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RESEARCH PAPER

Evaluation of anti- cancer and antioxidant properties of nanoemulsions synthesized by *Nigella Sativa* L. tincture

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ABSTRACT

Objective(s): Today, the use of medicinal plants for treating cancer is extremely important. Over the past few years, the anti-cancer properties of *Nigella Sativa* L. have been proven. The aim of the present study was to evaluate, the cytotoxic effect of a nanoemulsion synthesized using *N. Sativa* L. tincture, against a cancerous cell line as well as its and free radical scavenging activities.

Materials and Methods: The size and zeta potential of the nanoemulsion were determined using particle size analyzer and morphological shape of nano emulsion was visualized by transmission electron microscopy (TEM). The antioxidant activity of nanoemulsions was investigated by the DPPH assay. Cytotoxic effects of the nanoemulsions were assessed by MTT method against A2780 ovarian cancer and umbilical vein endothelial cells (HUVEC) as normal cells. To evaluate the probable molecular mechanism of cell death, acridine orange and propidium iodide staining methods were used for identifying apoptotic cells.

Results: The results obtained from this study showed that the synthesized nanoemulsion had a good and dose-dependent radical scavenging capacity in the DPPH assay (IC50 of about 47µg/ml). Also, the nanoemulsion significantly reduced the bioavailability of A2780 cancerous cells (IC50 of 0.72 µg/ml); however, its toxicity against HUVEC cells was much lower (IC50 > 25 µg/ml). The pro-apoptotic effect of the produced nanoemulsion was confirmed by acridine orange and propidium iodide staining.

Conclusion: Nano emulsions synthesized by *N. Sativa* L. tincture has a relevant potential antioxidant and anticancer effects and therefore they can be considered and studied as anticancer compounds in future experiments.

Key Words: *Apoptosis, Acridine Orange /Propidium Iodide staining, Cytotoxicity*

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INTRODUCTION

Using herbal medicines to treat diseases has been considered as a viable option for centuries [1-3]. Although most drugs used today are chemical, about one-third of them are of plant origin [4, 5]. *Nigella Sativa* L. has been known as an important medicinal plant for several thousand years and is of great importance due to its different healing properties [6]. Numerous studies have shown antioxidant, analgesic, anti-inflammatory, anti-cancer, anti-microbial, anti-parasitic, and anti-convulsant properties of *N. Sativa* L., as well as its effects on the cardiovascular system, gastrointestinal tract, blood circulation, immune system, kidneys, and the liver [7-9]. Studies to isolate and identify the ingredients of *N. sativa* L. seed have shown the presence of compounds such as essential and fixed oils, as well as saponin, alkaloids, and various proteins. The fixed oil which comprises 32 to 40% of the seed content consists of a combination of linolenic, almitoleic, eicosadienoic, myristic, arachidonic, stearic, oleic, palmitic, and linoleic acids. Cycloeucalenol, sterol glucosides, beta-sitosterol, sterol esters, and cycloartenol comprise other constituents of the fixed oil. The isoquinoline (comprising of nigellicimine and nigellicimine n-oxide) and pyrazol (nigellidine and nigellicine) are two main alkaloids of *N. sativa* L. The major component of the carbonyl fraction of the plant volatile oil (which comprises 0.4 to 0.45 % of the plant extract total content), is nigellone (a saturated fatty acid). Other ingredients of volatile oil comprise thymohydroquinone

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(THQ), thymoquinone (TQ), carvacrol, thymol, α and β-pinene, dithymoquinone, d-limonene, and d-citronellol. Furthermore, the compounds present in p-cymene volatile oil include carvacrol, p-cymene, 4-terpineol, t-anethole, and longifoline [10-12]. In traditional medicine, *N. Sativa* L. has been used for treating several diseases and ailments such as menstrual problems, asthma, diabetes, cough, and as a diuretic or laxative compound [13, 14]. In recent years, nanoparticle and nanoemulsions have received great attention from researchers [15, 16]. Nanoemulsions are very small emulsions at the dimensions of 20 to 200 nanometers. The very small size of droplets in nanoemulsions reduces the gravitational forces of the particles and thus prevents the cream from emulsifying and precipitating during storage [17]. In this way, the accumulation of emulsion droplets is prevented, and the system remains stable without the solution being biphasic or separated [18]. In comparison with other emulsions, nanoemulsions have more non-polar compounds due to their abilities to dissolve non-polar compounds and form covalent bonds and also have a greater potential for effective micro-coating of flavor-forming compounds due to the higher surface-to-volume ratio of droplets [19].

Besides, because nanoparticles (NPs) are much smaller than cancer cells, they can easily penetrate into blood vessels, and therefore, they can be widely used as carriers to deliver drugs to cancer cells [20-22]. Among NPs studied until today, nanoemulsions have shown important clinical and therapeutic applications. One of the promising strategies of cancer treatment is to target chemotherapy drugs toward cancer cells using specific carriers which using nanoemulsions in this strategy has been useful [23]. Anti-cancer properties of medicinal plants have been investigated in many studies in order to prevent tumor progression and induce apoptosis in cancer cells [24-26]. Tinctures are condensed vegetal extracts which produced by soakage the bark, seeds, leaves or roots of a plant in alcohol or vinegar. Tinctures are dietary additive in a condensed, shelf-stable, and fluid form. Similar to other herbal extracts, tinctures can be consumed to help a wide variety of wellness purpose [27]. However, a nanoemulsion of tincture has not been produced nor used for studies of cancer, in vitro or in vivo. In the present study, we formulated a nanoemulsion using *N. Sativa* L. tincture and assessed its antioxidant and anticancer properties against A2780 ovarian cancer cells.

MATERIAL AND METHODS

Materials

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). RPMI, FBS, trypsin, antibiotics, 3,4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), and acridine orange dye were purchased from Sigma-Aldrich Company, Ltd. (Poole, United Kingdom). Other reagents were from Merck (Germany). A2780 human ovarian cancer and HUVEC cell lines were purchased from Pasteur Institute of Iran (Tehran, Iran).

Preparation of N. Sativa L. tincture

Nigella Sativa L. seeds were provided from Pakan Bazer Company, Isfahan, Iran, and washed well with distilled water. It was then dried at room temperature and pounded well and powdered in a mortar. To prepare the tincture, first 10 g of N. *Sativa* L. powder were mixed with 75 mL of 96% ethanol and 25 mL of distilled water. After that, 5 mL glycerin was added to the mixture which was then transferred into a glass container and kept warm by being placed on a hot plate steer 3 times a day for 1 hour. After 21 days, the tincture was obtained [28].

Preparation of a nanoemulsion by N. Sativa L. tincture

An ultrasound method was used to prepare NPs. First, 5 mg of N. Sativa L. tincture was mixed with 100 µL of tween 20, 100 µL of tween 80, and 500 µL of ethylene glycol in a 50 mL Falcon. The final volume was reached to 50 mL by adding distilled water. Then the mixture was placed in an ultrasonic device for half an hour to generate a uniform solution [29].

Characterization of nanoemulsion

The synthetized nanoemulsion was analyzed using a particle size analyzer, dynamic light scattering (DLS), TEM, and Zeta potential methods. The size and zeta potential distributions of NPs were analyzed using the Zetasizer instrument (Malvern, UK). The prepared nanoemulsion shape was observed by TEM (JEOL, Japan).

DPPH scavenging activity

DPPH (1,1-diphenyl-2-picryl-hydrazyl) is a

method for measuring electron-based antioxidant properties, in which a purple solution with a maximum absorption at 517 nm is produced in ethanol. For performing this assay, DPPH powder was prepared from Sigma-Aldrich (USA). First, 0.1 mM DPPH solution in 95% ethanol was prepared and mixed in an equal proportion with either the nanoemulsion synthesized by *N. Sativa* L. tincture or BHA as a standard antioxidant compound. After 30 minutes for allowing adsorption, the samples were read at 517 nm. In order to calculate the concentrations of nanoemulsion and BHA required for scavenging 50% of the antioxidant agent (IC50), the experiment was performed using four different concentrations (6.25, 12.5, 25 and 50 µg/mL) of these substances. Each experiment was performed in three replications [30].

Cytotoxicity assay

A2780 and HUVEC cells were cultured in RPMI supplemented with 10% FBS, 100 µg /mL streptomycin, and 100 µg/mL penicillin and incubated at 37oC in a humidified 5% CO2¬ incubator. Then the cells were seeded in 96-well plates (5×103 cells/well). After overnight incubation, the cells were treated with different concentrations of the nanoemulsion and incubated in the humidified 5% CO2 incubator for 48 hours. Cell counting was performed using a hemocytometer slide after adding trypan blue dye to cell suspensions. The experiment was performed using six different concentrations (0.78, 1.5, 3.1, 6.2, 12.5, 25 µg/mL) of the nanoemulsion.The experiment was repeated for each concentration of the nanoemulsion three times. Finally, the viability of the cells was determined using MTT (3- and 4-Dimethyl thiazole-2-yl [-2-5-diphenyltetrazolium bromide) assay and a plate reader spectrophotometer (Epoch, Biotek, Winooski, VT, United Kingdom) at 570 nm. MTT assay is a test performed to study cytotoxic effects of drugs and various chemical and biological compounds against cells. Briefly, after removing the plates from the incubator and draining the supernatant, 20 µL of MTT solution was added to each well, and the plates were incubated at 37 °C for four hours. Next, the culture medium and MTT reagent were removed, and 100 µL DMSO was added to each well. DMSO changed the color of produced formazan crystals to purple, and the intensity of the dye was evaluated by an ELISA plate reader at 570 nm. Cell viability was calculated as following:

Cell viability (%) = (Testabs / Controlabs)×100

Where Testabs is the absorbance of treated cells, and Controlabs is the absorbance of cells without treatment [31].

Acridine orange (AO)/propidium Iodide (PI) staining

AO and PI as fluorescent dyes provide an easy and fast way for discerning living from non-living cells. This method was performed to determine the number of apoptotic cells in NP-treated and negative control groups. First, 5 mL of a cell suspension containing 1 million cells was cultured in flasks for 24 hr. After that, the culture medium was removed, and the cells were treated with different concentrations of NPs for 48 hours. Then the cells were centrifuged at 2700 rpm for 5 minutes, and after removing the supernatant, the cell pellet was diluted with 1 mL PBS. In the next step, 10 µL of the cell suspension was admixed with 10 µL AO and 10 µL PI, and the mixture was incubated at 37 for 5 min. Subsequently, 20 µL of the resulting mixture was poured on a slide, and a lamella was placed above the slide. Finally, the sample was photographed and examined by fluorescence microscopy [32].

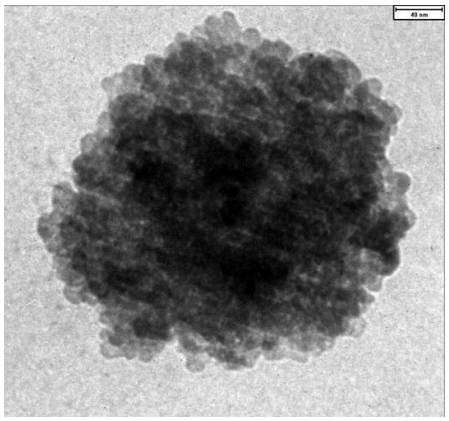

Fig 1. TEM image of nanoemulsions synthesized by *Nigella Sativa* L. tincture

Statistical analysis

In order to investigate antioxidant and cytotoxic activities, all the obtained ODs were first subjected to ANOVA analysis using the GLM procedure of SPSS V;22 (SPSS Software products, Marketing Department, SPSS, Chicago, IL, USA). Then one-way ANOVA was used to compare the means by the Least Significant Differences (LSD) method. One-way ANOVA was also used to evaluate the viability of the cells treated with the nanoemulsion

synthesized by *N. sativa* L. and compare it with that of untreated cells to check any significant difference. Analysis of variance and mean analogy were performed by the LSD method. P value of < 0.05 was used as the criterion for a statistically significant difference. All tests were performed triplicate and the results are expressed as mean values ± standard deviation (mean ± SD).

RESULTS

Characterization of nanoemulsion

The nanoemulsion was visualized by TEM. The results illustrated that the nanoemulsion had a spherical morphology (Fig 1).

DLS (Nano-ZetaSizer- HT, Malvern Instruments, and Malvern, UK) was done to determine the average size of the nanoemulsion (Fig 2).

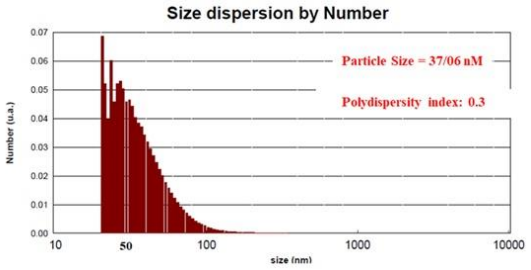

Fig 2. Particle size analysis of nanoemulsions synthesized by *Nigella Sativa* L. tincture

The particle size of the nanoemulsions ranged from 20 to 80 nm, and the average size was about 37 nm which is the best size for biological and biomedical applications. The polydispersity index (PDI) value of less than 0.7 indicated a single-phase and uniform dispersion of the synthesized nanoemulsions.

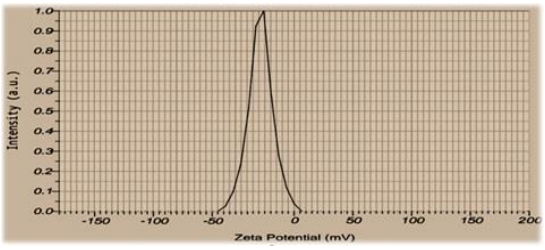

Fig 3. Zeta potential of nanoemulsions synthesized by *Nigella Sativa* L. tincture

The zeta potential of the surface of the nanoemulsion synthesized by *N. Sativa* L. tincture was -20 mV (Fig 3). According to Salopek et al., a zeta potential in the range of -30 to -16 reflects the stability of nanoemulsions [33]. Therefore, it can be said that the nanoemulsion prepared using N. Sativa L. tincture is a compound with acceptable stability.

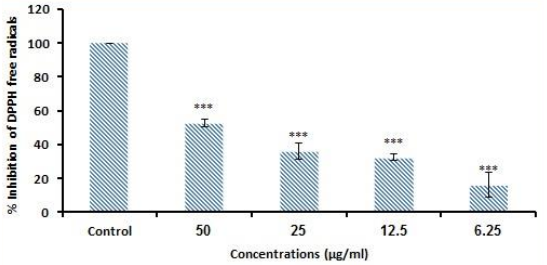

Fig 4. DPPH scavenging activity of nanoemulsions which synthesized by *Nigella Sativa* L. tincture. All tests were down in triplicate. *** p<0.001 indicated significant difference as compared to the BHA scavenging activity

DPPH scavenging assay

Fig 4. shows the DPPH free radical scavenging activity of the nanoemulsion synthesized by *N. Sativa* L. tincture compared to BHA as a positive control. The nanoemulsion removed DPPH free radicals in a concentration-dependent manner with higher concentrations showing stronger scavenging activities. The IC50 value was obtained as 47 µg/mL.

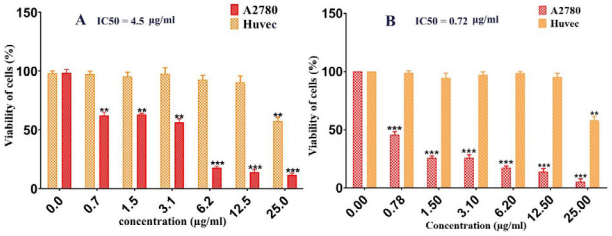

Fig 5. Cytotoxicity activity of *Nigella Sativa* L. tincture (A) and nanoemulsions synthesized by *Nigella Sativa* L. tincture (B) on the A2780 cell line and huvec cell line (as a normal cell) after 48h of incubation. Results are presented as the mean ± SD (n=3). ** represent p<0.01 and *** p<0.001 indicated significant difference as compared to the Control

Anticancer activity analysis

Fig 5 (A&B) shows the cytotoxicity of *N. Sativa* L. tincture and the synthesized nanoemulsion against A2780 ovarian cancer and normal HUVEC cells. The IC50 of the tincture and nanoemulsion for A2780 cancer cells was obtained 4.5 and 0.72 µg/mL at 48 hours after treatment, respectively. However, no cytotoxicity was observed against HUVEC normal cells at this concentration, indicating a selective toxicity against the cancerous cells.

The results showed that the nanoemulsion had potent concentration- and time-dependent toxicity against the cancer cells.

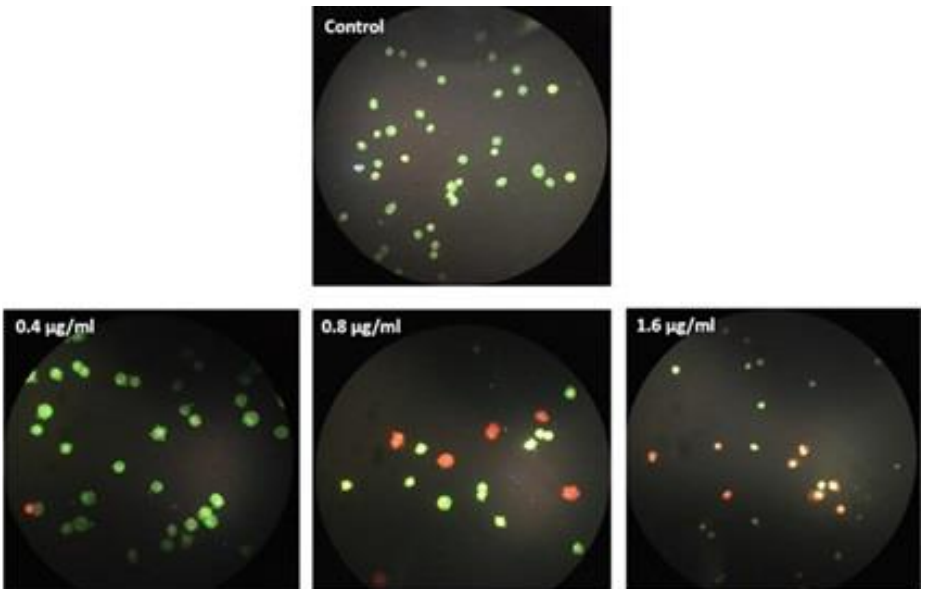

Fig 6. Acridine-Orange staning of A2780 ovarian cancer cells, treated with nanoemulsions synthesized by *Nigella Sativa* L. tincture. After incubation cells with different concentration of nanoemulsion, cells were stain using Acridine-Orange and seen under fluorescence microscopy (magnification 400). Green-colored cells repersent alive cells while the cells treated with the nanoemulsion underwent apoptosis. During apoptosis, cells stained with AO demonstrate nuclear shrinkage, cellular fragmentation, and loss of nuclear demarcation, appearing therefore as red cells

Evaluation of the apoptotic effects of synthesized nanoemulsion

In order to evaluate the apoptotic effects of the nanoemulsions synthesized by *N. Sativa* L. tincture against A2780 ovarian cancer cell line, AO/PI staining was performed. For this purpose, the cells were treated with the nanoemulsion at the concentrations of 0.4, 0.8, and 1.6 µg/mL. After 24 hours, the cells were stained with AO/PI fluorescent dyes. The results of fluorescence microscopy showed green-colored (i.e. alive) cells in the control sample while the cells treated with the nanoemulsion underwent apoptosis as evidenced by the absorption of PI by the cells, rendering them orange to brown under the microscope. The results showed that with increasing the nanoemulsion concentration, the intensity of orange color also increased, indicating a higher rate of apoptosis in the cancer cells (Fig 6). There was a significant positive correlation between the number of apoptotic cancerous cells and the nanoemulsion concentration.

DISCUSSION

Plant extracts are complex and completely natural volatile compounds without any harm or pollution toward the environment. Since synthetic materials cause many environmental problems, using organic materials is highly recommended in today's world. It showed that has been shown the health effects of some natural plants' essential oils were even better in some cases than that of synthetic materials [34, 35]. However, one of the main problems of plant extracts is their high volatility and instability. In order to deal with this problem, one of the best solutions is to use nanoemulsion formulations which dramatically increase the stability and efficiency of these compounds [36]. Nanoemulsions are promising compounds for overcoming the low solubility of plant extracts and to protect them from interactions with other compounds [37]. Nanoemulsions can also be used to design and manufacture lipid transport systems, and due to their smaller sizes than conventional emulsions, have a larger surface area delivering a more effective transfer process [37]. one of the advantages of using nanoemulsions is that they can obviate various problems encountered during the formulation of water-insoluble drugs, which increases drug availability in an aqueous environment and significantly improves their pharmacokinetics and pharmacodynamics [38]. *N. Sativa* L. is an important plant that has shown cytotoxicity against different types of tumor cells [39, 40]. In the present study, the anti-oxidative and cytotoxicity effects of the nanoemulsions

synthesized by *N. Sativa* L. tincture were investigated. For synthesize the nanoemulsions, we used Tween 80, Tween 20 and polyethylene glycol as surfactants. Also, in another study employing the essential oils of cumin, eucalyptus, and cinnamon to synthesize nanoemulsions, Tween 80 was used as a surfactant [41]. Oxidative stress is involved in a variety of human diseases such as atherosclerosis, diabetes, hypertension, inflammation, cancer, and AIDS [42]. Various studies have suggested an association between oxidative stress and the incidence of various cancers and carcinogenesis during which the level of reactive oxygen species (ROS) increases in oppose to antioxidants which decrease in cancer cells [43]. Elevated ROS in these cells which may occur intrinsically or under the influence of external factors can induce gene mutations and change transcriptional processes and signaling pathways ultimately leading to carcinogenesis [44]. An understanding of the oxidative status of tumor cells is essential in cancer therapy [45], and various studies have been conducted to investigate the effects of different nanoemulsions on the antioxidant capacity of cells [46]. We here evaluated the anti-oxidative effects of the synthesized nanoemulsions at the concentrations of 6.25, 12.5, 25, and 50 µg/mL using the DPPH radical scavenging assay. The results showed that this nanoemulsion had a good potential for inhibiting DPPH radicals with the IC50 of 47 µg/mL. In the other study showed that the clove essential oil nanoemulsion was able to inhibit free DPPH radicals with an IC50 of 32.55 µg/mL [47]. Previous studies reported nigellidine and nigellicine, and quinones such as volatile thymoquinone were the main chemical composition of *N. Sativa* L. which may be responsible for the antioxidant activities [48]. Today, cancer is one of the leading causes of death worldwide [49]. Ovarian cancer is one of the most common cancers among women [50]. Various studies have shown that nanotechnology provides a good alternative for cancer treatment [51]. On the other hand, natural compounds with cytotoxicity against cancer cells have also been considered as anti-tumor agents [52]. *N. Sativa* L. is one of the important natural compounds with cytotoxicity against different types of tumor cells. The results of MTT assay showed significant and dose-dependent cytotoxic effects of the nanoemulsion against A2780 ovarian cancer cell line, but not normal HUVEC cells. It was shown that *N. Sativa* L. nanoemulsion decreased the proliferation of A2780 in a dose- and time-dependent manner and delivered the IC50 values of about 0.72 µg/mL for the A2780 and >25 µg/mL for the HUVEC cells at 48 hours. Various studies have examined the anticancer effects of *N. Sativa* L. extract in human and animal models of leukemia, as well as tumors of breast, intestine, liver, lung, skin, kidney, and prostate [53-55]. In one study, the cytotoxic effects of N. Sativa L. extract were evaluated in 4T1 and CT26 cell lines, which significantly reduced the growth of these cell lines and delivered the respective IC50 values of 1000 µg/mL and 800 µg/mL after 48 hours [56]. Accordingly, the IC50 concentration of the nanoemulsion synthesized from *N. Sativa* L. tincture showed the higher toxicity of the nanoemulsion in comparison with black seed extract against cancer cells. In another study, the cytotoxicity of the aqueous extract of Trigonella foenum-graecum was investigated against SKVO3 ovarian cancer cell line. Assessing cellular viability by MTT assay, they showed that this aqueous extract had anti-cancer activities and decreased the growth of cancer cells with an IC50 of 5.99 µg/mL [57]. In comparison with the results of this study, the nanoemulsions synthesized by *N. Sativa* L. tincture had higher cytotoxicity against cancer cells than lemon alcoholic extract and Trigonella foenum-graecum aqueous extract. Various studies have shown that dill comprises different compounds such as thymoquinone and dithymoquinone, to which the antioxidant properties and cytotoxicity effects of this plant are attributed. In the investigation of *N. Sativa* L. essential oil components with GC-Mass analysis, the main substances included thymoquinone following by p-cymene, carvacrol, and longifolene, respectively [58].

More ever, to take more evidence about morphological alteration of cells which treated by nanoemulsion, AO/PI staining was performed. By this staining, normal and apoptotic cells obtain a different colors which could be seen under a fluorescence microscope [32]. The nanoemulsion synthesized using *Nigella sative* L. tincture obviously increased apoptosis the treated A2780 cells. Based on these findings, the synthesized nanoemulsion can be used as a potential drug for inhibiting the growth of ovarian cancer cells.

CONCLUSION

Recently, new nanotechnology-based treatment

strategies have emerged as potential alternatives to chemotherapy. Among various types of nano-products, nanoemulsions have several benefits to be used as anticancer drugs. In this study, the nanoemulsion synthesized by *N. Sativa* L. tincture showed potent antioxidant and cytotoxic activities against A2780 ovarian cancer cell line, indicating its potential as a therapeutic agent for this cancer. It is suggested to evaluate the toxicity of this compound toward other cancer cell lines.

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