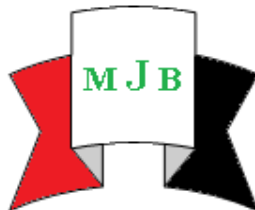


Effects of Black Seed on Lung Cancer/*in Vitro* Study

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Received 31 October 2013

Accepted 25 January 2015

Abstract

This study was done to evaluate the possible cytotoxic effects of black seed on lung cancer cells and to determine its IC₅₀ alone and in combination with cisplatin, and to study its effects on the expression of each of p53 and EGFR. QU-DB lung cancer cells were cultured in Eagle's MEM medium with 5% FBS and antibiotics. The cells were seeded in 96 well plate and the cytotoxic effects of each of cisplatin [25-0.195 µl/ml (or µg/ml)] and black seed [300-1.1719 µl/ml (each one µl is extracted from 25 µg of dried seed)] was determined using neutral red uptake (NRU) assay for 24, 48, and 72 hours in comparison with their corresponding control groups. Combined effect of black seeds with cisplatin was determined also using NRU assay. Cytotoxicity was further assessed by trypan blue exclusion assay at IC₅₀ of each agent for 48 hours duration. Immunocytochemistry assay was performed also to detect p53 and EGFR expression. Cisplatin induced a directly proportional, dose-dependent and time-dependant cytotoxic effect with an IC₅₀ of 8.5 µg/ml and 7.3 µg/ml after 48 hrs and 72 hrs of exposure respectively. Significant differences (p<0.05) were observed in optic density of cisplatin group from that of the control for all tested concentrations. Black seed extract induced a directly proportional, dose-dependent and time-dependant cytotoxic effect in experiments with 48 hrs and 72 hrs of exposure with an IC₅₀ of 149.5 µl/ml and 130 µl/ml respectively (each one µl is extracted from 25 µg of dried seed). While it produces a protective effect in 24 hrs exposure experiment. Significant differences (p<0.05) were observed in optic density of black seed from that of the control at concentrations of 75 µl/ml and above. Black seed produce an antagonistic action when combined with cisplatin, combination index (CI) >1.3. Cisplatin highly significantly (p<0.005) increased EGFR expression at different concentrations. While black seed extract highly significantly (p<0.005) reduced EGFR expression at 300 µl/ml (each one µl is extracted from 25 µg of dried seed). Cisplatin and black seed highly significantly (p<0.005) decreased the expression of P53. In conclusion: monotherapy of black seed have anticancer effects on lung cancer cell line, but an antagonizing effect to cisplatin when combined with it. Black seed may have a beneficial therapeutic effect in decreasing EGFR expression and decreasing mutant p53 expression.

Keywords: Cell culture, cell line, cisplatin, black seed, lung cancer, p53, EGFR, cytotoxicity, neutral red, immunohistochemistry.

تأثيرات الحبة السوداء على سرطان الرئة /دراسة في الزجاج

الخلاصة

الهدف من اجراء هذه الدراسة هو لتقييم التأثيرات السمية الخلوية المحتملة للحبة السوداء على خط خلوي لسرطان الرئة ولتقرير الجرعة السمية الخلوية للنصف في الزجاج لها على حدة وبالتمازج مع عقار السيسبلاتين. وكذلك لدراسة تأثيراتها على تعبير p53 و EGFR. زرعت خلايا سرطان رئة نوع QU-DB في مستنبت من نوع Eagle's MEM المكمل بمصل العجل الرضيع بنسبة ٥% وبالمضادات الحيوية. زرعت الخلايا في صفيحة زرع ذات ٩٦ حفرة وحددت التأثيرات السمية الخلوية لكل من السيسبلاتين بتركيز [٢٥-١٩٥، مايكرو لتر لكل مليلتر] (او مايكروغرام لكل مليلتر) والحبة السوداء [٣٠٠-١١٧١٩ مايكرو لتر لكل مليلتر (تم استخلاص كل مايكرو لتر من ٢٥ مايكروغرام من البذور الجافة)] باستخدام مقايصة قبط الأحمر المحايد (NRU) ل٢٤، ٤٨، و ٧٢ ساعة بالمقارنة بمجاميعهما القياسية المطابقة. كذلك حددت التأثيرات المشتركة للحبة السوداء مع السيسبلاتين باستخدام تمثيل مقايصة صبغة القبط الأحمر المتعادل (NRU). اجريت ايضا تجربة الكيمياء السيتولوجية المناعية لاكتشاف تعبير p53 و EGFR. احدث السيسبلاتين تأثيرات سمية خلوية نسبية مباشرة ومعتمدة على الجرعة والوقت وكانت الجرعة السمية الخلوية للنصف في الزجاج (IC₅₀) تساوي ٨،٥ مايكروغرام لكل مليلتر و ٧،٣ مايكروغرام لكل مليلتر بعد التعريض لمدة ٤٨ و ٧٢ ساعة

على التوالي. لوحظت إختلافات معنوية ($p < 0.05$) في الكثافة البصرية لمجموعة السيسبلاتين من تلك المماثلة لمجموعة السيطرة لكل التراكيز المُجرّبة. أحدث مستخلص الحبة السوداء تأثيرات سمية خلوية نسبية مباشرة ومعتمدة على الجرعة والوقت بعد التعريض لمدة ٤٨ و ٧٢ ساعة وكانت الجرعة السمية الخلوية للنصف في الزجاج (IC_{50}) تساوي ١٤٩,٥ مايكرو لتر لكل مليلتر و ١٣٠ مايكرو لتر لكل مليلتر على التوالي بينما أحدث المستخلص تأثيراً وقائياً بعد التعريض لمدة ٢٤ ساعة. لوحظت إختلافات معتد بها ($p < 0.05$) في الكثافة البصرية لمجموعة الحبة السوداء من تلك المماثلة لمجموعة السيطرة للتراكيز ٧٥ مايكرو لتر لكل مليلتر فما فوق. لقد قلل مستخلص الحبة السوداء من تأثير السيسبلاتين عند الاستخدام المشترك معه حيث كان مؤشر الشراكة (CI) أكبر من (١,٣). أحدث السيسبلاتين زيادة معتد بها ($p < 0.005$) بتعبير EGFR لعدة تراكيز مختلفة، بينما أحدث مستخلص الحبة السوداء نقصاناً معتد بها ($p < 0.005$) بتعبير EGFR خصوصاً عند التراكيز العليا (٣٠٠ مايكرو لتر لكل مليلتر). أحدث كل من السيسبلاتين و مستخلص الحبة السوداء نقصاناً معتد به ($p < 0.005$) بتعبير البروتين الطافر p 53. أظهرت الحبة السوداء تأثيرات مضادة للسرطان على خط خلوي لسرطان الرئة، ولكنها أظهرت تأثيرات مناهضة للسيسبلاتين عندما استخدمت معه شراكة. إن تخفيض تعبير البروتين الطافر p 53 و EGFR بواسطة الحبة السوداء لزمياً له تأثير علاجي مفيد.

Introduction

Lung cancer is the leading cause (18%) of cancer death in both men and women world-wide, causing 1.4 million deaths per year [1, 2]. The overall 5-year survival rate for all stages combined is a disappointing (15%) [3, 2, 4]. Chemotherapy with cisplatin is associated with many adverse side effects, such as nephrotoxicity, ototoxicity, bone marrow suppression and neurotoxicity [5]. The dominant oncogens that are frequently involved in lung cancer include c-MYC, K-RAS, EGFR (epidermal growth factor receptor), and HER-2/neu. The commonly deleted or inactivated tumor suppressor genes include p53 (protein 53 or tumor protein 53), RB, p16INK4a, and multiple loci on chromosome 3p [6, 3, 4].

Black seed (*Nigella sativa* L.) is one of the most highly valued for its medicinal properties [7] and low toxicity [8]. It was said that the holy prophet Muhammad (*piece on him and on his aal*) said: "In black seed, the cure from all illnesses except 'Al-Sam'" and 'Al-Sam' means death [9]. This famous saying indicates that black seeds contain many remedies against a wide range of diseases. The magnitude of EGFR expression correlated with increased tumor chemoresistance and radioresistance in a variety of *in vivo* tumors, including murine carcinoma, squamous cell carcinoma, ovarian adenocarcinoma, hepatocarcinoma, and adenosquamous carcinoma [10, 11, 12]. A number of human cancers, including

colon and lung carcinomas, as well as osteosarcomas, are associated with either a missing or mutated p53 gene.

The Aim of the study was to evaluate the possible cytotoxic effects of black seed on QU-DB lung cancer cell line when used alone and when used in combination to cisplatin, and to study the effects of black seed on the expression of p53 and EGFR in these cells.

Materials and Methods

Human lung cancer cell line "QU-DB" was purchased from national cell bank of Iran (NCBI), Pasteur Institute of Iran in Tehran/ Iran, (NCBI Code: C565) [13]. It is a large cell carcinoma cell line. It was cultured in DMEM + 10% fetal bovine serum (FBS) at the NCBI and was adopted in this study in Eagle's MEM +5% FBS [14].

Preparation of plants' and drugs' stock solution for cytotoxic investigation

Seeds of black seed were obtained from traditional market and were identified by the resources division of botany directorate/Abugraib/Baghdad/ Iraq. Plant extract was done according to the procedure mentioned by [15]. Briefly, crude plant seeds (black seed) were pulverized, weighed (2.5 g), macerated/homogenized and extracted in 10 ml of absolute ethanol for 7 days at 4 °C. The whole solution was then centrifuged for 2 minutes at 5000 rpm. Each 1 ml of the supernatant was subsequently diluted to 10 ml with Hank's balanced salt solution (HBSS) + 5 mM

HEPES, pre-adjusted to a pH of 7.4 with 0.1 N NaOH. The resultant solution was filtered through 0.45 micron and then through 0.2 micron millipore filters. Cisplatin was used as a solution taken from the provided vial for intravenous injection in a concentration of 1mg/ml (which contains sodium=30 mmol/L). Sodium chloride (SC) solution (0.18% which contains sodium=30 mmol/L) was prepared from sodium chloride solution (0.9% which contains 150 mmol/L sodium). Ethanol(Eth) control stock solution was prepared by diluting 1 ml of absolute ethanol (99.9 %) to 10 mL with HBSS + 5 mM {(N-[2-hydroxyethylpiperazine-N'-[2-ethanesulfonic acid]) (HEPES)}, pre-adjusted to a pH of 7.4 with 0.1 N NaOH [15]. The final stock solutions of all the above mentioned agents were then kept in sterile dark glass containers and kept in refrigerator at 4 °C until use. Seven serial dilutions of each experimental agent were prepared from the stock solution in order to span about 250-fold concentration gradient with the highest final plating concentration set at 300 µl/ml (each one µl is extracted from 25 µg of dried seed) for black seed and 25 µl/ml (µg/ml) for cisplatin.

Cytotoxicity was assessed by neutral red uptake (NRU) assay which was carried out as previously described [16, 17, 18]. Briefly, after incubation of cells with serial concentrations of tested agents for desired time interval, the medium was removed and the cells were incubated with fresh medium containing 40 µg/ml neutral red dye for 3 h. The medium was removed and the plate was rapidly rinsed with a mixture of 1% CaCl₂ / 0.5% formaldehyde. The dye was extracted into supernatant with 0.2 ml of solution of 1% acetic acid/50% ethanol. After shaking on a microtiter plate shaker (for few minutes), the optic density (OD) of the extracted dye was measured at 540 nm with a microplate spectrophotometers. The average of the results of the replicates for each concentration was then obtained.

The cytotoxic effect of each tested agent was evaluated based on percentage of inhibition values calculated according to the following formula:

$$\text{Percentage of inhibition (\%)} = \frac{\text{OD control} - \text{OD tested agent}}{\text{OD control}} \times 100\%$$

OD control

Cytotoxicity of each tested agent is expressed as 50% inhibitory concentrations (IC₅₀) value. The IC₅₀ value is the concentration of tested agents that causes 50% inhibition or cell death, averaged from the above mentioned experiments, and was obtained by plotting the percentage of inhibition versus concentration of tested agents [19]. In addition, (by using NRU assay) the interaction between cisplatin and black seed was evaluated by the isobolographic analysis (a dose-oriented geometric method of assessing drug interactions) [20, 21].

Immunocytochemistry (ICC)

Detection of EGFR and p53 expression was done using immunocytochemical procedure [22]. Briefly, tissue-culture flasks with cells in exponential phase of growth (~85% confluent) were selected each time for ICC as follows: After exposure to serial concentrations of tested agents, the plate was incubated for 48 hours. After incubation, medium was removed and cells were stained according to manufacturer's protocol {Expose mouse specific AP (red) detection IHC kit, 2012} with the use of Carazzi's haematoxylin preparation [23] as a counter stain. Few drops of glycerol were added to cover cells in each well and prevent drying until the time of photographing. Five sites for each concentration (each well) were photographed in 2 powers (10X and 40 X). The color intensity of 5 cells per each site (for EGFR) or 10 cells (for p53) was measured using digimizer software [24] and the average of all cells was taken as the final result for that concentration of the tested agent for comparison with those obtained from control group.

Statistical Analysis

The data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was done for data of 48 hrs exposure (n=6) using unpaired student's t-test. Values with $p \leq 0.05$ were considered significant [25].

Results

Cytotoxicity assay and combination test:

Cisplatin and black seed showed directly proportional cytotoxic effects on lung cancer (QU-DB) cells with increased concentration of each agent:

1. Effects of cisplatin {0.1953-25 $\mu\text{l/ml}$ ($\mu\text{g/ml}$)} on QU-DB lung cancer cells:

Microscopic examination of all experiments of cisplatin (i.e., after 24 hrs, 48 hrs, and 72 hrs of exposure), showed an obvious decrease in number of viable cells and an obvious increase in number of unviable cells in comparison with the control (SC) (figure 1).

Using NRU assay: at the end of each experiment, the measured optic density of the extracted dye was decreased with increasing concentration of cisplatin in comparison with the control (SC) group. For the 24 hrs experiment and 72 hrs experiment, there was an obvious decrease in mean optic density (n=3) for all concentrations when being compared to the corresponding concentrations of the control (SC) group, while after 48 hrs of exposure, a significant decrease ($p < 0.05$) in mean optic density (n=6) was detected for all concentrations when being compared to the corresponding concentrations of the control (SC).

Plotting the values of percentage of growth inhibition against cisplatin concentration for 24, 48, and 72 hrs experiments (Figure -2) reveals that about 90% inhibition was achieved in all experiments at cisplatin concentration of 25 $\mu\text{l/ml}$ ($\mu\text{g/ml}$), with IC_{50} of 8.5 and 7.3 $\mu\text{l/ml}$ ($\mu\text{g/ml}$) after 48 hrs and 72 hrs of exposure respectively.

2. Effects of black seed (1.17188-300 $\mu\text{l/ml}$) on QU-DB lung cancer cells:

Microscopic examination showed that 24 hrs exposure to black seed did not markedly affect cell viability except at the maximum concentration of 300 $\mu\text{l/ml}$, while examination after 48 hrs and 72 hrs of exposure revealed a marked decrease in number of viable cells and an obvious increase in number of unviable cells in comparison with the control (Eth) group (figure 3).

Using NRU assay: at the end of the experiment of 24 hrs exposure, the mean optic density (n=3) of the extracted dye of black seed (BS) group was increased in comparison with that of the control (Eth) group except at 300 $\mu\text{l/ml}$ giving negative values for percentage of growth inhibition which indicate that more viable cells are present after exposure to black seed. On the other hand, the optic density was decreased with increasing concentration of black seed in comparison with the control (Eth) group in 48 hrs and 72 hrs experiments. In 48 hrs experiment, (n=6) a significant decrease ($p \leq 0.05$) was detected at concentration of 75 $\mu\text{l/ml}$ and above when being compared to the corresponding dilutions of the control. Plotting the values of percentage of growth inhibition against black seed concentration for 24, 48, and 72 hrs experiments (figure-4) reveals that percentage of growth inhibition was directly proportional to black seed concentration for 48 hrs and 72 hrs experiments, with IC_{50} of (149.5 $\mu\text{l/ml}$) and (130 $\mu\text{l/ml}$) respectively. On the other hand, black seed partially affects the growth of cells after 24 hrs exposure. Figure (5) showed decreased number of viable cells with increasing concentration of black seed after 72 hrs exposure {in comparison with the dropped value of percentage of growth inhibition at 300 $\mu\text{l/ml}$ in figure (4). When combining black seed with cisplatin at IC_{50} (8.5 $\mu\text{l/ml}$) (table -1), there was an obvious decrease in mean optic density (n=4 for each concentration) from that of the control (Eth + SC at 8.5 $\mu\text{l/ml}$) group. Isobolographic analysis of Interactions

between cisplatin and black seed for 70% toxicity showed antagonistic effects of black seed when it is combined with cisplatin after 48 hrs of exposure as shown in table (2).

Immunocytochemistry (ICC)

Microscopic examination of the stained QU-DB lung cancer cells revealed that EGFR expression was totally cytoplasmic, while that of p53 was primarily nuclear, for that reason the color intensity was measured from these areas accordingly. Cisplatin obviously increased EGFR expression after 48 hrs of exposure, and this increment was highly significant ($p < 0.005$) at concentrations of 6.25, 25 and 50 $\mu\text{l/ml}$ ($\mu\text{g/ml}$) (figure -6). But EGFR expression was decreased in concentration 12.5 $\mu\text{l/ml}$ ($\mu\text{g/ml}$) in comparison to the control (SC) group. On the other hand, black seed generally decrease EGFR expression and this decrement was highly significant ($p < 0.005$) at a concentration of 300 $\mu\text{l/ml}$ (figure-7). Figure (8) shows the microscopically noticeable changes mentioned above at the highest concentrations of each tested agent after 48 hrs of exposure. Cisplatin decreased p53 expression, and this decrement was insignificant at concentration 3.125 $\mu\text{l/ml}$, but it was highly significant ($p < 0.005$) at concentrations of 25 and 50 $\mu\text{l/ml}$ ($\mu\text{g/ml}$). On the other hand, cisplatin causes an increased p53 expression at lower concentrations and this increment was significant at concentration of 12.5 $\mu\text{l/ml}$ ($\mu\text{g/ml}$) (figure-9). Black seed decreased p53 expression in all concentrations after 48 hrs of exposure and this decrement was highly significant ($p < 0.005$) at concentrations of 75 $\mu\text{l/ml}$ (figure-10). Figure (11) shows the microscopically noticeable changes mentioned above at the highest concentrations of each tested agent.

Discussion

Attention is now being directed to find novel anticancer agents (with their possible mechanisms of action) alone and

in combination with conventional anticancer agents [26, 27]. Cell culture provide a good tool for testing novel agents, as the results obtained from different experiments are both accurately and reproducible. In the US NCI (National Cancer Institute) plant screening program for testing crude extract, the recommended incubation time is between 48 and 72 hours [28, 30]. For that reason, the schedule of this study was focused mainly on 48 hours; finding a novel anticancer agent effective on cancer cells in 48 hrs duration of exposure is more significant than finding another one effective in 72 hrs. Fotakis and Timbrell [31] reported that NRU and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) are the most sensitive cytotoxicity assay that show statistically significant difference between the treated cells and the controls, especially in detecting early toxicity. Black seed contain a wide range of active phytochemicals including thymoquinone (most abundant component of black seed volatile oil), carvacrol (another volatile oil) and α -hederin (a major saponin in the defatted seeds of black seed). These chemicals shown to have antineoplastic activity against different types of cancer cells *in vitro* [32, 33, 34, 35, 36] while it is minimally toxic to normal cells [37]. The crude extracts of black seed possibly contain a mixture of these constituents tested together on a lung cancer cell line (QU-BD) to study the combined effect of these agents extracted from the original plant. The significant decrease ($p < 0.05$) in mean optic density readings of all applied concentrations of cisplatin from those of the control in 48 hrs experiment and the obvious changes in viable cell estimate (figure -1) and growth inhibition (figure-2) signify directly proportional dose-dependent cytotoxic effect of cisplatin. In addition to similarity in pattern (directly proportional, dose-dependent), the lower readings of cisplatin in 24 hrs experiment and the higher readings in 72 hrs

experiment (figure-2) from those of the 48 hrs experiment indicate the time-dependant effects of the drug. These results coincide with the known cytotoxic effects of cisplatin on lung cancer cells [5, 38].

In this study, the significant decrease ($p < 0.05$) in mean optic density readings of concentrations 75 $\mu\text{l/ml}$ and above of black seed from those of the control in 48 hrs experiment and the apparent changes in viable cell estimate (figure-3) and growth inhibition (figure-4) signify directly proportional dose-dependent cytotoxic effect of black seed. Furthermore, the noticeable decrease in mean optic density readings of black seed for concentrations less than 75 $\mu\text{l/ml}$ from those of the control in 48 hrs experiment supports the above finding. Similarly, studies showed the antiproliferative effects of thymoquinone (extracted from black seed) against lung cancer [39]. Also, alpha-Hederin (an ethanolic extract obtained from black seed) was identified as an active antitumor compound [40]. On other hand, the negative values of growth inhibition in 24 hrs experiment (figure-4) point to the growth enhancing effect of black seed. This result may point to the different nutrients that are available in the black seed including proteins, thiamin, riboflavin, pyridoxine, niacin, folacin, iron, copper and zinc [41]. In addition to similarity in pattern (directly proportional, dose-dependent), the higher readings in 72 hrs experiment (figure-4) from those of the 48 hrs experiment indicate the time-dependant effects of black seed. The odd result obtained at a concentration of 300 $\mu\text{l/ml}$ of black seed for 72 hrs is unexplainable (figure-4). However, the increment in growth inhibition is obviously detected by microscopical examination as shown in figure-5). Alpha-Hederin was identified as the active antitumor compound from a fraction of an ethanolic extract obtained from black seeds. In another study, alpha-hederin *in vitro* was 10 times more potent than

cisplatin against 3LL Lewis lung carcinoma [40].

It is important to investigate the combined effects of novel agents with standard therapy (cisplatin in this study) to know the expected behavior of new agents when they are rationally introduced in combination with conventional therapy. As the value of the combination index for the above combination was more than 1.3, this value indicates antagonistic action of black seed to cisplatin (table 1). The combination of thymoquinone (TQ) and clinically used anticancer drugs has been shown to improve the drug's therapeutic index, prevents nontumor tissues from sustaining chemotherapy-induced damage, and enhances the antitumor activity of drugs such as cisplatin and ifosfamide [42]. Jafriet *al.*, [39] claimed that TQ down regulated NF- κ B expression which may explain its various cellular activities and this activity may prove useful in overcoming cisplatin resistance from over expression of NF- κ B. On the other hand, Foudaet *al.*, [43] showed that TQ ameliorates renal oxidative damage and proliferative response induced by mercuric chloride in rats.

EGFR is highly expressed in lung cancer, and plays an important role in tumor growth, infiltration and metastasis [44]. EGFR tyrosine kinase inhibitors are known to contribute considerably to the extension of progression-free survival in EGFR-mutant non-small cell lung cancer. Nevertheless, a significant percentage of lung cancer patients do not respond to anti-EGFR agents and secondary resistance after initial benefit is a challenging reality faced by clinicians [45]. The highly significant ($p < 0.005$) increase in EGFR expression noticed mainly at higher concentration of cisplatin (figure-6) may give a clue to two possibilities; firstly: this increase in expression may indicate the resistance of QU-DB lung cancer cells to chemotherapy by cisplatin. Golding *et al.*, [46] and Baiet *al.*, [47] showed that the

up-regulation of the wild-type EGFR or the expression of its mutants is associated with resistance of tumor cells to both chemo- and radiotherapy and poor clinical outcomes. Secondly: it may indicate the potent cytotoxic action of cisplatin which induces a compensatory increase in EGFR expression to overcome the acute insult on the cells by cisplatin (treatment-induced repair mechanisms) [48]. EGFR modulates DNA repair after radiation-induced damage through association with the catalytic subunit of DNA protein kinase (DNA-PKcs) [49].

In comparison to cisplatin, black seeds showed opposite results [highly significant ($p < 0.005$) decrease in EGFR expression noticed mainly at higher concentration] (figure-7). The opposite effect of black seed to that of cisplatin in regard to EGFR expression may explain its antagonistic effect to cisplatin obtained from combination test by NRU assay (table 1 and 2). Normally functioning (wild-type) p53 gene protects the body from cells that contain DNA damage and mutations. For that reason, p53 gene and its product p53 protein have been described as "the guardian of the genome" [6]. After chronic exposure to tobacco-related carcinogens, p53 gene mutation within the bronchial epithelium is relatively common, leading to impaired function of the p53 protein (50). Wild-type p53 protein has a very short half-life and thus it is present in only minute amounts, generally below the detection level of immunocytochemical methods. In contrast, mutant p53 proteins are much more stable than wild-type p53 proteins, and have a much longer half-life and tend to accumulate to a high level in tumor cells; therefore, if a p53 protein is detectable by immunocytochemistry, it is generally considered to be a mutant form [51, 52]. Loss of the p53 function may cause resistance to apoptosis that leads to treatment failure to DNA-damaging agents [53]. Thus in the present study, the highly significant ($p < 0.005$) decrease in p53 expression for cisplatin and black

seed (figures-9 and 10) may point to the beneficial effects of these agents in reducing the mutant p53 and (possibly) the recovery of the normal wild type p53. Both p53 dependent expressions of caspases 6 and 7 and p53-independent activation of caspases through Bax/Bak mediated release of cytochrome C contribute to cisplatin induced renal tubular epithelial cell death [54]. In addition to cisplatin's known mechanism of action as an alkylating agent, it also elevated the levels of wild-type p53 and P21 in a dose-dependent manner [55]. Thymoquinone (TQ) extracted from black seed triggers apoptotic cell death in human colorectal cancer cells via a p53-dependent mechanism [32]. Anand, *et al.*, [42] stated that TQ induces apoptosis by p53-dependent and p53-independent pathways in cancer cell lines. It also induces cell-cycle arrest. TQ also down-regulated the expression of NF- κ B-regulated antiapoptotic (IAP1, IAP2, XIAP Bcl-2, Bcl-xL, and survivin), proliferative (cyclin D1, cyclooxygenase-2, and c-Myc), and angiogenic (matrix metalloproteinase-9 and vascular endothelial growth factor) gene products [56].

Conclusions

Black seed has an *in vitro* cytotoxic effect against lung cancer with an IC_{50} of 149.5 μ l/ml and 130 μ l/ml for 48 hrs and 72 hrs of exposure respectively. While it produces a protective effect in 24 hrs exposure experiment. Black seed produce an antagonistic action when combined with cisplatin, combination index (CI) > 1.3 . The reduced EGFR expression after exposure to black seed may point to their possible beneficial effects in reducing the resistance to chemotherapy and radiotherapy. The decreased expression of mutant p53 by black seed signifies its beneficial effect in restoring normal p53 functions.

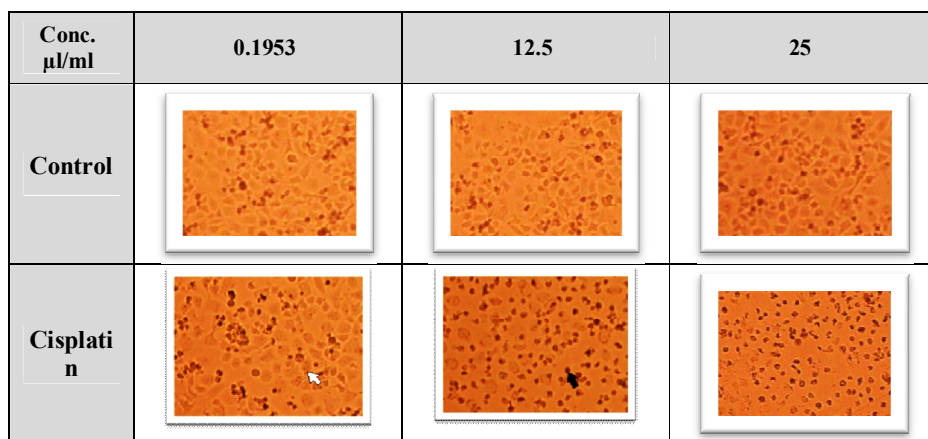


Figure (1): Effect of cisplatin on QU-DB* cells in comparison to control (SC) group 48 hrs after exposure to serial concentrations of cisplatin. White arrow (viable cell), black arrow (unviable cells)
* = large cell lung cancer cell line.

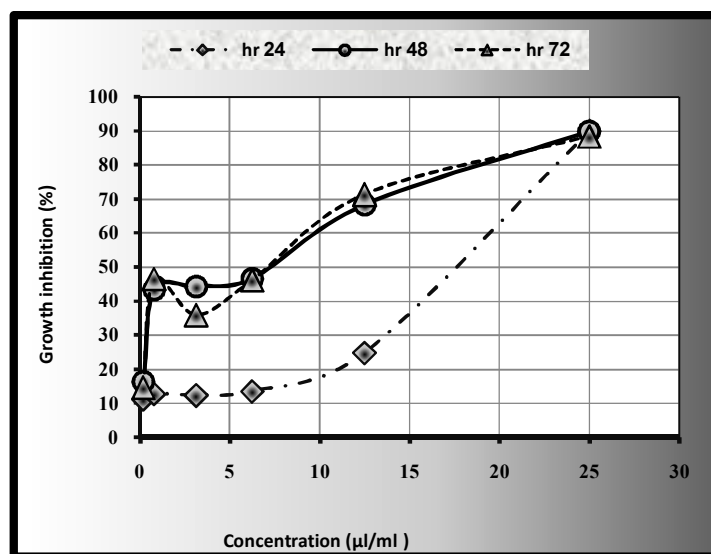


Figure (2): Effects of cisplatin on the growth of QU-DB* cells after 24, 48, and 72 hrs of exposure as evidenced by NRU¶ assay.

* = large cell lung cancer cell line.

¶ = Neutral red uptake

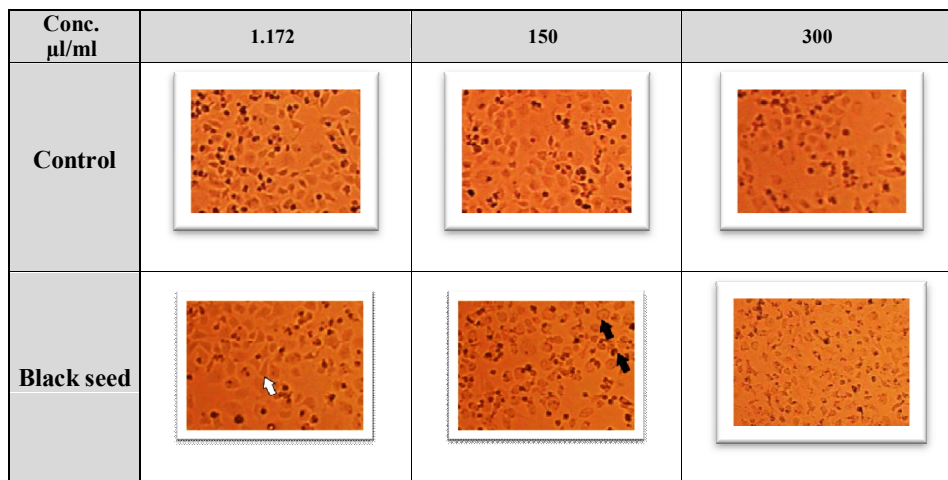


Figure (3): Effects of black seed on QU-DB* cells in comparison to the control (Eth) group 48 hrs after exposure to serial concentrations of black seed. White arrow (viable cell), black arrow (unviable cell)
* = large cell lung cancer cell line.

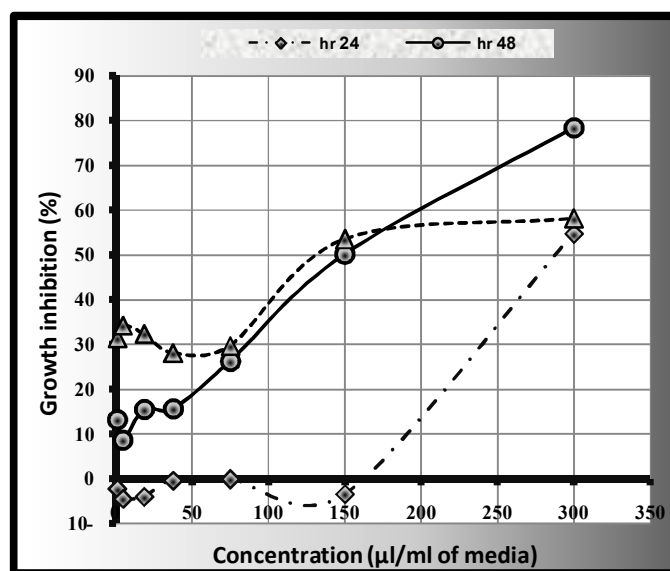


Figure (4): Effects of black seed on the growth of QU-DB* cells after 24, 48, and 72 hrs of exposure as evidenced by NRU¶ assay. (Negative value indicates growth enhancing effect)

* = large cell lung cancer cell line.

¶ = Neutral red uptake

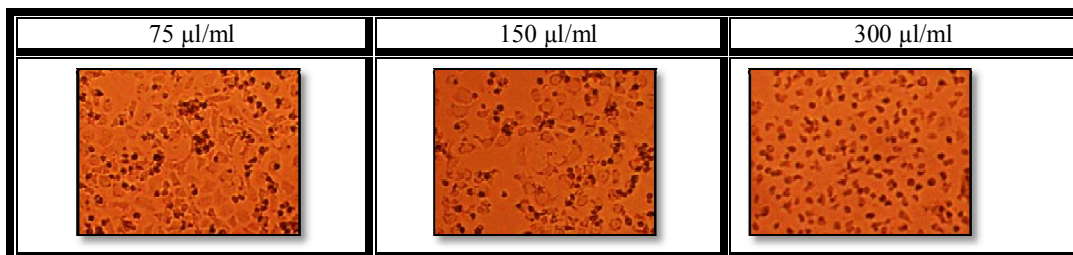


Figure (5): Effects of black seed on QU-DB* cells after 72 hrs of exposure to serial concentrations.

Table (1): Combined effects of black seed (BS) and cisplatin(CIS) at IC₅₀ of CIS (8.5 µl/ml) on the growth of QU-DB* cells in comparison to their controls (Eth and SC respectively) after 48 hrs as evidenced by NRU¶ assay.

Concentration of Eth and of BS (µl /ml)	Optic density (mean ± SEM)(n=4)		Growth Inhibition (%)
	Eth + SC at 8.5 µl/ml	BS+CIS at 8.5 µl/ml	
18.75	0.259 ± 0.009	0.105 ± 0.016	59.57
37.5	0.260 ± 0.015	0.103 ± 0.010	60.48
75	0.266 ± 0.021	0.072 ± 0.008	72.91
150	0.250 ± 0.021	0.042 ± 0.005	83.17

* = large cell lung cancer cell line.

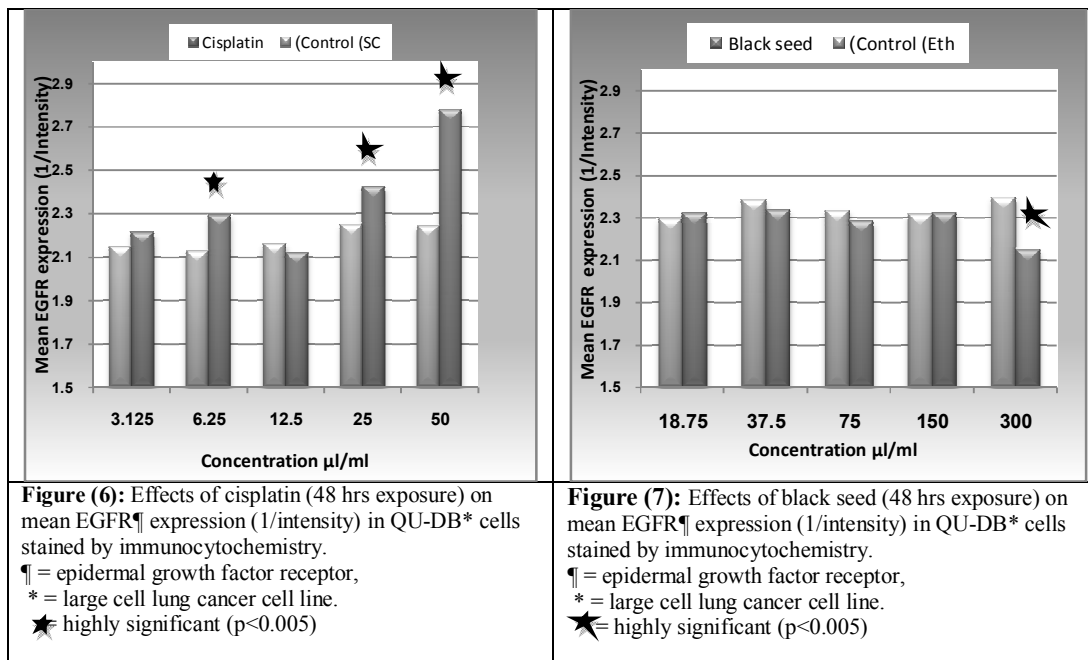
¶ = Neutral red uptake

Table (2): Combination index (CI) values of the interaction between cisplatin (CIS) with and black seed (BS), against QU-DB* cells after 48 hrs as evidenced by NRU¶ assay.

Agent	IC ₅₀	IC ₇₀	IC ₇₀ at IC ₅₀ of CIS	CI	Interpretation
BS	149.5	240.8	63	1.52	antagonism

* = large cell lung cancer cell line.

¶ = Neutral red uptake



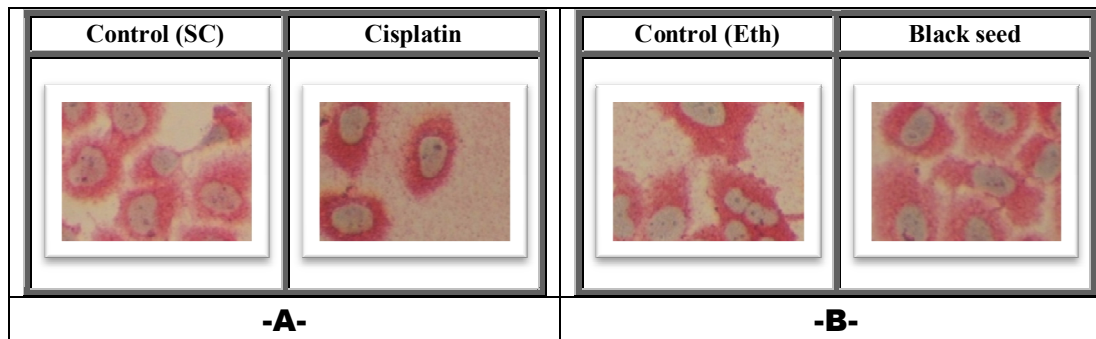


Figure (8): Effects of cisplatin 50 $\mu\text{l/ml}$ (A) and black seed 300 $\mu\text{l/ml}$ (B) on EGFR expression.

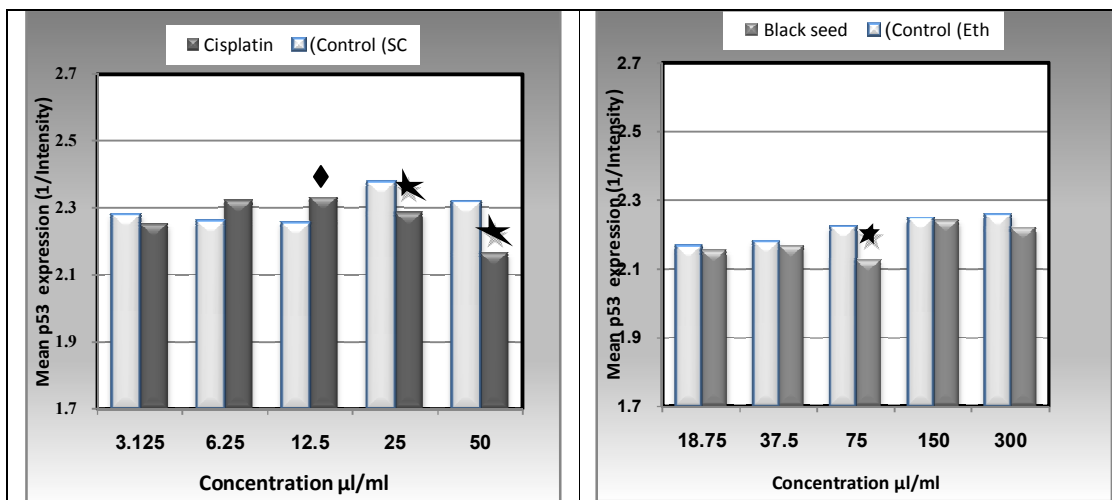


Figure (9): Effects of cisplatin (48 hrs exposure) on mean p53 $\uparrow$ expression (1/intensity) in QU-DB* cells stained by immunocytochemistry.

$\uparrow$ = protein 53,
 * = large cell lung cancer cell line.
 ★ = highly significant ($p < 0.005$)
 ◆ = significant ($p < 0.05$)

Figure (10): Effects of black seed (48 hrs exposure) on mean p53 $\uparrow$ expression (1/intensity) in QU-DB* cells stained by immunocytochemistry.

$\uparrow$ = protein 53,
 * = large cell lung cancer cell line.
 ★ = highly significant ($p < 0.005$)

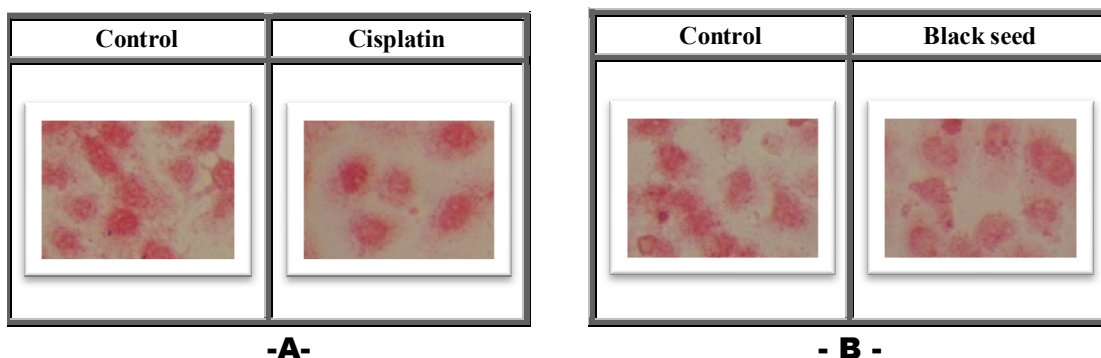


Figure (11): Effects of cisplatin 50 $\mu\text{l/ml}$ (A) and black seed 300 $\mu\text{l/ml}$ (B) on p53 expression

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