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Original Article

In vitro Valuation of Toxic Effects of Fennel (*Foeniculum vulgare* Miller.) Seed Decoctions on Plant and Human Cells

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Abstract

The medicinal plants have been used since ancient times and are still utilized by the majority of the population. Hence, it is essential to investigate the cytotoxic and genotoxic effects of these plants. The aim of this study was to evaluate the toxic effects of fennel seeds decoctions, traditionally used in Iran as an herbal remedy. The cytotoxicity was tested *in vitro* using *Allium cepa* L. roots and human cells and genotoxicity were evaluated using *A. cepa* L. roots. The seeds decoctions were prepared in the traditional method and in two concentrations, commonly used in Iran (CC) and 10 times concentration (10C). The *A. cepa* L. root tips were treated with the decoctions and the mitotic index (MI) and chromosome aberrations were assessed. Human lymphocytes and human bone marrow endothelial cell line (HBMEC) were also treated with the extracts and the cell viabilities were measured using trypan blue and lactate dehydrogenase (LDH) assays, respectively. Although both of the extracts decreased MI in the *A. cepa* L. root tip cells, only the 10C extract significantly increased chromosomal aberrations. In addition, dilutions 1:30, 1:62.5, 1:125 and 1:250 from the 10C extract were 100% cytotoxic to the human lymphocyte cells, however for the CC extract, only dilution 1:30 showed cytotoxic effects. The 1:30 dilution of the 10C extract caused 65% toxicity in HBMEC and none of the CC extract dilutions were toxic to these cells. The tested decoctions showed some cytotoxic and genotoxic effects and the safe dosage of the traditional decoctions needs to be precisely evaluated.

Key words: *Foeniculum vulgare* Miller., *Allium cepa* L., Cytotoxic, Genotoxic.

Introduction

Medicinal plants are widely used for remediation of diseases in different regions of the globe. About 80% of world's population use medicinal plants [1]. Although medicinal plant extracts have been used for many years, it has been shown that, some chemical compounds that exist in these plants may cause detrimental effects and carcinogenicity in human [2]. Therefore, it is essential to evaluate the safety and efficacy of the medicinal plants. Fennel is a valuable medicinal plant from Apiaceae family. Its habitat is extended from Europe to Asia [3]. Essential oils of fennel have shown antibacterial and antioxidant effects [4]. Fennel seeds are used as a traditional medicine in Iran for its estrogenic, lactogenic, diuretic, spasmolytic, antioxidant, immune booster and therapeutic effect on dyspepsia [5]. More than 30 different types of terpenes and terpenoids have been determined in fennel. The most important compounds are anethol, fenchone, estragole and methyl chavicol [6]. The aim of this research was to determine the toxic effects of fennel seeds boiled extract, traditionally prepared and consumed in Iran, on a number of plants and human cells.

Materials and Methods

Preparation of plant extraction

The fennel seeds were provided from Hamedan's Medicinal Plant Garden. To prepare common concentration (CC) fennel extract, 10 g seeds were

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added to 450 ml distilled water and the mixture was boiled until the volume was decreased to half (the extraction method was described by Mr. M. Khosravi, a traditional Iranian medicine professional advisor in Hamedan during a personal communication). The same procedure was carried out for 10 times concentration (10ºC) fennel extract, using 100 g fennel seeds and 450 ml distilled water. The prepared extracts were filtered and stored in sterilized containers in refrigerator prior to use.

Onion *(A. cepa* L.*)* root-tip cytotoxicity and genotoxicity tests

Onion bulbs, 15-20 g in weight and 3-5 cm in diameter, were procured from the local stores. The old roots and dry scales were removed and the bulbs were kept in the pots containing tap water and incubated at dark and room temperature (25±2 ºC). Containing water was replaced with fresh water every 24 h. When the young roots reached 1-2 cm, the plants were taken to grow in the treatment solutions (groups) for 24 h, six bulbs per group. The treatments were the fennel seeds decoctions. Drinking water was used as negative control. For each group, a recovery test was carried out as follows. After 24 h, a few root tips were removed and the bulbs were returned to distilled water for another 24 h in order to observe if there was any recovery from possible damage. The treated roots were fixed and stained by toluidine blue 1% (w/v) and mounted on permanent slides. The slides were analyzed using a microscope with 100 × objective. For each bulb, at least 1000 cells were examined, totaling 8400 for control, treatment and respective recovery. Some characteristics including roots and cell morphology, mitotic index (MI) and abnormality in chromosomes were evaluated to determine the cytotoxicity and genotoxicity of the fennel decoctions.

Trypan blue cell viability assay on human lymphocytes

Human peripheral blood lymphocytes were used to set up cultures for *in vitro* testing. Lymphocytes were obtained from three healthy young (about 24-26 years old) donors, apparently free from any pathogenic agents. They declared that they had not been under any treatment in the last six months prior to the test. The cells were grown in DMEM (Dulbecco's Modified Eagle Medium), from Sigma-Aldrich containing 5 µg/ml concavaline A (Sigma Chemical Co.) and 20% fetal calf serum, at 37ºC temperature, 95% humidity and 5% CO_2. Once the cells were grown enough, 1000-2000 cells were transferred to each well of 96-well plates. The cells were then treated with six dilutions of the plant decoctions (1:31.2, 1:62.5, 1:125, 1:250, 1:500 and 1:1000). After 24 h, a 1% trypan blue solution was added to each well and then viable and dead cells were counted.

Cell viability test with LDH assay on human bone marrow endothelial cells

A human bone marrow endothelial cell line (HBMEC) was kindly provided by Dr. Ali Mostafaie (Kermanshah University of Medical Sciences, Kermanshah, Iran). For the cell viability assay, 2000-3000 cells were cultured in each well of 96-well Microtiter plates containing 100 µl DMEM with 10% fetal calf serum for 24 h. The wells were then treated with 200 µl of six dilutions of the plant extracts (1:31.2, 1:62.5, 1:125, 1:250, 1:500 and 1:1000) in the serum free DMED and incubated at 37 ºC temperature, 95% humidity and 5% CO_2 for 24 h. The percentage of toxicity was estimated for each treatment according to the instruction manual of cytotoxicity detection kit (LDH) manufactured by *Roche applied science*. The light absorption was determined at 490 nm by an ELISA reader. Boiled and filtered water was used as negative control.

Statistical analysis

The data was statistically analyzed by SPSS v.10. The one-way analysis of variance (ANOVA) and Tukey test with the level of significance at $P \leq 0.05$ were applied for the analysis.

Results

The effect of fennel seed extract on onion root-tip cells

No morphological deformities were observed in the *A. cepa* L. root-tip cells treated with CC or 10C extracts. However, with the latter solution, the color of the onion root-tip cells changed from white to light brown and some degree of necrosis formation was observed.

The mitotic index was significantly decreased in the root-tip cells treated with both CC and 10C extracts. However, the mitotic index considerably decreased further in the cells treated with the more concentrated extract (Table 1). The root recovery data indicated that, the water recovery did not cause any significant increase in the mitotic index for both treatments. The analysis of *A. cepa* root chromosomal aberration showed that chromosome abnormalities slightly increased in the roots treated with CC fennel extract; however it was not significant (Table 2).

The abnormalities including chromosomal fragment, laggard chromosome, micronuclei, sticky chromosome, chromosomal loop, c-metaphase, orientation fault in equatorial plate and bridge significantly ($P \leq 0.05$) increased in the *A. cepa* L. root cells, treated with the more concentrated fennel extract compared to the control group (Table 2 and Fig 1).

Table 1 The effect of fennel seed extract on the mitotic index (MI) of *A. cepa* L. root tip cells.

	Group	MI (%) mean ± SD
Tr	Control	27.6±7.84
	CC	5.25***** ±3.73
	10C	3.3***** ±1.18
Re	Control	23.4±4.76
	CC	4.14***** ±3.4
	10C	0.21***** ±0.7

Note: The mitotic index was estimated as number of dividing cells over 8400 cells expressed in percentage. The experiment was repeated three times. * $P \leq 0.05$.
CC, common concentration of fennel extract. 10C, ten times concentration fennel extract. Tr, treatment for 24 h. Re, recovery after 24 h. and MI, mitotic index.

Table 2 The effect of fennel seed extract on total chromosomal aberrations of *A.cepa* L. root-tip cells.

	Group	Total chromosomal aberrations ± SD	Total micronucleated cells (MN) ± SD
Tr	Control	1±0.2752	0.416±0.193
	CC	3.75±0.4942	1.75±0.753
	10C	21.16***** ±2.99	17.166***** ±1.565
Re	Control	1.583± 0.287	0.666 ±0.188
	CC	3.91± 0.357	1.583± 0.312
	10C	13.33***** ± 1.068	2.416 ± 0.2599

Note: The number of total cells observed in each group was 8400. The experiment was repeated three times.* $P \leq 0.05$
CC, common concentration fennel extract. 10C, ten times concentration fennel extract. Tr, treatment for 24 h. and Re, recovery after 24 h.

Results for the recovery treatment showed that recovery with water decreased total chromosome abnormality caused by the more concentrated fennel extract (Table 2).

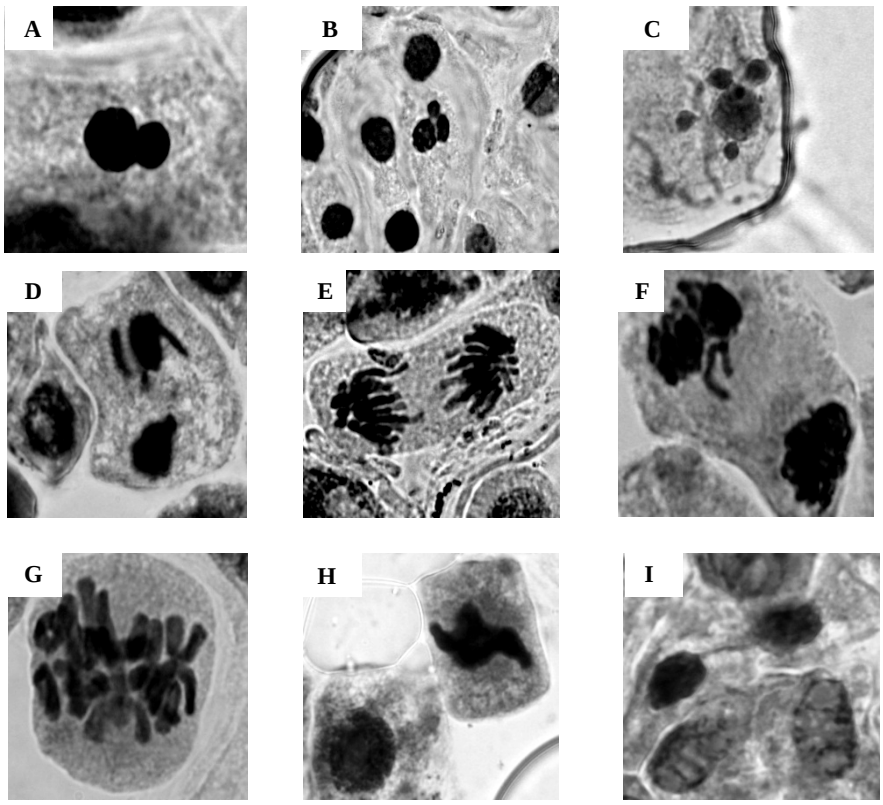

Fig 1 The root-tips of *A.cepa* L. showing different kinds of chromosome aberration (A), (B) and (C) micronuclei, (D) and (E) vagrant chromosome, (F) laggard chromosome, (G) c-metaphase, (H) orientation fault in equatorial plate and (I) bridge.

Investigating the micronuclei (MN) formation in the cells interphase revealed that although the frequency of MN formation did not increase in the *A. cepa* root-tip cells treated with CC fennel extract, in the cells treated with 10C extract, the frequency of MN formation significantly ($p \leq 0.05$) increased (Table 2). Recovery treatment with distilled water decreased the existence of MN caused by the 10 times concentration fennel extract (Table 2).

The cytotoxic effect of the fennel seeds extracts on human lymphocyte cells

The trypan blue cell viability assay showed that the first four dilutions made of the 10C fennel extract (1:30, 1:62.5, 1:125, 1:250) were 100% cytotoxic to lymphocyte cells. None of dilutions made of CC fennel extract showed any cytotoxic effect except the first dilution (1:30), which caused 100% cell death (Fig 2).

The cytotoxic effects of the fennel seeds extracts on HBMEC

The LDH assay on HBMEC revealed that about 65% of these cells were killed by the 1:30 dilution of the 10C fennel extract (Table 3). In addition, the microscopic observations showed that the shape of HBMEC was changed from fusiform to granular with decreasing dilution from 1:1000 to 1:30 (Fig 3). For the cells treated with CC fennel seed extract, microscopic evaluations showed none of the dilutions made from this extract was toxic against HBMEC and their normal shape was maintained.

Table 3 The result of LDH assay on HBMEC

Serial dilution of the boiled extracts	Light absorption (at 490 nm)		Toxicity%	
	CC	10C	CC	10C
1:30	0.263	1.006	0.263	65.40
1:62.5	0.257	0.621	0.000	31.66
1:125	0.254	0.422	0.000	14.00
1:250	0.246	0.374	0.000	10.00
1:500	0.245	0.305	0.000	03.90
1:1000	0.244	0.269	0.000	00.78

CC, common concentration fennel extract and 10C, Ten times concentration fennel extract.

Discussion

In the current study, the potential cytotoxic and genotoxic effects of two aqueous boiled extracts of *F. vulgare* Miller. on *A. cepa* L. were investigated. In addition, the cytotoxic effect of the extracts on human lymphocyte cells and HBMEC was evaluated. To test the effects of the medicinal plants in the form they were actually administered, the extracts were prepared in the same way and at the same concentrations as the therapeutic decoction used by the Iranian population at large. Hence, CC and 10C fennel decoctions were used to evaluate whether this stronger concentration had any toxic or mutagenic effects.

In this research, an anti-mitotic effect was observed in the *A. cepa* L. roots treated with both CC and 10C fennel extracts of the fennel seeds. The MI decreased and this effect was not reversible, as cell division did not recover within 24 h. Inhibition of root growth in *A. cepa* L. might be due to the presence of some toxic substances existed in the decoction of fennel seeds. Inhibition of cell division and MI reduction in this research were concurrent with the results obtained in other studies that reported the essence of fennel inhibited cell growth and cell division in microbial samples [7, 8]. In *A. cepa* L., whenever there is root growth inhibition, there is always reduction in the number of dividing cells [9-10, 11]. It has been reported that, the anti-microbial and anti-proliferation effect of fennel may be due to existence of dillapional, which is a phenyl propanoid derivative [12].

The chromosomal studies in the current study showed that, the *F. vulgare* Miller. seeds decoction significantly increased the total chromosomal aberration and particularly induced the formation of MN in *A. cepa* L. root cells when 10C fennel extract was applied (Table 2 and Fig 1).

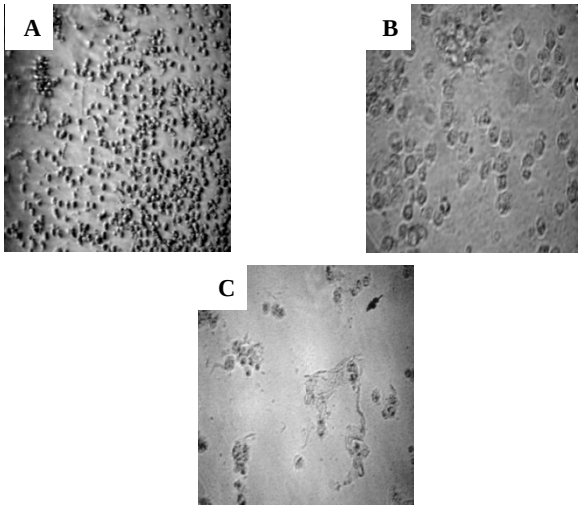

Fig 2 The effect of fennel seeds extracts on the shape of human lymphocyte cells (A) normal cells, (B) beginning of the cell disruption and (C) entirely disrupted cells.

The frequency of the cells with micronuclei is a good indicator of the cytogenetic effects of tested chemicals. Micronuclei often results from the acentric fragments or lagging chromosomes that fail to incorporate into the daughter nuclei during telophase of the mitotic cells and can cause cellular death due to the deletion of primary genes [13, 14]. Recent studies have suggested MN-induced effect of various

medicinal plant extracts. For instance, Askin Celik and Aslanturk (2010) reported MN-induced effect of *Lavandula stoechas* aqueous extract on *A. cepa* L. root tip meristematic cells [15]. In addition, it has been reported that, curcumin (the active component of turmeric) and aloin (the active component of aloe) caused chromosome aberrations in *A. cepa* L. root meristem cells [16]. The mutagenicity and antimutagenicity of fennel and some of its components have been subjected to some other investigations. A study indicated that trans-anethole, the main component of fennel oil, did not increase the mutant frequency in the Salmonella/microsome test. Moreover, trans-anethole did not induce chromosome aberrations in Chinese hamster ovary cells [17]**.** In addition, there was no significant increase in the genotoxicity of trans-anethole even when administered at high doses [18]**.** In a study conducted by Ebeed *et al.,* hot water crude extract of fennel seeds showed slight non-significant genotoxic effects on mice, using *in vivo* clastogenicity assay of mouse bone marrow cells [19]. In contrast, several studies showed antimutagenic effects of fennel. It is shown that pretreatment with trans-anethole, led to significant antigenotoxic effects induced by ethyl methane sulfonate (EMS), cyclophosphamide (CPH), procarbazine (PCB), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and urethane (URE) in the mouse bone marrow micronucleus test [18]. It can also be concluded that, fennel seed extract might contain toxic substances obtained from structural alterations of natural constituents in plant seeds due to decoction that might cause cytotoxic and genotoxic effects on *A. cepa* L. root tip cells.

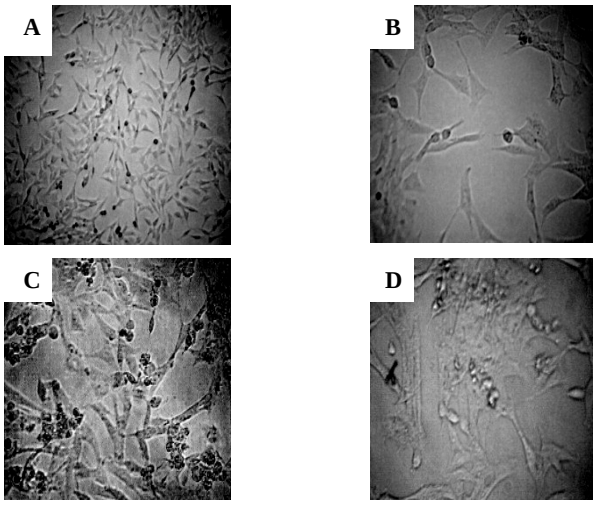

Fig 3 The effect of fennel seed extracts on the shape of HBMEC (A) normal cells, (B) beginning of the cell disruption, (C) development of cell disruption and (D) entire cell disruption.

In the present study, the cytotoxic effects of the fennel extracts on human cells (human lymphocytes and HBMEC) were also investigated. The trypan blue assay showed that 1:30, 1:62.5, 1:125 and 1:250 dilutions from 10C extract and a 1:30 dilution of CC extract were entirely toxic for human lymphocyte cells (Fig. 2). This data indicates the toxic effect of CC fennel seed extract and its 1:30 dilution. LDH assay on HBMEC revealed that the 1:30 dilution of fennel seed extract made from the 10C extract caused 65% toxicity and none of the dilutions made from the CC was toxic to the cells. Hence, it could be concluded that CC fennel seed extract would be toxic for HBMEC even in one third dilution. In addition, morphological studies on the human cells showed that with increasing concentration of the extract, the shape of the endothelial cells and lymphocytes changed (Fig. 2 and Fig. 3). For example, when HBMEC was treated with the higher concentration of the extract, their shape changed from natural fusiform to granular and finally the cell membrane was destroyed. Hence, these results indicated that the cytotoxic activity exerted by the seed extracts of *F. vulgare* Miller., partly due to apoptosis, apparently induced deformation of cell membrane and nuclei. It was possible that components in the seeds extract of 10C *F. vulgare* Miller. caused irreparable DNA damage and prohibition of mitotic division, which was observed in *A. cepa* L. root tip cells, and resulted in cell death. The results herein suggest that the tested extracts possess some cytotoxic and genotoxic effects and the safety of the fennel seed decoction, traditionally made and used in Iran, must be more accurately evaluated *in vitro* and *in vivo*.

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