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Abstract

Background: The main goals of our study were as follows: (1) to investigate whether the methanolic extract of the new cultivar (Gürarслан) of *Trigonella foenum graecum* L. seeds exhibit antiproliferative effects on the HeLa human cervix carcinoma, Mat-LyLu rat prostate carcinoma and 3T3 embryonic mouse fibroblast cell lines, (2) to determine how mitosis and DNA synthesis are affected in cancer cells by the extract, and (3) to observe the changes in cell morphology after treatment with the extract.

Materials and Methods: The effect on cell proliferation of these extracts was detected by using methyl thiazolyl diphenyl tetrazolium (MTT) assay. Mitotic index and labelling index was determined using the Feulgen staining and autoradiography methods, respectively.

Results: Our findings show that the methanolic extract of *T. foenum graecum* seeds might have antiproliferative properties on the cancer cell lines only, but not on the 3T3 embryonic mouse fibroblast cell line.

Conclusion: Our data show that the new variety of *T. foenum graecum* may have antitumoral properties.

Keywords: Anticancer effect, Cancer, Fenugreek, Cultivar Gürarслан, Plant extract.

Abbreviations: MI: Mitotic Index; LI: Labelling Index; NCCs: Normalized cell counts; TM: Methanolic extract of *Trigonella foenum graecum* (cultivar Gürarслан) seeds

Introduction

Epidemiological studies show that cancer is the second most common cause of death (after cardiovascular diseases) worldwide. According to estimates, the incidence of cancer will continue to increase, and cancer will become the first most common cause of death in the near future. For this reason, primary, secondary, and tertiary preventions for cancer are important. Primary prevention strategies entail blocking the formation of cancer and implementing precautions against risk factors for cancer. This is important from both the human health and economic (i.e., low cost) perspectives. Secondary prevention methods include early diagnosis and screening tests for the detection of the stages of cancer beginning at the cellular level, without clinical findings. In tertiary prevention, however, symptoms of cancer are present, and mortality can only be reduced with appropriate treatment. Secondary, and particularly, tertiary prevention strategies require significant financial funding (Tuncer, 2009). Recently, though the importance of primary prevention of cancer has been realized, considering the estimations of increase in the cancer incidence, developing new treatment approaches is essential. For instance, obtaining new chemotherapeutics from natural sources (i.e. plants, animals) may supply an advantage in terms of cost. Besides, the consumption of some herbal sources with diet may also contribute to primary prevention of cancer. Since several decades, many plants and plant derivatives have been used for the treatment of various diseases and as cosmetics (Baytop, 1999). In recent years, herbal treatments have become very popular and are being increasingly used by alternative and complementary/integrative medical practitioners. Because plants can be easily exploited, their activities should be the focus of scientific investigations.

In this study, we investigated the antitumoral properties of a new variety of *Trigonella foenum graecum* plant, which have a kind of therapeutic uses for humans. *T. foenum graecum* L. (common name: fenugreek, Family: Leguminosae) is cultivated for its seeds in the middle and southeastern Anatolia regions of Turkey, and worldwide (Secmen et al., 1995). It has been reported that the main components of fenugreek include fixed oil, mucilage, organic phosphorus compounds, choline, trigonelline, and a saponin, which yields diosgenin on hydrolysis (Baytop, 1999). It has been shown that the seeds of *T. foenum graecum* have blood glucose-reducing (Madar et al., 1988; Ali et al., 1995; Al-Habori and Raman, 1998), cholesterol-lowering (Al-Habori and Raman, 1998), antinociceptive (Javan et al., 1997), and nematocidal (Zia et al., 2001) effects. They are also used for the treatment of urinary system diseases, including prostate disorders and kidney stones, in humans (Ali- Shtayeh et al., 2000). A previous study found that the experimentally induced storage of renal calcium oxalate in rats could be cured with the use of *T. foenum graecum* seeds (Ahsan et al., 1989).

The aim of our study was to investigate the possible *in vitro* antitumoral effects of the methanolic extract of *T. foenum graecum* based on the kinetic parameters of the cancer cell lines (i.e. HeLa and Mat-LyLu). The fenugreek seeds used in the experiments are improved and registered in Turkey (*T. foenum graecum* cultivar Gürarслан, registration date: 2004) (Anon, 2010). To our knowledge, there was no investigation about the possible antitumoral effects of this cultivar of fenugreek. Therefore, it was selected this new variety of fenugreek to evaluate possible *in vitro* antitumor effects on cancer cells.

Materials and Methods

Cell culture

In the current study, HeLa (human cervix carcinoma, ATCC number is CCL-2), Mat-LyLu (rat prostate carcinoma, obtained from Prof. M.B.A. Djamgoz, Imperial College London) and 3T3 (embryonic mouse fibroblast, obtained from Istanbul University, Faculty of Medicine) cell lines were used. HeLa and 3T3 cell lines were cultured in DMEM/F12 (Invitrogen, California, USA) [supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, California, USA), 4×10^5 IU/L penicillin, and 2×10^5 µg/L streptomycin (Sigma, St. Louis, USA)], and Mat-LyLu cell line were cultured in RPMI-1640 (Invitrogen, California, USA) [supplemented with 1% heat inactivated fetal bovine serum, 1% L-glutamine, and 0.5% dexamethasone (Invitrogen, California, USA)] media. The cells were maintained under favorable cell culture conditions at 37°C and 5% CO₂ in a humidified chamber.

Preparation of extracts

Certified seeds of *T. foenum graecum* L. (cultivar Gürarşlan) were obtained from Prof. Bilal Gürbüz, Ankara University. The seeds were powdered mechanically and extracted with methanol (>99%, Riedel) (1:10 w/v) in an orbital shaker at room temperature for 24 h. Subsequently, the extracts were lyophilized at -40°C under a vacuum (Edwards, England).

Preparation of test medium

Stock solutions were prepared at a concentration of 10 mg/ml for all extracts dissolved in DMEM/F12 and RPMI-1640 media with supplements. These stock solutions were filtered using a disposable filter (Sartorius, Germany) with 0.45 µm or 0.20 µm pore sizes. The final concentrations of test media, with methanolic extract of the sample, were 10, 15, 20 and 25 µg/ml. Dilutions were achieved in the conditioned media.

Proliferation assay

The effects of the extracts on the proliferation of cell lines were assayed using a tetrazolium reagent. A total of 3×10^4 cells/well were plated in the cell culture medium in a 96-well plate. At 24 h after plating, the medium in the control dishes was replaced with fresh medium (plus supplements). The medium in the test dishes was renewed with fresh test medium of different concentrations. In order to determine cell proliferation, absorbances were measured using the MTT method, as previously described (Mosmann, 1983), at 24 and 48 h after the addition of the test medium by using an ELISA plate reader (Bio-Tek µQuant, USA). Absorbance and reference wavelengths were 570 and 690 nm, respectively. Furthermore, cytotoxicity of the extracts in the 3T3 cell line at maximum concentrations was measured using the MTT assay.

Mitotic activity

The mitotic index was measured to observe the effects of the extracts on the mitotic activity of the HeLa and Mat-LyLu cell lines. For this purpose, the cells were plated on cover slips and treated with the control and test media for 24 h. After incubation, the cells were fixed with 3:1 ratio of ethanol:glacial acetic acid and stained using the Feulgen method (Bancroft et al., 1990). The number (n) of cells counted during the different phases of mitosis (late prophase, metaphase, anaphase and telophase) totaled 4000–6000 cells (C). All of the slides were evaluated by the same person. The mitotic index (MI%) was calculated using the following formula (Ozalpan, 2001): $MI = (n/C) \times 100$.

DNA synthesis

The effect of the extract on DNA synthesis in the cancer cell lines was evaluated using the autoradiography method with tritiated thymidine (³H-TdR). The method was conducted as previously described (Baserga, 1995). The cells were plated on cover slips and treated with the control and test media for 24 h. At the end of the experiment, all groups were treated with a conditioned medium supplemented with 5 µCi/ml ³H-TdR (185 MBq/5 mCi, Amersham, England) for 20 min. at 37°C. Subsequently, the cells were fixed with 3:1 ratio of ethanol: asetic acid as previously described and stained by the Feulgen method (Bancroft et al., 1990). The remaining radioactive materials, not linked to the DNA, were removed with 2% perchloric acid. For autoradiography, the cells on the slides were coated with autoradiographic Hypercoat EM-1 emulsion (Amersham, England) by the dip-coating method. After 8 d of exposure, the slides were developed using a Kodak D 19 b developer. The labelled cells (n) per 1000 cells (C) were counted on each slide. The same person evaluated all slides by counting at least 4000 cells from each group. The labeling index (LI%) was calculated with the following formula (Ozalpan, 2001): $LI = (n/C) \times 100$.

Experimental schedule

In our study, cell kinetics, including proliferation, mitotic activity, and DNA synthesis, of the HeLa and Mat-LyLu cell lines were investigated. The 3T3 embryonic mouse fibroblast cell line was used to evaluate the cytotoxic effect of the extracts on normal cells. We also conducted morphologic observations in cell lines using light microscopy.

Statistical analysis

The experiments repeated triple times at least. Data obtained from the experiments are presented as the mean of the groups ± standard error of means (S.E.M.). Student's *t*-test, a comparison test of 2 ratios, was used to compare the data. A p-value of <0.05 was considered statistically significant. Analysis were performed on a PC using the MS Office Excel and SPSS 11.0 programs.

Results and Discussion

Traditionally, many plants have been used for the treatment of different diseases. Some studies have investigated the effects of medicinal plants and their derivatives. Our study describes the possible antitumoral effects of crude extract, which was prepared with the methanol extract of *T. foenum graecum* cultivar Gürarşlan. The extract was obtained from a new variety of a cultivated plant in Turkey.

According to the experimental results, methanolic seed extract of Gürarşlan fenugreek (TM) inhibited proliferation of HeLa cells, and the inhibition rates typically increased with an increase in concentration. The inhibition at all concentrations for TM were statistically significant at 24 h. However, at concentrations of only 20 and 25 µg/ml for TM were statistically significant at 48 h ($p < 0.05$). Normalized cell counts (NCCs) of HeLa cells in experimental (TM-treated) groups were reduced by the extract (Table 1, Figure 1A). The half-maximal (50%) inhibitory concentration (IC_{50}) of TM on cell growth at 48 h was calculated as 84.0 ± 0.34 µg/ml (Figure 1B). On the other hand, the proliferation of Mat-LyLu prostate cancer cell line was not affected by TM (Table 2, Figure 2). The main components of fenugreek seeds were saponin derivatives such as trigonelline (Kinsky et al., 1967), diosgenin, yamogenin (Taylor et al., 1997) and protodioscin (Hibasami et al., 2003). Hibasami et al. (2003), found that protodioscin, isolated from fenugreek seeds, inhibited cell growth of human leukemia HL-60 cell line by inducing apoptosis. Raju et al. (2004), indicated that diosgenin was shown to suppress proliferation of HT29 human colon cancer cells. In another study, these researchers showed that diosgenin led to the dose-dependent inhibition of HCT116 human colon cancer cell line proliferation and induced apoptosis (Raju and Bird, 2007). Moalic et al. (2001), showed that diosgenin inhibited proliferation of the human osteosarcoma 1547 cell line through cessation of the cell cycle during the G1 phase and the induction of apoptosis. Another group of researchers showed that diosgenin arrested the cell cycle of the same cell line and induced apoptosis by increasing expression of the p53 tumor suppressor oncoprotein (Corbiere et al., 2003). In our study, we showed that the mitotic activity of HeLa cells was suppressed by the TM at 24 and 48 h. The mitotic index (%) was determined as 7.75 ± 1.17 and 5.84 ± 1.27 in the HeLa cell control group at 24 and 48 h, respectively. After TM treatment, mitotic activity significantly decreased to 3.19 ± 0.42 , and 2.99 ± 0.73 (25 µg/ml of TM) at 24 and 48 h, respectively ($p < 0.05$) (Figure 3A). In addition, the decrease of DNA synthesis of this cell line with TM was statistically significant only at 24 h (Figure 3B). The reason for reducing the cell growth and the mitotic activity of HeLa cells by TM, without significant decrease of DNA synthesis at 48 h, might be related the doubling time of the cell line. Contrary to HeLa cell line, the mitotic activity of Mat-LyLu cells was not affected by TM, in similar with cell proliferation. (Figure 4A). However, DNA synthesis rate (LI%) of this cell line significantly decreased at 48 h after TM treatment. The labelling index (%) decreased from 38.3 ± 2.13 (control group) to 25.6 ± 1.27 (25 µg/ml of TM) (Figure 4B). Considering the data obtained from this 3 parameters, if the duration of treatment is extended, it may predict that Mat-LyLu cell proliferation might decrease. Indeed, Shabbeer et al. (2009), prepared the crude extract of fenugreek with a combination of ethanol, hexane, petroleum ether, chloroform, and ethyl acetate, and administered the extract on pancreas (MiaPaCa, HS766T, Panc1, L3.6PL, and BXPC3), breast (MCF, MDA-MB-231, T47D, and SKBR3), and prostate (DU145, PC-3, and LNCaP) cancer cell lines, and the hTertPrEC human normal prostate cell line for 72 hours. The crude extract inhibited cell proliferation at concentrations of 10 and 15 µg/ml, whereas it did not have an effect on the hTertPrEC cell line.

The data indicate that TM did not exhibit cytotoxicity on the proliferation of the 3T3 embryonic mouse fibroblast cell line (Figure 5). Vacuoles in the cytoplasm of HeLa (not Mat-LyLu) cells and a disruption of normal patterns, as determined by light microscopy, were observed at 24 h after application of TM. An increase in the extract concentrations and treatment duration led to an increase in deterioration of HeLa cell patterns. In addition, vacuoles in 3T3 cells appeared, but this appearance occurred to a lesser extent and later than that observed for HeLa cells.

Table 1: The means of Normalized cell counts (\pm S.E.M.) and inhibition rates (%) of cell proliferation in HeLa cells which treated with fenugreek seed extract (TM).

	Normalized cell counts		Inhibition rates (%) of cell proliferation	
	24 th hour	48 th hour	24 th hour	48 th hour
Control	1	1	--	--
TM10	$0.90 \pm 0.03^*$	1.01 ± 0.04	$10.4 \pm 2.88^*$	-1.09 ± 0.30
TM15	$0.84 \pm 0.01^*$	0.95 ± 0.02	$15.7 \pm 0.95^*$	4.63 ± 1.82
TM20	$0.87 \pm 0.05^*$	$0.85 \pm 0.09^*$	$12.6 \pm 5.04^*$	$14.7 \pm 1.88^*$
TM25	$0.84 \pm 0.05^*$	$0.85 \pm 0.03^*$	$16.3 \pm 5.03^*$	$15.3 \pm 3.11^*$

*: Significant difference compared to the control group ($p < 0.05$).

Table 2: The means of Normalized cell counts (\pm S.E.M.) of Mat-LyLu cells which treated with fenugreek seed extract (TM).

	Normalized cell counts	
	24 th hour	48 th hour
Control	1	1
TM10	1.15 ± 0.06	1.32 ± 0.05
TM15	1.14 ± 0.04	1.34 ± 0.09
TM20	1.12 ± 0.06	1.21 ± 0.04
TM25	1.07 ± 0.03	1.09 ± 0.03

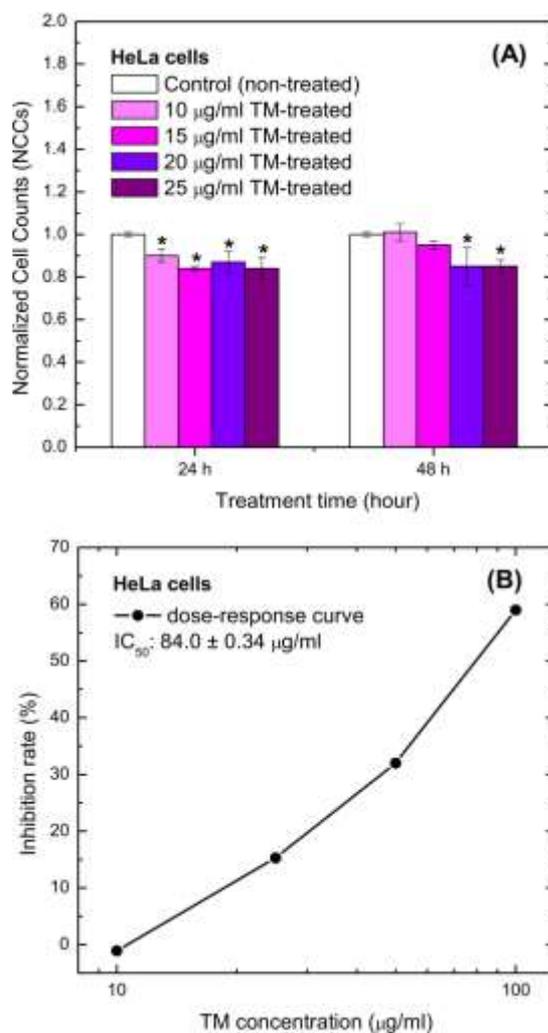


Figure 1: The effects of *T. foenum-graecum* seed extract (TM) on cell proliferation of HeLa cell line. Data are presented as mean ± standard error of the mean (S.E.M.) (A) Normalized cell counts (NCCs) of HeLa cell line after treatment with TM for 24 and 48 h. (B) The inhibition rates (%) of HeLa cell proliferation at 48 h. The half-maximal (50%) inhibitory concentration (IC₅₀) is 84.0 ± 0.34 µg/ml. *: Significant difference compared to the control group (p < 0.05).

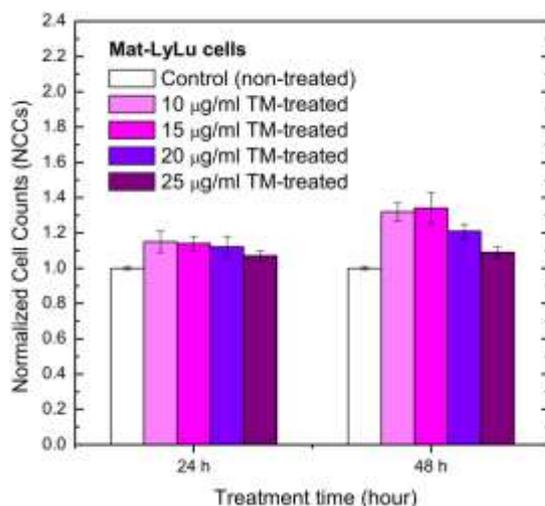


Figure 2: Normalized cell counts (NCCs) of Mat-LyLu cell line after treatment with TM for 24 and 48 h. Data are presented as mean ± standard error of the mean (S.E.M.). There is no statistically significant difference between TM-treated and control groups.

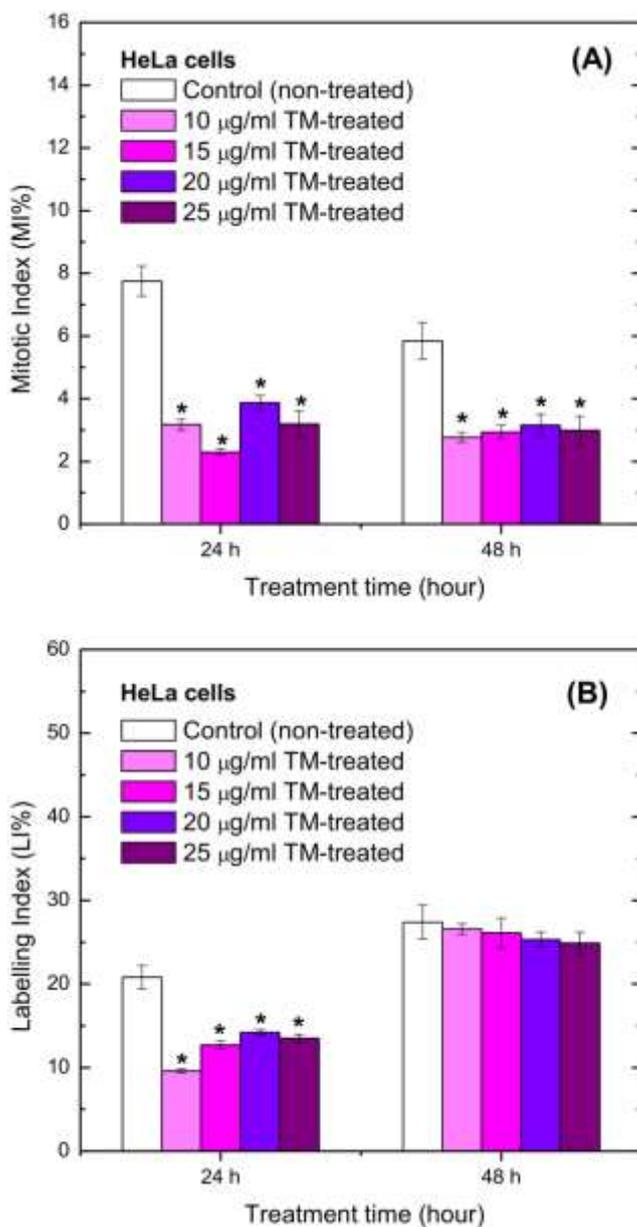


Figure 3: The effects of *T. foenum-graecum* seed extract (TM) on (A) Mitotic index (MI%) and (B) Labelling index (LI%) values for HeLa cells. Data are presented as mean \pm standard error of the mean (S.E.M.). Mitotic activity and DNA synthesis were suppressed by TM in this cell line.

*: Significant difference compared to the control group ($p < 0.05$).

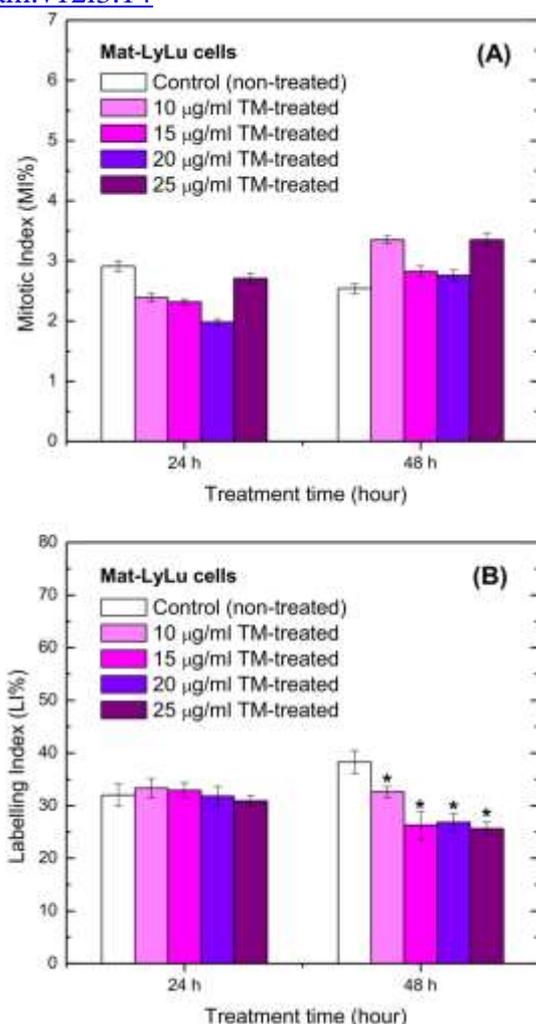


Figure 4: The effects of *T. foenum-graecum* seed extract (TM) on (A) Mitotic index (MI%) and (B) Labelling index (LI%) values for Mat-LyLu cells. Data are presented as mean ± standard error of the mean (S.E.M.). TM did not effect the mitotic activity of Mat-LyLu cells, but DNA synthesis was suppressed by TM at 48 h after treatment.

*: Significant difference compared to the control group ($p < 0.05$).

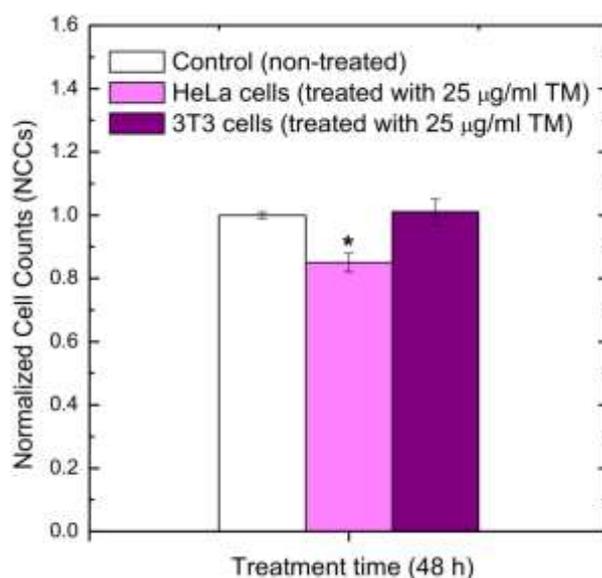


Figure 5: Normalized cell counts (NCCs) of 3T3 and HeLa cell lines at 48 h after treatment with the TM at concentration of 25 µg/ml. TM inhibited the proliferation of cancer (HeLa) cells only, but not normal (3T3) cells.

*: Significant difference compared to the control group ($p < 0.05$).

Conclusions

Our findings can be briefly summarized as follows:

1) According to the experimental data, the methanolic extracts from *T. foenum graecum* cultivar Gürarlan seeds inhibited cell proliferation and DNA synthesis, and reduced the mitotic index of the HeLa cell line. Moreover, this extract inhibited only DNA synthesis in Mat-LyLu prostate cancer cell line.

2) The methanolic seed extract of this new fenugreek cultivar did not toxic against 3T3 embryonic mouse fibroblast cell line.

In conclusion, the methanolic seed extract of *T. foenum graecum* (cultivar Gürarlan) may exhibit antitumor effects, as shown by the inhibition of proliferation of the cancer cell lines but not normal cells. Additionally, the antitumor effects may get stronger with repeated doses and extended treatment time. In particular, *T. foenum graecum* seeds can play an important role in the prevention or treatment of various cancer types, particularly because they are edible, cultivable, easily accessible and can be combined with chemotherapeutics. Therefore, active substances that can inhibit kinetic parameters of the cancer cell lines should be isolated and purified from this extract, and the molecular mechanisms underlying the effects of these active substances should be investigated. Additional *in vitro* and *in vivo* experiments should be conducted using different cancer cell lines. Novel and effective pharmacological agents or chemotherapeutics derived from natural resources, such as plants, will have a positive effect on the development of drugs when considering the cost of the process and its negative impact on the environment. Therefore, we hope that the results obtained from our study will contribute to the development of new pharmacological agents, chemotherapeutics or supplements.

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