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RESEARCH ARTICLE

Silver nanoparticles: biosynthesis and cytotoxic performance against breast cancer MCF-7 and MDA-MB-231 cell lines

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ABSTRACT

Objective(s): A study was performed on the possibility of synthesizing silver nanoparticles (Ag-NPs) from *Ducrosia Anethifolia* Aqueous Extract for treating breast cancer MCF-7 and MDA-MB-231 cell lines.

Methods: The application of Ducrosia Anethifolia Aqueous Extract caused a reduction in silver ions and facilitated the production of silver nanoparticles. Different analyzing procedures were exerted to evaluate the qualities of synthesized NPs by *Ducrosia Anethifolia* Aqueous Extract including UV-Vis spectroscopy, transmission electron microscopy (TEM), and dynamic light scattering (DLS). In the following, subsequent to assessing the viability of MCF-7 and MDA-MB-231 cell lines, their exposure to AgNPs was evaluated by the usage of MTT assay. Moreover, the Bax, Bad, Bcl-2, and c-FLIP expression levels of treated cells were examined by real-time PCR analysis.

Results: The observance of an absorption peak at 460 nm through the obtained data from a spectrophotometer affirmed the production of silver NPs. The images of electron microscope displayed the spherical shape of NPs in an average size of around 9.41 nm. Moreover, this product caused a robust reduction in the viability of both MCF-7 and MDA-MB-231 cell lines that was mainly pointed out by the improved expressions of Bax and Bad gene, while minimizing the gene expressions of Bcl-2 and c-FLIP.

Conclusions: This experiment approved the applicability of Ducrosia Anethifolia Aqueous Extract for performing the green synthesis of NPs. In addition, the synthesized NPs exhibited anti-tumor effects on the applied breast cancer cell lines through the up-regulation of pro-apoptotic proteins concomitant along with the down-regulation of anti-apoptotic proteins expression.

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INTRODUCTION

As a global health concern, the inducement of cancer is dependent on aberrant mutation genes, which proliferate and differentiate the normal cells and turn them into unregulated proliferation cells with the ability to travel throughout healthy organs and cause tumor metastasis and malignancy. The exertion of customary treatments, similar to

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chemotherapy and radiotherapy, is very costly and commonly results in extreme side effects such as bone marrow obstacles, hair loss, nausea, emesis, and fatigue[1, 2]. In this regard, the world demands novel approaches to surpass the restraints of traditional therapies.

The interest of many scientists has focused on the interesting features of nanoparticles (NPs), including efficient surface area and high reactivity. Nanomaterials are used for the production of varying industrial and medical products due to their diverse sizes and shapes achieved through the quick progresses of nanotechnology. Next to different medical fields such as drug delivery and imaging of organs and cells, NPshave the potential of being utilized for the diagnosis and treatment of cancer. The binding of NPsto nano-sized pharmaceuticals can be facilitated through their nanometer-scaled sizes, while this feature results in their adsorption by cancer cells as well. Consequently, the exposure of normal cells to anticancer drugs would be prohibited and the inducement of common side effects would be avoided[3].

Among the available metal based NPs, including gold, silver, zinc, iron, titanium, magnesium, and alginate, silver can up-regulate or down-regulate cellular mechanisms along with taking the role of a medium in the course of detecting and diagnosing body illnesses. The type of applied synthesizing routes and strategies mainly determines the exhibition of exceptional qualities of Ag NPs. The conduction of certain physical and chemical procedures were reported for the formation of silver nanoparticles (Ag-NPs), which involved the exertion of using chemical agents and devices, such as photochemical reduction, evaporation- condensation, laser ablation, electrochemical routines, chemical reaction by organic and inorganic agents, gamma irradiation, thermal decomposition of silver oxide within water and ethylene glycol, and microwave processing. The application of toxic and dangerous materials in the roles of surfactant and capping agents is required in chemical procedures, while the physical techniques process on the exertion of high energies (such as microwave and UV irradiation) and complex devices to complete the synthesis of Ag NPs. Therefore, the threatening impacts of these methods to the wellbeing of environment and their contradiction to the principles of green chemistry are entirely evident. Meanwhile, reports indicated the interesting properties of biologically-composed AgNPs that include containing a high yield, as well as being soluble highly stable. In comparison to the other synthesizing routines for AgNPs, biological procedures proved to be uncomplicated, quick, lack toxicity, qualify as reliable, and stand as green approaches with the ability to manufacture well-defined morphology and sizes throughout optimized circumstances in regards to translational research. Considering these facts, it can be a very promising attempt to achieve the synthesis of AgNPs through a green route [4-6].

As a member of Apiaceae family, *Ducrosia anethifolia* plant mainly contains the chemical compositions of n-decanal, ndodecanal, chrysanthenyl acetate, myrcene-limonene, and α-pinene. According to the common knowledge, the aliphatic compounds of this herb can function as antibacterial agents in the cases of Grampositive and Gram-negative bacteria [7, 8]. Categorically, medical plants incorporate bioactive molecules with the ability to facilitate the green synthesis of Ag-NPs. These molecules are capable of taking the roles of reducing and capping agents throughout synthesizing processes and produce applicable NPsfor biomedical implementations [9-13]. For instance, Hashemi et al. reported the exertion of Sambucus ebulus (S. ebulus; AgNPs @ SEE) extract for synthesizing Ag-NPs and evaluating the obtained antibacterial, anticancer, and photocatalytic properties. According to the outcomes, their synthesized product through a green method resulted in exhibiting a great potential for medical applications[14].

Therefore, the objective of this work was set to investigate the synthesis of Ag-NPs by the application of Ducrosia anethifolia plant extract and its anti-cancer properties against breast cancer cell lines.

MATERIALS AND METHODS

Materials

Silver nitrate (AgNO3, 7761-88-8) was acquired from Merck (Germany). MTT reagents and mRNA Isolation Kit was procured from Gibco (Germany), while the High Capacity cDNA Reverse Transcription Kit was obtained from Thermo Scientific (USA). Nuclease-free water was purchased from Invitrogen™ (Germany) and SYBR Premix Ex Taq kit was ordered from TAKARA (Japan).

Characterizations of silver nanoparticles

The conduction of TEM assessment required the preparation of certain specimens of synthesized AgNPs. For this purpose, the ultrasonic dispersion of NPs was performed in ethanol to have the suspensions settled on a carbon-coated copper grid. The process of TEM was completed by the usage of a (CM30 3000Kv). A computerized inspection system (MALVERN Zen3600) with DTS® (nano) software was exerted in order to distinguish the size distribution of Ag NPs through a DLS procedure. The next step involved the performance of UVVis spectroscopy (UV-Vis) analyses by the application of a Varian Cary 50 UV–vis spectrophotometer, while the spectra were recorded throughout a range of 350-800 nm.

Preparation of aqueous extract of Ducrosia Anethifolia plant

To obtain an aqueous extract, about 2 grams of dried plant was appended to 10 cc of deionized water to be shaken for 24 hours. Then, the extract was centrifuged (500 rpm ,15 min) and washed twice.

Synthesis of silver particles using aqueous extract of Ducrosia Anethifolia plant

To begin the green synthesizing process of Ag-NPs, 3 mL, 0.05 mM of AgNO3 was appended to 7mL of the extract to be stirred by a magnetic stirrer for 6 hours at ambient temperature. The observance of an alteration in the color of suspension subsequent to 15 of interaction, which turned from light-yellow to brown, referred to the production of Ag NPs. Once the obtained brown Ag suspension was chilled at room temperature, it was stored to undergo the upcoming characterization techniques.

Cell culture

The cultivation of human breast cancer cells, MCF-7 and MDA-MB-231 (ATCC), was performed in a high glucose Dulbecco's Modified Eagle's Medium (DMEM) that was augmented with fetal bovine serum (FBS) 10%, 100 units/ml of penicillin, and 100 µg/ml of streptomycin (PAA, Austria) within a humidified air with 5% CO2 at 37 °C.

MTT assay

We evaluated the cytotoxicity of AgNPs towards MCF-7 and MDA-MB-231 by the means of MTT assay. In brief, the seeding of MCF-7 and MDA-MB-231 cells within 96-well plates was completed at a density of 5×10^4 cells per well. Then, the cells were treated with 0-120µg/ml concentrations of AgNPs for 12, 24, 48, and 72 hours of exposure. Upon the four time intervals of 12, 24, 48 and 72 hours of treatment, 20 µL of 5 mg MTT/ml medium was appended to the wells that contained MCF-7 and MDA-MB-231. Subsequent to storing the cells at 37°C for 4 hours, the OD of wells was measured at the wavelength of 570 nm by the application of an ELISA reader.

RNA Isolation

The total RNA of MCF-7 and MDA-MB-231 was procured through the usage of mRNA Isolation Kit (Gibco, Germany). In addition, Nanodrop-2000 was exerted to assess the quality and quantity of extracted RNA content.

Reverse transcription and cDNA synthesis

In this section, we attempted to evaluates the primers by using RT-PCR and also estimate the expression levels of Bax, Bad, Bcl-2 and c-FLIP genes in MCF-7 and MDA-MB-231 cells through the application of real-time PCR (quantitative PCR, qPCR). For this purpose, the transcription of isolated total RNA into cDNA was completed by the utilization of High Capacity cDNA Reverse Transcription Kit (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific, USA). Concisely, nuclease-free water (Invitrogen™, Germany) was appended up to a concentration of 10 µL to the mixture of RNA (1 µg) with random hexamer primer (1 µL). Then, once the tubes were positioned in a thermocycler at 65 ˚C for 5 minutes, all of the boxes were maintained on ice to perform the final step of adding other reagents. The platform of 5 minutes at 25 ˚C, 60 minutes at 42 ˚C, and 5 minutes at 70 ˚C was exerted to amplify the First-strand cDNA.

Real Time-PCR

The performance of Real Time-PCR required the usage of a StepOnePlus Real-Time PCR system (Applied Biosystems, USA) and a SYBR Premix Ex Taq kit (TAKARA, Japan). The gathering of standard reaction mixture (20 µl) was completed with 10 µl of SYBR Premix Ex Taq 2x, 1 µl of template cDNA, 5 µl of ultra-pure water, and 200 nM concentrations of primers. Table 1 displays the primer sequences exploited in Real-Time PCR. GAPDH was applied

Table 1. Primer pairs used for Real-time PCR

Gene		Primer Sequence (5′-3′)
Bax	F	TTTGCTTCAGGGTTTCATCC
	R	GCCACTCGGAAAAAGACCTC
Bad	F	CATTTGTTCTGTGTCTCCAGTTG
	R	GCTCGTCTTGCTTCATGTCAC
Bcl-2	F	GGATTGTGGCCTTCTTTGAG
	R	CAGCCAGGAGAAATCAAACAG
c-FLIP	F	GCAGCAATCCAAAAGAGTCTCA
	R	ATTTCCAAGAATTTTCAGATCAGGA
GAPDH	F	GAGTCAACGGATTTGGTCGT
	R	TTGATTTTGGAGGGATCTCG

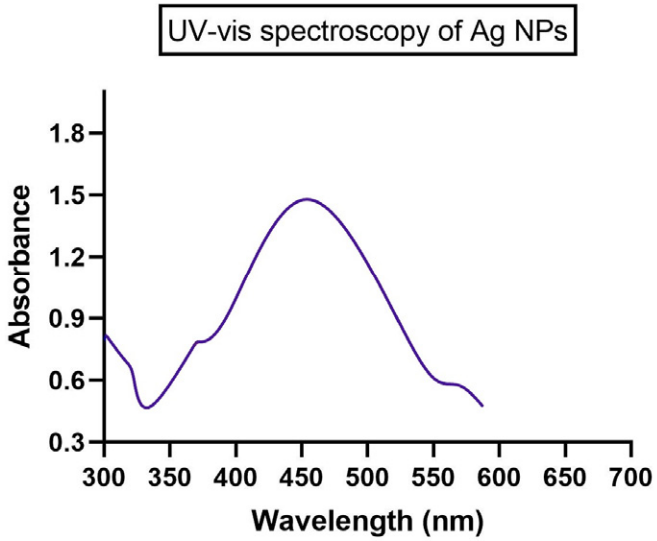

Fig. 1. UV-vis spectroscopy of Ag NPs

as the internal control and the tests were conducted in triplicate for each data point.

Statistical Analysis

The conduction of statistical analyses required the usage of GraphPad Prism version 8.01. The results were preventive of means ± SEM from three independent tests. The statistical differences of experimental groups were defined by the application of Student's t-test. The statistically significant values contained P-value <0.05.

RESULT AND DISCUSSION

UV-Vis Assessment

The appending of silver nitrate cause an alteration in the color of plant extract that turned from light yellow to brown, which was caused by surface plasmon resonance and mainly relies on the size and form of NPs. In conformity to Fig. 1, the observed sharp peak at around 460 nm related to the synthesis of Ag-NPs. The data of previous literature indicated the correspondence of the detected bond to the induced absorption by colloidal silver nanospheres throughout the range of 450-500 nm[15].

TEM and DLS investigation

The morphology and size distribution of NPs is demonstrated in Fig. 2. The spherical morphology of AgNPs is displayed by the TEM images (Fig.

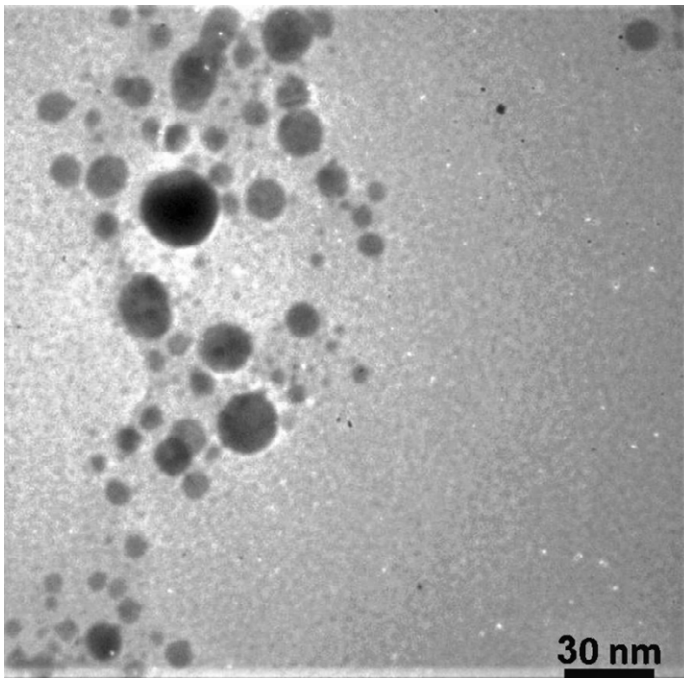

Fig. 2. TEM image of synthesized AgNPs

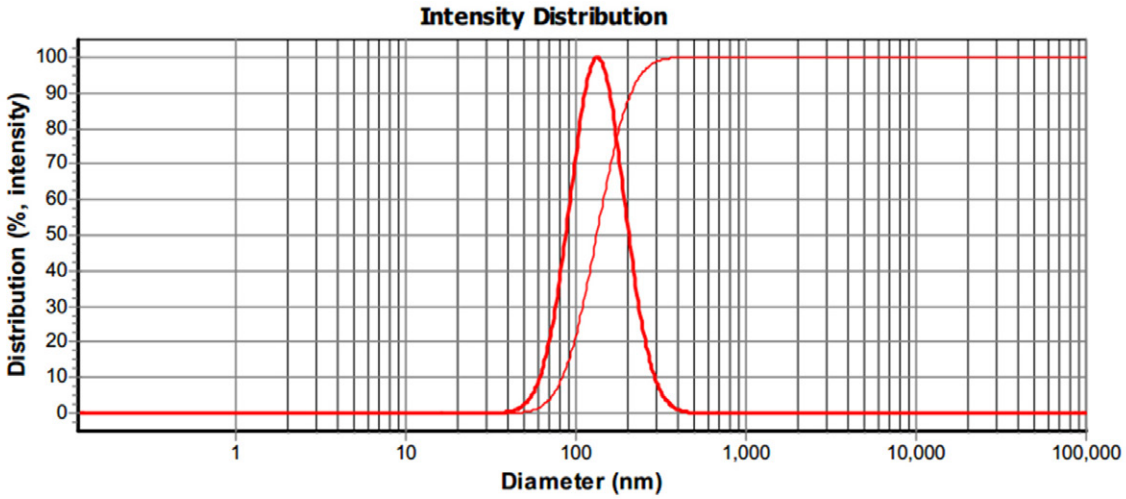

Fig. 3. DLS of green synthesized Ag NPs

2), while their size distribution was in the range of 3.02 to 20.8 nm in an average size of 9.41 nm. A histogram of NPs size distribution was attained by the means of DLS technique and provided in Fig. 3. The data of analyses reported the average size of Ag-NPs to be 135 nm. Furthermore, the outcomes of DLS and TEM were observed to be dissimilar due to their surrounding cover, as well as the fact that DLS revolves around the measurement of hydrodynamic diameter of NPs. Moreover, since an aqueos environment can force certain NPs to connect and induce an aggregation, the performed DLS provides the measurement of overall diameter of the set of NPs. The particle size distribution of nanoparticles in DLS measurement implicates a highly narrow to a highly broad range due to its dependence on the size of NPs and the magnitude of biological components connected to various plant extract NPs. This difference in particle size distribution has been also observed in other similar studies[16].

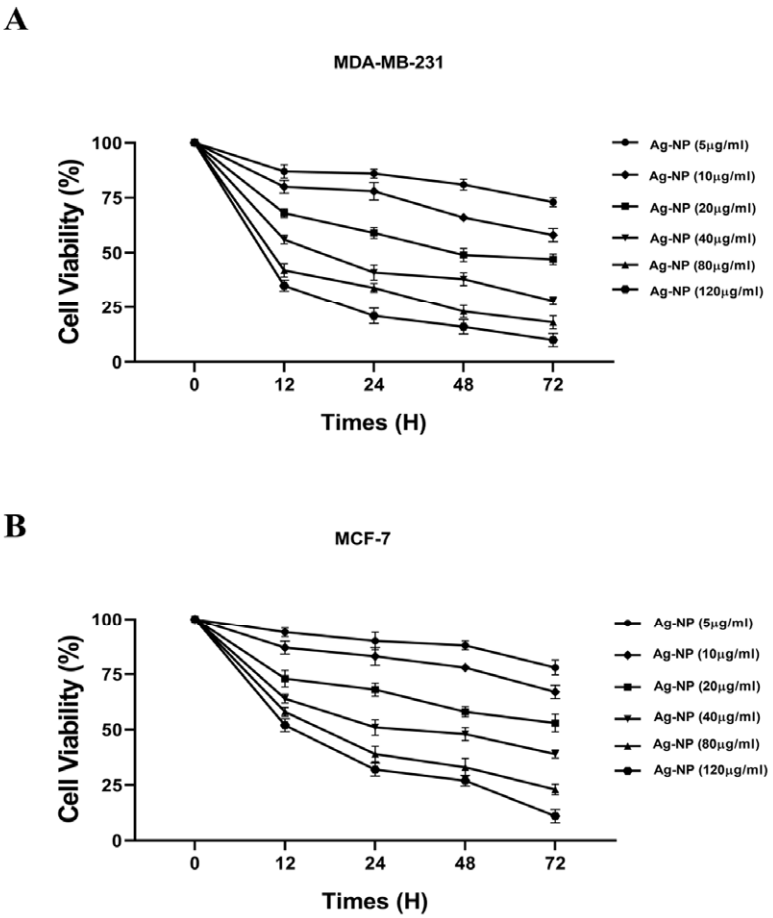

Fig. 4. MTT assay results concerning the AgNPs impacts on MDA-MB-231 (A) and MCF-7 and cell lines viability (B). Data implicates three independent tests, while the values are expressed in mean ± SEM.

Table 2. AgNPs IC50 value (Mean±SEM) (μg/ml)

Cell line	IC50 value (12 h)	IC50 value (24 h)	IC50 value (48 h)	IC50 value (72 h)
MDA-MB-231	61.43±2.46	33.78±3.54	19.87±1.51	16.87±2.76
MCF-7	124±3.16	45.24±2.96	36.56±2.29	27.9±2.06

Ag NPs induced cytotoxicity against MCF-7 and MDA-MB-231 cell lines

With respect to the results of MTT assay, AgNPs 5, 10, 20, 40, 80 and 120 μg/ml attenuated the viability of MCF-7 and MDA-MB-231 cell lines with 12, 24, 48 and 72 hours of treatment (P<0.05) (Fig. 4A, B). Although AgNPs 5 μg/ml diminished MCF-7 cell lines viability at 12 hours of treatment, this decrease was not significant (P<0.05) (Fig. 4 B). Based on results, te inhibitory effects of AgNPs on cell viability were time-dependent and dose-dependent. (P<0.05) (Fig. 1A, B). The IC50 values of AgNPs in MCF-7 and MDA-MB-231 cell lines are listed in Table 2.

The obtained outcomes of this work were parallel to the results of other related reports. Gopinath et al. exhibited that 10-15 nm size of AgNP induced robust cytotoxicity against human colon cancer HT29 cells in vitro [17]. In addition, AgNPs with median 30.71 nm size impaired the proliferation and viability of liver carcinoma HepG2 cell lines with 75 μg/mL the IC50 value [18]. Moreover, there

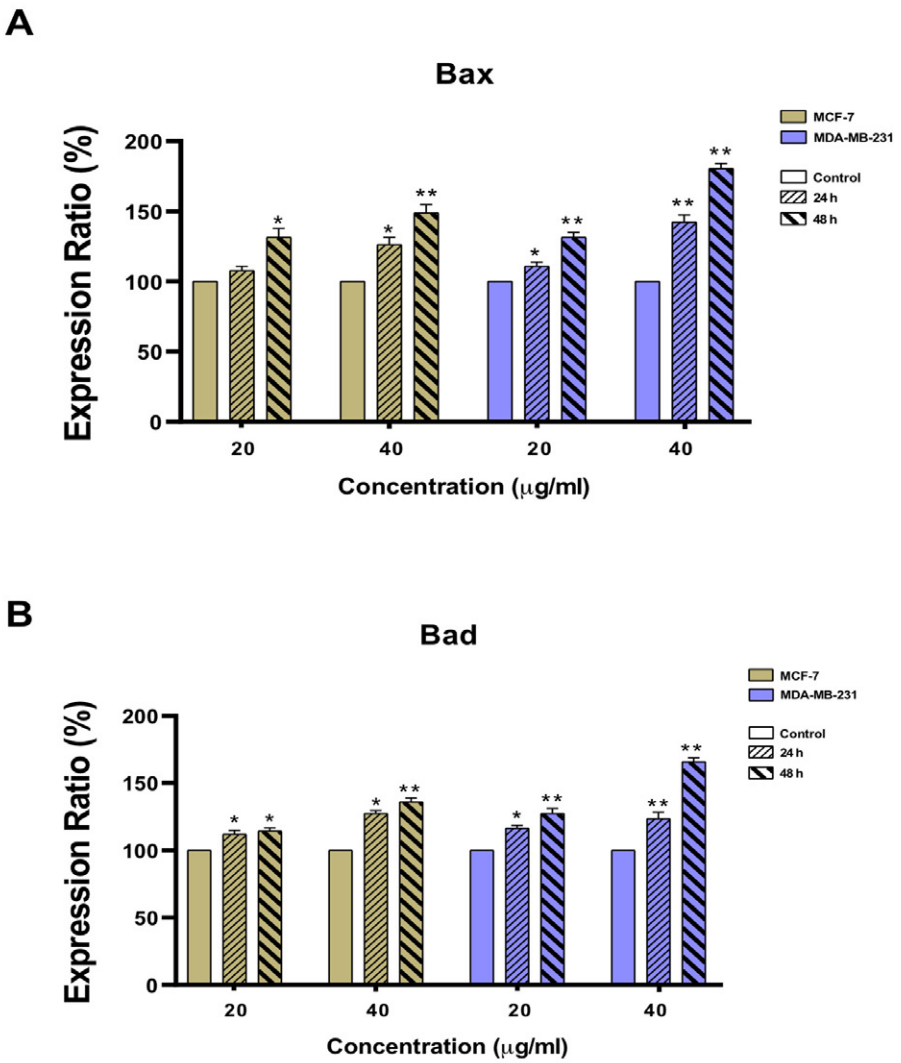

Fig. 5. Real Time-PCR results for Bax (A) and Bad (B) expression in MDA-MB-231 and MCF-7. Data is presented as means ±SEM from three independent tests. GAPDH was applied in the role of the internal control. The Student's t-test was conducted to define observed statistical differences. P values <0.05 were recorded as statistically significant. (* ; $p < 0.05$, ** ; $p < 0.01$)

is clear evidences signifying that AgNP reduced the viability of human lung cancer cell line, A549 by up-regulation of reactive oxygen species (ROS) levels [19]. Furthermore, Syed et al reported the usage of thermophilic fungus Humicola sp to accomplish the biosynthesis of AgNPs [20] and also provided data on the cytotoxicity of this product on NIH3T3 mouse embryonic fibroblast cell line and MDA-MB-231 cell line [20].

Ag NPs up-regulated pro-apoptotic protein expression

The expression levels of pro-apoptotic proteins Bax and Bad genes at mRNA levels were estimated through the means of Real-Time PCR in MCF-7 and MDA-MB-231 cell lines following treatment with AgNPs 20 and 40µg/ml within 24 and 48 hours of exposure (Fig. 5A, B). Based on results, 20 and 40µg/ml concentrations of AgNPs up-regulated expression levels of Bax in both MCF-7 and MDA-MB-231 cell lines ($P<0.05$) (Fig. 5A). However, this improvement was not significant in MCF-7 cell treated with 20 µg/ml concentration of AgNPs within 24 hours of treatment. Also, in comparison to MCF-7 cell lines, the enchantment of Bax expression was more evident in MDA-MB-231 cell lines (Fig. 5A). Likewise, 20 and 40µg/ml concentrations of AgNPs brought about an increases in Bad expression levels in both MCF-7

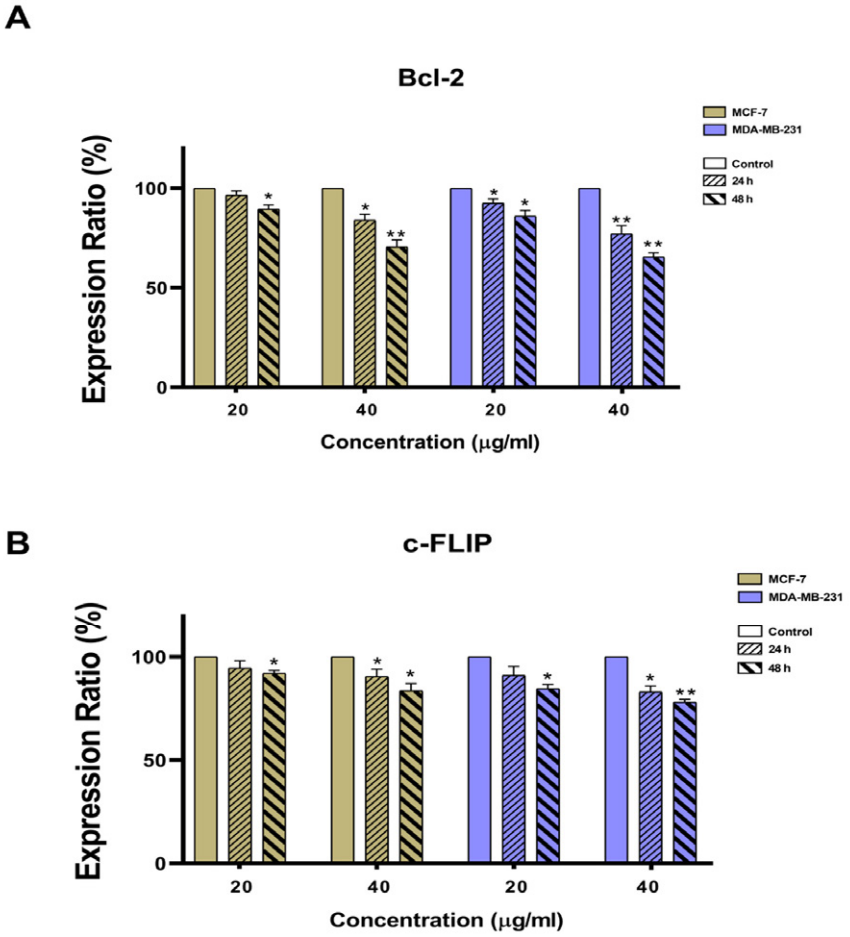

Fig. 6. The outcomes of Real Time-PCR for Bcl-2 (A) and c-FLIP (B) expression in MDA-MB-231 and MCF-7. Data is exhibited as means ±SEM from three independent tests. The role of internal control was filled with GAPDH, while the statistical differences were determined through the performance of Student's t-test. P values <0.05 were regarded as statistically significant. (* ; $p < 0.05$, ** ; $p < 0.01$)

and MDA-MB-231 cell lines (P<0.05) (Fig. 5B).

Other studies also indicated that AgNPs could stimulate up-regulation of pro-apoptotic gene expression leading to impaired viability and provoked apoptosis. For instance, Baharara et al. AgNPs biosynthesized utilizing Achillea biebersteinii flower extract could elicit cytotoxicity against cancer cell by improving Bax expression [21]. AgNPs also stimulated reduction of Bc-2/Bax expression ration and thus causes Dalton's Lymphoma tumor cell death in mice [22]. In addition, improved expression of Bax and caspase 3 upon cancer cell treatment with AgNPs reflected their key role in inspiring anti-cancer effects [23].

Ag NPs down-regulated anti-apoptotic protein expression

The expression levels of anti-apoptotic proteins Bcl-2 and c-FLIP genes at mRNA levels in MCF-7 and MDA-MB-231 cell lines were evaluated through the usage of Real-Time PCR following treatment with AgNPs 20 and 40µg/ml within 24 and 48 hours of tretment (Fig. 6A, B). Analysis demonstrated that 20 and 40µg/ml concentrations of AgNPs down-regulated the expression levels of Bcl-2 and c-FLIP in both MCF-7 and MDA-MB-231 cell lines (P<0.05) (Fig. 6A, B). Nonetheless, there was a mellow reduction in MCF-7 cell upon exposure with AgNPs 20 µg/ml during 24 hours of treatment (P<0.05) (Fig. 6A, B).

Likewise, Zhang et al. revealed that AgNPs caused a reduction in Bcl-2 expression in ovarian cancer cells [24]. In addition, AgNPs biomimetically synthesized by Date Palm Pollen extract triggered robust cytotoxicity versus cancer cell in part by down-regulation of Bcl-2 expression

[25]. Down-regulation of Bcl-2 expression results in activation of caspase cascade, thus culminating cell death [21]. In addition, AgNPs synthesized by Mushroom extracts induced marked cytotoxicity against HepG2 and MCF-7 cell line mainly by up-regulating Bax/Bcl-2 ratio [26].

CONCLUSION

Next to their numerous applications in industry, metal NPs can stand as a solution for the desperate demand of the world for the design of a low-risk and safe treatment that would lack the occurrence of adverse consequences on both humans and environment. In this regard green synthesizing methods can offer amazing qualities such as being less costly, provide more safety, and function in eco-friendly manners. Considering these facts, we exerted the aqueous extracts of Ducrosia anethifolia in the form of a reducing agent to reduce the Ag+ cation into the AgNO3 to Ag0. The reaction of AgNO3 in the appearance of Ducrosia anethifolia aqueous extracts led to the synthesis of Ag-NPs. Several identifying procedures were performed to characterize the NPs including TEM, DLS, and UV, which proved the success of the process. In addition, the obtained data of this study indicated the great potential of synthesized AgNPs for suppression of breast cancer cells viability mainly by up-regulating the expression of Bax and Bad and causing the down-regulation of Bcl-2 and c-FLIP expression levels.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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