h are shown in Fig. 3a. The cell proliferation was found to be decreased with increasing concentration of AgNPs. From the study results, the IC_{50} value of AgNPs against HepG2 cells was calculated and it was found to be 57 ± 1.5 µg/ml. The study result was agreed with Sivalokanathan et al. who reported earlier on the cytotoxic effect of *T. arjuna* against HepG2 cells with IC_{50} value 60 mg/L. This combined effect of metal nanoparticle and the supporting matrix was responsible for the significant antiproliferative activity of AgNPs [42].

3.2.1. Flow cytometry analysis

Apoptosis or programmed cell death is a fundamental cellular process that plays a significant role in the regulation of cells growth and homeostasis of tissue by eliminating unnecessary cells, and this cellular process are grave for organ development, tissue remodeling and regulation of immunity and several disease conditions [43]. Initiation of apoptosis due to the DNA damage, cleavage in proteins and over expression of caspase activate proteolytic cascade. Several of techniques have been used to detect the apoptosis based several cellular measures in the apoptotic process. On the earliest measures, cells which are undergoing apoptosis reorient phosphatidylserine from the inner side of the plasma membrane to its outer leaflet. Under this circumstance, cells can bind with Annexin V and this process can be used as vital apoptosis

marker. In this experiment, the HepG2 cells were treated with various concentrations of synthesized AgNPs for 48 hr and the apoptosis was performed by using Annexin V-FITC/PI assay. The Annexin V-FITC/PI assay results are showed in Fig.4. Fig. 4b exhibited that the addition of AgNPs (40 µg/ml) to the cell growing media marginally induced the cell necrosis (0.5%), early apoptosis (8.0%) and late apoptosis (1.7%) percentage than that of untreated cells (Fig. 4a). Interestingly, the results clearly showed that AgNPs at 50 µg/ml treatment significantly increased the necrosis (2.5%), early apoptosis (24.5) and late apoptosis (51.1%) in HepG2 cells (Fig.4c). This quantitative measure suggested that the AgNPs encouraged most of the cells into late apoptosis and induced apoptosis. The present study result was concordance with an earlier report that the biological syntheses of AgNPs induce the late apoptosis [44].

3.2.2. Western blot analysis

As developed AgNPs showed a significant reduction in cell viabilities on the HepG2 cells, then investigated the mechanism of AgNPs on apoptotic related proteins including Bax, cytochrome-c, caspase-3, caspase-9, PARP and anti-apoptotic associated proteins such as Bcl-2 and Bcl-xl expression in liver cancer cells by western blot method. The activations of Bax, cytochrome C, Caspase-3, caspase-9 and PARP were determined in cells after treatment with AgNPs at different concentrations for 48 h incubation. Expression of apoptotic proteins Bax, cytochrome-c, caspase-3, caspase-9 and PARP were upregulated in HepG2 cells after AgNPs treatment (Fig. 4a). Furthermore, the anti-apoptotic proteins Bcl-2 and Bcl-xL expressions were upregulated in HepG2 cells. In contrast, cells treated with AgNPs at different concentrations significantly reduced expression of Bcl-2 and Bcl-xL (Fig. 4b), suggest that the AgNPs triggered the apoptosis of HepG2 cells by increased apoptotic proteins and declined anti-apoptotic proteins in a time and dose dependent manner.

Apoptosis is programmed cells death process characterized by diverse morphological properties and energy based biochemical mechanisms. In general, apoptosis includes major component of various processes such as normal cell turnover, immune-modification, hormone-based atrophy, embryonic development and chemical-induced cell deaths [45]. Apoptosis is triggered via various mechanisms; mitochondrial mediated cascade plays major role in apoptosis induction. It has well established Bcl-2 members of proteins could act as both apoptotic and anti-apoptotic process by regulating mitochondrial membrane permeability. Bcl-2 and Bcl-xL are the anti-apoptotic proteins that inhibit the apoptosis by inactivation of the BH3 domain of pro-apoptotic proteins [46]. In contrast, Bax is an apoptosis inducer that found in the outer cell membranes of mitochondria [47], which facilitates the cytochrome-c release; it is an essential event in the execution cell damage via intrinsic pathway [48, 49]. Variations in pro-apoptotic and apoptotic proteins in cells lead to cause damage of mitochondrial membrane, as resulting cytochrome c release which stimulates caspase-9 activations [50], Caspase-3 is activated by proteolytic cleavage of caspase-9, key apoptotic executive caspase. Caspase-3 activates PARP by cleavage of PARP protein at the well-preserved region, indicating the potential role of PARP cleavage in apoptosis [51, 52]. In the current study involvement of mitochondrial apoptotic pathway in AgNPs induced cell death was detected as the changes in the Bcl-2 and Bax expressions. AgNPs treatment caused a considerable reduction in Bcl-2 and elevation in Bax expressions in cancer cells, therefore ultimately preferring the apoptosis process. Also, we noted AgNPs treatment at different concentrations induced time-dependent activation of caspase-3, and caspase-9 as well as cleaved PARP, which key players in mitochondrial-mediated apoptosis. AgNPs could perform well as anticancer therapy for its capacity to disrupt mitochondrial respiratory chain, which induces the reactive oxygen species (ROS) productions cause DNA

damage and reduction in ATP synthesis [53]. ROS production by AgNPs may also be required mitochondrial involvement for initiating apoptotic pathway. Our results are concurrent with this statement, AgNPs treatment-induced cytochrome-c release which eventually changed the mitochondrial potential and leads to programmed cell death.

4. Conclusion

To discover the new therapeutic concepts for treating cancer therapy with nano-silver formulations has got great attention as an attractive option. Characterisation study shows the spherical shape of particle, as well as intrinsic capping on the surface of chitin derived nanoparticles, enables its direct use for cancer treatment. The synthesized AgNPs showed significant cytotoxic effects against HepG2 cell line. Furthermore, AgNPs treatment increased apoptosis related proteins such as Bax, cytochrome-c, caspase-3, caspase-9, and PARP and decreased anti-apoptotic protein Bcl-2 & Bcl-xL expression in HepG2 cells. Therefore, our study findings suggest the anticancer activity of biologically synthesized AgNPs against HepG2 cells and might play a key role in the development of new therapeutic agent to treat cancer in future.

Author statement

Mayakrishnan Vijayakumar: Data curation, Writing- Original draft preparation, Writing -Review & Editing. **Kannappan Priya**: Data curation, Methodology, Investigation, Validation. **Soundharrajan Ilavenil**: Data curation, Methodology, Software. **Balakarthikeyan Janani**: Data curation, Investigation. **Vadanasundari Vedarethinam**: Data curation, Visualization, Software. **Thiyagarajan Ramesh**: Investigation, Software. **Mariadhas Valan Arasu**: Conceptualization, Funding acquisition. **Naif Abdullah Al-Dhabi**: Conceptualization, Funding acquisition, Writing-Re-view & Editing. **Young-Ock Kim**: Investigation, Software. **Hak-Jae Kim**: Investigation, Validation, Software.

Acknowledgements

We acknowledge the Management, Principal and Secretary of PSG College of Arts & Science, Coimbatore, Tamilnadu, India for their continuous support to carry out this research. Authors also extend their appreciation to the researcher supporting project number (RSP-2020/20) King Saud University, Riyadh, Saudi Arabia.

Conflict of interest

The authors declare that they have no potential conflict of interests.

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Figure legends

Fig.1. (a) UV-vis spectra of synthesized AgNPs using shrimp shells extracted chitin. **(b)** FTIR spectra of the chitin and synthesized AgNPs using shrimp shells extracted chitin. **(c)** EDX pattern of synthesized AgNPs using shrimp shells extracted chitin. **(d)** XRD spectra of synthesized AgNPs using shrimp shells extracted chitin.

Fig.2. (a) SEM analysis of synthesized AgNPs using shrimp shells extracted chitin. **(b)** TEM analysis of synthesized AgNPs using shrimp shells extracted chitin. **(c)** Zeta potential of synthesized AgNPs using shrimp shells extracted chitin. **(d)** PDI of the synthesized AgNPs using shrimp shells extracted chitin.

Fig.3. Cytotoxicity effect of various concentrations of chitin, $AgNO_3$, doxorubicin and synthesized AgNPs, using shrimp shells extracted chitin on cell proliferation of human hepatocellular carcinoma HepG2 cells. $^{a, b, c, d, e f}$ $p<0.05$ alphabets within a line indicates significance between different concentration of samples.

Fig.4. Biologically synthesized AgNPs mediated induction of apoptosis in human hepatocarcinoma (HepG2) cells. Flow cytometry analysis of HepG2 cells after 48 h treatment with 40 µg/ml and 50 µg/ml of AgNPs. Representative figures showing the population of live cells (Q3), early apoptosis (Q4), late apoptosis (Q2), and necrotic (Q1) cells. $^{a, b, c d}$ $p<0.05$ alphabets within bar graph indicates significance between control and treatments.

Fig.5. (a) Western blot analysis and densitometric determination of apoptotic proteins, full length of caspase-3, caspase-9 and PARP in experimental cells **(b)** Western blot analysis and densitometric determination of apoptotic proteins, Bax, cytochrome-c, cleaved caspase-3, cleaved caspase-9 and cleaved PARP in experimental cells **(c)** Western blot analysis and

densitometric determination of anti-apoptotic proteins Bcl-2 and Bcl-xL in experimental cells.

Fig. 1.

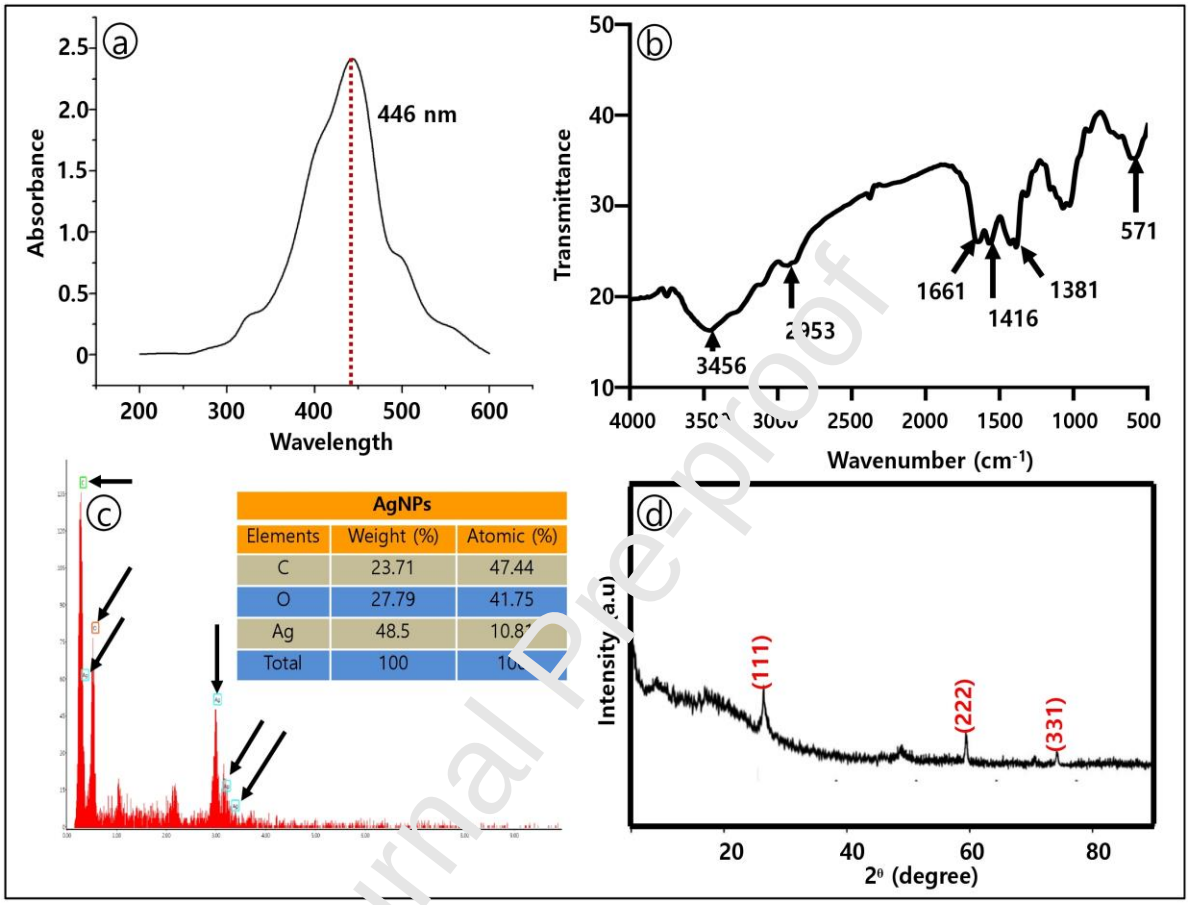
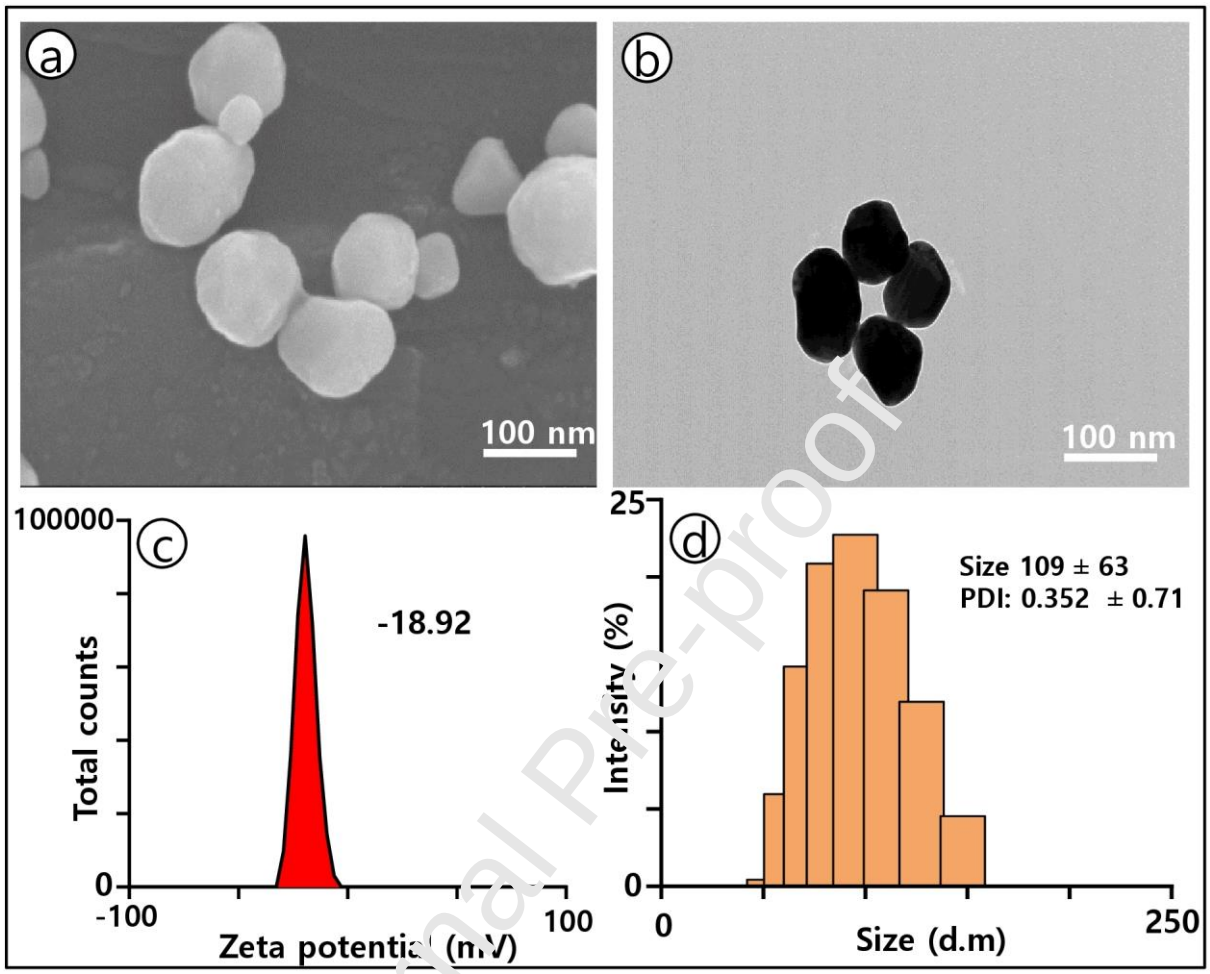


Fig. 2.



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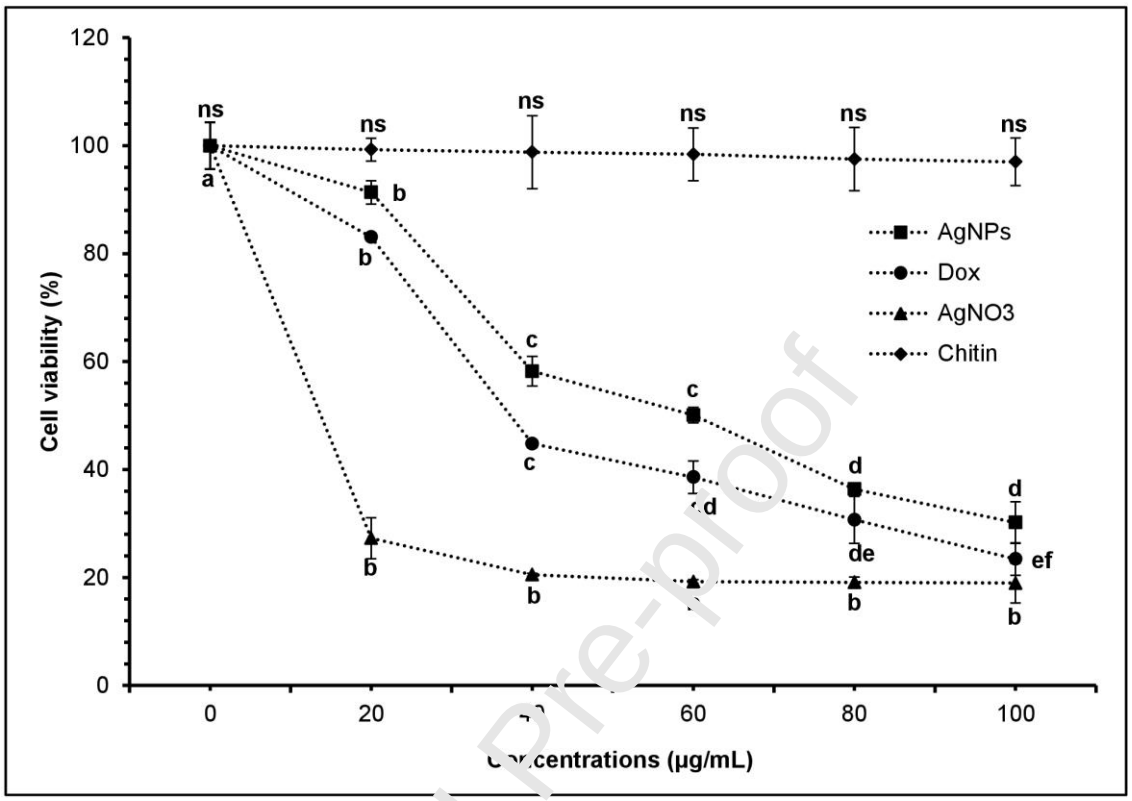

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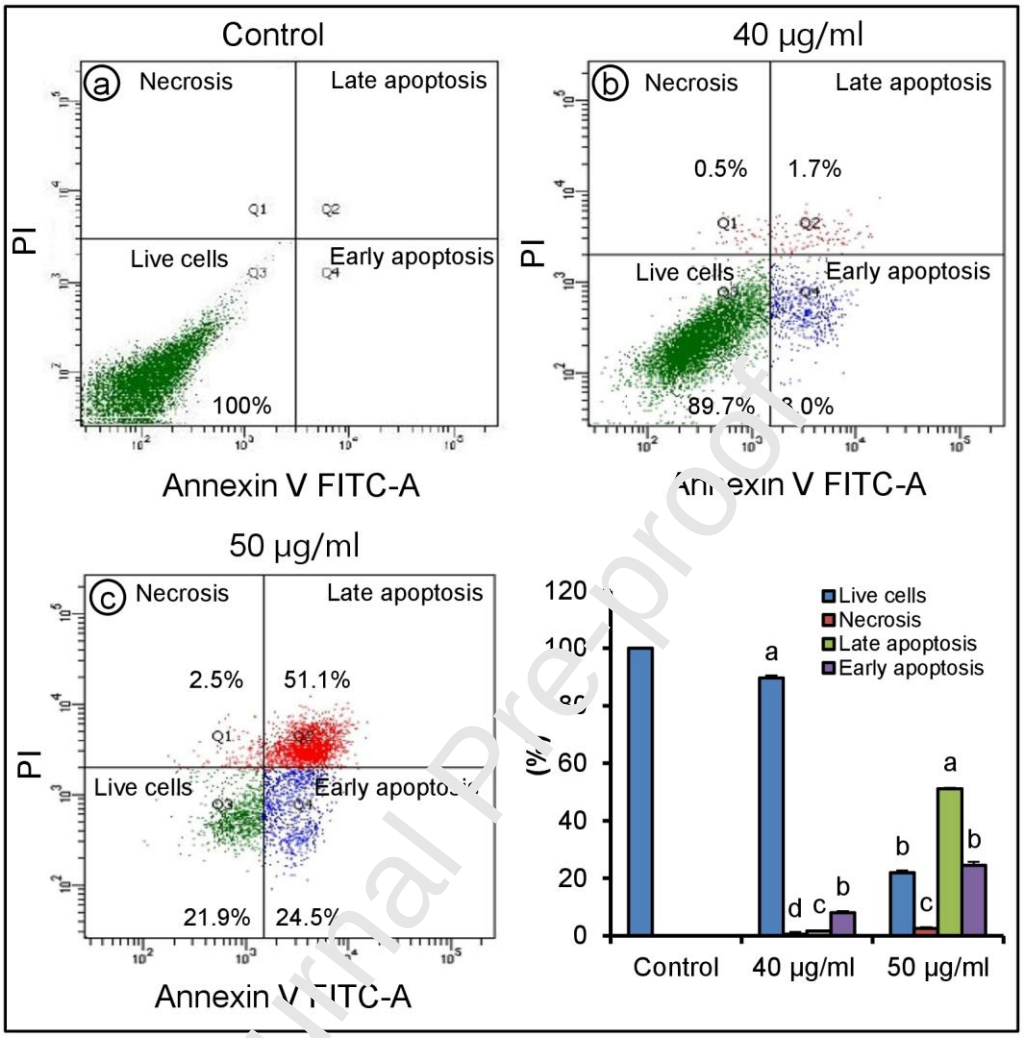

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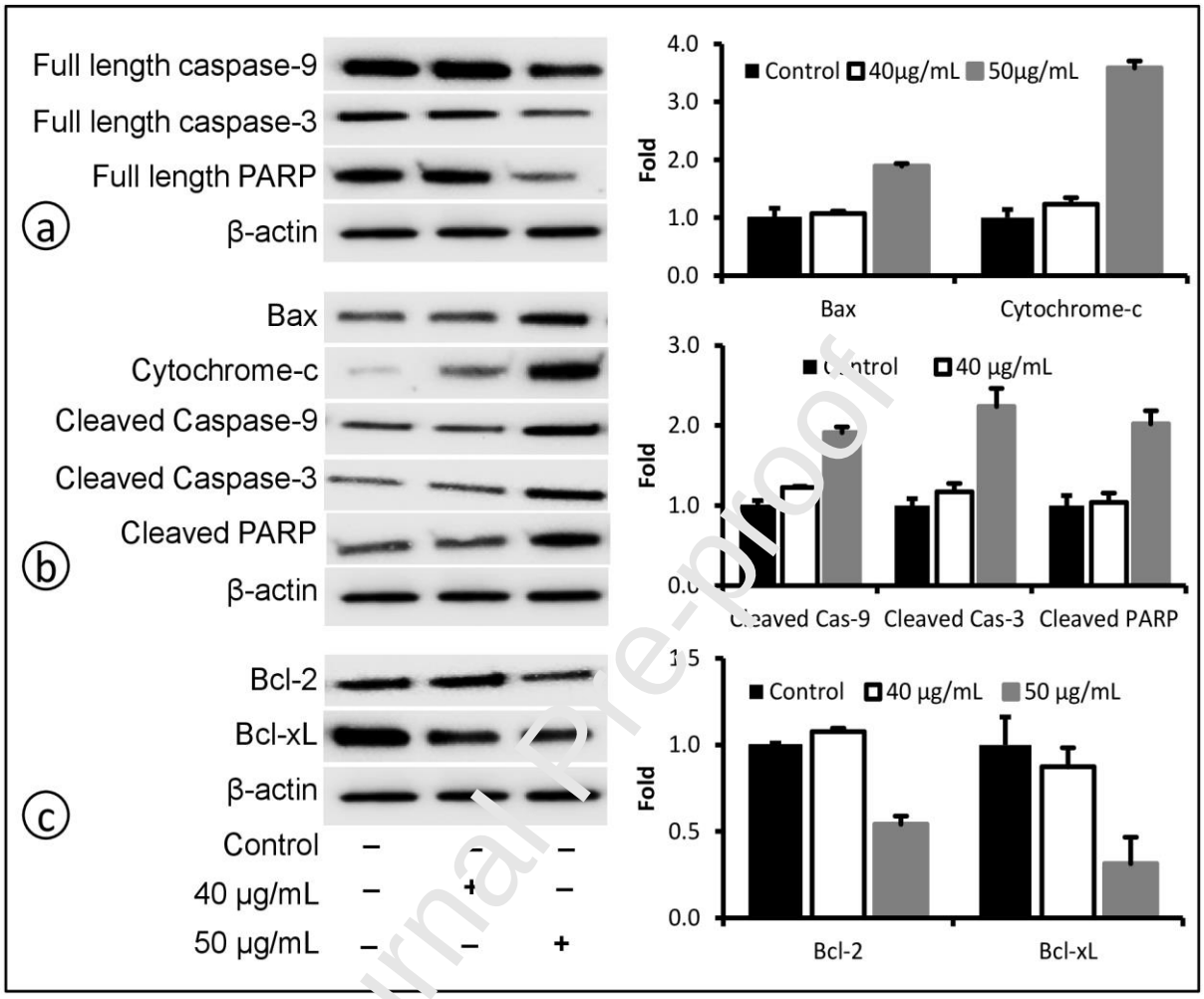

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