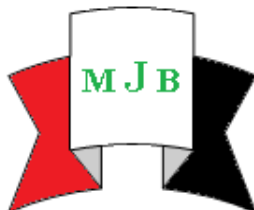


Effects of Fenugreek on Lung Cancer / *In Vitro* Study

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Abstract

Lung cancer is the leading cause (account for 18%) of cancer death in both men and women world-wide. The overall 5-year survival rate for all stages combined is disappointing (15%).

The aim of this study is to evaluate the possible cytotoxic effects of fenugreek on lung cancer cell line and to determine its IC₅₀ alone and in combination with cisplatin. And to study the effects of fenugreek on the expression of each of p53 and EGFR

QU-DB lung cancer cells were cultured in Eagle's MEM culture media supplemented 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 fenugreek [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 each of fenugreek and 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 EGFR and p53 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.

Fenugreek extract also 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 88.25 µl/ml and 125 µl/ml respectively (each one µl is extracted from 25 µg of dried seed). While it produces a growth enhancing effect in 24 hrs exposure experiment. Significant differences (p<0.05) were observed in optic density of fenugreek from that of the control at concentrations of 37.5 µl/ml and above.

Fenugreek produces 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 fenugreek 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 fenugreek highly significantly (p<0.005) decreased the expression of P53.

Monotherapy of fenugreek have anticancer effect on lung cancer cell line, but an antagonizing effect to cisplatin when combined with it.

Fenugreek may have a beneficial therapeutic effect in decreasing EGFR expression and decreasing mutant p53 expression.

Further study is recommended to explore the effect of fenugreek on other cell cycle proteins and to study their potential beneficial therapeutic effects in vivo.

تأثيرات الحلبة على سرطان الرئة /دراسة في الزجاج

الخلاصة

ان سرطان الرئة هو السبب القيادي (حوالي ١٨ %) للوفاة بسبب السرطان في كلا من الرجال والنساء حول العالم، حيث انه يُسبب ١,٤ مليون حالة وفاة بالسنة، وان معدل البقاء الاجمالي لخمس سنوات لكل مراحل المرض مشتركة مخيب للآمال (١٥ %).

لتقييم التأثيرات السمية الخلوية المحتملة للحلبة على خط خلوي لسرطان الرئة ولتقرير الجرعة السمية الخلوية للنصف في الزجاج لكل منها على حدة وبالتمازج مع عقار السيسبلاتين. وكذلك لدراسة تأثيرات كل منها 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 السيبلاتين بتركيز [٢٥-١٩٥، مايكرو لتر لكل مليلتر (او مايكروغرام لكل مليلتر)] والحلبة [٣٠٠-١٧١٩ مايكرو لتر لكل مليلتر (تم استخلاص كل مايكرو لتر من ٢٥ مايكروغرام من البذور الجافة)] باستخدام مقايصة تمثيل صبغة القبط الأحمر المتعادلة (NRU) ل٢٤، ٤٨، و٧٢ ساعة بالمقارنة بمجاميعهم القياسية المطابقة. كذلك حددت التأثيرات المشتركة للحلبة والسيبلاتين باستخدام مقايصة قبط الأحمر المحايد (NRU).

ان التأثيرات السمية الخلوية قُيِّمت بشكل أبعد باستخدام تجربة استبعاد التريبان الازرق (trypan blue) عند الجرعة السمية الخلوية للنصف في الزواج لكل عامل لمدة ٤٨ ساعة. اجريت ايضا تجربة الكيمياء السيتولوجية المناعية لإكتشاف تعبير EGFR و p 53.

احدث السيبلاتين تأثيرات سمية خلوية نسبية مباشرة ومعتمدة على الجرعة والوقت وكانت الجرعة السمية الخلوية للنصف في الزواج (IC₅₀) تساوي ٨،٥ مايكروغرام لكل مليلتر و ٧،٣ مايكروغرام لكل مليلتر بعد التعريض لمدة ٤٨ و ٧٢ ساعة على التوالي. لوحظت إختلافات معتد بها (p < 0.05) في الكثافة البصرية لمجموعة السيبلاتين من تلك المماثلة لمجموعة السيطرة لكل التراكيز المُجرَّبة.

احدث مستخلص الحلبة تأثيرات سمية خلوية نسبية مباشرة ومعتمدة على الجرعة والوقت بعد التعريض لمدة ٤٨ و ٧٢ ساعة وكانت الجرعة السمية الخلوية للنصف في الزواج (IC₅₀) تساوي ٨٨،٢٥ مايكرو لتر لكل مليلتر و ١٢٥ مايكرو لتر لكل مليلتر على التوالي (تم استخلاص كل مايكرو لتر من ٢٥ مايكروغرام من البذور الجافة) بينما احدث المستخلص تأثيرا محسنا للنمو بعد التعريض لمدة ٢٤ ساعة. لوحظت إختلافات معتد بها (p<0.05) في الكثافة البصرية لمجموعة الحلبة من تلك المماثلة لمجموعة السيطرة للتراكيز ٣٧،٥ مايكرو لتر لكل مليلتر فما فوق. لقد قلل مستخلص الحلبة من تأثير السيبلاتين عند الاستخدام المشترك معه حيث كان مؤشر الشراكة (CI) اكبر من (١،٣). احدث السيبلاتين زيادة معتد بها (p<0.005) بتعبير EGFR لعدة تراكيز مختلفة، بينما احدث مستخلص الحلبة نقصانا معتد بها (p<0.005) بتعبير EGFR خصوصا عند التراكيز العليا [٣٠٠ مايكرو لتر لكل مليلتر (تم استخلاص كل مايكرو لتر من ٢٥ مايكروغرام من البذور الجافة)]. احدث كل من السيبلاتين و مستخلص الحلبة نقصانا معتد به (p<0.005) بتعبير p 53 الطافر. اظهرت الحلبة (على حدة) تأثيرات مضادة للسرطان على خط خلوي لسرطان الرئة، ولكنها اظهرت تأثيرات مناهضة للسيبلاتين عندما استخدمت معه شراكة. ان تخفيض تعبير p 53 الطافر و EGFR بواسطة الحلبة لربما له تأثير علاجي مفيد. نوصي بدراسات ابعد لإستكشاف تأثير الحلبة على بروتينات اخرى من دورة الخلية ولدراسة تأثيراتهم المحتملة العلاجية المفيدة داخل الجسم.

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, p16^{INK4a}, and multiple loci on chromosome 3p. (6, 3,4).

Published reports reveal that DG inhibits proliferation and induces apoptosis in a wide variety of human tumor cells: colon, breast, prostate and liver as well as osteosarcoma and leukemia (7, 8).

Trigonelline has been found to be an efficient inhibitor of a transcription factor called nuclear factor E2-related factor 2 (Nrf2), capable of blocking Nrf2-dependent proteasome activity and thereby apoptosis protection in pancreatic cancer cells (9).

It was said that the holy prophet Muhammad (*piece on him and on his aal*) said: "treat yourselves with fenugreek; if my nation knows what is in fenugreek they would treat themselves with it even if it cost an equal weight of gold to its weight" (10). This famous saying indicates that fenugreek contain valuable 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 (11, 12, 13).

A number of human cancers, including colon and lung carcinomas, as well as

osteosarcomas, are associated with either a missing or mutated p53 gene.

Aims of the study:

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

Materials and Methods

Lung cancer cell line:

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 (15).

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

Seeds of fenugreek 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 (16). Briefly, crude plant seeds (fenugreek) 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 (16).

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 fenugreek and 25 µl/ml (µg/ml) for cisplatin.

Assessment of cytotoxicity by neutral red uptake (NRU) assay

The NRU assay was carried out as previously described (17, 18&19). 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 agitation 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 inhibition values calculated according to the following formula:

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

Cytotoxicity of each tested agent is expressed as 50% inhibitory

concentrations (IC_{50}) value. The IC_{50} 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 inhibition versus concentration of tested agents (20).

Combination test using (NRU) assay

Interaction between cisplatin and fenugreek was evaluated by the isobolographic analysis (a dose-oriented geometric method of assessing drug interactions) (21, 22).

Immunocytochemistry (ICC)

Detection of EGFR and p53 expression was done using immunocytochemical procedure (23). 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 (24) 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 (25) 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 in tables [as mean $\pm$ standard error of the mean (SEM)] and in figures. 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 (26).

Results

Cytotoxicity assay and combination test:

Cytotoxicity assay:

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

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

Microscopic assessment:

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) see figure (1).

Neutral red uptake (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.

After 48 hrs of exposure, a significant decrease ($p < 0.05$) was detected in mean optic density (n=6) 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 μ l/ml (μ g/ml), with IC_{50} of 8.5 and 7.3 μ l/ml (μ g/ml) after 48 hrs and 72 hrs of exposure respectively.

2. Effects of fenugreek (1.17188-300 μ l/ml) on QU-DB lung cancer cells:

Microscopic assessment

Microscopic examination showed that 24 hrs exposure to fenugreek did not obviously affect cell viability, while

examination after 48 hrs and 72 hrs 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 as shown in figure (3).

Neutral red uptake (NRU) assay

At the end of 24 hrs experiment, the mean optic density (n=3 for each concentration) of the extracted dye of fenugreek (FG) group was increased in comparison with that of the control (Eth) group giving negative values for percentage of growth inhibition (except for the first concentration 1.172 µl /ml) which indicate that more viable cells are present after exposure to fenugreek.

On the other hand, the optic density was decreased with increasing concentration of fenugreek 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 37.5 µl/ml and above when being compared to the corresponding dilutions of the control ($p \leq 0.05$).

Plotting the values of percentage of growth inhibition against fenugreek concentration for 24, 48, and 72 hrs experiments (figure-4) reveals that percentage of growth inhibition was directly proportional to fenugreek concentration for 48 hrs and 72 hrs experiments, with IC_{50} of (88.25 µl/ml) and (125 µl/ml) respectively. On the other hand, fenugreek did not affect the growth of cells after 24 hrs exposure.

Combination tests:

When combining fenugreek with cisplatin at IC_{50} (8.5 µl/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 µl/ml) group.

Isobolographic analysis of Interactions between cisplatin and fenugreek for 70% toxicity showed antagonistic effects of fenugreek when combined with cisplatin 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.

1.Effects of cisplatin and fenugreek on the expression of EGFR:

Cisplatin obviously increased EGFR expression, and this increment was highly significant ($p < 0.005$) at concentrations of 6.25, 25 and 50 µl/ml (µg/ml) (figure -5). On the other hand, fenugreek generally decrease EGFR expression and this decrement was highly significant ($p < 0.005$) at a concentration of 300 µl/ml (figure-6).

Figure (7) shows the microscopically noticeable changes mentioned above at the highest concentrations of each tested agent.

2.Effects of cisplatin and fenugreek on the expression of p53:

Cisplatin generally decreased p53 expression, and this decrement was highly significant ($p < 0.005$) at concentrations of 25 and 50 µl/ml (µg/ml). On the other hand, cisplatin causes an increased p53 expression at lower concentrations and this increment was significant at concentration of 12.5 µl/ml (µg/ml) (Figure -8).

Fenugreek generally decrease p53 expression and this decrement was highly significant ($p < 0.005$) at concentrations of 75 µl/ml (figure -9).

Figure-10 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 (27 and 28).

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 plant screening program for testing crude extract, the recommended incubation time is between 48 and 72 hours (29, 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 is more significant than finding another one effective in 72 hrs.

Fotakis and Timbrell (32) 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.

The two important chemical constituents of fenugreek with medicinal value; i.e. diosgenin and trigonelline together with many other active phytochemicals were demonstrated to have anticancer activity when applied separately on different cell lines including lung cancer cell lines (7 and 9).

Diosgenin, furanones, dioscin, protodioscin and trigonelline have been shown to have anticancer activity in mice, against breast, and colon cancer (33).

Cytotoxic effects of cisplatin on QU-DB lung cancer cells

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, 34).

Cytotoxic effects of Fenugreek on QU-DB lung cancer cells

In the present study, the significant decrease ($p < 0.05$) in mean optic density readings of concentrations 37.5 $\mu\text{l/ml}$ and above of fenugreek from those of the control in 48 hrs experiment and the apparent changes in viable cell estimate and growth inhibition signify directly proportional dose-dependent cytotoxic effect of fenugreek. Furthermore, the noticeable decrease in mean optic density readings of fenugreek for concentrations less than 37.5 $\mu\text{l/ml}$ from those of the control in 48 hrs experiment supports the above finding

Chen, *et al.*, (7) and Li, *et al.*, (8) showed that diosgenin, a steroidal saponin present in fenugreek can inhibit proliferation, and induce apoptosis in various tumor cells. These effects are mediated through cell-cycle arrest, disruption of Ca^{2+} homeostasis, activation of p53, release of apoptosis-inducing factor, and modulation of caspase-3 activity (35 and 36). Furthermore, Shishodia and Aggarwal, (37) stated that diosgenin inhibits osteoclastogenesis, invasion, and proliferation through the downregulation of Akt, I κ B kinase activation and NF- κ B-regulated gene expression.

Shabbeer, *et al.*, (38) showed that protodioscin (Another active agent identified in fenugreek extract) induces cell death and morphological change indicative of apoptosis in the leukemic cell line H-60, but not in gastric cancer cell line.

On other hand, the negative values of growth inhibition in 24 hrs experiment point to the growth enhancing effect of fenugreek (because optic density is proportional to the number of viable cells). This result may point to the different nutrients that are available in fenugreek seeds including proteins, amino acids and vitamins (39). In contrast, another study (40) showed that fenugreek alone did not enhance cell viability; yet, the combined therapy decreased the toxic effects of cisplatin on Vero cells.

Furthermore, another study (41) demonstrate the potential protective effect of fenugreek seeds against 7,12-dimethylbenz (α) anthracene (DMBA)-induced breast cancer in rats. Also, a chloroform extract of fenugreek seeds was found to stimulate the proliferation of MCF-7 (estrogen receptor positive breast carcinoma) cells, and the latter action is possibly due to the *in vitro* estrogenic activities of fenugreek (42). Another study detected that trigonelline has similar results on breast cancer cells (43).

Growth inhibition results for 72 hrs experiment were consistently lower than the corresponding results in 48 hrs experiment (Figure-4). This time-dependent decrement in cytotoxicity is similar to the dose-dependent decrement in cytotoxicity found by Shabbeer, *et al.*, (38); they found that the growth inhibitory effects of fenugreek extract on primary prostate or htert-immortalized prostate cells was lost when the dose was increased. These two findings may again support the growth enhancing effect of different nutrients that are available in fenugreek seeds including proteins, amino acids and vitamins.

Combination test using (NRU) assay

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 fenugreek to cisplatin (Table 2).

Sakret *al.*, (44) showed that aqueous fenugreek seed extract ameliorates adriamycin-induced cytotoxicity and testicular alterations in albino rats.

Immunocytochemistry (ICC) for p53 and EGFR

Epidermal growth factor receptor (EGFR) is one of the dominant oncogenes that are frequently involved in lung cancer

(3and 4). EGFR is highly expressed in lung cancer, and plays an important role in tumor growth, infiltration and metastasis (45). 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 (46).

The highly significant ($p < 0.005$) increase in EGFR expression noticed mainly at higher concentration (figure -5) 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.*, (47) and Baiet *al.*, (48) 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) (49). EGFR modulates DNA repair after radiation-induced damage through association with the catalytic subunit of DNA protein kinase (DNA-PKcs) (50).

The highly significant ($p < 0.005$) decrement in EGFR expression for fenugreek (figures-6) may point to its beneficial effect in reducing the resistance to chemotherapy and radiotherapy. This is the first report about the effect of fenugreek on the expression of EGFR.

The opposite effect of fenugreek to that of cisplatin in regard to EGFR expression may explain its antagonistic effect to cisplatin obtained from combination test by NRU assay (Table -2).

The oncogen *p53* is one of the commonly deleted or inactivated tumor suppressor genes involved in lung cancer. *p53* mutations are common to both small cell

and non-small cell lung carcinomas (3 and 4).

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 (51).

Wild-type p53 protein has a very short half-life and thus 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 (52 and 53).

Loss of the p53 function may cause resistance to apoptosis that leads to treatment failure to DNA-damaging agents (54).

Thus in the present study, the highly significant ($p < 0.005$) decrease in p53 expression for cisplatin, and fenugreek (figures 3-20, 3-22 and 3-23) 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 expression of caspases 6 and 7 and p53-independent activation of caspases through Bax/Bak mediated release of cytochrome C

contribute to cisplatin induced tubular epithelial cell death (55).

In addition to cisplatin's known mechanism of action as an alkylating agent, it also causes elevated levels of wild-type p53 and P21 in a dose-dependent manner (56).

Shabbeer, *et al.*, (38) showed that a fenugreek extract down regulates mutant p53 in DU-145 cells. Raju, *et al.*, (57) stated that possible mechanisms that could be involved in the inhibition of HT-29 (human colon cancer) cells by diosgenin (a fenugreek extract) could be those relating to modulation of cyclooxygenase-2 and the activation of nuclear factor- κ B, p53, or p21 expression.

Conclusion

1. Fenugreek has an *in vitro* cytotoxic effect against lung cancer with an IC_{50} of 88.25 μ l/ml and 125 μ l/ml for 48 hrs and 72 hrs of exposure respectively. While it produces a protective effect in 24 hrs exposure experiment.
2. Fenugreek produces an antagonistic action when combined with cisplatin, combination index (CI) > 1.3 .
3. The reduced EGFR expression after exposure to fenugreek may point to its possible beneficial effect in reducing the resistance to chemotherapy and radiotherapy.
4. The decreased expression of mutant p53 by fenugreek signifies its beneficial effect in restoring normal p53 functions.

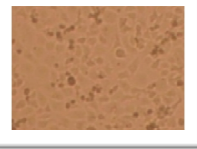
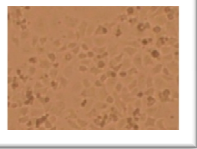
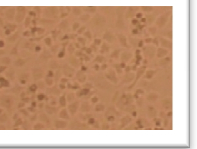
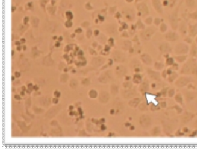
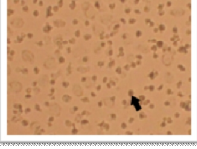
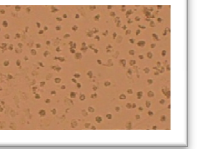
Conc. μl/ml	0.1953	12.5	25
Control			
Cisplatin			

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.

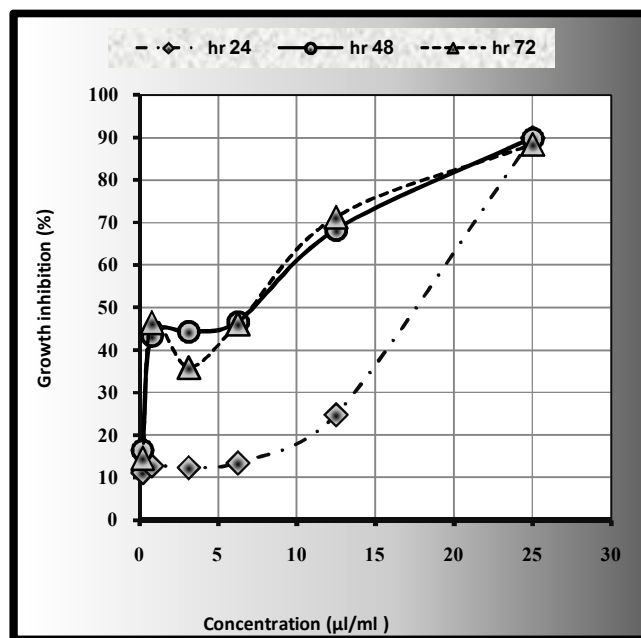


Figure (2): Effects of cisplatin on the growth of QU-DB* cells after 24, 48, and 72 hrs of exposure as evidenced by NRU¶ assay.

* = large cell lung cancer cell line.

¶ = Neutral red uptake

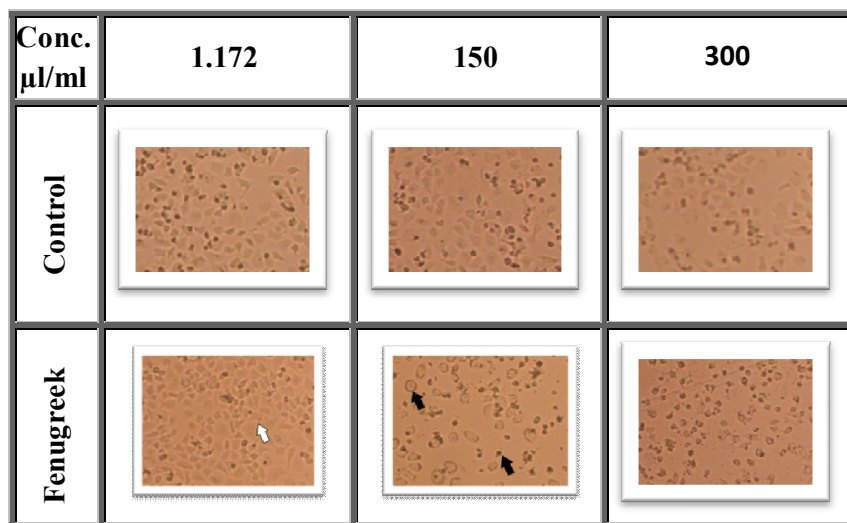


Figure (3): Effect of fenugreek on QU-DB* cells in comparison to the control (Eth) group 48 hrs after exposure to serial concentrations of fenugreek. White arrow (viable cell), black arrow (unviable cell)

* = large cell lung cancer cell line.

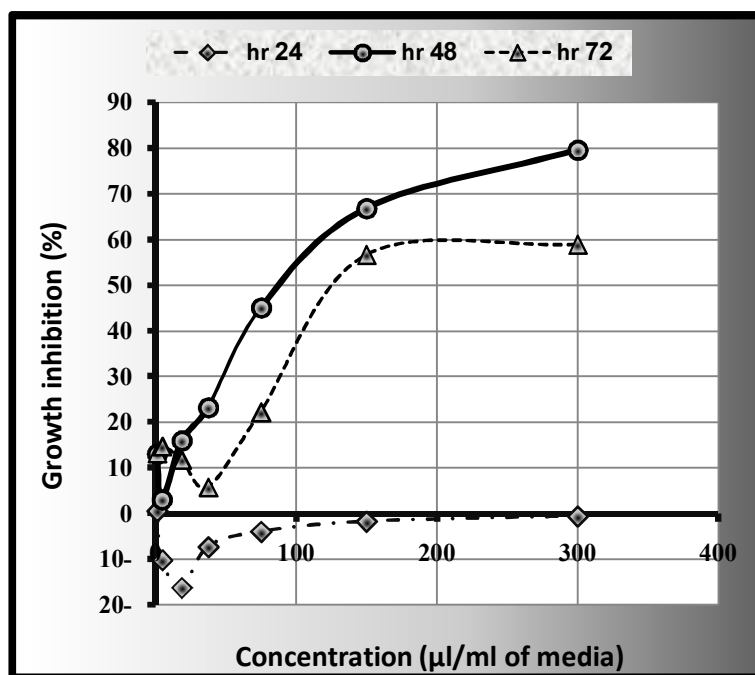


Figure (4): Effects of fenugreek on the growth of QU-DB* cells after 24, 48, and 72 hrs of exposure as evidenced by NRU¶ assay. (Negative value indicates growth enhancing effect)

* = large cell lung cancer cell line.

¶ = Neutral red uptake

Table 1: Combined effects of fenugreek (FG) 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 FG (µl /ml)	Optic density (mean ± SEM) (n=4)		Growth inhibition(%)
	Eth + SC at 8.5 µl/ml	FG+CIS at 8.5 µl/ml	
18.75	0.259 ± 0.009	0.091 ± 0.007	64.89
37.5	0.260 ± 0.015	0.088 ± 0.010	66.35
75	0.266 ± 0.021	0.077 ± 0.005	71.03
150	0.250 ± 0.021	0.066 ± 0.010	73.45

* = large cell lung cancer cell line.

¶ = Neutral red uptake

Table 2: Combination index (CI) values of the interaction between cisplatin (CIS) and fenugreek (FG) 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
FG	88.25	173.6	62.5	1.72	antagonism

* = large cell lung cancer cell line.

¶ = Neutral red uptake

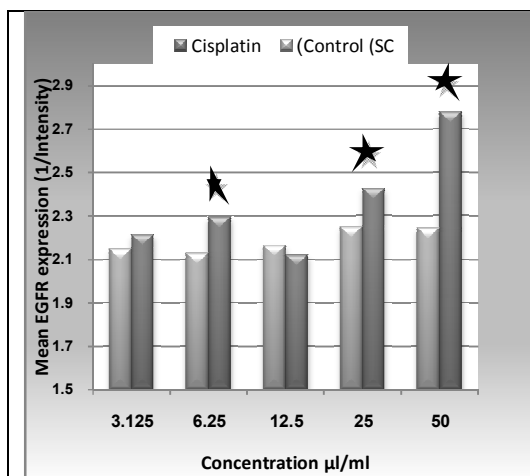


Figure (5): Effects of cisplatin (48 hrs exposure) on mean EGFR¶ expression (1/intensity) in QU-DB* cells stained by immunocytochemistry.

¶ = epidermal growth factor receptor,

* = large cell lung cancer cell line.

★ = highly significant (p<0.005)

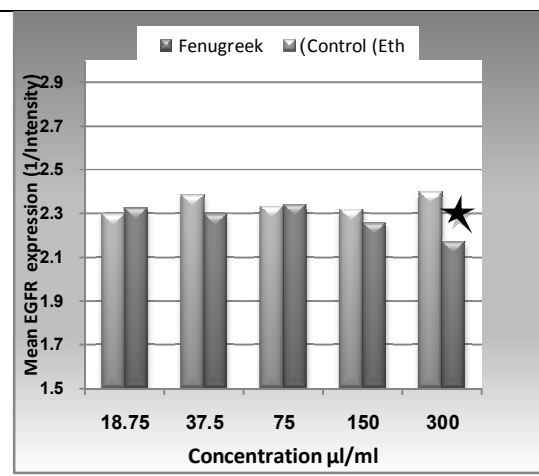


Figure (6): Effects of fenugreek (48 hrs exposure) on mean EGFR¶ expression (1/intensity) in QU-DB* cells stained by immunocytochemistry.

¶ = epidermal growth factor receptor,

* = large cell lung cancer cell line.

★ = highly significant (p<0.005)

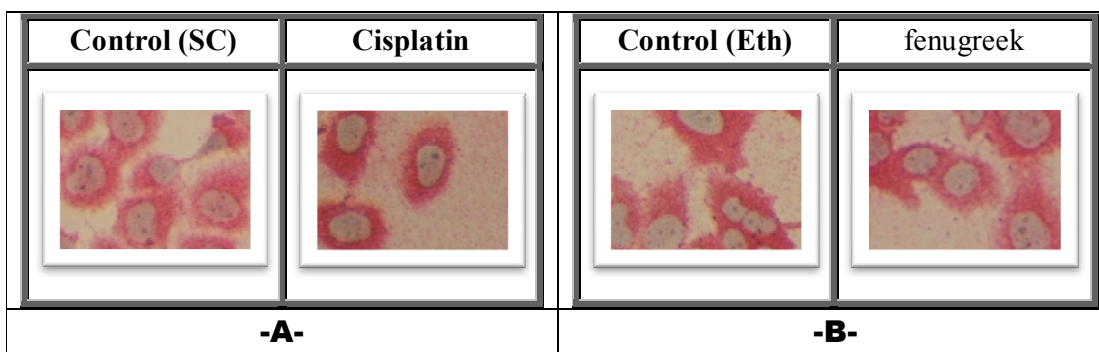


Figure (7): Effects of cisplatin 50 μ l/ml (A) and fenugreek 300 μ l/ml (B) on EGFR expression.

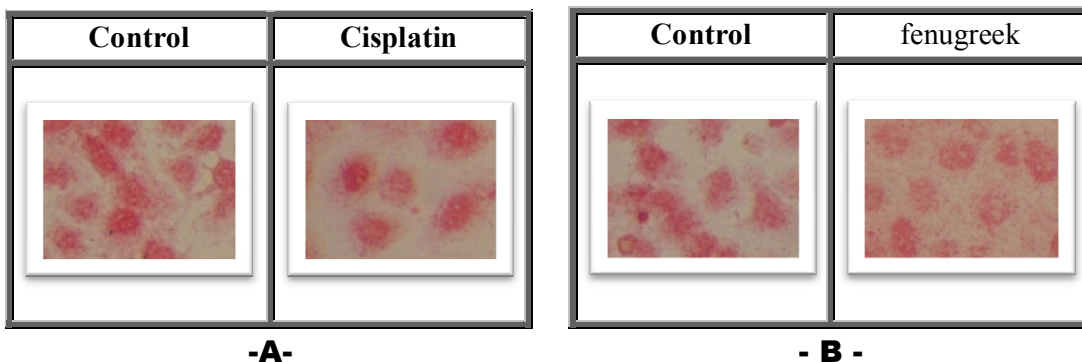
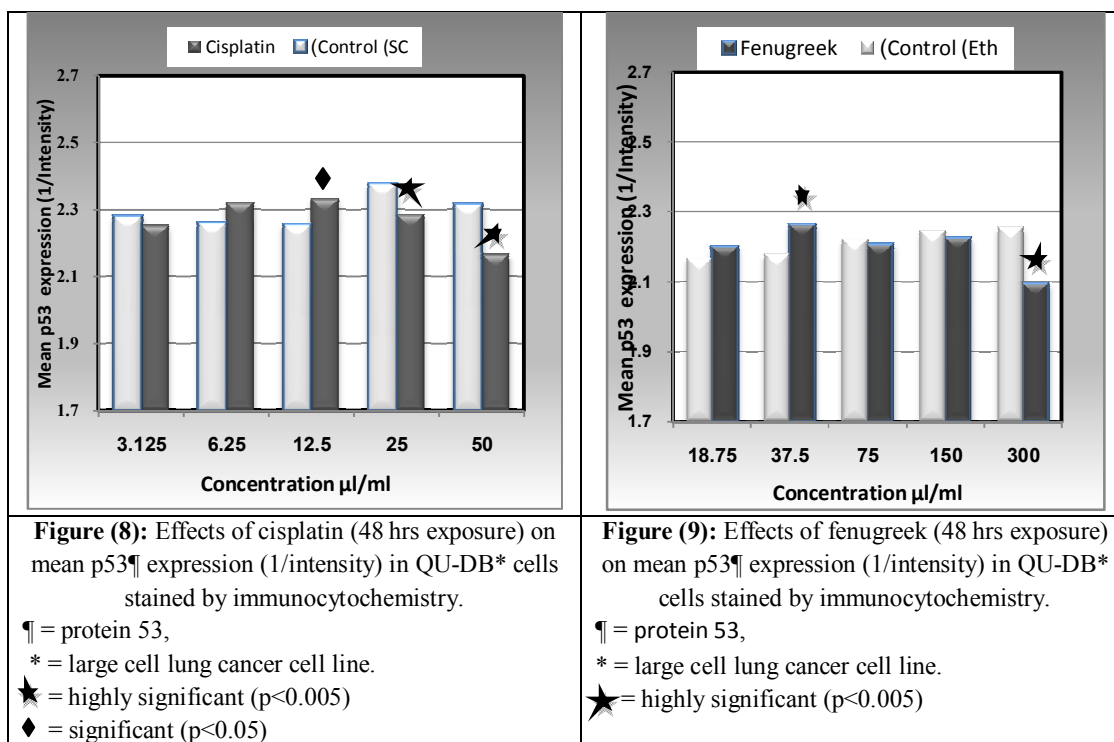


Figure (10): Effects of cisplatin 50 μ l/ml (A) and fenugreek 300 μ l/ml (B) on p53 expression

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