Investigating The Anticancer and Antioxidant Activity of Silver Nanoparticles from *Rosmarinus officinalis*, *in vitro* Analysis

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ABSTRACT

Silver nanoparticles have emerged as anticancer agents due to their ability to generate free radicals which induce damage to the cellular membrane. In this study, the *Rosmarinus officinalis* (*R. officinalis*) leaf extract was used for the biosynthesis of silver nanoparticles (ROAgNPs). The potency of anticancer and antioxidant effect of ROAgNPs was demonstrated. UV-vis spectrophotometer was used to measure the absorption peaks of the ROAgNPs. The chemical substances present on the nanoparticle surface were determined using Fourier-transform infrared spectroscopy (FTIR). Zeta potential was used to determine the surface charge of ROAgNPs for understanding the interaction of the ROAgNPs with the cancer cell wall. Field Emission Scanning Electron Microscopy (FESEM) was used for imaging of the ROAgNPs and size analysis. The anticancer effect of ROAgNPs was performed using lung cancer cells (A549) and cervical cancer cells (HeLa), and the antioxidant activity was performed using DPPH. The absorption peak showed a position at 420 nm, and zeta potential exhibited a value of -5.72mV. FTIR analysis divulged the chemical compounds in plant extracts that act as reducing and capping agents during ROAgNP synthesis. FESEM images showed that ROAgNPs were monodispersed with a size of 15.60 nm and displayed typical anticancer and antioxidant effectiveness.

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1. Introduction

Nanotechnology is an interdisciplinary cutting-edge field of science, covering a wide range of disciplines such as engineering, biology, physics, and chemistry [1]. Remarkable progress has been made about the biomedical applications of nanoparticles because of their enhanced surface area, high reactivity, and chemical stability [2]. In contrast to the bulk materials, nanoparticles exhibit fantastic and unique physico-chemical characteristics such as large surface area, high surface charges, surface plasmon resonance, fluorescence, catalyst and photothermal effect when their dimensions are in the nanoscale (>100 nm) [3]. Noble metals have been used for many purposes [4]. For example, silver metal was initially used as an antibacterial and later as an anti-inflammatory to treat several ailments [5,6]. However, advances in nanotechnology brought about the transformation of the bulk metals to the nanometric scale whose characteristics are uniquely different from the bulk. Interest in metal nanoparticles has grown rapidly due to their wide range of potential uses [7,8]. For example, silver nanoparticles have been used as food preservative,[9], cosmetic and water disinfection [10,11], antimicrobial and antifungal [5,12-15], and the more interesting, anticancer [16]. Silver nanoparticles can be synthesized by physical, chemical and biological means broadly group into top-down and bottom up methods [17]. The top-down method is the systematic disintegration of the bulk silver metal to the nanometer scale by physical means. This method requires the use of equipment that may exert huge financial burden and time consuming, making the method unattractive [18]. On the other hand, bottom up method involves the systematic assembly and nucleation of nanoclusters of silver atoms to nanoparticles through chemical or biological means. The chemical means are primarily characterized by safety concerns such as toxicity to human beings, living organisms and the environment [18]. The use of toxic chemicals and expensive equipment is not needed for the biological methods synthesis [19-22]. Biosynthesized silver nanoparticles are eco-friendly, very simple synthesis protocol and cost effective [23]. Biological methods require plants extract and microorganisms that environmentally friendly and can be sourced locally, thus, reducing the cost of synthesis of silver nanoparticles [24-27]. Plant extracts for example, are widely used in the nanoparticle synthesis protocols due to their contents with active chemical compounds such as polyphenols, flavonoids, and tannins as reducing and stabilizing agents [24,28-30]. Thus, *Rosmarinus officinalis* leafs were chosen for fabricating and stabilizing AgNPs. *Rosmarinus officinalis* dry leafs are locally available in Iraq at Basrah city markets and their potentials for biosynthesis of silver nanoparticles have not explored,

hence, the utilization of *Rosmarinus officinalis* dry leaves for biosynthesis of silver nanoparticles. Most interestingly, the anticancer effect of silver nanoparticles has been demonstrated in several studies [31]. For example, Ganesan *et al*. utilized extract from the leave of *Solanum trilobatum* for synthesis of silver nanoparticles, which inhibited the viability of oral cancer cells [32]. Algotimi *et al* demonstrated that silver nanoparticles synthesized with Red Sea marine algal induced anticancer effect against MCF-7 cancer cells line [31]. The anticancer effect of silver nanoparticles stems from their ability to generate reactive oxygen species (ROS) that exert oxidative stress on the cancer cells leading to apoptosis [33,34]. The current study aims to explore the antimicrobial, anticancer and antioxidant potency of silver nanoparticles using extract from the dry leafs of *Rosmarinus officinalis.* The scheme of the current study is displayed in Figure 1.

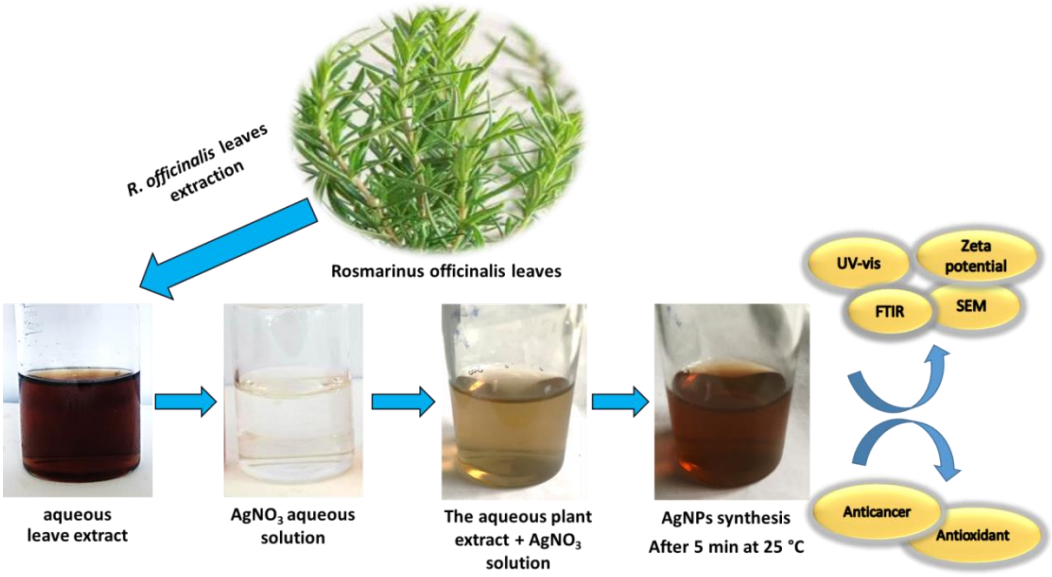

Figure 1: A scheme of the eco-friendly synthesis of ROAgNPs, characterization and applications

2. Experimental

2. 1 Materials

Silver nitrate ($AgNO_3$) (99%), ascorbic acid, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) 97% were purchased from Sigma aldrich. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide and Dimethyl sulfoxide (DMSO) were purchased from fisher scientific.

2. 2 Methods

2. 2. 1 *R. officinalis* leafs collection and aqueous extract preparation

R. officinalis dry leafs were collected from Basrah city markets, Iraq. The leaves were blend using electrical grinder. The aqueous extract was prepared following a protocol of [35], Shortly, 100 ml of sterile DW was added to 5 g of dried leaf powder in a conical flask of 250 ml, then heated at 30 °C for 30 min and then left to cool. The extract was then filtered and stored at 4 °C until used.

2. 2. 2 ROAgNPs synthesis approach

ROAgNPs were synthesized via green method using an aqueous extract of the *R. officinalis* leave [36]. In a 250 ml beaker, 95 ml of silver nitrate aqueous solution ($AgNO_3$) (0.001 M) was put, and then 5 ml of plant extract was added while rapidly agitated for 5 minutes at room temperature. The mixture was then kept in a dark place at room temperature for 15 min to complete nanoparticle synthesis.

2.3 Characterization of ROAgNPs

A UV-Vis spectrophotometer of wavelength range (200-800nm), was utilized to measure the surface plasmon resonance (SPR) band . Zeta potential (Nano ZS) was used to ascertain the surface charge of ROAgNPs. Furthermore, the chemicals composition of the plant extract and the ROAgNPs were determined with FTIR instrument (BRUKER, chemistry department, the University of Basrah) in the mid-IR wavelength range (400 and 4000 cm^{-1}). Field Emission Scanning Electron Microscopy (FESEM) (MIRA III) (Tescan, Czech) was used to capture images of ROAgNPs, with a magnification of 350.00 kx, electron window of 5.01mm, and high resolution units at a voltage of 15.00 kV [37].

2.4 MTT cytotoxicity assessment (Anticancer) of ROAgNPs

The anticancer investigation (the cytotoxicity) of ROAgNPs was performed using an MTT assay to investigate nanoparticle toxicity in living cells. Two types of cell lines were used, (A549 and HeLa). Cells were cultivated at the $5x10^3$ cell in 96-well plates, while media only was used as a background. After 18 hrs of seeding, media was discarded and cells (3 replicates), were incubated with ROAgNPs at different concentrations (0.002, 0.004, 0.008, 0.016, 0.032, 0.063, 0.125, 0.250,

0.5, 1 and 2 (mg/ml) of medium (100 µl per well) and incubated for 24 hrs. cells (3 replicates) also were left without treatment with ROAgNPs and used as control. After the incubation, the media from each well was discarded, and 100 µl of MTT solution was added to treated and control cells and incubated for 1 hr. Next, the MTT solution was removed, and DMSO solvent (100 µl) was added and the cells were set in incubation for 5 min. Finally, the plates were placed for absorption using a plate reader (FLUOstar Optima (BMGLabtech, USA), λexc = 545 nm, λem = 595 nm to display the cell viability % comparing to control cells (untreated cells). Further, the morphological variations in the A549 and HeLa cells before and after incubating cells with the highest concentration of ROAgNPs, 2 mg/ml, were determined using Olympus 41 inverted microscope with 60X magnification. The cell viability (%) was calculated using the following:

$$\text{The cell viability (\%)} = \frac{\text{The absorbance mean of treated cells}}{\text{absorbance mean of untreated control} \times 100}$$

2.5 Antioxidant of ROAgNPs

The antioxidant consequences of ROAgNPs were explored utilizing a free radical scavenging assay following the protocol described by [38], using the 2, 2-Diphenyl-2-picrylhydrazyl (DPPH). The ROAgNPs and Ascorbic acid (A. A.), were, separately prepared at various concentrations (50, 100, 200, 400, 600, and 800 µg/ml), where A. A., dissolved in distilled water, was used as a positive control, while the DPPH, dissolved in methanol, was considered a negative control. To conduct the assay, DPPH solution (1 mM), was separately added to the ROAgNPs samples, positive, and negative tubes with a volume ratio of (1:1) and mixed thoroughly. The tested tubes were then incubated in a dark place, at room temperature for half an hour. After incubation, the absorption wavelength of the tubes was measured at 517 nm with (UV-1900i-Shimadzu). Free radical scavenging was assessed using the following: Free radical scavenging % = [(Ac−As) ÷Ac] × 100, Ac = OD of the control; As = OD of ROAgNPs [39].

2.6 The statistical Analysis

The Graph Pad Prism program version 21, was used to assess the statistical significance between variants. The statistics were introduced depending on the mean ± SD using Two-way ANOVA, $P \leq 0.0001$.

3. Results and discussion

3.1 Synthesis of ROAgNPs

Figure 2 demonstrate changing colour of the mixture of plant extract and silver nitrate from light yellow (A) to brown (B) post 15 minutes of incubation at 25°C. Figure 2 describes the reduction of Ag^1 ions to Ag^0 ions, which accumulate to form small clusters to produce Nano-sized metallic Ag particles. This result is similar to the previous reports [40,41]. The exhibiting of ROAgNPs a brown colour is due to such a surface Plasmon Resonance phenomenon [42]. Stated that *R. officinalis* leaves possess many active materials like what? that act as reduction and capping agents during the synthesis of silver nanoparticles [43].

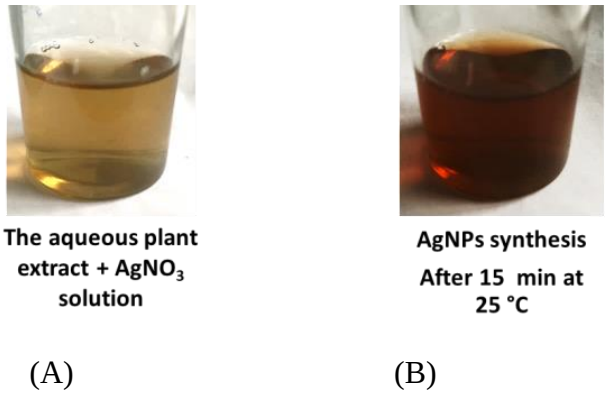

Figure 2: Synthesis of ROAgNPs (A) reaction mixture at time 0, and (B) reaction mixture post 15 min incubation

3.3 ROAgNPs Characterisation

3.3.1 Peak optical absorbance of ROAgNPs

Figure 3. demonstrates the optical absorption pattern of ROAgNPs. As we can see the peak of SPR is concentrated at a position of 420 nm of wavelength. Our finding is similar to previous studies, which show that absorption peaks of ROAgNPs at wavelengths of 420-450 nm [44,45]. The patterns of absorption of ROAgNPs are due to the resonant oscillation of surface electrons of these nanoparticles [30,46,47]. In addition, the surface electrons supply great information regarding NPs size [48].

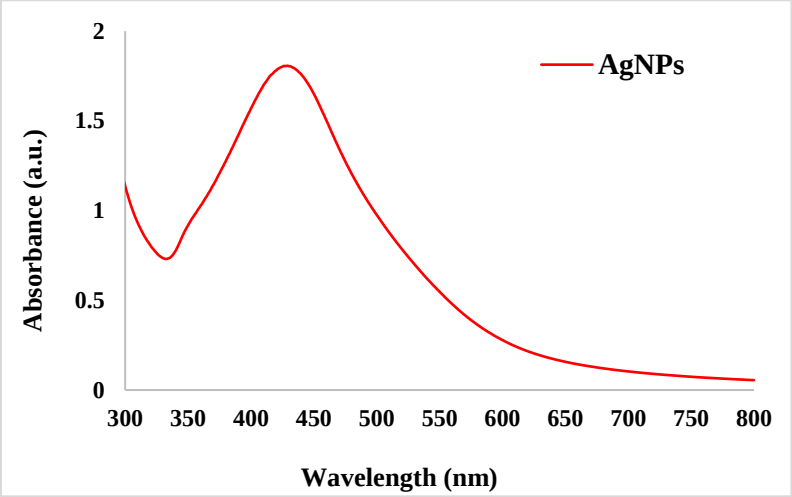

Figure 3: The optical absorbance from ROAgNPs

3.3.2 Zeta potential of ROAgNPs

Figure 4 shows the zeta potential of ROAgNPs. The zeta potential of AgNP synthesized using *R. officinalis* was -5.72mV [8,49]. Zeta potential estimation of ROAgNPs presents a single peak with a ratio of peak intensity 46.58 (mV)/ 85.8 (Area%), as shown in Figure 4. It was reported that the lowest zeta potential value of ± 30mV is required for stabilized nanoparticles [50]. The negative zeta potential of ROAgNPs may be due to the capping of the organic biomolecules found in the *R. officinalis* extract. The weak negative value of the ROAgNP surface charge could play a role in nanoparticle cytotoxicity and their ability to enter cells and affect the cellular organelles.

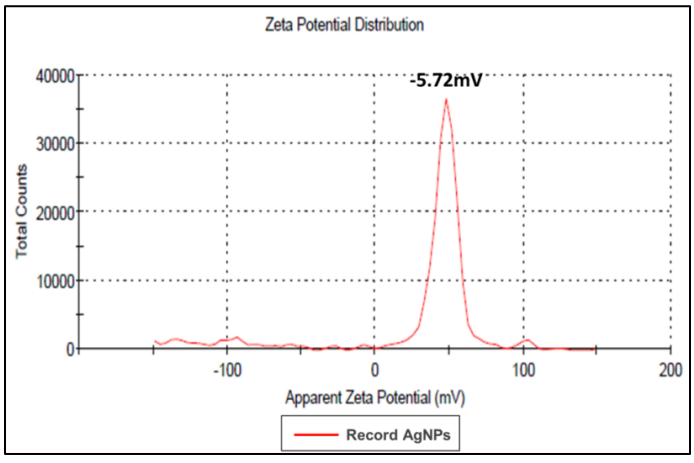

Figure 4: Size potential of ROAgNPs

3.3.3 Fourier-transform infrared spectroscopy (FTIR) measurements of ROAgNPs

FTIR spectra within the range of 4000-400 cm^{-1} were acquired to determine the chemical composition of the ROAgNPs. The FTIR analysis of ROAgNPs is shown in Figure 5. A broad, sharp absorption peak has been detected at 3347.90 cm^{-1}, suggesting a hydroxyl group ($^{-}$OH) in the alcoholic substances of *R. officinalis* extract. Furthermore, three anticipated peaks with wavelengths of 2356.02, 1636.65, and 565.68 cm-1 are observed from the FTIR analysis. The three peaks might be due to the interaction of silver (I) ions with *R. officinalis* active groups that act as reducing and capping agents of ROAgNPs. These findings are consistent with previous report [51].

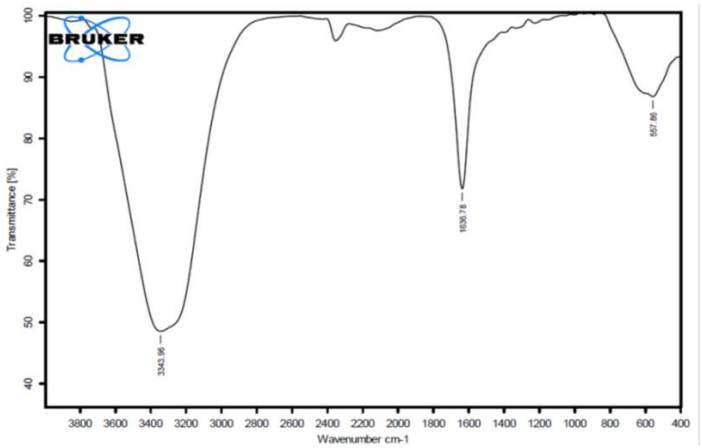

Figure 5: FTIR analysis of *R. officinalis* extract synthesised AgNPs

3.3.4 Field Emission Scanning Electron Microscopy (FESEM) measurements of ROAgNPs

Figure 6 represents image of ROAgNPs. The images of ROAgNPs were visualized under Field Emission Scanning Electronic Microscope (FESEM), to acquire a visual structural image of the AgNPs. FESEM image analysis indicates that ROAgNPs had a sphere shape with sizes ranging 10-15 nm using a scale bar of 100 nm. The FESEM shows monodispersed ROAgNPs with clear structural and morphological characteristics. Four images of ROAgNPs were taken showing homogenous nanoparticles without visible aggregation.

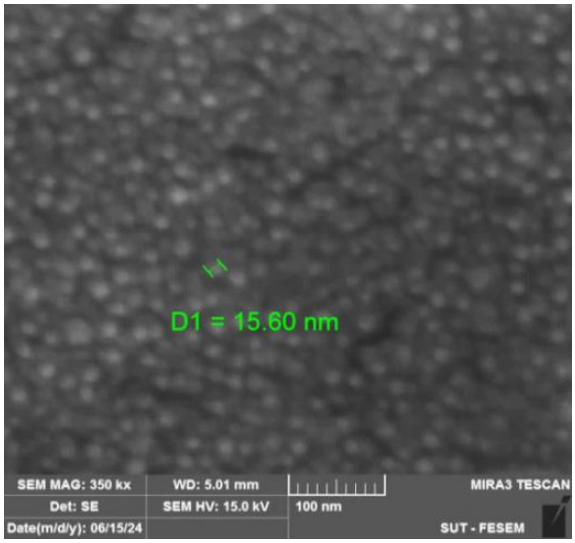

Figure 6: FESEM image of ROAgNPs, at SEM HV:15.00 kV, 5.01mm imaging window, 350.00 kx resolution, and scale bar 100nm

3.4 MTT cytotoxicity assessment (Anticancer) of ROAgNPs prepared with *R. officinalis* leafs extract

Figure 7 shows the viability percentages of A549 and HeLa cells treated with ROAgNPs. It was noticed that ROAgNPs caused a decrease in cell viability (%) of both types of cells with an increase in the concentration of ROAgNPs compared to the control (untreated cells). Regardless of the concentration of ROAgNPs (Figure 7), both cell types were affected by nanoparticles which indicate similar responses to ROAgNP treatment. Even so, HeLa cells were more affected by nanoparticles compared to A549, however, there were no significant differences in the cell viability (%) between the two types of tested cells, only at the concentrations of 0.032, ($P<0.01$) and the concentrations of 0.5 mg/ml, ($P < 0.05$) using Two-way ANOVA, $P \leq 0.0001$. This could specify that the ROAgNPs possess a high toxicity to the tested cells which was dose-dependent. The cytotoxicity of ROAgNPs can be described by different standards. The first criterion is related to the raised Ag^+ ion levels inside cancer cells due to the oxidation of AgNPs by O_2 [52]. Ag^+ produces strong bonds with N and S and forms free radicals that react to the proteins that hold sulfur such as glutathione and superoxide dismutase [53]. The collection of reactive oxygen species (ROS) in cancer cells creates oxidative stress harm [54,55]. This leads to a decrease in the efficiency of cellular enzymes and proteins and transcription factors [56]. In addition, the disbanding of AgNPs in the cells conducts to decreases the pH inside cells resulting in lysosomal

unsteadiness [57]. Furthermore, in our study, the toxicity of ROAgNPs could result from their small size (15 nm), which was found to be a critical factor for the toxicity of AgNPs compared to larger sizes [58]. Images of A549 and HeLa cells were acquired using inverted microscope with 60X magnification (Figure 8). As we can see that the ROAgNPs induced a huge effect on HeLa cells by inducing apoptosis, that found to be slightly less apparent in A549 cells. It has been found that most HeLa cells were exposed to apoptosis, while some A549 cells remained alive after overnight incubation time with 2 mg/ml as the highest concentration of the ROAgNPs was applied in the MTT assay.

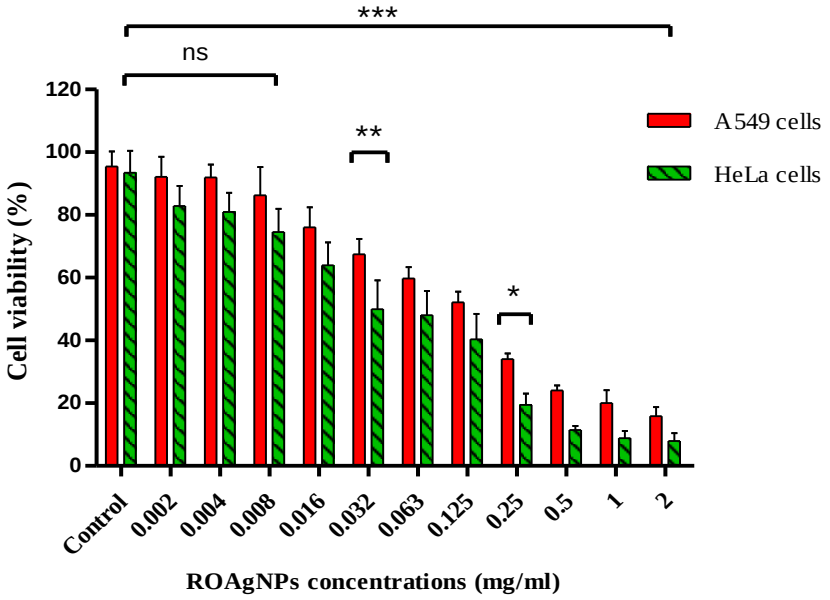

Figure 7: MTT cytotoxicity assessment (Anticancer) of ROAgNPs against A549 and HeLa cell lines exposed to different concentrations (mg/ml) for 24 hrs

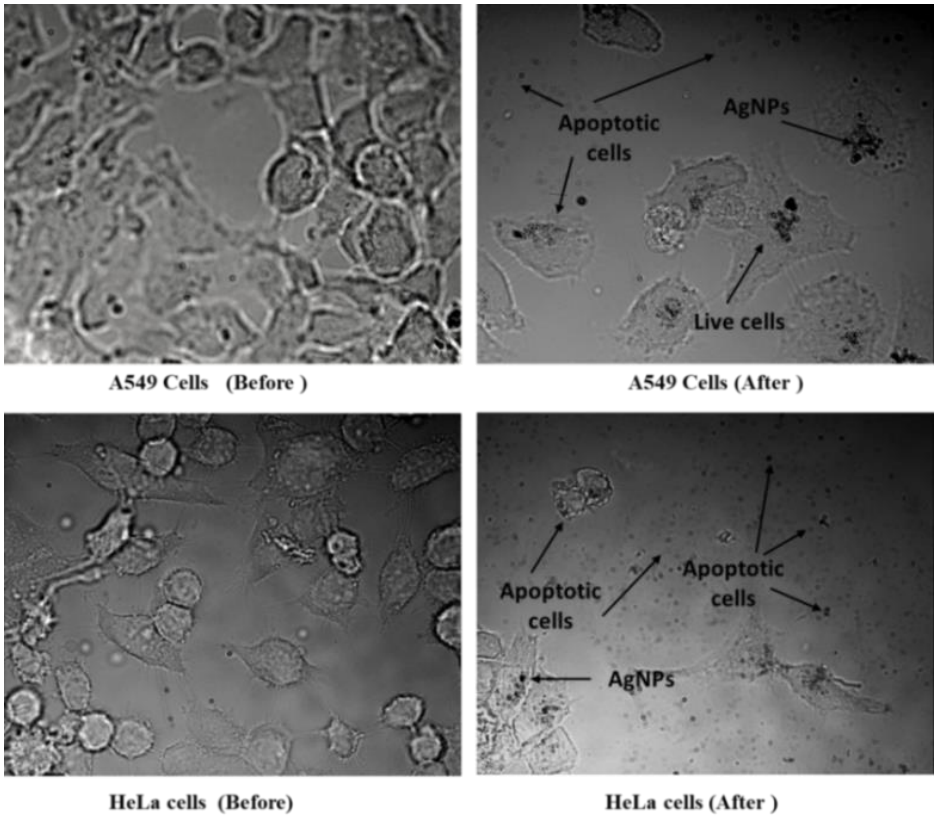

Figure 8: A549 and HeLa cell lines exposed to ROAgNPs (2 mg/ml) for 24 hrs. Top images for A549 cells and down images for HeLa cells, Before and After treatment, (60X magnification).

3.4 Antioxidant evaluation of ROAgNPs

The extract of *R. officinalis* was used for the synthesis and capping of AgNP. The antioxidant effect of ROAgNPs was investigated with DPPH scavenging assay [38]. It was found that the antioxidant effect of ROAgNPs was more effective than that of Ascorbic acid (Figure 9). The absorbance of DPPH changed and it's dark purple colour disappeared following mixture with ROAgNPs and Ascorbic acid. The DPPH scavenging activity of ROAgNPs was 50 per cent at 197.92ug/ml while that of Ascorbic acid was 50 per cent at 95.49ug/ml.

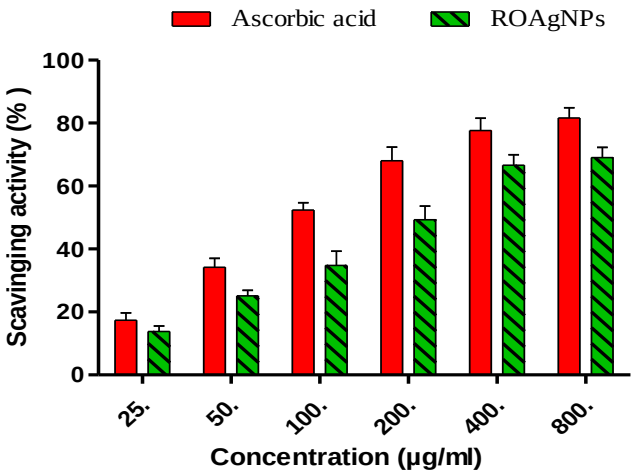

Figure 9: Antioxidant activity of ROAgNPs

4. Conclusions

In the present study, the aqueous extract of *R. officinalis* leafs was used as a reducing agent to enhance the synthesis of ROAgNPs in a safely and ecological friendly manner. In addition, MTT assay conforms the cytotoxicity activity of ROAgNPs against two types of cancer cells. In vitro analysis demonstrates the effectiveness of ROAgNPs as antioxidant and anticancer agents.

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التحقيق في النشاط المضاد للسرطان والمضاد للأكسدة لجسيمات النانو الفضية المصنعة من نبات إكليل الجبل، تحليل في المختبر

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المستخلص

ظهرت الجسيمات النانوية الفضية (AgNPs) كعوامل جيدة مضادة للسرطان بسبب قدرتها على توليد الجذور الحرة التي تسبب تلف الغشاء الخلوي. في هذه الدراسة، تم استخدام مستخلص أوراق إكليل الجبل (*R. officinalis*) لتخليق الجسيمات النانوية الفضية (ROAgNPs)، وإثبات فعاليتها في التأثير المضاد للسرطان ومضاد الأكسدة. تم استخدام مطياف الأشعة فوق البنفسجية المرئية لقياس قمم امتصاص ROAgNPs. تم تحديد المركبات الكيميائية الموجودة على سطح الجسيمات النانوية باستخدام مطياف الأشعة تحت الحمراء بتحويل فورييه (FTIR) . كذلك تم استخدام جهد زيتا لتحديد الشحنة السطحية للجسيمات النانوية ROAgNPsلفهم آلية تفاعل ROAgNPs مع جدار الخلايا السرطانية. تم استخدام المجهر الإلكتروني الماسح (FESEM) لتصوير ROAgNPs وتحليل الحجم. تم إجراء النشاط المضاد للأكسدة باستخدام اختبار الـ DPPH ، وتم إجراء التأثير المضاد للسرطان لجسيمات النانو ROAgNPs باستخدام خلايا سرطان الرئة A549 cells وخلايا سرطان عنق الرحم HeLa cells . أظهرت ذروة الامتصاص لجسيمات الفضة النانوية موضعًا عند 420 نانومتر. وامتلكت الجسيمات النانوية جهد زيتا بقيمة -5.72mV. كشف تحليل FTIR عن المركبات الكيميائية لمستخلص النبات والتي تعمل كعوامل اختزال وتغطية أثناء تخليق ROAgNPs. أظهرت صور المجهر الإلكتروني الماسح بنية ROAgNPs والتي وجد انها جسيمات احادية التشتت وبشكل كروي بحجم صغير يبلغ 15.60 نانومتر. وأظهرت جسيمات ROAgNPs فعالية نموذجية مضادة للسرطان ومضادة للأكسدة.