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In vitro investigation of anticancer activities of green synthesized gold nanoparticles prepared from extracted leaf of *Olea europea L*

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The emergence of nanoparticles with remarkable physical and chemical properties has provided a fertile ground for solving modern problems, such as the limitations of cancer treatment. The current study is designed to investigate the anticancer activity of the gold nanoparticles synthesized from *Olea europea L.* leaf against human lung cancer cell line (A549), and induction of apoptosis. AuNPs are prepared from *Olea europea L*. leaf, and their morphological and physical features are characterized. Followed by the examination of their anticancer, toxic activities against human lung cancer cell line (A549), and DNA fragmentation is evaluated. The results suggest that the synthesized AuNPs are spherical in shape and highly dispersible, with an average size ranging between 32-48 nm. In vitro investigation confirms that AuNPs show anticancer and toxic activities against human cancer cells, the A549 cell line. The anticancer and toxic activities are concentration-dependent. Induction of apoptosis in A549 is approved using fluorescent staining ethidium bromide (EB)/acridine orange (AO) method and DNA fragmentation. Our study concluds that small size nanoparticles that induce apoptosis process in cancer cells lead to decrease cells viability. It could be considered AuNPs as a promising anticancer therapy in the future. Future research should include in vivo investigation of AuNPs to confirm their activities.

Keywords: Apoptosis; AuNPs; Cancer cell line; MTT; *Olea europea L.*

1. INTRODUCTION

Humans have accomplished significant progress in developing different scientific fields, such as molecular and biology nanoscience. Nanotechnology, which relates to the Greeks and the Democritus era, is considered one of the prominent fields that evolved to overcome recent problems. It is important to highlight the difference between nanotechnology and nanoscience. Nanotechnology refers to the technology employed in the practical field to identify, measure, and manipulate objects, such as devices; while the science that deals with small-sized particles ranging between 1-100 nm is called nanoscience [1]. Recently, scientists have utilized nanoscience in different aspects, such as the development of novel therapeutic agents, like antimicrobial and anticancer [2,3]. Rivlin *et al*., have estimated that 50% of human cancers are associated with unfunctional P53 protein [4], uncontrolled growth of cells in the living body is defined as malignant growth leading to cancer [5]. The cell cycle is regulated by specific checkpoints before they are allowed to cycle. It is estimated that the genetic mutations of two key cell cycle *P53* and *P21* lead to uncontrolled growth of cells. It is suggested that the two protein suppressor genes P53 and P21, arrest the cell cycle.

According to the World Health Organization, in addition to increasing the incidence of cancer worldwide, it is the second leading cause of death after cardiovascular diseases[6] However, ReFaey *et al*., have expected cancer incidence may exceed the cardiovascular disease in the future [7]. Conventional anticancer have shown therapeutic limitations, and therefore great efforts have been made to develop a novel anticancer, such as utilizing microorganism's metabolite and nanoparticles [8,9]. The delivery of anticancer to targeted cancer cells and binding to the cell ligand are one of the prominent limitations.

Green nanoparticles are synthesized using plant extract and characterized as eco-friendly, biocompatible, safe and inexpensive[10]. Synthesized nanoparticles possess particular properties enable them to tackle these problems [11]. The anticancer activities of nanoparticles against cancer cells are demonstrated previously through different mechanisms, such as mitochondrial dysfunction, oxidative stress, and trigger of apoptosis [10]. These nanoparticles interfere with cell membrane permeability and have proven effective in delivering them to targeted cancer cells. They also exhibit selectivity for cancer cells than for healthy ones, making them useful for diagnostic puropses. Common examples of nanoparticles include gold nanoparticles (AuNPs), silver nanoparticles (AgNPs), titanium dioxide (TiO2), and selenium nanoparticles (SeNPs) [12-14].

AuNPs are an inorganic nanomaterial that is considered a remarkable medication. AuNPs are known as the noblest among other metals owing to their physical and chemical unique characteristics, such as difficulty of separation into particles due to their inactivity, as well as optoelectronic and surface Plasmon Resonance (SPR). Their circular shape and positive charge enable AuNPs to be internalized by the cell membranes. Au particles show stability against oxidation under specific physiological conditions, such as temperature and PH [11]. The physico-chemical properties of AuNPs enable them to penetrate the cell membrane and trigger the apoptosis mechanism in cancer cells, leading to cell death [15]. Previous works have indicated the effectiveness of green-synthesized nanoparticles against cancer cells. These NPs offer great advantages; in addition to their selective cytotoxicity against cancer cells; they are affordable, eco-friendly and easily accessible [16]. Few studies considered the synthesis of AuNPs from Olea europaea leaf extract and their activity against lung cancer cell line (A549) [17-19]. Therefore, the current study sets out to investigate the biological activity of AuNPs against cancer cells. To achieve this aim, the objective of the current study is to synthesize gold nanoparticles following green synthesis approach, using the leaf extract *Olea europea L.,* identify physicochemical properties and

assess their cytotoxicity and activities against the human lung cancer cell line (A549) in vitro. The study evaluates the ability of AuNPs to trigger apoptosis through DNA fragmentation and AO/EB staining analysis.

2. EXPERIMENTAL

2.1 Sample collection and preparation of extract

Olive leaves are obtained from the local area in Basrah. The leaves are washed, dried, and ground into a fine powder. 10g of prepared powder is mixed with the 100 ml D.W, boiled for 1h, and cooled down at room temperature. Later, the mixture is filtered through filter paper and centrifuged at 3000 rpm for 10 min. The supernatant is kept in a fresh container until used.

2.2 Gold Nanoparticle biosynthesis

About 0.04 g of the precursor material $H.AuCl_4.3H_2O$ is added to 100 ml of prepared leaf extract. The mixture is then transferred into a dark place overnight to allow triggering the reaction. The control tube containing 100 ml of leaf extract is used. Afterward, the tubes are centrifuged at 3000 rpm for 10 min, washed three times with D.W and dried in the oven at 45℃. The change in color from transparent yellow to dark brown indicates the reaction execution compared to the control. Finally, the synthesized nanoparticles are stored for further analysis.

2.3 Characterization of gold nanoparticles

The physical properties of AuNPs are identified by the following techniques, including Ultrasonic frequencies (>20 kHz), UV-visible spectroscopy at a wavelength of about (500-580) nm, and morphological features, such as shape, are determined using Scanning electron microscopy (SEM).

2.4 Biological activity of biosynthesis AuNPs against cancer cell lines

Human lung cancer cell line (A549) is purchased from the National Cell Bank of Iran (Pasteur Institute, Iran). Cells are grown and maintained in RPMI-1640 medium (Gibco) (containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin). Flasks are incubated at 37°C with 5% CO_2 using a Sanyo CO_2 incubator (MCO-17A).

2.4.1 Investigation cells viability in vitro by MTT assay

The MTT assay is used to investigate the biological activities of AuNPs against the cell line. Cells are seeded at 1.4×10^4 cells/well, incubated for 24 h at 37 °C with 5% CO_2, and washed with phosphate-buffered saline (PBS). Then, cells are treated with AuNPs (12.5, 25, 50, 100, 200 µg/ ml), incubated for 48h at 37℃, and washed with PBS three times. 200 µl/well of MTT solution (0.5 mg/ml in phosphate-buffered saline [PBS]) is added to cells and incubated for 4h. Cells are washed, and 100 µl per well of dimethyl sulfoxide is added to remove crystals. The plate is transferred to ELISA reader (Model wave xs2, Bio Tek, USA) to measure optical density (OD) utilizing a spectrophotometer at 570 nm [20]. The experiment is triplicated to obtain accurate and reliable results. The following equation is used to calculate the inhibition of cell growth:

$$\text{Inhinition rate} = \text{A} - \text{B/A} * 100 \quad (1)$$

A) is the OD of the control, and (B) indicates the OD of the sample. Linear regression (dose-response curves) is used to evaluate IC_{50} value. The cytotoxicity of AuNPs graph is composed of two axes plotting the serial of AuNPs concentrations against cell viability. IC_{50} refers to the accurate concentration that inhibited 50% of cells growth.

2.4.2 Examination of apoptotic efficacy of AuNPs against cancer cell line by AO/EB Staining

Further analysis is carried out to investigate cell viability following fluorescent staining ethidium bromide (EB)/acridine orange (AO). Fluorescent staining ethidium bromide (EB)/acridine orange (AO) stain is obtained from Sigma-Aldrich. 1.4×10^4 cells/well of A549 cell lines are incubated with IC_{50} concentration of prepared AuNPs for 24 h. Later, the incubated cells are washed with PBS and stained with a solution containing EB/AO. Then, the stained cells are immediately examined under a fluorescence microscope (Axioskop 2 plus, Ziess, Germany) and images are captured. Cells are categorized based on their color appearance. Green cells represent viable cells and are stained only with AO, green and orange cells with condensed chromatin represent early and late apoptotic cells and are stained with both AO and EB (with moderate alteration in membrane permeability), finally, necrotic cells are orange and stained with EB [21].

2.4.3 DNA fragmentation assay

A549 lung cancer cell line is incubated with the IC_{50} concentrations of AuNPs alongside with the control (untreated cells) for 24h at 37°C with 5% CO_2. GDNA of treated and control cells is isolated following phenol/chloroform protocol Sambrook *et al*., [22]. Isolated DNA is separated on 0.8% agarose gel stained with ethidium bromide.

3. RESULTS AND DISCUSSION

3.1 Biosynthesis of AuNPs

A variety of resources are utilized to synthesize AuNPs, including plants, algae, microorganisms, animals, and polymers [13,23,24]. Increased environmental pollution and economic cost have urged the scientists to seek eco-friendly resources, affordable and easily accessible. It is found that green plant resources are one of a prominent resource solved these obstacles [25]. Considering the above, the current study is designed to investigate the biological activities of AuNPs on cancer cells. In the vitro biosynthesis reaction is carried out by adding olive plant extract with the precursor $H.AuCl_4.3H_2O$ in aqueous solution. The reaction is resulted in color alteration from yellow to dark, indicating the formation of AuNPs. This observation corresponds to a previous finding Basavegowda *et al*. [26]. Meanwhile, Khalil *et al*. observed color alteration from yellow to violet to dark pink and green [27]. Color degree variations may relate to solutions concentrations. The AuNPs are synthesized from *Olea europea L*.; different nanoparticles are synthesized from olive oil, such as AgNPs and AuNPs [28]. It is demonstrated that small size of nanoparticles may aggregate together forming large mass [29].

3.2 Characterization of AuNPs

3.2.1 UV- Visible spectrophotometer

UV–visible spectra of Au nanoparticles are measured to monitor the reaction between metal ion and plant extract in aqueous solution. The maximum absorption peak of the samples is estimated 520 nm owing to the surface plasmon resonance (SPR) phenomenon, as has shown in Figure 1.

Apart from source of AuNPs, the UV spectrum of AuNPs is found to be about 530± 2 nm [30,31]. Previous works suggest that the plant extract concentration and particles size have affected the peak value of surface plasmon resonance (SPR) [27,32]. Our results suggest that *Olea europea L. i*s a productive source used to yield AuNPs.

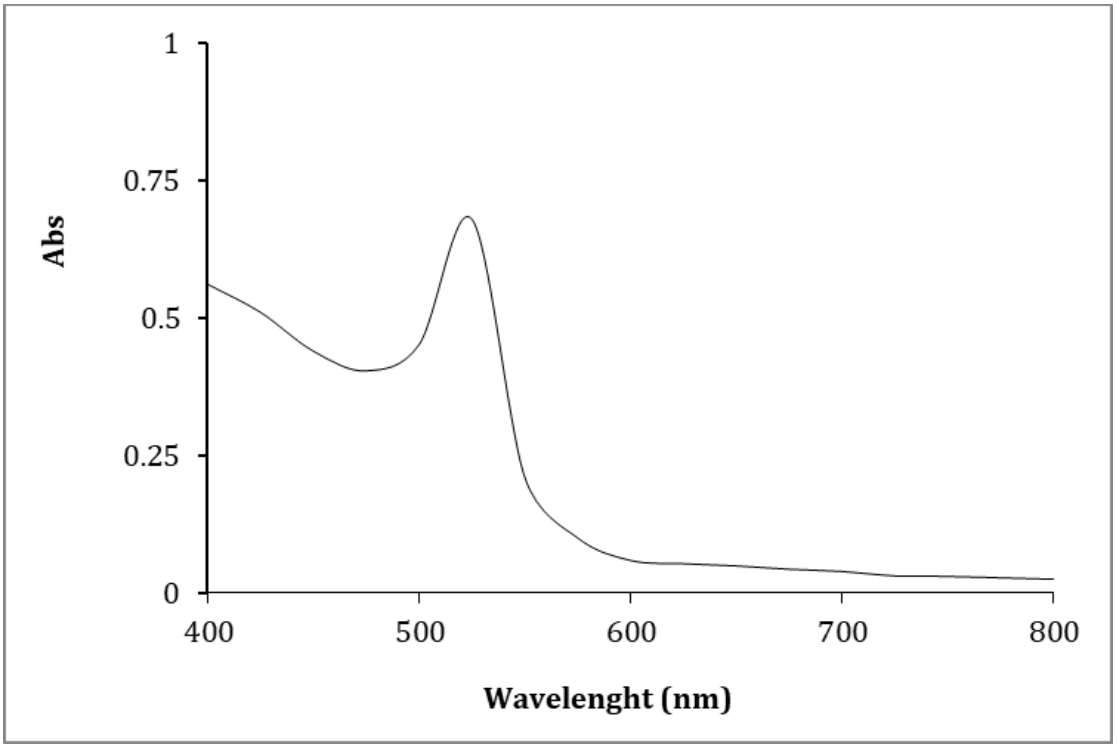

Figure 1 UV-Vis absorption spectrum of green synthesized AuNPs**.**

3.2.2 Scanning electron microscope (SEM)

The morphological features of synthesized gold nanoparticles are evaluated using Scanning Electron Microscope (SEM), as has shown in Figure 2. The AuNPs are observed to be spherical with high dispersibility, and their average size has ranged from 32-48 nm, which falls within the nanoparticle size range (1–100 nm), indicating the successful synthesis of the gold nanoparticles. In other words, particles size confirms their classification as Nanoparticles, which is consistent with previous findings. Similarly to our finding, Aljabali *et al*. have indicated that AuNPs under SEM examination are appeared as a spherical shape with high dispensability [33]. Previously, the range size of synthesized AuNPs from *Cordia myxa L.* extracts is estimated (56.49–89.38) nm, while others have suggested that they are ranging from 31.26–58.06 nm [34]. Variations in the morphological appearance may be related to the workflow preparation method or source.

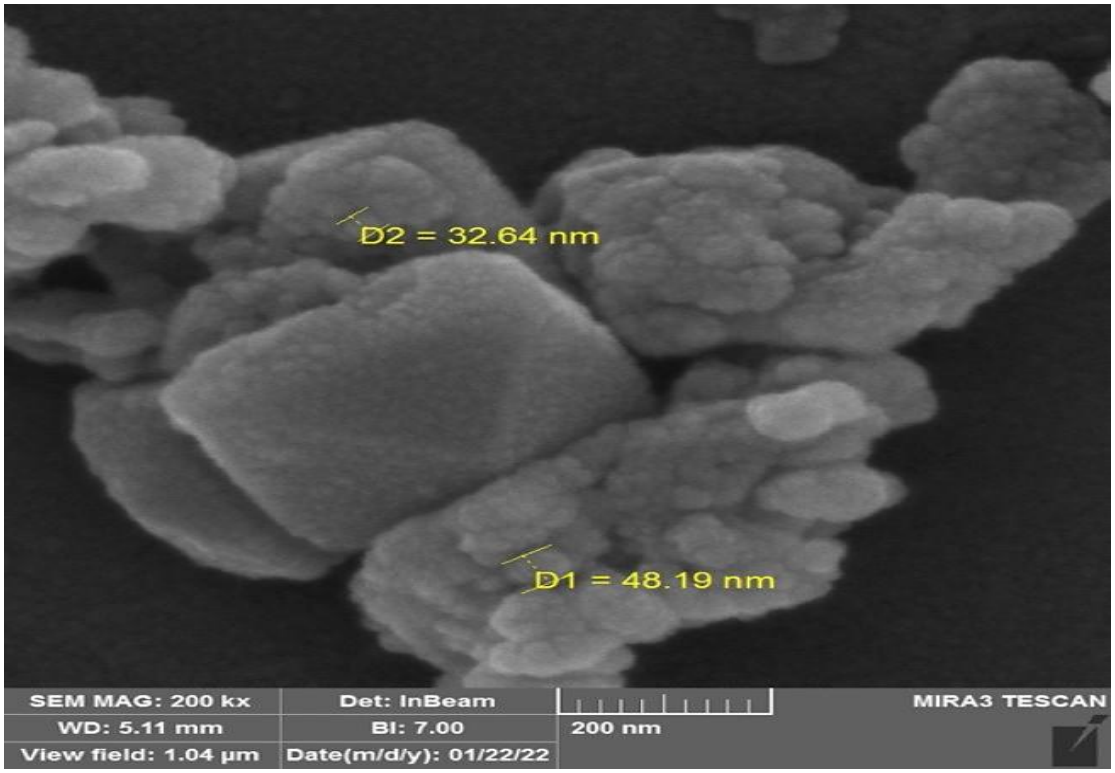

Figure 2 SEM photographs of gold nanoparticles synthesized from reacting *Olea europea L.* with precursor H.AuCl4.3H2O in aqueous solution. The particles are spherical shape and their average size ranging (32-48 nm). D1 and D2 are the measured size of gold nanoparticles 48.19 nm and 32.64 nm, respectively.

3.3 In vitro examination of AuNPs activity against cancer cells

Human lung cancer cell line (A549) is involved in investigating the anticancer activities of AuNPs. Cells are treated with different concentrations of synthesized AuNPs (50, 100, 150, and 250 µg\ml) and incubated 72h at 37 °C with 5% CO_2 (Figure 3). Morphological appearance of cells is scrutinized after treating cells with IC_{50} concentration of nanoparticles for 24 h. Cancer cells show morphological appearance and cell proliferation alternation is observed after incubation with above concentrations. The alternations include cellular shrinkage, rounding and clumping and suppression of proliferation.

3.4 Cytotoxicity of the AuNPs on MCF-7 and A549 cancer cell line

The cytotoxicity of synthesized AuNPs on A549 cell line is determined using MTT assay. The results report that AuNPs showed anticancer activity against A549 with the IC_{50} concentration equal to 24.2691 µg/ml. Figure 3 shows the percentage of cells viability pattern, which is inversely proportional with AuNPs concentrations. This result indicats that the concentration of AuNPs affect cells viability. The percentage average of cell viability is decreased dramatically (86.88%, 70.92%, 37.35%, 40.9%, 27.42%) for the 12.5, 25, 50, 100 and 200 µg/mL, respectively. Cell viability is relatively high at (12.5 and 25 µg/mL) concentration of AuNPs. Viability reduction is observed at 50-200 µg/mL. Further

analysis to support our finding, Strong anticancer activity is demonstrated at IC50 value (24.2691 µg/mL). It could be concluded that the AuNPs inhibit cancer cell viability.

Similarly to our results, high toxicity of AuNPs particles against cancer cells is observed previously [35]. Kus-Liśkiewicz *et al.* have stated NP characteristics, such as functional groups, NP size and charge, affect AuNPs cytotoxicity [36]. AuNPs properties, such as small size, resulted in cytotoxicity to cancer cells, binding to proteins, penetration and deposit on the tumor tissue [37].

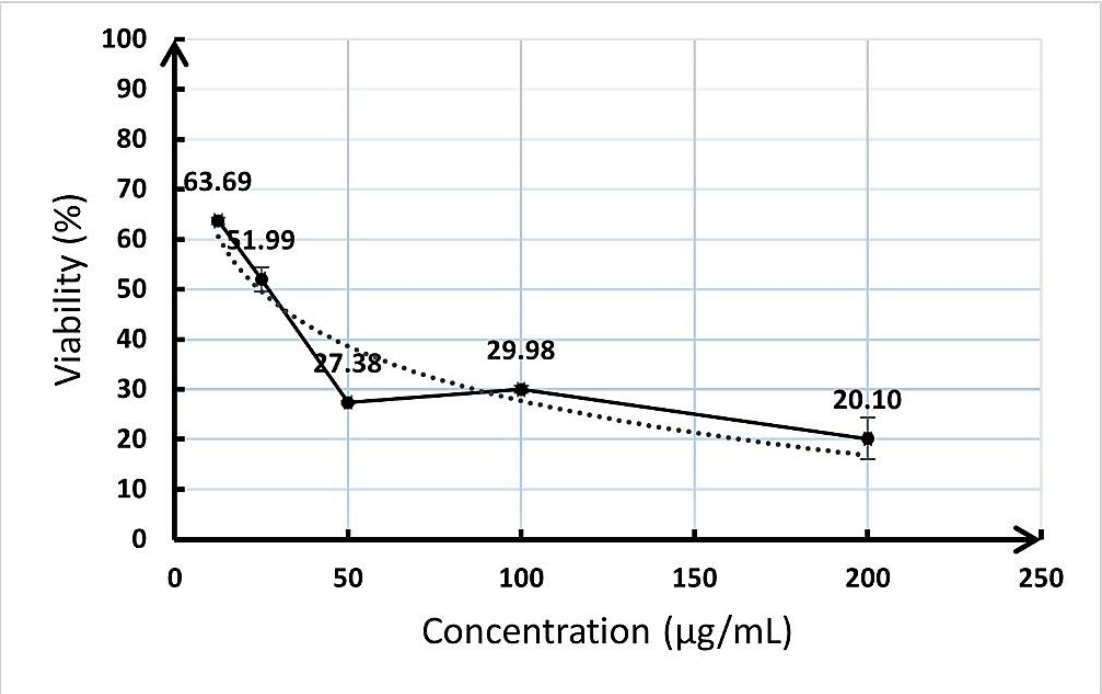

Figure 3 Cell viability percentage of A549 cell line after 48 and 72 h incubation with different concentration of AuNPs is synthesized by aqueous extract of *Olea europea L*. leaf. Cell viability is dose-dependent. Cell viability reduces with high concentration.

Inhibition of cell viability could be linked to trigger of apoptotic mechanism. This result is supported by additional observations, such as morphological alterations (cells shape), AO/EB staining, and DNA fragmentation assay. AuNPs properties are associated with cellular uptake and cellular biological processes, such as oxidative stress and cell integrity, including DNA fragmentation leading to initiated cells apoptosis [38].

Further analysis of cell viability is performed using fluorescent staining Ethidium bromide (EB)/acridine orange (AO). A549 cells stained 24 h with AO were green, whereas orange cells, stained with both AO and EB, represent early and late apoptotic cells. Cells are stained with EB showed orange hue and represented necrotic cells, as has shown in Figure 4. The result indicats that the viability of treated cells with IC_{50} concentration of the gold nanoparticles within 24 h is reduced. The mechanism of AuNPs against cancer cells is associated with binding to the membrane, oxidative stress, mitochondrial, and DNA damage [39].

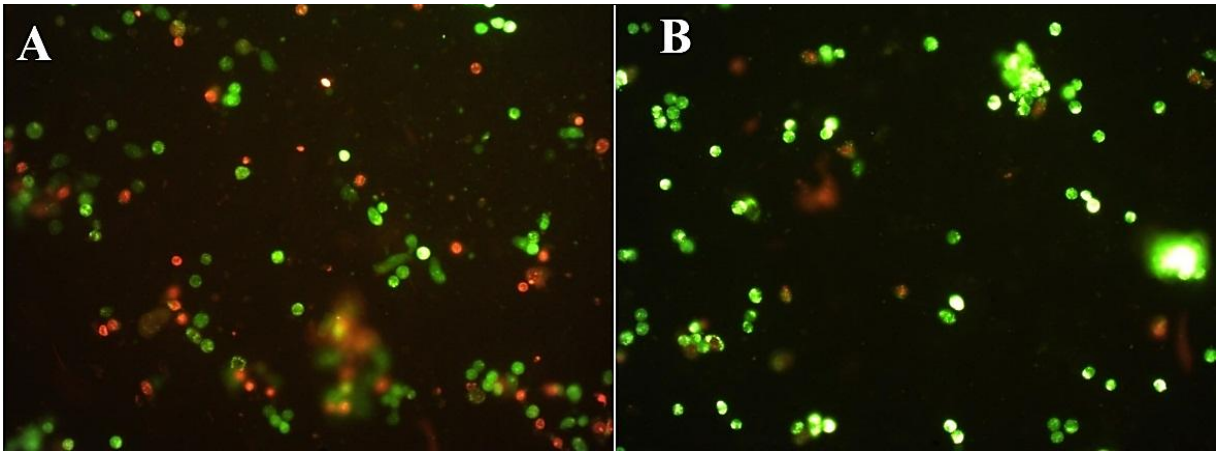

Figure 4 Apoptotic analysis. A549 cell line treated with IC_{50} concentration of green synthesized AuNPs and stained with fluorescent staining ethidium bromide (EB)/acridine orange (AO). A. shows the treated A549 cell line, Necrotic cells are orange stained with EB. B. are untreated viable normal cells (Control).

3.5 DNA fragmentation

Further analysis is carried out to confirm apoptotic induction by investigating DNA fragmentation assay. Isolated DNA of the A549 cells line treated with IC_{50} concentration of AuNPs are separated on agarose gel comparing with untreated control cells. The results demonstrat the ability of AuNPs to induce apoptosis in A549 cell line. Lane.1 in Figure 5 shows a fragmented DNA pattern, where the separated DNA resembles a smear or ladder, referring to apoptotic cell suicide. The DNA is fragmented to pieces as a result of the endonucleases activation.

Previous work observs the induction of apoptosis of cancer cells treated with NPs [40]. The interaction between NP and DNA is demonstrated; different ways of interaction are observed, including absorption on the surface of the NMs, covalent and hydrophobic interaction. It is observed that factors affect AuNPs binding to nucleic acid strand, such as type of nucleic acid, DNA or RNA, NPs charge, and nucleic acid sequence[41]. Induction of apoptosis by nanoparticles is observed previously by Tawfeeq [40]. Gold nanoparticles (AuNPs) can bind to single-stranded DNA (ssDNA) and RNA, and the degree of binding depends on the charge of the NPs and the sequence and type of the nucleic acid [42].

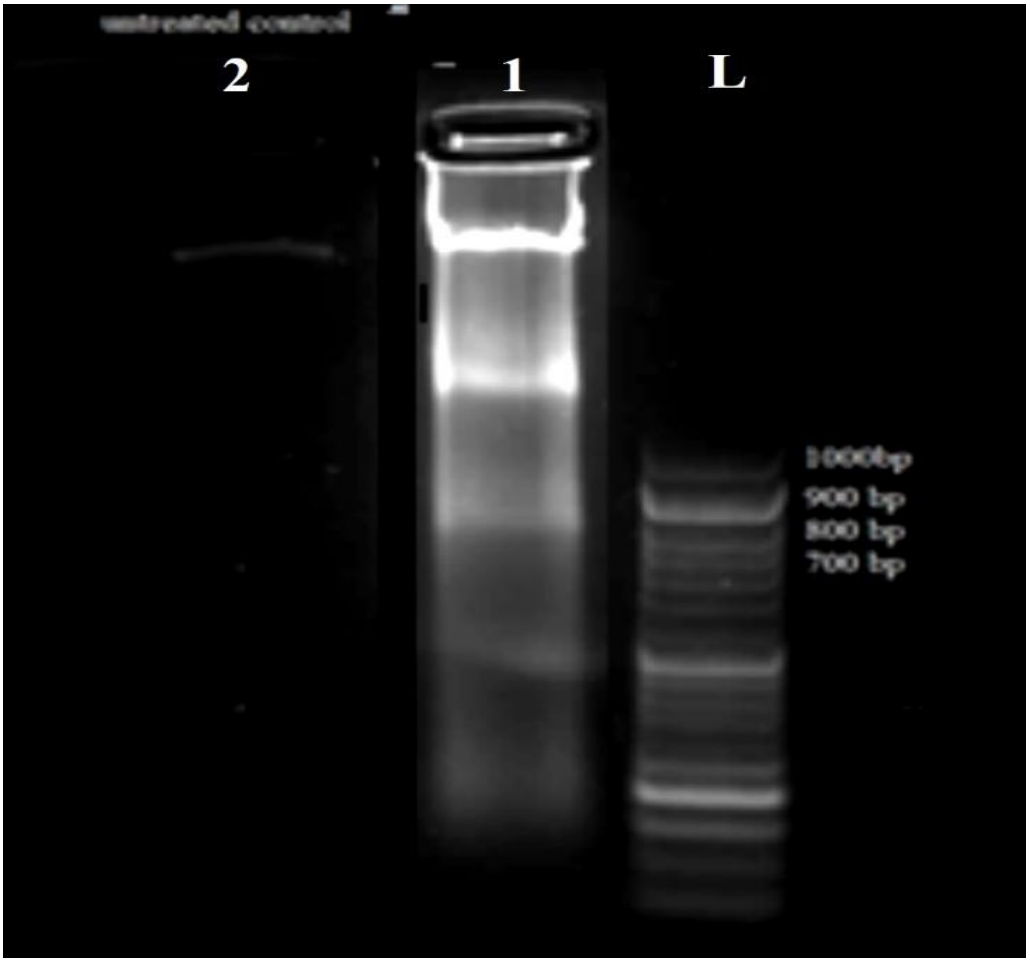

Figure 5 The DNA fragmentation system of A549 cell line treated with the IC_{50} concentration of AuNPs compared to untreated control cells. Gel electrophoresis of GDNA isolated from treated A549 cells with IC_{50} concentration of AuNPs. Lane L. is Ladder, Lane 1 represents fragmented DNA isolated of treated A549 cell line, Lane 2 is untreated A549 cell line (control).

4. CONCLUSIONS

In the current study, a green, eco-friendly and inexpensive method was utilized to synthesize gold nanoparticles (AuNPs) using *Olea europaea L.* leaf extract. Morphologically, AuNPs were spherical in shape, ranging in size from 32–48 nm, with a surface plasmon resonance peak at 520 nm. The synthesized particles exhibited a remarkable anticancer activity against the A549 lung cancer cell line in a dose-dependent manner, with an IC_{50} value of 24.2691 µg/mL. Indications of apoptosis in the treated cancer cells were confirmed as a mechanism of anticancer activity against cancer cells. Our results, collectively, indicated that AuNPs could be considered a potential promising anticancer therapy.Usage of single cell line in the current study was considered as one of the limitations. Another one was the absence of XRD analysis, Scherrer's equation, and a table of structural properties. Future analysis should add, particularly the analysis of crystallite size. In future, further analysis need to investigate AuNPs activity against cancer cells in vivo. Moreover, investigation of the active compound in *Olea europea L*

plant and study binding by using docking technique. Comparative study to AuNPs prepared from different multi-cell line to study their apoptosis induction.

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