RESEARCH ARTICLE

Green Synthesis of Stable Silver Nanoparticles Using Teucrium polium Extract: In-vitro Anticancer Activity on NALM-6

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ABSTRACT

The therapeutic effect of silver nanoparticles on leukaemia cancerous cells has been demonstrated in several studies. However, most of these nanoparticles are toxic to normal cells as well as cancerous cells. In the present study, green chemistry has been applied for the synthesis of silver nanoparticles by Teucrium polium (T.P) extract. The synthesized nanoparticles were spherical with an average diameter of 14.3 ± 9.7 nm and a surface charge of -0.84 mV. Based on the FTIR results, the silver nanoparticles have been coated with T.P extract phytochemicals. The extract was not toxic toward cancerous cells. However, the T.P extract coated silver nanoparticles (T.P@AgNPs) with concentrations ≥ 50 µg/mL could eradicate the NALM-6 cancerous cells in a significant amount. Based on the flow cytometry analysis, the predominant mechanism of cancerous cell death is the apoptosis in NALM-6 cancerous cells; and the T.P@AgNPs had no toxic effect on normal PBMC cells.

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INTRODUCTION

Acute lymphoblastic leukaemia (ALL) is an invasive form of hematologic malignancy that affects lymphoid progenitors [1]. This leukaemia is caused by the proliferation and accumulation of lymphoid cells in the peripheral blood, bone marrow, and other tissues (extramedullary areas). ALL is the most common malignancy among children aged 2-5 years. It can also affect adults, therefore, suffering from ALL around the age of 60 can be very deadly. The annual prevalence of ALL in the United States has been reported to be 1.7 per 100,000. Hence, the resulting death toll in 2018 was estimated at 1,470 [1, 2].

Nanotechnology has created a promising domain in the field of cancer treatment. Among various nanostructures, metal nanoparticles were applied for different cancer treatment strategies such as chemotherapy, radiation therapy, and

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hyperthermia [3, 4]. So far, various compounds of colloidal solution of noble metals have been applied for their therapeutic values for different diseases such as cancer [5]. In recent years, the anticancer and anti-angiogenic effects of silver nanoparticles have been investigated and the results have shown that silver nanoparticles could be considered a potential anticancer agent [5, 6].

The wet chemical method is the main technique for the silver nanoparticles synthesis. This technique requires the reduction of silver ions through various chemical agents including, hydrazine, amino-boranes, oxalic acid, oleylamine, sodium borohydride, and citrate. Some other chemicals such as 4-(3-phenyl propyl)pyridine (PPP), N, N-dimethylformamide (DMF), Cetyltrimethylammonium bromide (CTAB), PVA (poly(vinyl alcohol)), and PVP (polyIJvinylpyrrolidone) could also be applied for stabilization of the synthesized silver nanoparticles [5]. Many other chemicals might be applied for adjusting the synthesis condition. The presence of these toxic chemicals and their by-products in the nanoparticle solution could hinder the silver nanoparticles application due to some safety considerations. Thus, there is a lot of interest to apply safe techniques for the synthesis of silver nanoparticles without toxic chemicals.

Currently, it has become a primary goal for scientists to create efficient techniques for the synthesis of silver particles with suitable properties based on green chemistry. One of the best practices of green chemistry is to apply the aqueous extracts of medicinal plants such as lotus for silver ion reduction and stabilizing the synthesized silver nanoparticles [7]. Green synthesized metal nanoparticles have been applied for various cancer treatments including gastric [8], cervical [9, 10], breast [11], lung [12], and colorectal [3] cancer cell lines. It seems that the anticancer effect of the green synthesized nanoparticles was related to increased ROS formation and depletion of antioxidants [10]. Also, it has been shown that biosynthesized AgNPs induce SubG1 arrest and apoptotic/necrotic cell death in cancerous cells [9].

Teucrium polium (*T.P*) is a wild-growing herb that belongs to the Lamiaceae species, and it is found abundantly in various regions including South-Western Asia (including Iran), North Africa, and eastern Europe [13]. Phytochemical investigations have shown that *T. Polium* contains various compounds such as terpenoids, flavonoids and iridoids [14]. Flavonoids which are polyphenols detected in medicinal plants have biological activities such as antioxidant and anticancer [15]. Cirsiliol, cirsimaritin, cirsilineol, salvigenin, and 5-hydroxy-6,7,3′,4′ –tetramethoxyllavone are flavonoids isolated from different *Teucrium* species [16]. The anticancer effect of *T.P* extract has experimented in different tumours such as colon, lung, and breast cancer. The *T.P* extract inhibits cell proliferation and deregulates cell cycle progression in H322 and A549 lung cell lines. Haïdara et al. suggested that this plant extract could have a therapeutic role in the treatment of human non-small cell lung cancer [17]. Also, it appeared that *T.P* showed anticancer activity by increasing ROS levels, Sirt3 activity, and cell death in HT-29 colorectal cancer cells [18]. It has also been demonstrated that the methanol extract of *Teucrium* species such as *Teucrium polium L.* and *Teucrium montanum L.* extracts could induce apoptosis in breast and colon cancer cells. [19]. In the current work, *T.P* extract was applied for the green synthesis of silver nanoparticles. Then the anticancer effect of these nanoparticles was evaluated in B cell precursor leukaemia cell line (NALM-6) at the *in-vitro* situation.

MATERIALS AND METHODS

Materials

Silver nitrate ($AgNO_3$, 7761-88-8), carboxy-methylcellulose(CMC, low viscosity, MW), hydr-ochloric acid, and nitric acid were purchased from Merck (Germany). Glassware was washed by diluted aqua regia ($3HCl\text{-}1HNO_3$). Fetal bovine serum (FBS), RPMI 1640, penicillin and streptomycin were supplied by Gibco (USA). 3-(4, 5- dimethyltiazol-2-ly)-2, 5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) were purchased from Sigma. The flow cytometry analysis was performed using Apo FlowEx® FITC kit (ExBio Company, ED7044). B cell precursor leukaemia cell line (NALM-6) which has been used in this study, was purchased from the Pasteur Institute of Iran.

T.P extract preparation

Teucriumpolium plants were harvested from the Rangelands of Jiroft, (Kerman, Iran). To prepare the extract, aerial parts of the plant were washed with distilled water and were thoroughly dried. Then, 20 grams of *T.P* plant was flooded into 400 mL of distilled water and boiled for 15 minutes. The resulting solution was centrifuged and the

supernatant was passed through filter paper. Finally, the obtained extract was freeze-dried and stored in the refrigerator as a lyophilized powder.

Green synthesized of silver nanoparticles

For the green synthesis of silver nanoparticles, 0.5 mL, 15 mM of $AgNO_3$ was poured into 10 mL of the extract (1.5 mg/mL) and placed on a magnetic stirrer for two hours at room temperature under dark condition. The yellow color of the reaction solution is a representation of nanoparticle formation. To stabilize the synthesized nanoparticles, CMC polymer (0.1% w/v) was added and placed on a magnetic stirrer for another hour. For removing unreacted materials, the nanoparticles were washed with deionized water twice through centrifugation and decantation of the supernatant at 9000 rpm for 20 minutes. The final silver nanoparticles were freeze-dried and stored at room temperature for future experiments.

Characterizations of silver nanoparticles

The formation of silver nanoparticles was investigated using the UV-Vis array spectrophotometer (PhotonXAr2015, Iran) at a wavelength of 200 to 800 nm through observing the characteristic surface plasmonic resonance of silver nanoparticles. Fourier-transform infrared spectroscopy (FTIR, Thermo Nicolet AVATAR, USA) with a resolution of 4 cm^{-1} and a scanning range of 400 to 4000 cm^{-1} was applied for the analysis of phytochemicals coating of green synthesized silver nanoparticles. The hydrodynamic diameter of nanoparticles was measured by Dynamic Light Scattering (QudixScatteroscope I, South Korea). Zeta potential measurements were performed with a zeta potential analyzer (Cordouan Technologies). The morphology of nanoparticles was observed using TEM analyses (Philips CM120) with a voltage of 80 kV.

Cell studies

NALM-6 cells were cultured with RPMI 1640 cell culture supplemented by L-glutamine, FBS (10%) and Pen/Strep (1%) and different concentrations of nanoparticles (1, 2.5, 5, 10, 25, 50, 100, and 200 µg/mL) and *T.P* extract (10, 20, 50, 100, 250, and 500 µg/mL) in density of 10000 cells per well (in 96 well-plates) in a standard condition (37°C, 5% CO_2). After 24 and 48 hours, MTT assay was conducted for cell viability assessment. Each well was supplemented by 0.5 mg/mL MTT dye and kept in an incubator for 2-4 hours. Then 100 µL DMSO was added to the wells and the absorbance of each well was measured at 570 nm using a microplate reader (ELX808, Biotek, USA). The viability percentage of each group was analyzed based on the control group[20].

Annexin V-FITC/propidium iodide (PI) apoptosis assay

The NALM-6 cells were exposed to the silver nanoparticle (200 µg/mL) in RPMI-1640 at a density of 10^6 cells/sample in a 6-well plate for 48 hours. The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Mabtaq) was used to distinguish the population of apoptotic and non-apoptotic cells. The 100 µLcell suspension (1×10^6/mL)was stained with 5 µl of Annexin V and propidium iodide (PI), vortexed, and incubated for 20 min at room temperature in dark condition. After the staining, flow cytometry was performed using flow cytometer (Sysmex, CyFlow® Space, Sysmexpartec) to quantify the population of apoptotic and non-apoptotic cells. The results are expressed as the rate of apoptosis (the percentage of early and late apoptotic cells).

Statistical analysis

Statistical significance was evaluated using unpaired Student's t-test and one-way analysis of variance (ANOVA). All data presented as mean ± standard deviation and a P-value <0.05 regarded to be significant.

RESULTS AND DISCUSSION

Synthesis and characterizations

After adding the silver nitrate solution to the *T.P* extract, the color of the solution began to turn yellowish-brown, which is due to the reduction of silver ions to the silver nanoparticles. This change in color represents the surface plasmon resonance (SPR) in the synthesized nanoparticles [20, 21].Fig. 1-a shows the UV-Vis spectrum of the synthesized silver nanoparticles in *T.P* extract with concentrations of 1.5 mg/mL. The presence of the SPR peak in the range of 430 nm confirmed the formation of silver nanoparticles[20].

The silver nanoparticles that have been synthesized by various plant extracts are inherently unstable and tend to aggregate. In general, the aggregation of nanoparticles synthesized with plant extracts is due to the slow kinetics of reaction or poor bonding of the extract phytochemicals to the

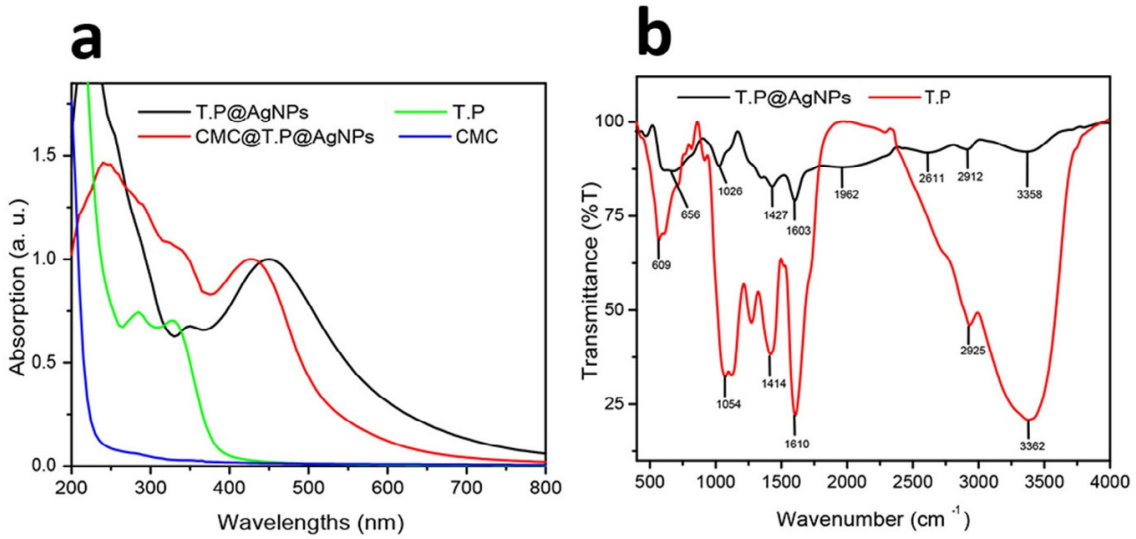

Fig. 1. The spectroscopic investigation of *T.P@AgNPs*. (a) UV-Vis spectra of *T.P*. extract, CMC, and *T.P@AgNPs* before and after stabilized by CMC. (b) FTIR spectra of *T.P@AgNPs* and *T.P* extract.

surface of the nanoparticles which could cause a low surface charge of the nanoparticles [22, 23]. However, many functional groups have existed in various phytochemicals in every plant extract. Carboxylic and catechol groups which existed in various natural phenolic compounds prevent the aggregation and increase in nanoparticle size by forming a negative coating on the surface of the synthesized nanoparticles[23, 24].

Silver nanoparticles are composed of mineral silver in the crystalline form. Nanoparticle surface coating is a crucial factor for nanoparticle stability and depends on the type of material used to reduce the Ag ions and stabilization of the nanoparticle surface. In the present experiment, silver nanoparticles prepared with TP extract were stable for one week, and after this period the color of the colloidal solution was changed which indicates the aggregation of the nanoparticles. Hence, CMC was applied to increase the stability of green synthesized silver nanoparticles. CMC, as a chemical derivative of cellulose, is widely used as a stabilizer in the synthesis of metal nanoparticles in aqueous media. This polymer inhibited the growth of FeS agglomerates and increased the stability of FeS nanoparticles[25]. The CMC can form a negatively charged layer on the surface of the nanoparticles to provide an intra-particle electrostatic repulsion, thereby dispersing the nanoparticles[26]. Stabilizers play an important role in controlling the size and stability of particle dispersion. Abdel-Halim et al. applied CMC as a reducing and stabilizing agent in the synthesis of silver nanoparticles [27]. As shown in Fig. 1-a, a slight change in λ_{max} was observed in the sample containing 0.1 % CMC, indicating that the particles have been covered by CMC. Based on our observation, by adding CMC to the silver solution, the colloidal stability of nanoparticles was increased for one month.

The FTIR was performed to investigate the *T.P* extract existence on the surface of the AgNPs. As demonstrated in Fig. 1-b, the FTIR spectra of the *T.P* extract is similar to the AgNPs that have been synthesized with *T.P* extract. However, few differences are clear which could help understand the responsible biomolecules for reducing and possible stabilization of synthesized nanoparticles.

Unlike green synthesized *T.P@AgNPs*, most of the bands are very clear in the *T.P* extract samples. However, the spectrum of each one is very similar to those of the others that indicates the coverage of *T.P* extract phytochemicals on the surface of *T.P@AgNPs*. For example, the peak at 3360 cm^{-1} for both samples is attributed to the various hydroxyl compounds in alcohol and phenolic phytochemicals. Some of these hydroxylsexist on the surface of the AgNPs. Probably some of these hydroxyls are deprotonated for the reduction of silver ions. The bands at 2912 and 2924 cm^{-1} were coming from symmetric stretching of –CH. The band at 1600 cm^{-1}in both samples could be attributed to the ketone functional groups of C=O

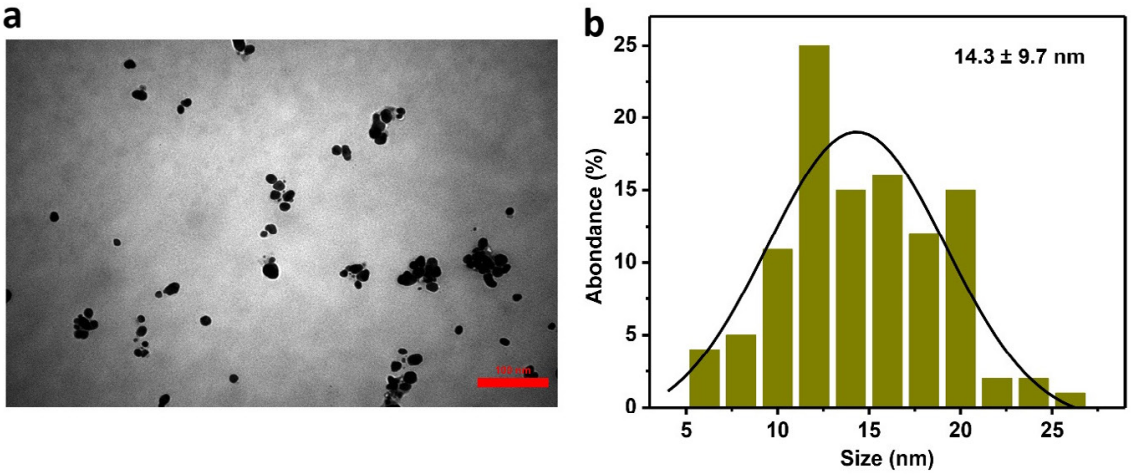

Fig. 2. TEM micrograph of *T.P@AgNPs* (a) and corresponding size distribution diagram. Scale bars represent 100 nm.

stretching or stretching of C=C double bond in alkene compounds. The bands at 1020 and 1050 cm^{-1} are attributed to the phosphate compounds. The clear C-Br stretch band at 610 and 655 cm^{-1} can result from aliphatic bromo compounds. These bands indicate the presence of various chemical constituents on the surface of synthesized silver nanoparticles.

The TEM micrograph was analyzed to investigate the average diameter and shape of *T.P@AgNPs*. Based on the acquired TEM micrographs, most of the synthesized nanoparticles are circular, and the analyzed average diameter was 14.3 ± 9.7 nm (Fig. 2). Most of the green synthesized silver nanoparticles are heterogeneous in size and multifaceted in shape. However, *T.P@AgNPs* are spherical and homogenous in size based on provided TEM micrographs. The hydrodynamic diameter of the *T.P@AgNPs*was 28.1 nm which is larger than the average size obtained from an electron microscope (Fig. 3a). In this test, larger particles have a very high impact on the results because they create a higher light scatter and have a high impact on the test result [28]. We washed the *T.P@AgNPs* after the synthesis to remove unreacted material. Due to the washing procedure, the surface charge of the particles shows a value of -0.84 (Fig. 3b), which is a neutral value. This value is too low for the electrostatic stability of the particles [29]. However, the nanoparticles were covered by CMC that provided steric stability[30].

Cellular studies

In recent years, the use of metal nanoparticles, especially silver nanoparticles, has expanded dramatically. The chemical, physical and antimicrobial properties of these particles have been confirmed in many studies [20, 31, 32]. The viability of NALM-6 cells with 24 and 48 hours treatment of *T.P*extract and silver nanoparticles with various concentrations was studied by MTT assay (Fig.4). Our results showed that *T.P*extract had no toxic effect up to 500 µg/mL on NALM-6 cells. Fig.4a and 4b show a comparison of cell viability after treatment with different concentrations of extracts alone. In contrary, our MTT results indicate that the viability of the NALM-6 cells depends on the concentration of silver nanoparticles and incubation periods. With increasing concentration of silver nanoparticles or treatment duration, the viability of NALM-6 cancer cells was decreased (Fig.4 c, d).

The apoptosis of NALM-6cells under *T.P@AgNPs* treatment was also evaluated by flow cytometry using FITC Annexin-V apoptosis detection kit. To confirm the non-toxicity of nanoparticles on normal blood cells, the PBMC cells (peripheral blood mononuclear cell) were incubated (48 hours) with 200 µg/mL of*T.P@AgNPs*. As presented in Fig.5, this exposure did not significantly increase the percentage of Annexin-V and Annexin-V/PI double-positive cells. In contrast, after 48h treatment of the NALM-6 cells with AgNPs, the percentage of apoptotic cells was significantly higher in comparison with the untreated cells ($P < 0.01$). It has also been shown that silver nanoparticles can increase apoptosis and prevent cellular proliferation by producing reactive

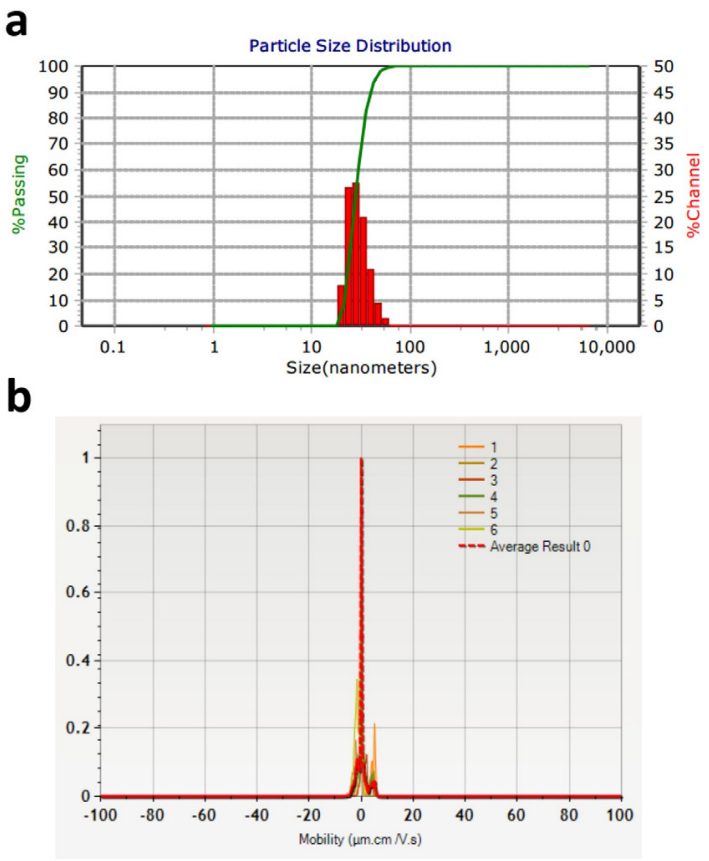

Fig. 3. Investigation of hydrodynamic diameter (a), and zeta potential (b) of silver nanoparticles. The hydrodynamic diameter of *T.P@AgNPs* is 28.1 nm and the surface charge of the particles shows a value of -0.84 mV.

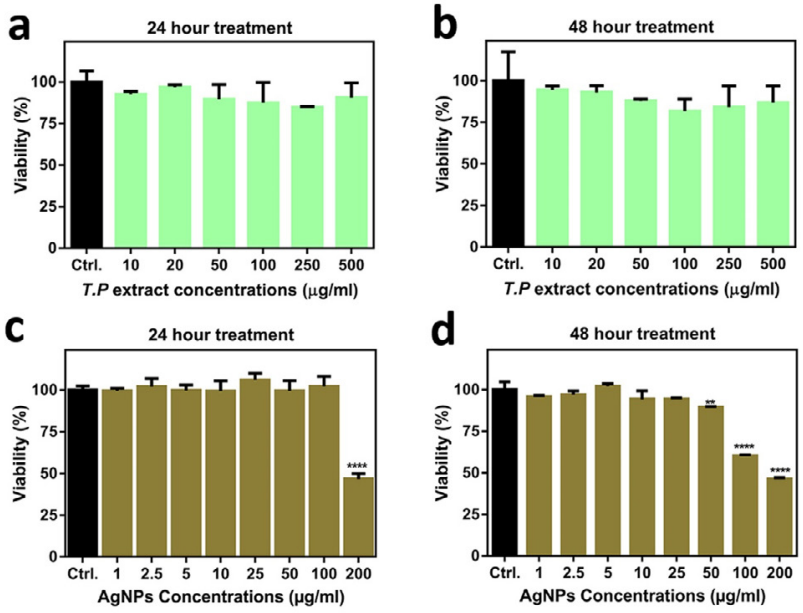

Fig. 4. The NALM-6 cells viability after 24-and 48-hours incubation with *T.P* extract (a-b) and *T.P@AgNPs* (c-d). Ctrl: Control. *T.P@AgNPs*: Ag nanoparticles. *T.P* extract: *Teucrium polium* extract. One representative experiment of three performed is shown (n=3, $P < 0.01$ relative to untreated cells). The concentration of 200 µg/mL of *T.P@AgNPs* after 48 hours was selected for flow cytometry analysis.

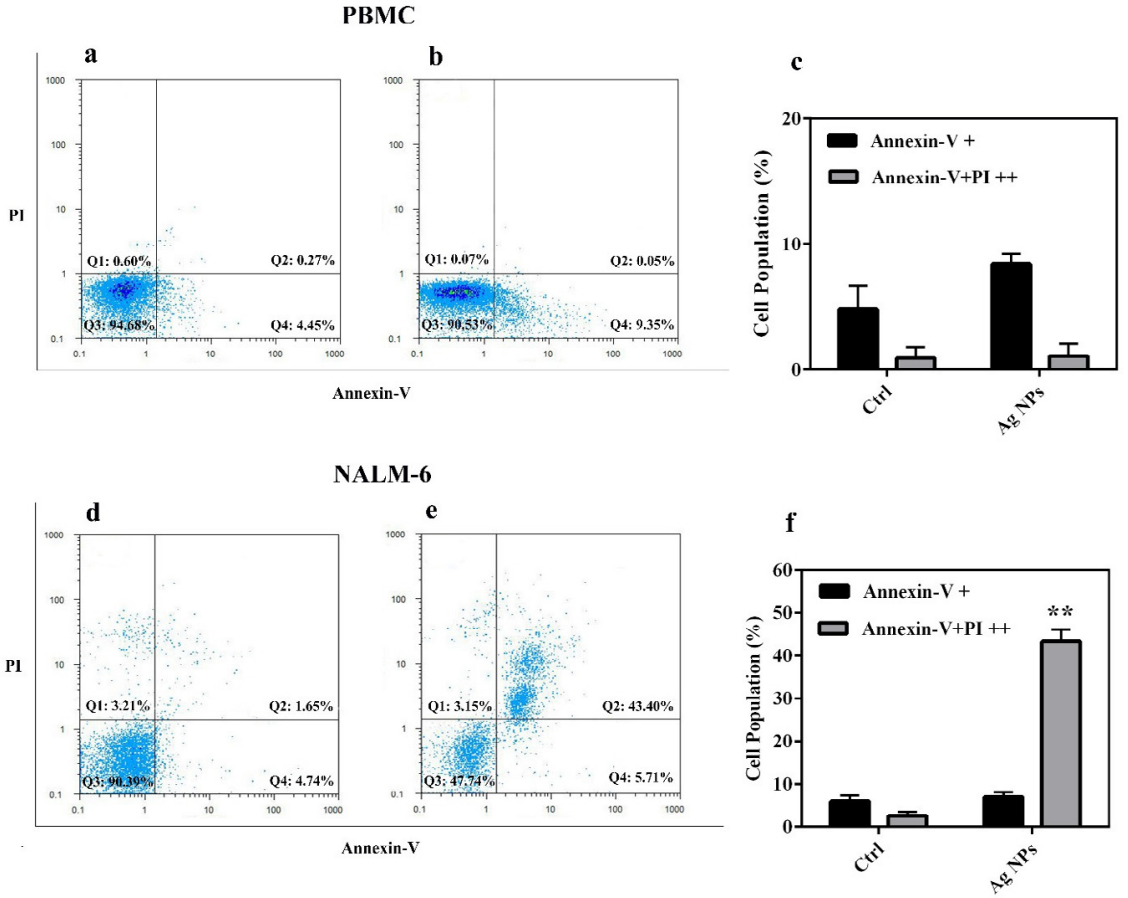

Fig. 5. Flow cytometry analyses demonstrated that the AgNPs significantly promotes apoptosis in NALM-6 cells. PBMC and NALM-6 cells were treated with AgNPs (200 µg/mL) for 48 h. Then, cells were analyzed for Annexin-V and PI staining by flow cytometry and showed as PBMC control cells (a), PBMC AgNPs treated cells (b), NALM-6 control cells (d), and NALM-6 AgNPs treated cells (e). Q1, Q2, Q3 and Q4 indicate PI-positive, Annexin-V/PI double-positive, Annexin-V/PI double-negative and Annexin-V positive cells, respectively. (c), and (f) shows a quantitative analysis of staining. Ctrl: control. AgNPs: Ag nanoparticles. One representative experiment of three performed is shown (n=3, **P < 0.01 relative to untreated cells).

oxygen species (ROS) and damaging DNA[33]. This mechanism is important for inhibiting tumor cells[34]. Therefore, the anti-tumor properties of silver nanoparticles can be used in the treatment of many cancers [33]. Farahani et al. examined the cytotoxic effect of silver nanoparticles on human lymphocytes and HPB-ALL cell line and showed that these particles significantly cause cytotoxic effects on HPB-ALL cell line [35]. The study by DaweiGuo et al. also suggested that silver nanoparticles could induce cytotoxicity and apoptosis through producing reactive oxygen species in a chronic lymphoblastic leukaemia cell line (K562). The results of this study showed that the proper use of silver nanoparticles could be widely used in the treatment of ALL in the future [36]. Also, in two other studies, the apoptotic and necrotic effects of silver nanoparticles on a human acute monocyticleukaemia cell line (THP-1), and human T lymphocyte cells (Jurkat) were confirmed [37, 38].

CONCLUSION

Based on the provided results, the *T.P@AgNPs* were synthesized in a green chemistry technique with a proper characteristic. The average diameter of the particles is 14.3 ± 9.7 nm and represents a natural surface charge. These particles were very biocompatible in the face of the normal PBMC cells. However, *T.P@AgNPs* could have a therapeutic effect against NALM-6 cancerous cells. The *T.P@AgNPs* could eradicate the cancerous cells through the apoptotic route. The more molecular and cellular analysis could be performed to shade

light on the unknowns.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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