



IN VITRO EVALUATION OF THE EFFECT OF A BOTANICAL COMBINATION ON CANCER CELL CONTROL

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ABSTRACT

The present century has witnessed the development of green synthesis of silver nanoparticles (AgNPs) and its wide application in the medical field. The green synthesis method is the most stable, highly effective and easy to prepare method for producing AgNPs. Figs, olives and moringa trees are grown in Iraq and have been mentioned in the Holy Qur'an several times and their benefits, as well as the proof of intensive scientific studies of the benefits of figs, olives and moringa, whether their leaves or fruits against microbes, oxidation, cancer and various therapeutic applications. Accordingly, the study aimed to examine the plant composition of leaves (fig, olive and moringa) and the possibility of forming AgNPs nanoparticles against the activity of cancer cells. The surface properties of AgNPs were confirmed and described by UV-Vis, XRD, SEM, TEM, AFM and FTIR spectrophotometry. And also FESEM, TEM technique was used to find the average size and the morphological shape of AgNPs. And the TEM results showed the average size of the nanoparticles of the plant synthesis of fig, olive and moringa (FOM) between 44.08-71.57 nm. Also, the ability of FOM mixture extract with silver nanoparticles to inhibit a type of FCM 7 cancer cell lines was studied. The results of the study showed the significant presence of the biological active components of the plant synthesis. Also, the ability of FOM mixture extract with silver nanoparticles to inhibit a type of FCM 7 cancer cell lines was studied. The results of the study showed the significant presence of the biological active components of the plant synthesis. Nanomaterials of synthetic nanoparticles have also demonstrated the size and shape of the nanoparticles and the presence of reactive chemical bonding sites. The study also proved that using concentrations higher than 50 µg/mL that there is a possibility to kill and inhibit the growth of cancer cells of type FCM 7 in later study.

Keywords: Silver nanoparticles AgNPs, (fig- olive- moringa FOM) combination, anticancer.

In Vitro تأثير توليفة نباتية على مكافحة الخلايا السرطانية مختبريا

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الخلاصة

شهد القرن الحالي تطور التخليق الأخضر لجسيمات الفضة النانوية (AgNPs) وتطبيقه الواسع في المجال الطبي. وتعد طريقة التوليف الخضراء هي الطريقة الأكثر استقرارًا وذات فعالية عالية وسهولة التحضير لإنتاج AgNPs. تزرع أشجار التين والزيتون والمورينجا في العراق وقد ورد ذكرها في القرآن الكريم عدة مرات وفوائدها وكذلك إثبات الدراسات العلمية المكثفة لفوائد التين والزيتون والمورينجا سواء أوراقها أو ثمارها ضد الميكروبات والأكسدة والسرطان والتطبيقات العلاجية المختلفة. وبناء على ذلك، هدفت الدراسة إلى فحص التركيبة النباتية لأوراق (التين والزيتون والمورينجا) وإمكانية تكوين الجسيمات النانوية AgNPs ضد نشاط الخلايا السرطانية. تم التأكيد ووصف الخصائص السطحية لـ AgNPs بواسطة القياس الطيفي UV-Vis و XRD و SEM و TEM و AFM و FTIR. وكذلك تم استخدام تقنية FESEM، TEM للعثور على متوسط حجم والشكل لـ AgNPs. وأظهرت النتائج TEM، ان معدل حجم الجسيمات النانوية للتوليفة النباتية FOM يتراوح بين 44.08-71.57 نانومتر. وكذلك تمت دراسة قدرة مستخلص مزيج FOM مع جزيئات الفضة النانوية في تثبيط نوع من خطوط الخلايا السرطانية FCM 7. أظهرت نتائج الدراسة الوجود المعنوي للمكونات البيولوجية النشطة للتوليفة النباتية. كما أثبتت الدراسات النانوية للجسيمات النانوية المخلفة الحجم والشكل للجسيمات هي نانوية ووجود مواقع الروابط الكيميائية الفعالة. وكذلك أثبتت الدراسة أنه باستخدام تركيزات أعلى من 50 µg/mL هناك إمكانية للقتل والتثبيط نمو الخلايا السرطانية من النوع FCM 7 في دراسات لاحقة.

الكلمات المفتاحية: جزيئات الفضة النانوية AgNPs، مزيج اوراق (التين-الزيتون- المورينجا FOM)، مضاد للسرطان.

INTRODUCTION

Treating many diseases with wild herbs and plants medicinal and known as one folk medicine, one of these diseases is cancer (Cataldo et al., 2017). Surgical, radiological, chemical, or combination treatments are used. In general, the two surgical treatments radiation is used in cases of local tumors chemotherapy is used when cancer cells have spread to the body (Aman & Dipak, 2020). Despite the benefits of these treatments, they have side effects that negatively affect the health of the patient, especially chemotherapy and radiotherapy that are toxic to tissues natural in the body or by causing genetic mutations to its cells and this in turn its immune system is weakened (Sutradhar & Amin, 2014; Alsharairi et al., 2019). Fig, olive and moringa trees were cultivated widely and used in the Mediterranean countries in the human diet and in traditional medicine (Marianna et al., 2019; Rania et al., 2019). Moringa leaves have also been used for the same purpose in many Asian and African countries as a treatment for various diseases, including cancer of all kinds (Khor et al., 2018; EI behairy et al., 2019). Olives, figs and moringa leaves are thrown away during the production of olive oil or the fig and moringa fruits are harvested and are unexploited by-products. Recent and intensive studies have indicated that the leaves of the olive tree, moringa and figs indicate their high absorption capacity and the possibility of creating nanoparticles using extracts of these leaves with various noble minerals such as gold, silver... etc. of these mineral elements in small quantities and their biological ability to kill many different agricultural pests (Delgado and Romero, 2017; Al-Hamdani et al., 2021). Also, many scientific studies have proven that most medicinal and food plants possess biologically active plant compounds and can be used to reduce many food products instead of industrial sources and to avoid their harmful side effects on human health (Boss et al., 2016; He et al., 2017). In addition, olive leaf and fig fruit

extracts reduced the fat oxidation of baked goods (Difonzo *et al.*, 2018). Several studies revealed that olive polyphenols act as an anti-inflammatory and antioxidants, polyphenols also play a protective role against cancer caused by DNA damaged by reactive radicals (Boss *et al.*, 2016). Many medicinal plants have proven their therapeutic ability for cancerous tumors, and medicinal plants that have an important effect in treating many different types of cancer are olive leaf extracts, figs, as well as moringa (Shahinuzzaman *et al.*, 2019). In the current century, silver nanoparticles (AgNPs) have been used with interest by researchers because of their important applications in biomedicine (Cataldo *et al.*, 2017), agriculture, food industry, catalysts, and biosensors (Huq, 2020). AgNPs nanosynthesis from plants has been successfully employed in various pharmaceuticals, biomedicine, and in many other fields domains, such as antimicrobial and anticancer (Kaushik *et al.*, 2021), antibiotic, antifungal, anti-inflammatory, antioxidant, antiviral and wound healing (Shahinuzzaman *et al.*, 2019). Scientific studies have proven synthesis of AgNPs using plant extracts is promising for ease of method, availability of various plants, non-toxicity, simplicity and cost-effectiveness of the method and high reduction ability (Li-Xu *et al.*, 2020). So, the aim of this study to prove the effect of silver Nano synthesis with blend (FOM) extract on two types of carcinogenic cell in vitro treatments.

MATERIALS AND METHODS

Plant material and extract preparation

The leaves of blend (fig, olive and moringa (FOM) plants were collected from one of the private orchards located on the outskirts of Baghdad. Then only the leaves were picked without the stems, and equal weights were taken from each of the three above-mentioned plants, and the surface was cleaned with running tap water to remove dust and other contaminated organic contents. By double distilled water, the leaves washed again and were dried using the conventional method after rolling the leaves in stainless steel trays in the shade and under the ceiling fan for five days in the laboratory at room temperature (20-25 °C). Then dried blend was grounded in a Wiley mill to pass through 1 mm sieve. Then about 10 grams of the dried and grounded leaves were placed in a beaker containing 200 ml of distilled water and boiled for 30 minutes. Then, an incubator shaker (Model SI 50) was used at 240 rpm to increase the efficiency of extracting the largest possible amount of the active compounds from the sample. Then the process was centrifuged at 4000 rpm for 10 minutes using Laboratory centrifuge machine, then the supernatant was filtered with Whitman No.1 filter paper, and then the extract was stored at 4 °C until used directly to synthesis and assay of Ag-NPs synthesized as described by (Ahmed *et al.*, 2020; Hamdia & Sundus, 2019), to determine the antibacterial, antioxidant and (Shahinuzzaman *et al.*, 2019) and against cancer cells activities.

Green synthesis of silver nanoparticles

The green silver nanoparticles were made by bio-reduction of Ag + using plant-blend extracts of each of the leaves (fig, olive, and Moringa (FOM)), and the process was done by adding about 5 mL of the pre-prepared extract drop by drop to an aqueous solution of AgNO₃ (50 mL, 0.1 M/ mL) and stirred at 45-50 °C for 30 minutes. Then, the mixed solution was subjected to ultra-sonication over the solution for 3 hours. The complete synthesis and formation of Ag-NPs was confirmed by changing the colorless silver nitrate solution to a dark brown one, after which the residual AgNO₃ was removed by hemodialysis against deionized water at 4 °C (Song & Kim, 2009). The stability of Ag-NPs (NFOM) was also examined by observing the color of the solution after 20, 40, 50 and 60 days of storage at 4 °C as (Sriram *et al.*, 2012; Pacioni *et al.*, 2015).



Chemical composition of FOM blend

The chemical analyzes of the plant mixture were carried out in the Chemical Laboratory for Market Research and Consumer Protection / University of Baghdad, as moisture, ash, protein, fat and crude fiber were measured using standard methods (AOAC, 2000).

Phytochemical screening

The extract of ethanol and hexane of blend (FOM) prepared for phytochemical detection to reveal the various biological components of the plant such as: Alkaloids, tannins, flavonoides, total phenols, saponines, glycosides, comarines, ratingen, trypsin and steroid was done using standard protocols and according to (Lalitha et al., 2012; Aman & Dipak, 2020).

Characterization of silver nitrate nanoparticles

UV-Visible spectroscopy

Average size is determined for the silver nanoparticles created (NFOM) by performing UV-Vis spectroscopy by pumping one milliliter of the sample into a test tube and analyze them at room temperature with a resolution of 1 nm and wavelength ranges between 200-800 nm and after being decomposed by exposure to dynamic light using (Spectroscatter 201) Shimadzu type Ultraviolet Visible Spectrophotometer (UV-1800, Japan).

X-ray Diffraction (XRD) determination

Crystal forms of the phytosynthesized AgNPs were carried out by using X-ray diffraction (XRD) method (Hamdia et al., 2021). XRD test was carried out by Phillips X'Pert Pro powder X-ray diffractometer (XRD) (PANalytical, Almelo, The Netherlands) with Cu Ka radiation in the range of $2^{\theta} = 10^{\circ} - 80^{\circ}$. All Structure confirmations of nanoparticles were done in Kashan University/Iran.

Atomic force microscopy (AFM)

AFM is also used to analyze volume, surface morphology, structural and physical mechanical properties by silicon probe saturated with phosphorous (Reddy, et al., 2014). The AgNPs sample was prepared by dissolving it in water or ethanol and a drop was placed on a silicon substrate, then, it was left for a period of minutes until it dried and then analyzed by AFM technology using a probe according to (Jain et al., 2021).

Scanning electron microscopy (SEM)

To study the cell surface topography and characterize the elemental composition of synthesized AgNPs, SEM combined with EDX were analyzed by using Sigma 300 (Carl Zeiss, Germany), operated at a 20 KV. A small amount of AgNPs (10 μ L) was drop-cast on a clean coverslip and dried. The samples were then sputtered with gold and observed under a FESEM and EDX (Sudad et al., 2020; Al-Hamdani et al., 2021).

Transmission Electron Microscopy (TEM)

TEM was applied to depict the morphology, size and particle distribution of the green synthesized AgNPs. The images were taken by JEOL 2100 Electron Microscope (JEOL, Peabody, MA, USA). For TEM analysis, a single drop (10 μ L) of AgNPs suspension was placed on the carbon-coated copper grid, dried for 2 h at room temperature (24 $^{\circ}$ C) and loaded into the specimen holder before performing analysis at 120 kV accelerating voltage.



Fourier Transform Infrared (FTIR)

To study the functional group present on the synthesized AgNPs surface, FTIR analysis was performed by using an FTIR spectrophotometer (Perkin Elmer spectrum 100 FTIR, 710 Bridgeport, CT, USA). The FT-IR spectra were scanned with wave numbers ranging between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1} in the transmittance mode (Sastry *et al.*, 2013; Malathi *et al.*, 2014; Al-Hamdani *et al.*, 2021).

Cell viability and cytotoxicity

MCF 7 was kindly provided from the Iraqi Center for Cancer and Medical genetics Research/Al-Mustansiriyah Univ. /Baghdad. Thawing of cells was performed quickly in a 37 °C water bath for 2 minutes. Then, the cells was transferred immediately into a culture flask containing pre-warmed RPMI culture medium was supplemented with 10 % FCS 7 and incubated at 37 °C with 5% CO₂. The next day, the medium was replaced with fresh medium to remove traces of DMSO. The cells were grown to 60-70 % confluence and harvested by trypsinization. To determine the cell proliferation rate of extract treatment, crystal violet assay was conducted as on 96-well plates (Falcon). Breast cancer cells (MCF-7) were seeded at 1×10^4 cell/well and incubated at 37 °C for 24 hr. or until 70% confluent is achieved. Cells were treated with each extract at 50, 25, 12.50, 6.25, 3.125 and 1.56 $\mu\text{g/mL}$ using two fold serial dilutions reaching. The extract added to MCF-7 cells for 72 hr. at 37 °C. After the dedicated time, cells were washed with PBS. Cell viability was measured after removing the medium, adding 100 μl of solution of 5 mg crystal violet (BDH-England) + 200 ml methanol + 50 ml formaldehyde 37 % and incubated for 30 min. at 37 °C. After removing the crystal violet solution, cells were washed with water three times. The absorbency was determined on a micro plate reader (Organon Teknika Reader 230S, Austria) at 492 nm (test wavelength), the assay was performed with modification in triplicate. Endpoint parameters that are calculated for cell line include cell proliferation rate, which is the percentage of control absorbance. The inhibiting rate of cell growth (the percentage of cytotoxicity) was calculated as $(A-B)/A \times 100$, Where A is the mean optical density of untreated wells and B is the optical density of treated wells.

STATISTICAL ANALYSIS

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

RESULTS AND DISCUSSION

The chemical composition of the plant mixture and each component

The chemical composition of the plant blend and each component of the blend are represented in the Table 1. The results of the study showed the high and significant ($P \leq 0.05$) content of ash, fiber and carbohydrates for the plant mixture, as well as containing significant amounts of protein and fat as shown in Table 1. These results are identical to what was found by (Rania *et al.*, 2019).

Table (1): Chemical composition of the dried leaves of the fruit trees used, the combination and the production ratio of their extracts.

Compenents%	Moringa	Olive	Fig	Plant blend (FOM)	LSD value
Yield	14.50	13.00	13.67	6.72	2.58 *
Moisture	0.90	0.85	0.92	0.90	0.207 NS
Ash	4.65	5.68	5.80	5.28	0.693 *
Protein	2.85	2.65	2.56	1.50	0.622 *
Fat	2.5	3.06	2.90	2.86	0.501 *
Fiber	19.6	22.06	20.50	22.60	2.197 *
Carbohydrate	38.5	25.66	30.65	31.66	4.731 *
* (P<0.05), NS: Non-Significant					

Phytochemical screening

Plants contain vital organic compounds such as flavonoids, amino acids, carboxylic acids, ketones, phenols and proteins (Attia & Elsheery, 2020). These substances play an important role in restore mineral salts and produce nanoparticles in easy, fast and environmentally safe ways. Results of this study presented the content of each fig, Moringa, olive and botanical blend (Fig, Olive and Moringa) as shown clearly as in Table 2. It was founded each plant separately or in a combination state were contained alkaloids, tannins, flavonoids, phenols, glycosides, comarins, trypsin and steroids. While, it was founded the saponin not founded in olive leaf extract. Also, Ratingen and trypsin content not significantly founded in extract of blend plants. The leaves extract of FOM plant blend act as reducing and capping agents in the synthesis of AgNPs and may be possesses anticancer activity (Jang, et al., 2016). Also, it was presented these compounds have been confirmed for antioxidant, anti-inflammatory, anticancer, and antimicrobial properties (Patel et al., 2015)

Table (2): Phytochemical detection of the blend and each type of combination

Phytochemicals	Aqueous extract of moringa	Aqueous extract of fig	Aqueous extract of olive	Aqueous extract of blend (FOM)	P-value
Alkaloids	+	+	+	+	NS
Tannin	+	+	+	+	NS
Flavones	+	+	+	++	0.044 *
Total phenol	+	+	+	+++	0.006 **
Saponin	+	-	+	+	0.048 *

Glycosides	+	+	+	+	NS
Comorians	+	+	+	++	0.044 *
Ratingen	+	+	+	+	NS
Trypsin	+	+	+	-	NS
Steroids	+	+	+	+	NS
* (p<.05), ** (p<.01), NS: Non-Significant.					

Nanosynthesis of silver nitrate using plant (FOM) blend

UV-Visible

Changing the color of the prepared solution from clear to yellow and then to dark brown is an indicative of conversion of silver ions into silver nanoparticles as shown clearly in Figure 1a. Figure 1b was showed the spectra of surface Plasmon resonance absorption for solutions of silver nanoparticles were prepared with FOM plants were examined at a different wavelength as shown in Figure 1b. UV–Vis spectrum of silver nanoparticles result indicated the presence of peak at 450 nm represents the complete synthesis of silver nanoparticles Figure 1b. This is an interesting optical property; it is a characteristic of metallic nanoparticles, the colors of the solutions change depending on the particle size of nanoparticles and their concentration (**Devatha & Thalla, 2018**). Changing the color of silver indicates the possibility of an aggregation condition for silver nanoparticles, and then the color of colloidal solutions mainly determines the size distribution and aggregateon of nanoparticles (**Roy et al., 2019**). Phytoconstituent of the plant act as reducing agents for silver ions by oxidizing and capping Nano synthetic particles. In the presence of oxygen in silver nitrate ($AgNO_3$), these compounds lose their electron and oxidize via common cellular actions, thus acting as reducing agents (**Jang et al., 2016**).



b

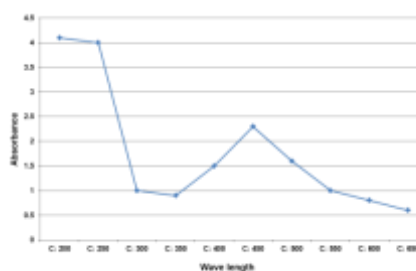


Figure 1. UV-Spectroscopy of plant blend (FOM) nanoparticles

a

Figure 1 (a, b): Biosynthesized of silver nanoparticles using FOM blend.

AFM

AFM is also used to analyze volume, surface morphology, structural and physical mechanical properties (Silva *et al.*, 2019; Al-Hamdani *et al.*, 2021). Results of this study showed AFM imagination the average size range 18.0 nm and the surface shape of nanoparticles is barbed or pointed as shown in Figure 4.

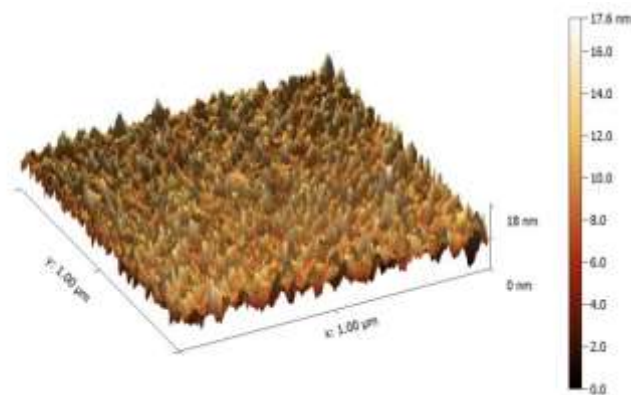


Figure (4): AFM of synthesised AgNPs with blend FOM extract.

FTIR

The FT-IR spectrum analysis of the AgNPs using leaf extract of *FOM* confirmed the presence of biomolecules. The presence of a broad and strong peak in 3829 cm^{-1} is belonged to the stretching vibration of the phenolic and alcoholic O–H groups in the extract. The absorption peak at 2955 cm^{-1} refer to the stretching vibrations of the aromatic group C–H of benzene rings and aliphatic groups, respectively. Also, strong absorption peak was observed at 1633 cm^{-1} , associated with stretching vibrations of the C=O bond of the carbonyl amide protein group. The absorption peaks at 1116 and 1407 cm^{-1} related to the bending vibration of the CH₂ group. While, the bending vibrations of C–H bonds in the C=C–H groups appeared in the region below 1000 cm^{-1} . These results are close to what was found (Bita *et al.*, 2018).

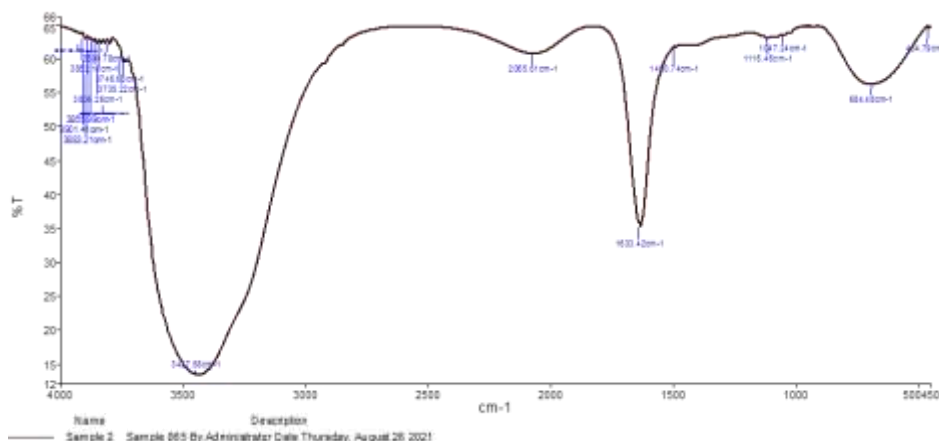


Figure (5): FT-IR spectra of FOM silver nano particles FOM leaf extract



Effect of blend silver nanoparticles on breast cells carcinoma

MCF 7 cells were treated with 50, 25, 12.5, 6.25, 3.125 and 1.56 $\mu\text{g/mL}$ of the plant nano combination in comparison with the control and commercial AgNPs treatment for 24 h. The results of studying the effect of different concentrations of particles silver nanoparticles using aqueous extract of the plant synthesis FOM and its effectiveness on inhibiting cancer cells live for breasts (MCF 7) shown in (Table 5). It was found that increasing the concentration of the Nano-extract of the plant combination had a stronger effect on the death of cancer vital cells (MCF 7), the percentage of its effect and effectiveness was approximately 30% compared to low concentrations and through Table 5. The highest activity of silver nanoparticles using 50, 25, 12.5 and 6.25 $\mu\text{g/mL}$ aqueous extract of the plant synthesis FOM against growth percentage of cancer cells MCF 7 were to 72.2, 89.5, 119.9 and 133.3 respectively, which was statistically significant ($p < .01$) compared to the control cells. While using 25, 12.5 and 6.25 $\mu\text{g/mL}$ aqueous extract of the plant synthesis FOM against cancer cells MCF 7 growth percentage were increased significant ($p < .01$) to 89.5, 119.9 and 133.3 respectively compared to the control cells. From this it can be concluded that using high concentrations above 50 $\mu\text{g/mL}$ may give good positive results for killing and inhibiting breast cancer cells MCF 7 or the plant combination can be used for other types of cancers in later studies. These results came close to what was proven (Vasanth et al., 2014; Nancy et al., 2021) using the nano extract of *Moringa oleifera* plant (Kang et al., 2018), its toxicity to cancer cells (Lalitha et al., 2012). Photochemical content in FOM blend may be acted as anticancer activity. Also, Photochemical compounds have been confirmed for antioxidant, anti-inflammatory, anticancer, and antimicrobial properties (Alam et al., 2013; Patel et al., 2015).

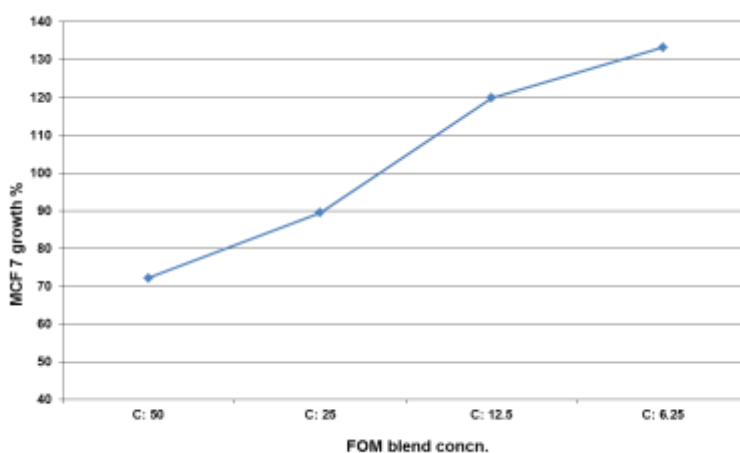


Figure 1. Growth % of MCF7 of breast cancer in vitro treating with different concentration of (FOM) bend



Table (6): Growth % of breast cancer MCF7 *in vitro* treating with different concentration of (FOM) bend

FOM blend Concentration (µg/mL)	MCF 7 growth %
Zero (control)	100
50	72.2
25	89.5
12.5	119.9
6.25	133.3
3.125	110.0
1.56	99.0
LSD value	12.493 **
** (P≤0.01).	

CONCLUSION

Several methods have been discovered to prepare nanoparticles mineral such as physical, chemical and biological since the end of the last century until now. Particles were applied nanoparticles have many uses based on their physical and optical properties and electrical. The nanoparticles of the noble elements such as silver and gold...etc of the elements were used where they found their way. In biomedical applications, these metallic nanoparticles enter in the form of cell assemblies due to their small size and different physical properties. The chemical may affect different cells through various cellular processes. Silver nanoparticles were widely selected to know the toxicity of these materials against various tumors and cancers from most of the previous studies. There are many studies, which gave positive results when integrating silver nanoparticles alone or with capers of different plant extracts in the treatment of different types of cancer. The current study showed the relative effect of different concentrations of the synthesized silver nanoparticles on the plant mixture of figs, olives and moringa on the growth of FCM 7 breast cancer cells if the inhibition rate is 30% at the concentration 50 µg/mL. Further studies of anticancer mechanisms of AgNPs are essential to develop economical, reliable, and broad-spectrum anticancer agents. AgNPs with plants led to a good biological activity with less toxicity to normal cells and moderate toxicity to cancer cells, and this filters AgNPs particles in high concentrations to plants promising for cancer treatment in the future. Therefore, the method of nanosynthesis using the plant blend of (figs, olives and moringa) is characterized by its cost-effectiveness, time saving and synthesis tiny-sized particles that helped prevent the spread of MCF 7 for breast cancer cells and the induction of programmed cell death. So there may be a useful potential in the treatment and targeting of drug delivery for these types of cancers.

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