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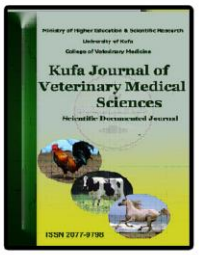

The cytotoxic effect of olive oil, nigella sativa and their combination on Hela cancer cell line (*in vitro*)

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Abstract

This study was done to comparison among the cytotoxicity of olive oil, nigella sativa oil and the mixture of olive oil and nigella sativa oil on Hela cancer cell line, the results reveled to the maximum cytotoxic concentration of each olive oil and nigella sativa oil on Hela cancer cell line started from (1 µg/ml – 10000 µg/ml) at 72 hr. incubation periods, which reflect the pattern of cytotoxicity was time depending.

While the result the mixture cytotoxicity shows an increasing in cytotoxicity occurred with increasing in the incubation periods especially at 72hr. at concentration equal to 10000 µg/ml , which give indication about the mixture cytotoxicity was time and concentration depends ,

The cytotoxicity of the mixture was less when comparing with the cytotoxicity of olive oil and nigella sativa oil reflection an antagonism may occurred between the active ingredients of each olive oil and nigella sativa oil causing decreasing in the cytotoxicity of the mixture toward Hela cancer cell line .

key words: black seed ,cervical cancer, thimoquinon, phenols, caspase.

التأثير السمي الخلوي لزيت الزيتون او زيت الحبة السوداء او مزيجهما على خط خلايا سرطان عنق الرحم البشري (هلا) في الزجاج

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الخلاصة:

أجريت الدراسة بهدف مقارنة التأثير السمي الخلوي لخلايا سرطان عنق الرحم البشري (هلا) لزيت الزيتون أو زيت الحبة السوداء أو مزيجهما في الزجاج بتركيز (1 مكغم / مل – 10000 مكغم/ مل).

أظهرت النتائج إن التأثير السمي الخلوي الأقصى لكل من زيت الزيتون أو زيت الحبة السوداء على خط سرطان عنق الرحم البشري (هلا) يبدأ من 1 مكغم / مل الى 10000 مكغم /مل عند 72 ساعة من زمن التعرض والذي يعكس نمط السمية الخلوية بالإعتماد على وقت التعرض.

أما نتائج المزيج فقد اظهر بأن التأثير السمي الخلوي يزداد عند زيادة فترة التعرض للزيتين وخصوصآ عند 72 ساعة وبتركيز 10000 مكغم / مل والذي يدل على ان السمية الخلوية للمزيج تعتمد على عاملي التركيز والزمن الخاص للتعرض في نفس الوقت.

إن التأثير السام للخلايا في حالة المزيج كان أقل مما هو عليه من التعرض الى زيت الزيتون أو زيت الحبة السوداء كل على حده وهذا ممكن ان يعكس حالة التضاد بين المواد الفعالة الموجودة في زيت الزيتون مع زيت الحبة السوداء والتي أدت الى تقليل التأثير السمي الخلوي على خط سرطان عنق الرحم (هلا) .

Introduction:

Cancer is defined as an abnormal mass of tissue which its growth is uncoordinated, persists in excessive manner after the cessation of the stimuli which evoked the change , In 2007, cancer was the second leading cause of death in economically developed countries (following heart diseases) and the third leading cause of death in developing countries (following heart diseases and diarrheal diseases) (1).

Cervical cancer is the second leading cause of cancer deaths in women worldwide and is the most prevalent female malignancy in many developing countries (2). The human papilloma virus (HPV) has been consistently identified as the major causal factor of this disease (3). In addition, other specific genetic abnormalities may also play an important role in carcinogenesis and the aggressiveness of cervical tumors.

The natural products, including plants (vegetables, herbs and spices) used in folk and traditional medicine, have been found to be a potential source of novel anticancer drugs over the decades and have much contributed to cancer chemotherapy (4,5). Among natural products, the oil from *N. sativa* have attracted the interest of medical scientists. *N. sativa* is an annual herbaceous plant with black seeds, commonly known as black seeds, belongs to Ranunculaceae family and grows in countries bordering the Mediter-ranean Sea, Pakistan and India (6). It has been used for many centuries as a food flavor and natural remedy to promote health and to treat a broad array of diseases in many countries in the Middle East, South Asia and the Far East (7).

Black seed is one of the most extensively studied plants both phytochemically and pharmacologically; numerous studies have shown that the seeds and oil of this plant are characterized by a very low degree of toxicity (6). Many studies have been reported for its antimicrobial, anti-hyperlipidaemic, anti-hyperglycaemic, diuretic and anti-oxidant effects (8) as well as anti-neoplastic activities in different types of cancer (9). The biological activities of *Nigella sativa* seed are related to the main active components, thymoquinone, or TQ in short, a crystalline substance that has been isolated from the essential oil and is considered the major component of the essential oil (6,10). TQ has been considered as a potent, anti-carcinogenic, antioxidant and anti-mutagenic agent (11,12,13). TQ has been shown to exert anti-neoplastic effects both *in vitro* and *in vivo* (7,13).

The growth Inhibitory effects of TQ is specific to cancer cells leading to improvements in therapeutic index while it is less toxic to and prevents non-tumor normal cells from sustaining chemotherapy-induced damaged .

An ethanol extract from *Nigella sativa* has been found to inhibit proliferation

and induces apoptosis in the human cervical cancer HeLa cell lines , However, despite knowledge of these potential anti-neoplastic effects, the mechanism by which TQ in inducing apoptosis on two different human cervical cell lines differing in HPV and p53 status (14).

Polyunsaturated fatty acids (PUFA) from n-6 family display a strong promoting effect, this may be partially due to the especially prone to lipid peroxidation of PUFA that leads to formation of aldehydes, which react with DNA bases, forming genotoxic exocyclic etheno(epsilon)-adducts. On the contrary, there are growing evidences that monounsaturated oils, like olive oil, may be associated with a decreased risk of some cancers. However, the epidemiological data do not fully agree with the experimental ones previously published. Minor compounds from (extra virgin) olive oil, mainly phenolics like hydroxytyrosol and tocopherol, are antioxidants and radical scavenging. They can minimize the amount of reactive oxygen species (ROS) generated by fatty acid peroxidation and in the case of monounsaturated fatty acids (MUFA) the DNA damage can be reduced by a lower lipid peroxidation (15)

Material & methods:

The olive oil and *Nigella sativa* oil were extracted mechanically from original sources **,** where Human cervical cancer Hela cell line was purchased from tissue culture unit/ Iraqi Centre for Cancer and Medical Genetics Research (ICCMGR), The cells were cultured in 75 cm2 tissue culture flasks under humidified 5% CO2 atmosphere at 37°C in RPM**I**-1640 medium (Sigma chemicals, England) with 10% fetal bovine serum (FBS), and penicillin- streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) during the course of the experiment (16).

Cytotoxicity Assay

Cells cultures in microtiter plate (96wells) were exposed to range of (olive oil , nigella sativa , and mixture of equal amounts of olive oil and nigella sativa oil) at a concentration of (1,10,100,1000 and 10000) µg/ml during the log phase of growth and the effect determined after several incubation time. (16) , every well contain $1X10^4$cells/well, Serum calf medium 10% used for seeding, Plates were then incubated for 24hrs in 37°C for achieve cell attachment, then By using maintenance medium, fivefold serial dilution were prepared starting from (0. 1-1000 µg/ml) for each olive oil , nigella sativa oil and for the mixture of olive oil and nigella sativa oil)

After incubation for 24 hrs, cells were exposed (Six replicate at 200µl for each tested concentration), 200 µl of maintenance medium added to each well of control group, the times of exposure were 24, 48 and 72 hrs. The plates were sealed with self adhesive film then returned to incubator, cells where staining with MTT stain.

The optical density of each well was read by using a micro-ELISA reader at a transmitting wavelength on 550 nm (16).

The inhibitor rate measuring according to (17) as follows:

$$IR\% = \frac{A - B}{A} \times 100$$

IR=inhibitor rate, A= the optical density of control, B= the optical density of test.

Statistical Analysis

Data were analyzed Statistically by using SAS (2012) on the basis of two-way analysis of variance (ANOVA)at level (P<0.05),least significant differences (LSD) test was used to significant compare between means in this study (18).

Results:

1- The cytotoxicity of olive oil on Hela cancer cell lines:

The result revealed to the maximum cytotoxic effect occur at (10, 100, 1000, and 10000) µg/ ml at72hr. Incubation periods with a significance variation comparing with control at level (p<0.05), without a significant variation between the cytotoxic effect of each (1.10, 100, 1000 and 10000) µg/ ml at 72hr Incubation periods, and a significant variation between incubation period 72hr. as compared with 24 and 48 hr, for all the concentration that included (1.10, 100, 1000 and 10000) µg/ ml , with a maximum cytotoxic effect occurred at concentration 10000 µg/ ml in incubation period 72 hr. These results are shown in table (1).

Table (1) Effect of concentration and time in growth inhibition rate for olive oil on Hela cancer cell line

Con.	24 hr. Mean ± SE	48hr. Mean ± SE	72hr. Mean ± SE
0 µg	0±00 C a	0±00 B a	0±00 B a
1 µg	25±0.72 B b	30±0.36 A b	86±0.55 A a
10 µg	28±1.69 B b	32±0.04 A b	90±0.64 A a
100 µg	35±2.06 B b	32±0.51 A b	90±0.26 A a
1000 µg	58±0.15 A b	38±2.12 A c	91±0.04 A a
10000 µg	59±0.84 A b	41±0.70 A c	92±0.87 A a

Different capital letter represents significant differences (P<0.05) between means of the same column; Different small letters represent significant differences (P<0.05) between means of the same row.

2- The cytotoxicity of nigella sativa oil on Hela cancer cell lines:

The result revealed the maximum cytotoxic effect occur at (1. 10, 100, 1000, 10000) µg/ ml at 72 hr. incubation period with a significance variation comparing with control at level (p<0.05), with a significance variation. Between (24 and 48) hr as compared with 72 hr incubation period for (1. 10, 100, 1000, 10000) µg/ ml , with a maximum cytotoxic effect occurred at 10000 µg/ ml at 72 hr. incubation periods These results were shown in table (2).

Table (2) Effect of concentration and time in growth inhibition rate for nigella sativa oil on Hela cancer cell line

Con.	24 hr. Mean ± SE	48hr. Mean ± SE	72hr. Mean ± SE
0 µg/ml	0±0.00 B a	0±0.00 C a	0±0.00 B a
1 µg/ml	31±0.82 A b	18±1.14 B c	87±0.67 A a
10 µg/ml	35±0.52 A b	29±1.88 A b	89±1.14 A a
100 µg/ml	36±1.88 A b	29±2.94 A b	89±1.58 A a
1000 µg/ml	38±1.67 A b	30±1.48 A b	90±0.74 A a
10000 µg/ml	40±1.14 A b	31±2.58 A b	91±1.90 A a

Different capital letter represents significant differences (P<0.05) between means of the same column; Different small letters represent significant differences (P<0.05) between means of the same row.

Table (3) Effect of concentration and time in growth inhibition rate for A mixture of olive oil and nigella sativa oil on Hela cancer cell line

Con.	24 hr. Mean ± SE	48hr. Mean ± SE	72hr. Mean ± SE
0 µg/ml	0±0.00 C a	0±0.00 C a	0±0.00 D a
1 µg/ml	10±0.35 B a	0±0.00 C b	5±0.36 CD ab
10 µg/ml	14±0.33 AB a	0±0.00 C b	10±0.05 C a
100 µg/ml	15±0.38 AB a	10±0.26 B a	11±0.22 C a
1000 µg/ml	15±0.05 AB b	10±0.22 B b	27±1.09 B a
10000 µg/ml	18±1.06 A c	28±0.39 A b	41±1.74 A a

Different capital letter represents significant differences (P<0.05) between means of the same column; Different small letters represent significant differences (P<0.05) between means of the same row.

3- The cytotoxicity of a mixture of nigella sativa oil and olive oil on Hela cancer cell lines:

The result revealed the maximum cytotoxic effect occur at (10000) µg/ ml at 72 hr. incubation period with a significance variation comparing with control at level ($p<0.05$), without a significance variation between each (1, 10, 100) µg/ ml for (24 , and 72) hr incubation period , These results are shown in table (3).

These results revealed to their was an antagonism occurred between the nigella sativa oil and olive oil, when was compared between the cytotoxicity of nigella sativa and olive oil with the mixture cytotoxicity showed the cytotoxicity of the mixture was less than the cytotoxicity of each nigella sativa and olive oil for each 24 , 48 , and 72 hr. incubation period table (4,5,6) .

Table (4) comparison among the cytotoxicity of olive oil , nigella sativa and the mixture on Hela cancer cell line in 24 hr. incubation period

Con.	Olive oil Mean ± SE	Nigella sativa Mean ± SE	Mixture Mean ± SE
Control	0±00 a	0±0.00 A	0±0.00 a
1 µg	25±0.72 b	31±0.82 A	10±0.35 c
10 µg	28±1.69 b	35±0.52 A	14±0.33 c
100 µg	35±2.06 a	36±1.88 A	15±0.38 b
1000 µg	58±0.15 a	38±1.67 B	15±0.05 c
10000 µg	59±0.84 a	40±1.14 B	18±1.06 c

Different small letter represents significant differences (P<0.05) between means of the same column.

Table (5) comparison among the cytotoxicity of olive oil , nigella sativa and the mixture on Hela cancer cell line in 48 hr. incubation period

Con.	Olive oil Mean ± SE	Nigella sativa Mean ± SE	Mixture Mean ± SE
control	0±00 a	0±0.00 a	0±0.00 A
1 µg	30±0.36 a	18±1.14 b	0±0.00 C
10 µg	32±0.04 a	29±1.88 a	0±0.00 B
100 µg	32±0.51 a	29±2.94 a	10±0.26 B
1000 µg	38±2.12 a	30±1.48 b	10±0.22 C
10000 µg	41±0.70 a	31±2.58 b	28±0.39 B

different small letter represents significant differences (P<0.05) between means of the same column.

Table (6) comparison among the cytotoxicity of olive oil , nigella sativa and the mixture on Hela cancer cell line in 72 hr. incubation period

Con.	Olive oil Mean ± SE	Nigella sativa Mean ± SE	Mixture Mean ± SE
Control	0±00 A	0±0.00 a	0±0.00 a
1 µg	86±0.55 A	87±0.67 a	5±0.36 b
10 µg	90±0.64 A	89±1.14 a	10±0.05 b
100 µg	90±0.26 A	89±1.58 a	11±0.22 b
1000 µg	91±0.04 A	90±0.74 a	27±1.09 b
10000 µg	92±0.87 A	91±1.90 a	41±1.74 b

Different small letter represents significant differences (P<0.05) between means of the same column.

Discussion:

Several studies were done for evaluating the anticancer activity of olive oil as anticancer, where (19), demonstrated that it has a great tumor growth inhibition effect by inhibiting the growth of colon tumors that implanted in mice subcutaneously , Starting in third week of treatment. Tumor volume in the treated group was approximately reduced by 50% as compared with control group, while, (20), find that the ethanolic olive cake extract exerted an *in vitro* cytotoxic activity in a dose-dependent manner, and the IC50 values were ranging from 20 to 40 µg/mL. Interestingly, compared to the conventional antitumor drug methotrexate, where the cytotoxic effect on normal human peripheral blood mononuclear cells were not observed , in addition to that extracts showed an apoptotic effect against P815 tumor cell line depending on the dose and the extract's phenolic content.

The anticancer effect of olive oil may related to its contains a several active compounds as oleic acid which consider as the major component of olive oil, where oleic acid (omega-9) is a mono-unsaturated fatty acid found in olive oil that contains about 55-80% of the acid, as glyceride1, in quantity greater than any other fatty acid . Oleic acid blocks the action of HER-2/neu, a cancer-causing oncogene which found in about 30% of breast cancer patients oleic acid suppressed the action of the oncogene and also synergistically improved the effectiveness of the breast cancer drug, herceptin, a targeted therapy made by Swiss drug maker Roche Holding that works against the HER-2/neu gene (21).

Sterols contains of olive oil have also a potential anticancer activity , where phytosterols considered as a anticancer dietary components (22). Studies in human breast cancer cells showed that b-sitosterol inhibits tumour cell invasion and cell growth by 70% compared with controls and induces cell cycle arrest at the G2/M phase (23). The mechanistic regulation of growth was through MAPK pathway , Moreover, b-sitosterol induces apoptosis by the activation of caspases 8, 9 and 3 (24). b-Sitosterol may offer protection from breast cancer metastasis by inhibiting cell invasion of the basement membrane (laminin and collagen IV), which may be mediated by its ability to limit the adhesiveness of the tumour cells.

Tocopherols also that founded in olive oil showed a Potential anticancer activity, where investigated about the effects of α-tocopherol on the growth of human prostate and colon cancer cells showed that, although all of them inhibit the cell growth, was a-tocopherol which strongest induced a significant growth inhibition (86%) in androgen-independent prostate cancer cell line DU-145 (50% by a-tocopherol , These observations were basically due to a decrease in the levels of cyclin D1 and E, and an increase in the levels of three cdk inhibitors (CKI): p21, p27 and p16 ,

Squalene also has a Potential anticancer activity where Olive oil contains 0.2-0.7% of squalene. , Squalene may have chemopreventive activity against colon carcinogenesis (25,26). Its mechanism of action lies on the strong inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, which reduces the availability of farnesyl pyrophosphate for the phenylation of the ras oncogene. Experiments in animals show that consumption of squalene significantly inhibits the formation of colonic crypt foci (ACF) by 46% (26) and lung hyperplasia by 70% (27). Normal human bone marrow (BM) derived colony-forming unit (CFU) growth can be increased by squalene in a dose-dependent manner . Squalene treatment can significantly protect the CFUs from cisplatin-induced toxicity; the protective effect was equivalent to reduced glutathione (GSH), development of safe and non-toxic cytoprotective agents for the adequate management of cancer chemotherapy. A phase I trial demonstrated that oral squalene is safe and tolerable (28). There is several result concluded a symmetrical result s where (14) find that EENS significantly inhibited proliferation and colony formation and induced apoptosis in HeLa cells. It exerted its apoptotic potentiality through increasing the Bax: Bcl-2 protein ratio leading to release of cyt *c* into cytosol and activation of downstream caspases (3, 9 and 8). In addition, it modulated expression levels of the cell cycle-

related proteins including c-Myc, hTER, cyclin D1, CDK-4, p53 and p21. These findings suggest that EENS could potentially be a new therapeutic option in anticancer treatment for cervical cancer.

The most pharmacologically important components within the n.sativa oil structure are thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ), and thymol (THY), Much of the biological activities of the seeds have been shown to be due to thymoquinone, which is consider as the major component of the n. sativa essential oil , TQ thymoquinone is considered as potent anti-oxidant (29), anti- carcinogenic and anti-mutagenic agent (30,31) . TQ is a relatively safe compound, particularly when given orally to experimental animals (32). Alpha (α)-hederin, a pentacyclic triterpene saponin isolated from the seeds of *N. sativa*, was also reported to have potent *in vivo* antitumor activity (33).

The results of combination reveled to either an antagonism occurred between the activity of each olive oil and nigella sativa oil or chemical reaction occurred in the mixture between the active ingredients of each olive oil and nigella sativa oil that responsible for anticancer activity , or the antagonism may occurred due to the anticancer mode of action of each nigella sativa and olive oil, where together have the same target through induced apoptosis by activation of caspase (3.8 and 9) , where olive oil phytosterol (b-sitosterol) have ability to inhibits tumor cell invasion and cell growth by 70% compared with controls and induces cell cycle arrest at the G2/M phase (23). The mechanistic regulation of growth was through MAPK pathway, and induces apoptosis by the activation of caspases 8, 9 and 3 (24), on the other side nigella sativa oil significantly inhibited proliferation and colony formation and induced apoptosis in HeLa cells. It exerted its apoptotic potentiality through increasing the Bax: Bcl-2 protein ratio leading to release of cyt *c* into cytosol and activation of downstream caspases (3, 9 and 8) , theses symmetry in the mode of action may lead to impairments the activity olive oil or nigella sativa oil by interaction between there mode of action especially through inducing of apoptosis .

Interaction also can be occurred by a chemical interaction occurred between the chemical constituents that responsible for anticancer activity in each nigella sativa and olive oil which may lead to minimized the action of the chemical compound that responsible for the anticancer captivity in each nigella sativa and olive oil.

In Conclusions:Nigella sativa and olive oil were a type of medical plants that have a promising role in cancer treatment future, where nigella sativa contains several types of phytochemicals that have a promising effect as anticancer, the most important type was thymoquine , which have ability to induce apoptosis through activation of caspase (3,8 and 9)in different types of cancer cell lines *in vitro* as in acute lymphocytic leukemia and human cervical carcinoma cancer cell lines .

While olive oil contains also several compounds that related to it anticancer ability as oleic oil and phytosterol which also have ability to fight cancer cells by apoptosis induce through activation of caspase (3, 8 and 9).

The result of the present study also conclude a mixture between the two oil causing minimizing their cytotoxicity ,

that may related to occurrence of antagonism between the two oils that may related to the competition between them on the same target that related to it the anticancer activity as activation of caspase (3, 8 and 9) where the mechanism of anticancer activity for each oils , as a final conclusion of the study the mixture of two oils became less effective as anticancer activity comparing with each oils activity alone .

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