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THE PROTECTIVE EFFECT OF (*FOENICULUM VULGARE*) OIL ON ETOPOSIDE-INDUCED GENOTOXICITY ON MALE ALBINO RATS

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ABSTRACT

Etoposide is an anticancer drug that belonging to topoisomerase II inhibitors. Fennel (*Foeniculum vulgare*) is a medicinal plant belonging to the family Apiaceae. The present study evaluated the possible protective potential of oral simultaneous treatment of fennel oil (0.5 &1 mg/kg body weight) every 48 hours for 21 days against the genotoxic effects of etoposide intraperitoneal administration (5 mg/kg body weight) every 72 hours for 21 days on bone marrow and testis in male albino rat (*Rattus norvegicus*). Seventy two adult male albino rats were used as the following, 36 rats (6 for each group) were prepared for DNA, sperm head abnormalities and total protein (they were received the treatments for 21 days). The other 36 rats were used for chromosomal aberrations in bone marrow in addition to mitotic index in bone marrow and testis evaluation (they were received the treatments for 72 hours only). Animals treated with etoposide showed DNA fragmentations on agarose gel electrophoresis and a significant increase ($P \leq 0.001$) in the percentage of bone marrow total chromosomal aberrations (TCA: 230.3±1.9) with marked decrease ($P \leq 0.01$) in mitotic index in both bone marrow and testis (39.6±10.9 & 17.8±4.3) respectively. Sperm head abnormalities were significantly ($P \leq 0.001$) increased also after etoposide treatment (68.9±2.17). Total Protein contents in testis were increased after etoposide treatment. While simultaneous treatment of fennel oil (either 0.5 or 1 mg/kg b. wt.) improved the protein levels. In conclusion, the results of the present study indicated that fennel oil exerted a protective effect against genotoxicity and cytotoxicity induced by etoposide that may be due to its antioxidant effects. Consequently, we recommended that fennel oil can be suggested to be administrated as co-medicine in chemotherapeutic treatments of cancer.

KEYWORDS: Etoposide, Fennel oil, DNA, Chromosomal aberrations, Mitotic index.

INTRODUCTION

Etoposide is a semisynthetic derivative from podophyllotoxin that extracted from roots and rhizomes of American Mayapple (*Podophyllum sp)*.[1] Etoposide used as antineoplastic drug for treatment of lung cancer, malignant lymphoma (Hodgkin's and non-Hodgkin's types), acute non-lymphocytic leukaemias and solid tumors as testicular, ovarian, bladder cancer, carcinoma of the breast and childhood malignancies.[2,3] For testicular germ cell cancer bleomycin and cisplatin are used in combination with etoposide.[4] It was found that etoposide induced adverse effects such as low blood pressure, hair loss, pain, nausea, vomiting, diarrhea and loss of appetite.[5] Etoposide induced molecular germ cell death and induced apoptosis to spermatocytes that inhibited by adding a general caspase inhibitor along with etoposide.[6] In addition, etoposide caused damage in developing ovarian germ cells in mice.[7] Etoposide administration caused embryo toxicity and teratogenicity to pregnant rodents with DNA fragmentation in neuroepithelial cells.[8] More observations showed that mating of normal female rats with etoposide-treated male rats resulted in all offspring mortality within 7 days after birth.[9] Concerning the effect of etoposide on DNA, it was found that etoposide was genotoxic and induced DNA breaks on mouse cells.[10,11] Etoposide induced DNA fragmentation, chromatin alternation and oxidative damage to human sperm (invitro studies).[12] Etoposide could induce cytochrome c released from isolated mitochondria in vitro.[13] Cytotoxicity of etoposide on cervical carcinoma HeLa and colon carcinoma HCT116 cells was examined. It was found that one hour treatment with 25 μm etoposide was highly toxic causing DNA double strands damage.[14] Human colon cancer cell line HT-29 treated with etoposide loss clonogenicity, extent of G2/M arrest and DSBs (double strand breaks) appeared.[15] Etoposide is a genotoxic agent which caused chromosomal instability, micronuclei formation, chromosomal aberrations and suppressed the proliferated erythroblast in bone marrow cells of mice.[16]

In the present study, etoposide was used as genotoxic material and on the other hand, fennel oil was used to reduce etoposide toxicity.

Large number of plants are used in medicine and treatment of various disease as fennel plant. Fennel (*Foeniculum vulgare*) is a medicinal plant belonging to the family Apiaceae (Umbelliferae). Essential fennel oil was extracted from fennel seed using steam distillation and the main components of fennel oil were analyzed which are anethole 50–60%, estragole 22.4%, α- fensone 21.9% and other components including α- pinene 2.45%, champene 0.17%, β-myrcene 0.26%, limonene 3.73%, δ-carene 1.53%, champor 0.22%, vitamin C, calcium, magnesium, copper and phosphorus ions.[17] The most important compounds of fennel essential oil are anethole and fenchone[18] that responsible for antioxidant activity of fennel oil. Essential oil of fennel used as flavoring agent in food product. Fennel oil has a hepatoprotective effect[19,20,21,22,23], anti-cancer effect[24], antibacterial and antiviral activities.[25,26] Fennel oil has antioxidant activity including inhibition of hydrogen peroxidase H_2O_2, iron chelating and radical scavenging[27], Antitumor, chemopreventive, cytoprotective, hypoglycemic[28] and oestrogenic activities, memory enhancing and reduce stress. Fennel oil inhibited acute and sub-acute inflammatory diseases and allergic reactions due to its contents of vitamin C.[29] Fennel oil has a protective potential activity against radiation induced biochemical disturbances and oxidative stress in rats.[30] Fennel oil has antimutagenic and protective effects against genotoxicity and oxidative stress induced by cyclophosphamide in mice. Treatment with fennel oil inhibited aberrant metaphases, chromosomal aberration, micronuclei formation and cytotoxicity in mouse bone marrow cells induced by cyclophosphamide and restored levels of super oxide dismutase (SOD), glutathione (GSH), catalase (CA) and malondialdehyde (MDA) to normal levels.[31]Moreover, fennel extract has large amounts of antioxidants that give fennel it's chemo-protective effect against chemicals induced DNA damage and it's antimutagenic effect which enhance DNA repair system. [32]

MATERIAL AND METHODS

Materials

Etoposide **(**etopophos®**),** $C_{29}H_{32}O_{13}$ (4'-Demethylepipodophyllotoxin9-[4,6-O-(R)-ethylidene-β-D glucopyranosi- de], 4'-(dihydrogen phosphate) was purchased from a local pharmacy.

Fennel oil (*Foeniculum vulgare*) belongs to family Apiaceae. Fennel oil was obtained from Cap Pharm for Extracting Natural Oils and Herbs, Cairo, Egypt.

Experimental animals

A.2.1- Housing of the animals

Healthy adult male albino rats (*Rattus norvegicus*) weighting 130±10 g were obtained from the Serum and Antigen Laboratories, Helwan, Egypt. Animals were kept under constant condition of temperature (25±2 ºC) for at least two weeks before and throughout the experimental work. They were maintained on a stander rodent diet composed of 20% casein, corn oil, 55% corn starch, 5% salt mixture and 5% vitaminized starch obtained from Egyptian Company for Oils and Soup Kafr- Elzayat, Egypt. Water was available *ad libitum*. The experimental protocol was approved by Zoology Department, Faculty of Science, Menoufia University.

A.2.2 Groups of animals under investigation

In the present study 72 adult male albino rats were equally divided in to 6 groups used as the following:
Group (1) animals were served as negative control (untreated). **Group (2)** animals were injected intraperitoneally with etoposide (5 mg/kg b. wt.) every 72 hours.[33,34] **Group (3)** animals were orally given (0.5 mg/kg b. wt.) fennel oil every 48 hours as low dose of plant.[20] **Group (4)** animals were orally given (1 mg/kg b. wt.) fennel oil every 48 hours as high dose of plant.[35] **Group (5)** and **Group (6)** animals were injected intraperitoneally with etoposide (5 mg/kg body weight) every 72 hours for and simultaneously, rats were orally given (0.5 and 1 mg/kg b. wt.) fennel oil respectively, every 48 hours.

Thirty six rats (6 for each group) were prepared for DNA, sperm head abnormalities and total protein. These groups were received the treatments for 21 days. While the other 36 rats (6 for each group) were specified for chromosomal aberrations in bone marrow and mitotic index in bone marrow and testis evaluation. These groups were received the treatments for 72 hours.

Methods

1- Molecular investigations

1.1- Total genomic DNA extraction and apoptosis detection

Nucleic acid extraction was done according to extraction method of[36] with some modifications had been introduced by[37] in which the direct staining of DNA sample was done. Apoptotic bands of DNA fragmentation appeared and located at 180 bp and its multiples 360, 540 and 720 bp against therteen bands of DNA marker (100–3000 bp, Fermentas).[38] The intensity of released DNA fragments was measured by image J software, as a mean of optical density values.

2- Cytogenetic investigation

2.1- Chromosomal Preparation in bone marrow and testes

The method was described by.[39] Control and treated animals were used for the evaluation of chromosomal aberrations and mitotic index in bone marrow and testes.[40]

2.1.1-Chromosomal aberrations in bone marrow

For each animal 100 metaphase spreads were scored for chromosomal aberrations. Only cells with well spread chromosomes were selected for scoring. Slides were examined at (×1000) magnification by light microscope (Olympus BX41, Japan) and the representative photos were captured using digital camera.

2.1.2- Mitotic index in bone marrow

The same slides of chromosomal aberrations were used to evaluate the mitotic index in bone marrow. For mitotic examination, 500 cells of each animal were examined at **(**×200) magnification by light microscope (Olympus BX41, Japan) and the representative photos were captured using digital camera. Cells were classified according to their division to resting cells (non-dividing cells), prophase and metaphase. The percentage of metaphase was calculated according to the following equation.

Mitotic index in bone marrow (%) =

$$\frac{\text{(metaphase)} \times 100}{\text{(resting cells + prophase+ metaphase)}}$$

2.2- Mitotic index in testes

This test evaluates the percentage of dividing cells in testis. For studying mitotic index, slides were examined at (×200) magnification by light microscope (Olympus BX41, Japan) and the representative photos were captured using digital camera.

Mitotic index in testis (%) =

$$\frac{\text{(metaphase + prophase)} \times 100}{\text{(resting cells+prophase+metaphase)}}$$

3- Evaluation of sperm head abnormalities

This test was used for determination and evaluation either normal or abnormal morphology of sperm head according to.[41] Slides were examined at (×1000) magnification by light microscope (Olympus BX41, Japan) and the representative photos were captured using digital camera.

4- Estimation of testicular total protein content

For total protein estimation, testicular tissue homogenized in lysis buffer on ice was used. Testes were rinsed on ice cold 0.175 M KCl /25 mM Tris – HCl (pH 7.4) to remove the blood. Then testes homogenated by homogenizer with a Teflon pestle. Testis homogenates were centrifuged at 5000 rpm for 15 min. The supernatants were used for total protein estimation. The protein concentration in testicular homogenate was detected by the method of[42] as modified by.[43]

5- Protective effect

Protective effect percentage of fennel oil against etoposide was calculated according to the following equation. [44]

Protective effect (%) =

$$\frac{\text{(toxicant gp – antioxidant gp)} \times 100}{\text{(toxicant gp – control gp)}}$$

Statistical Analysis

In the present work, the result were represented as Mean ± Stander Deviation. Comparisons were made between the untreated and treated groups. All numerical data were statistically analyzed using Statistical Program of Social Science (SPSS) software for windows, version 10. ($P \leq 0.01$) were considered statistically significant with mitotic index of bone marrow, mitotic index in testis and total protein in testis. ($P \leq 0.001$) were considered statistically significant with total released DNA fragmentation, total chromosomal aberrations and sperm head abnormalities.

RESULTS

1- Molecular investigation results

1.1- Analysis of total genomic DNA damage

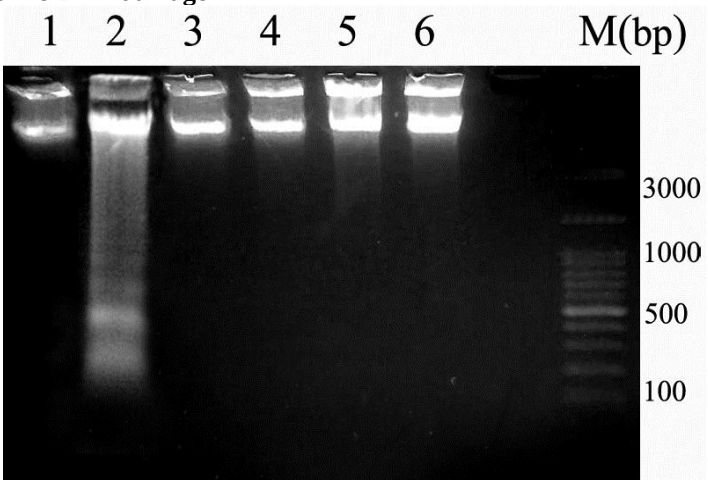

Figure (1): Digital photograph of DNA electrophoresis of rat testis tissue showing the protective effect of fennel oil against etoposide. Where L1: control; L2: etoposide (5 mg/kg b. wt.); L3: fennel oil (0.5 mg/kg b. wt.); L4: fennel oil (1mg/kg b. wt.); L5: combination of etoposide with fennel oil low dose; L6: combination of etoposide with fennel oil high dose and M: DNA marker.

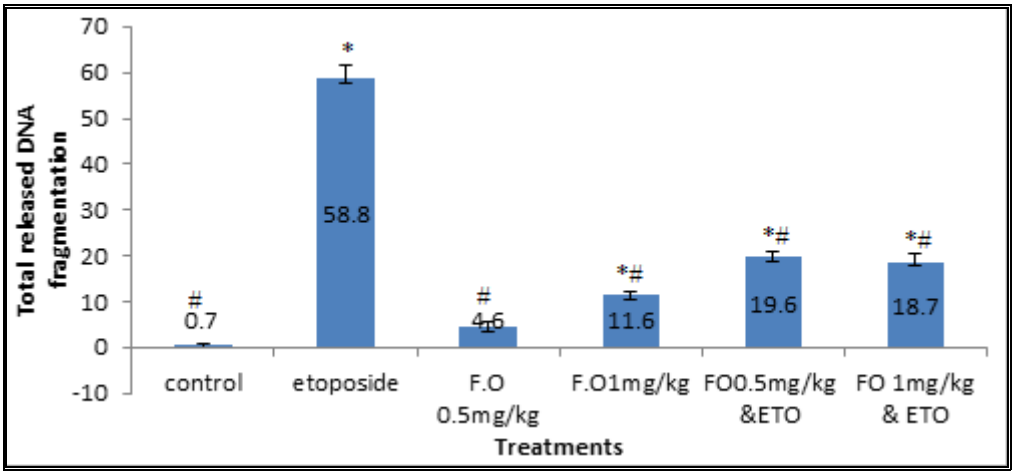

Figure (2): Total released DNA fragmentation in testis tissues of rats treated with etoposide and the protective effect of fennel oil doses. Data were presented as Mean± S.D. *Statistically significant (*P*≤0.001) with respect to control. # Statistically significant (*P*≤0.001) with respect to etoposide. ETO: etoposide; F.O: fennel oil. n=6.

It is cleared that etoposide was able to damage DNA and induced apoptosis appeared as laddering pattern of DNA fragments at 200, 400, 600 and 800 base pair (diagnostic pattern of apoptosis) (Fig. 1). Optical denisity value of fragmented DNA extracted from testes of etoposide-treated rats was (58.8±2.6) (Fig. 2). It showed significant increase (*P*≤0.001) when compared with control (0.7±0.1). While animals treated with fennel oil low dose (0.5mg/kg b. wt.) showed normal appearance of intact DNA (optical denisity 4.6±1.2) that was non-significant when compared with control (0.7±0.1) and significant (*P*≤0.001) when compared with etoposide released DNA fragmentation (58.8±2.6). Whereas, animals treated with high dose of fennel oil showed a slight increase in total DNA released fragmentation (11.6±0.6) which can be neglected when compared with etoposide damage (58.8±2.6). On the other hand, combination of etoposide with fennel oil doses (0.5 or 1mg/kg b. wt.) showed an improvement DNA fragmentations (19.6±1.4 and 18.7±1.7) respectively, when compared with etoposide alone. Low and high doses of fennel oil showed a protective effect reached to (67.5 and 69%) respectively.

2- Cytogenetic investigation results

2.1- Chromosomal aberrations in bone marrow

Chromosomal aberrations are changes in chromosome structure or number. In this study only structural chromosomal aberrations were scored. These aberrations involve alternation of the genetic material and detected by light microscope during cell division at metaphase. Normal chromosomes showed in (Fig. 3A).

The structural aberrations include end to end association (Fig.3 B), breaks (Fig. 3C), deletions (Fig. 3D), centromeric attenuation (Fig. 3D), ring chromosome (Fig. 4A), centric fusion (Fig. 4C), chromatid gaps (Fig. 4C) and sticky chromosomes (Figs. 3C and 4B).

Data in Table (1) showed that etoposide significantly (*P*≤0.001) increased total chromosomal aberrations (TCA: 230.3±1.9) when compared with control (TCA: 47.3±5.0). Moreover, etoposide statistically (*P*≤0.001) decreased the percentage of structurally normal metaphases to (8.2±1.9) when compared with control (63±1.16). Data showed that administration of low dose of fennel oil (0.5 mg/kg b. wt.) was non-significant (TCA: 42.6±3.2) when compared with control group (47±5.0), and significant (*P*≤0.001) when compared with etoposide group (230.3±1.9). In addition, fennel oil low dose showed statistically non-significant increase (*P*≤0.001) in the percentage of structurally normal metaphases (64.5±1) when compared with control (63±1.16). While the high dose of fennel oil (1 mg/kg b. wt.) increased the mean values of total chromosomal aberrations to (99±5.8) that increase was significant (*P*≤0.001) when compared with control (47.3±5.0) or with etoposide (230.3±1.9). The percentage of structurally normal metaphases in animals treated with high dose of fennel oil showed statistically significant (*P*≤0.001) decrease (43.2±2.3) when compared with control (63±1.16). Animals treated with fennel oil low dose (0.5mg/kg b. wt.) and etoposide at the same time showed improvement in total chromosomal aberration (TCA: 88.3±5.2) when compared with etoposide group (230.3±1.9). This group showed good protective effect of fennel oil (low dose) on chromosomes against etoposide that reached to (77.5%). Moreover, the percentage of structurally normal metaphases was increased (52.1±1.7) when compared with etoposide alone (8.2±1.16). Whereas, the combination of etoposide with high dose of fennel oil (1mg/kg b. wt.) showed a decrease in total chromosomal aberrations (114.6±7.4) when compared with etoposide alone (230.3±1.9) with a protective effect reached to (63.1%). In addition, the percentage of structurally normal metaphases were increased (41.8±1.9) when compared with etoposide alone (8.2±1.9).

Table (1): The chromosomal aberrations in bone marrow of rat treated with etoposide and the protective effect of fennel oil treatment.

Groups	%Structurally normal metaphases	Structural chromosomal aberrations									
		Deletion	Fragment	Centric attenuation	Centric Fusion	End to end	Break	Ring	Sticky chromosomes	Gap	T C A
Control	63±1.16$^{\#}$	14.3±0.48	0.66±0.36	8.4±0.44	0.65±0.57	0.63±0.2	4.4±0.41	19.1±1.31	0.62±0.1	0.68±0.40	47.3±5$^{\#}$
ETO 5mg/kg	8.2±1.9*	35.8±2.3	60±2.15	63.6±1.7	26±1.6	19.2±0.81	0.6±0.34	12.3±0.30	4±0.32	8±0.18	230.3±1.9*
F.O.0.5mg/kg	64.5±1$^{\#}$	6±2	4±0.38	17.5±0.64	2.5±0.41	0.65±0.36	2.4±0.5	8.1±0.56	0.63±0.16	0.68±0.23	42.6±3.2$^{\#}$
F.O 1mg/kg	43.2±2.3$^{*\#}$	15.2±0.39	19.2±1	20.2±1.9	2.58±0.43	0.65±0.55	12.2±0.54	26±2.2	0.64±0.49	1.4±0.30	99±5.8$^{*\#}$
F.O.0.5mg/kg & ETO 5mg/kg	52.1±1.7$^{*\#}$	18.5±0.43	8.4±0.86	45.1±1.22	2.5±0.35	0.65±0.85	1.3±0.44	4.9±1.1	1.33±0.32	2.4±0.33	88.3±5.2$^{*\#}$
F.O 1mg/kg & ETO 5mg/kg	41.8±1.9$^{*\#}$	24±1.4	1.3±0.34	44±2.3	5.2±0.28	4±0.33	9.6±0.68	19.1±1.4	0.6±0.44	2.5±0.32	114.6±7.4$^{*\#}$

Data were represented as Mean ±S.D. *Statistically significant ($P \leq 0.001$) with respect to control. # Statistically significant ($P \leq 0.001$) with respect to drug. ETO: Etoposide; FO: Fennel oil; TCA: Total chromosomal aberration, n=6.

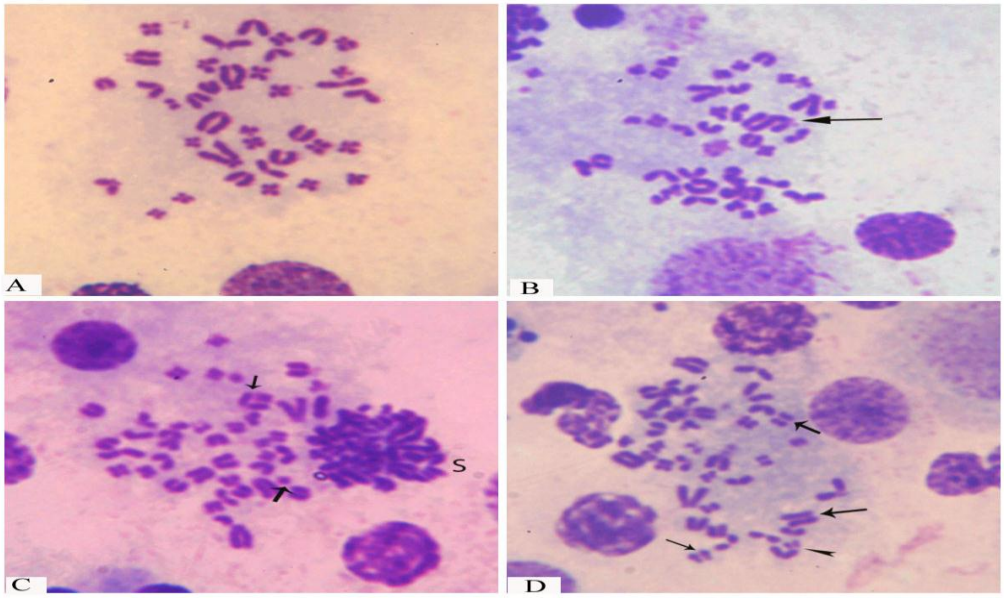

Figure: (3) Photomicrograph shows bone marrow chromosomal preparations of control and treated rats (Giemsa stain, ×1000) (A): Normal metaphase spread in bone marrow cells of control rats. (B): Metaphase spread showing end to end association (arrow) in bone marrow cells of etoposide-treated rats. (C): Metaphase spread showing break (thin arrow), fragment (thick arrow) and sticky chromosomes (S) in bone marrow cells of etoposide-treated rats. (D): Metaphase spread showing centric attenation (arrows) and deletion (head of arrow) in bone marrow cells of etoposide treated rats.

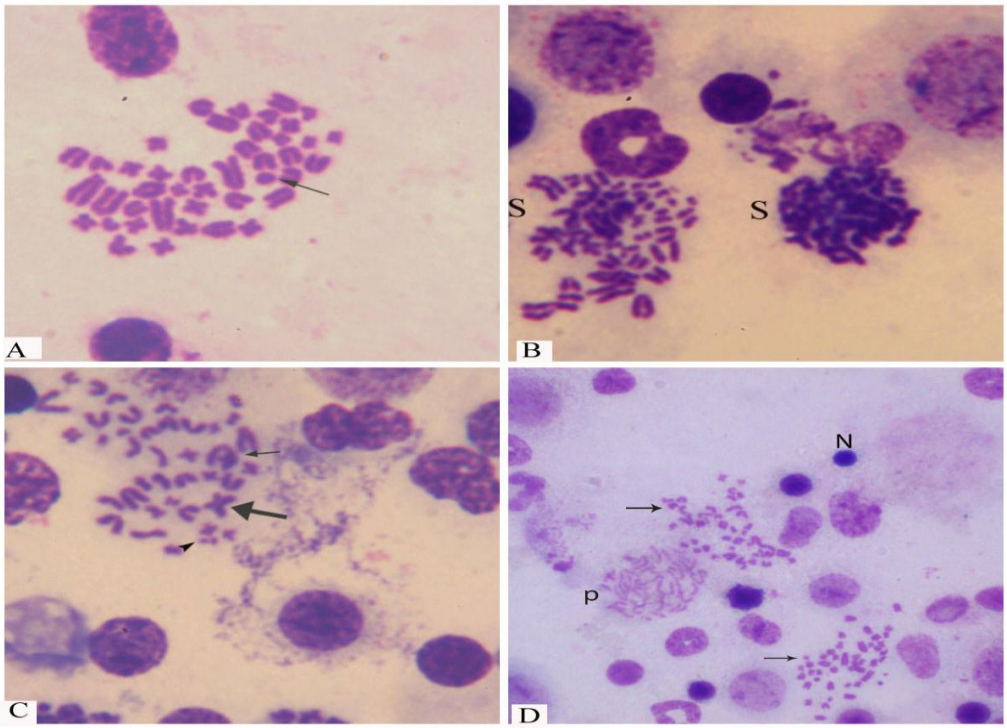

Figure: (4) Photomicrograph shows bone marrow chromosomal preparations of control and treated rats (Giemsa stain) (A): Metaphase spread showing ring chromosome (arrow) in bone marrow cells of etoposide-treated rats (×1000). (B): Metaphase spread showing sticky chromosomes (S) in bone marrow cells of etoposide-treated rats (×1000). (C): Metaphase spread showing fragment (head arrow), centric fusion (thick arrow) and gap (thin arrow) in bone marrow cells of etoposide-treated rats, (×1000). (D): Mitotic index of bone marrow showing stages of cell cycle in bone marrow metaphase (arrows), prophase (P) and nucleus (N) (×400).

Mitotic index in bone marrow

Figs. (5 & 4D) showed the mean values of mitotic index and stages of cell cycle in bone marrow cells of treated male rats. The results showed that etoposide significantly ($P \leq 0.01$) decreased the mean values of mitotic index (39.6±10.9) when compared with control group

(63.9±5.1). While fennel oil low and high doses (0.5 and 1 mg/kg b. wt.) were non-significant ($P \leq 0.01$) when compared with control (63.9±5.1) and significant ($P \leq 0.01$) when compared with etoposide group (39.6±10.9). The mean values of mitotic index of fennel oil low and high doses were (60.1±4.7 and 58.8±4.7) respectively. Animals received low dose of fennel oil (0.5 mg/kg b. wt.) in combination with etoposide showed an increase in mitotic index values (48.3±8.5) when compared with etoposide group (39.6±10.9) and showed a protective effect reached (35.8%). While animals treated with fennel oil high dose (1 mg/kg b. wt.) in combination with etoposide showed an increase in mitotic index values (59.2±2.8) which was non-significant ($P \leq 0.01$) when compared with control (63.9±5.1) and significant when compared with etoposide group. The high dose of fennel oil showed a protective effect against etoposide reached (80.6%).

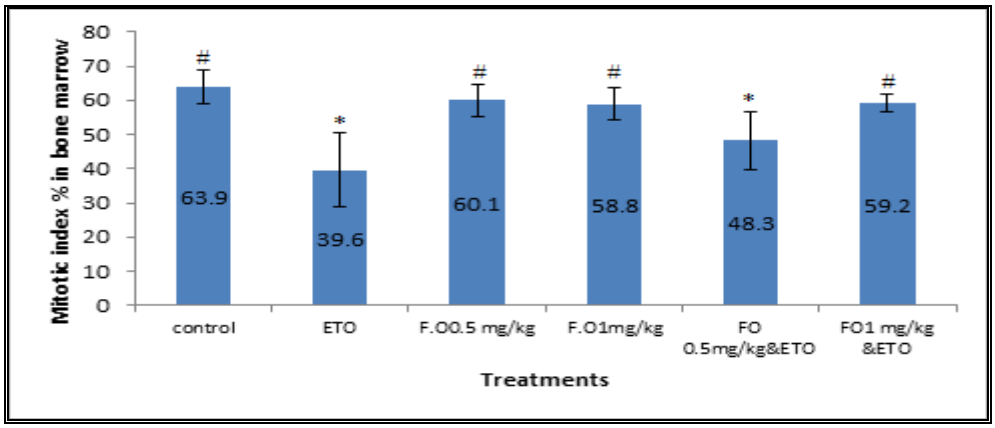

Figure (5): The effect of etoposide on mitotic index in bone marrow of rats and the protective effect of fennel oil. Data were presented as Mean ± S.D. *Statistically significant ($P \leq 0.01$) with respect to control. #Statistically significant ($P \leq 0.01$) with respect to etoposide. F.O: fennel oil; ETO: etoposide. n=6.

Mitotic index in testes

Figs. (6 & 7A) revealed the mean numbers of mitotic index and stages of cell cycle in testis cells of treated male rats. The rate of mitotic index in etoposide treated rats significantly ($P \leq 0.01$) decreased (17.8±4.3) (Fig. 7D) when compared with control group (62.7±8.29). While fennel oil low and high doses (Figs. 7 B &C) respectively, showed statistically significant ($P \leq 0.01$) increase (59.7±6.1 and 64.1±9.8) respectively, when compared with etoposide group and non-significant difference when compared with control group. The combination groups (low dose of fennel oil with etoposide and high dose of fennel oil with etoposide) showed increased in the mitotic values (31.4±3.4 and 36.7±4.9), (Fig. 7 E & F) respectively, when compared with etoposide group. Testes tissues restored their normal division rate although, some cells still arrested. The protective effect of fennel oil low dose against etoposide in combination group was (30.2%), while the high dose of fennel oil showed more protection in rat testis reached to (42%).

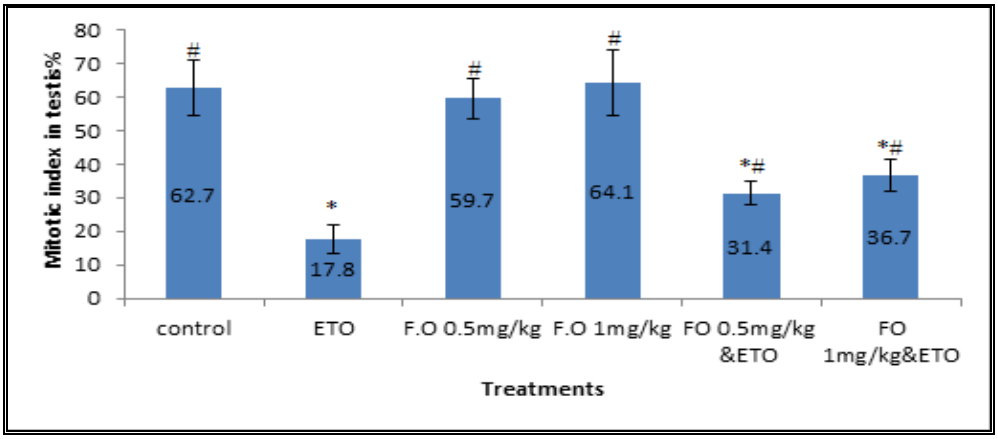

Figure (6): The mean value of testis mitotic index of rats treated with etoposide and the protective effect of fennel oil treatment. ETO: etoposide; F.O: fennel oil. Data were presented as Mean ± S.D. *Statistically significant ($P \leq 0.01$) with respect to control. # Statistically significant ($P \leq 0.01$) with respect to etoposide. F.O: fennel oil; ETO: etoposide.(n=6).

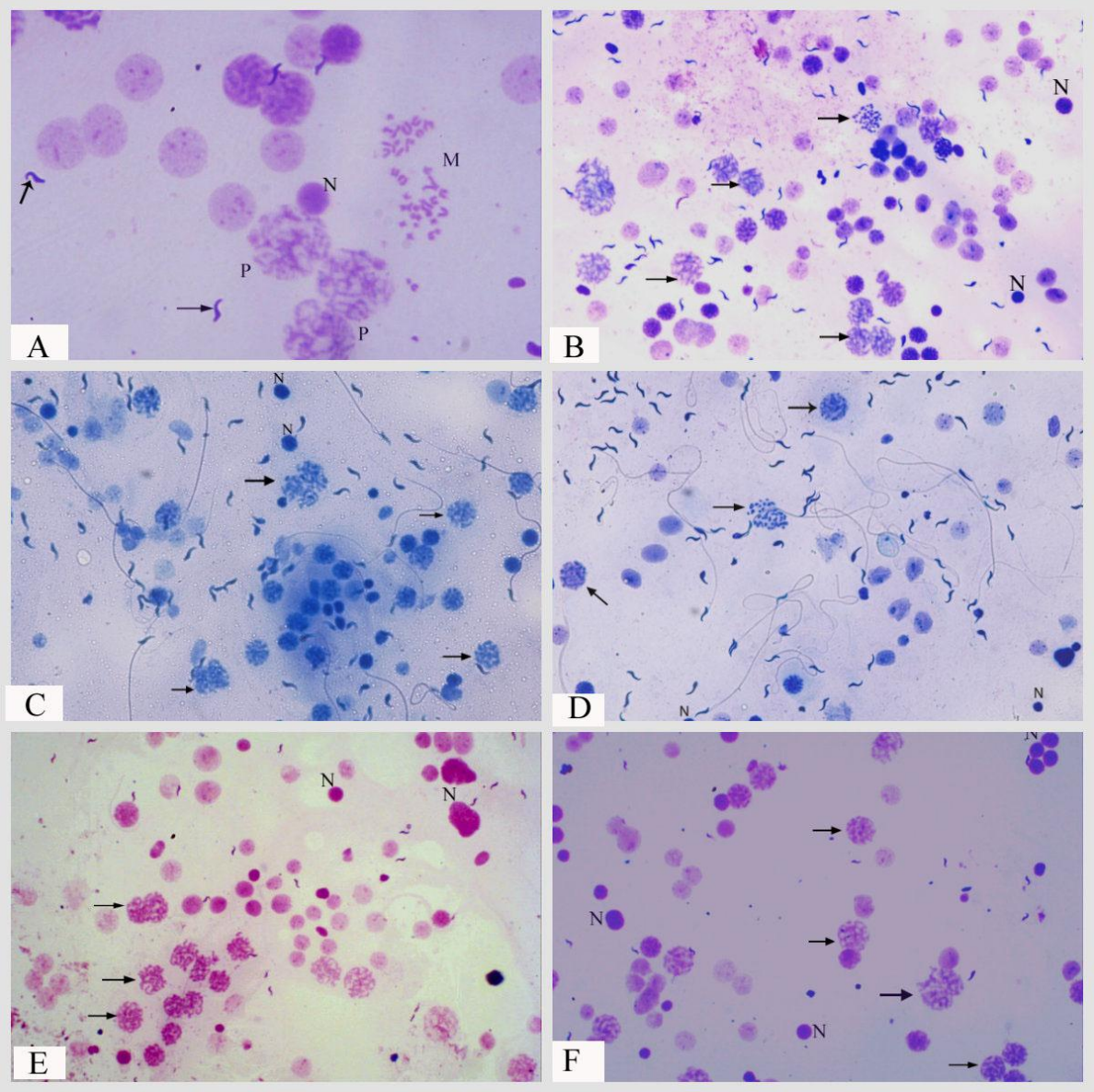

Figure (7): Photomicrograph shows mitotic index of testis of control and treated rats (Giemsa stain) (A): Mitotic index of testis of control rat showing stages of cell cycle in testis M: metaphase, P: prophase, N: nucleus and sperm head (arrows), (×400). (B): Mitotic index of testis in rats treated with fennel oil low dose showing many dividing cells (arrows) and resting cells (nucleus) (N), (×200). (C): Mitotic index in testis of rats treated with fennel oil high dose showing dividing cells (arrow) and nucleus (N), (×200). (D): Mitotic index in testis in rats treated with etoposide showing many nuclei (N) and few number of dividing cells (arrows), (×200). (E): Mitotic index in etoposide treated rat and fennel oil low dose (0.5 mg/kg b. wt.) at the same time. Many cells restored their normal division (arrows), some cells still resting (nucleus) (N), (×200). (F): Mitotic index of etoposide treated rats and fennel oil high dose (1mg/kg b. wt.) at the same time. Many cells restored their normal division (arrows). Some cells still arrested (nucleus: N), (×200).

3- Sperm head abnormalities

Normal sperm head (Fig. 9A). The evaluated head abnormalities were banana (Fig. 9A), without hook (Fig. 9B), hummer shape (Fig.9C) and amorphous in shape (Fig.9D).

Table (2) illustrated that etoposide increased sperm head abnormalities (68.9±2.17) and showed statistically significant difference ($P \leq 0.001$) when compared with control (18.8±1.27). There was no significant difference of abnormal sperm heads between control and fennel oil groups (low 16.9±0.69 and high 17.8±0.23). Fennel oil doses showed significant difference ($P \leq 0.001$) when compared with etoposide group (68.9±2.17). The combination of etoposide with fennel oil low dose decreased the ratio of sperm head abnormalities (28.6±2.3) when compared with etoposide group (68.9±2.17). The protective effect of fennel oil low dose against etoposide reached to (81.6%). The high dose of fennel oil was able to decrease percentage of abnormal

sperm heads to (30±2.45) in combination group compared with etoposide group (68.9±2.17). The protective effect of fennel oil high dose against etoposide reached (77.6%).

Table (2): The effect of etoposide on rat sperm head morphology and the protective effect of fennel oil treatments.

Groups	Sperm head abnormalities					
	Normal	Banana	Amorphous	Hummer	Without hook	% of total abnormal sperm head
Control	836.6±1.25	22.33±1.24	10±0.82	114±2.16	20.33±0.47	18.8± 1.27 #
Etoposide 5mg/kg	294.9±1.9	114.7±2.05	12.67±2.05	520.7±0.94	57.67±2.62	68.9±2.17*
Fennel oil 0.5mg/kg	861.6±3.29	20±0.82	3.46±0.38	96.67±1.24	19.5±0.82	16.9±0.69#
Fennel oil 1mg/kg	822±2.16	24.9±2.6	8.33±0.47	139.7±1.25	5.33±0.47	17.8±0.23#
F.O 0.5mg/kg and ETO 5mg/kg	720±0.81	20±3.2	23.9±2	180±4.08	57±1.6	28.6±2.3*#
F.O 1mg/kg and ETO 5mg/kg	701.3±3.6	24.6±0.81	28±3.2	185±1.6	63±2.4	30±2.45*#

Data were represented as Mean ±S.D. *Statistically significant ($P \leq 0.001$) when compared with control. #Statistically significant ($P \leq 0.001$) when compared with etoposide. F.O: fennel oil; ETO: etoposide. n=6.

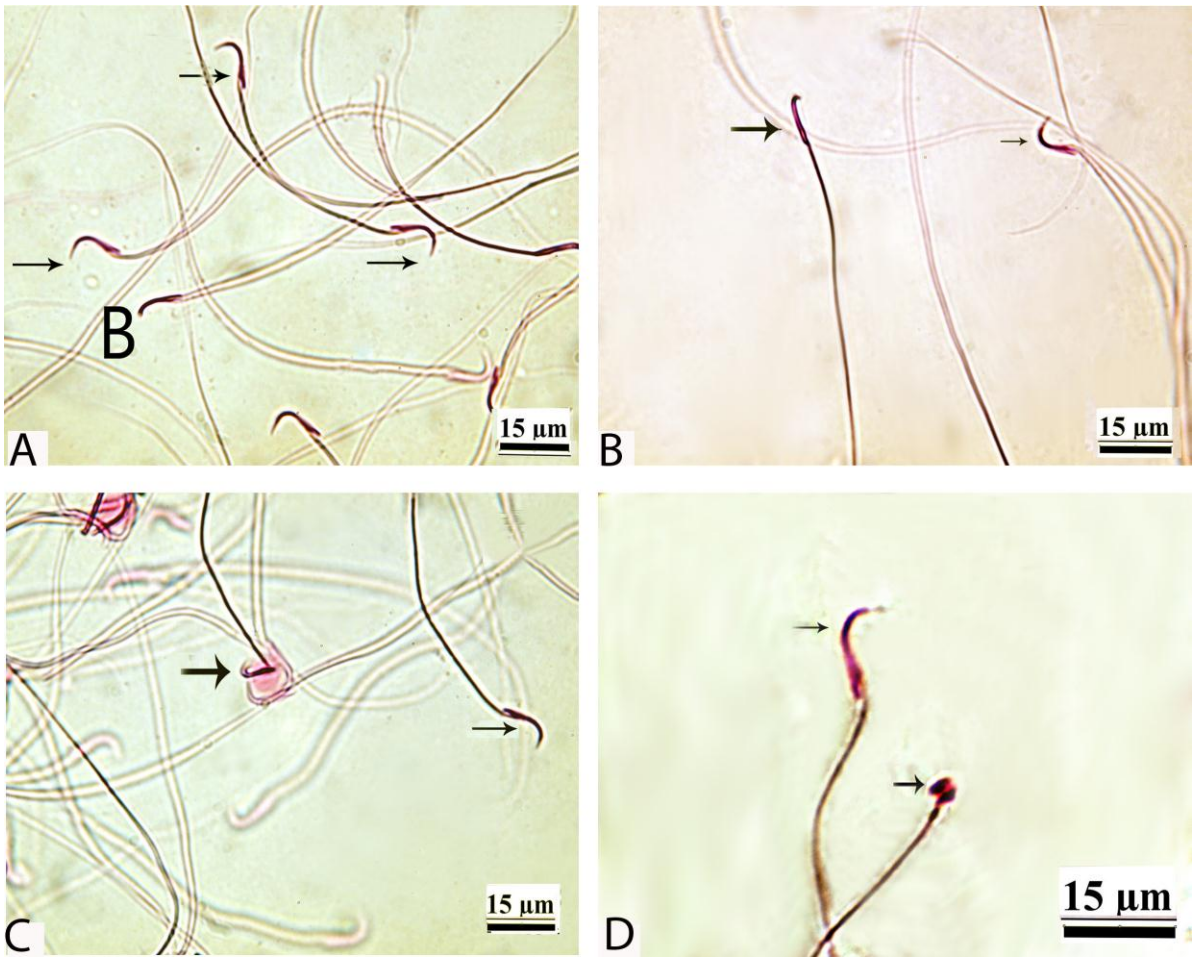

Figure (9): Photomicrograph shows sperm head of control and treated rats (eosin stain). (A): Normal structure of rat sperm (arrows) and banana shape sperm (B) (B): Abnormal shape of sperm head without hook (thick arrow) and normal one (thin arrow). (C): Abnormal shape of sperm head with hummer head (thick arrow) and normal one (thin arrow) D): Abnormal shape of sperm head with amorphous shape (thick arrow) and normal one (thin arrow).

Total protein contents in testis tissue

A marked increase in the level of total protein content in testis tissues of animals treated with etoposide (5.1±0.17) that showed statistically significant ($P \leq 0.01$) increase when compared with control (4.6±0.16) (Fig. 10). In the present study, animals treated with either doses of fennel

oil (low dose or high dose) showed non-significant increase in the level of total protein content in testes (4.6±0.26 and 4.7±0.21) respectively when compared with control group (4.6±0.12). While animals treated with fennel oil low dose (0.5mg/kg b. wt.) with etoposide was (4.4±0.20) that showed non-significant difference ($P \leq 0.01$) when compared with control group (4.6±0.18) and significant when compared with etoposide group. Animals treated with fennel oil high dose (1mg/kg b. wt.) with etoposide (4.6±0.08) showed non-significant difference ($P \leq 0.01$) when compared with control and significant difference when compared with etoposide.

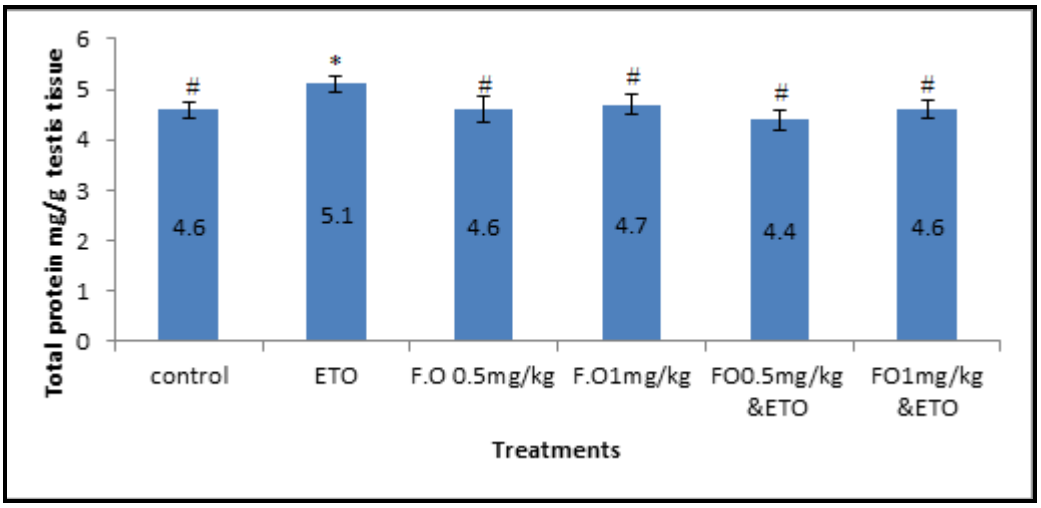

Figure (10): The mean values of protein contents in rat testis treated with etoposide and the protective effect of fennel oil. ETO: etoposide; F.O: fennel oil. Data were presented as Mean ± SD. *Statistically significant ($P \leq 0.01$) with respect to control. # Statistically significant ($P \leq 0.01$) with respect to drug. F.O: fennel oil; ETO: etoposide. n=6.

DISCUSSION

Cancer is one of serious diseases that lead to death all over the world. Chemotherapy is the treatment of cancer by cytotoxic drug which act by killing cells (that divide rapidly) non-selectively that causing injury to normal cells.[64]

In the present study, etoposide-treated animals showed alternations in extracted DNA from testis which associated with release of DNA fragmentation (appeared as DNA laddering pattern at diagnostic pattern of apoptosis). This may be due to toxic effect of etoposide on DNA that included inhibition of DNA synthesis by forming topoisomerase II (top II) DNA complex. Stabilization of this complex lead to formation of DNA strand breaks and DNA fragmentations yield from inhibition of DNA re–ligation that lead to cell death by apoptosis.[45,46,47,48] In addition, etoposide able to damage DNA due to formation of free radicals that induced oxidative damage to DNA.[49] Etoposide has highly affinity for chromatin and histone H1 protein, so chromatin is also considered as a target for etoposide.[50]

Cytoprotective substances were used in combination with chemotherapeutic drugs for preventing the damage caused to non-cancerous cells. In the present study, fennel oil was used for reducing etoposide toxicity and reclaims its side effects. Fennel oil low dose showed normal intact DNA, while the high dose of fennel oil showed a slight DNA fragmentation released and caused improvement in DNA fragmentation which has been induced by etoposide. There were many studies that suggested the mechanism of fennel oil to protect cells against toxicity. Fennel oil has anticancer and anti-inflammatory properties that appeared in its effect on intracellular signaling pathway called tumor necrosis factor TNF, fennel eliminates inflammatory cascade molecules called nuclear transcription factor NF-kappaB which regulate the expression of various genes that have important role in apoptosis.[51,52] In addition, fennel contain powerful compounds which enhance human immunity by enhancing natural killer cells that prevent cancer.[53,54,55] Anethole (the main components of fennel oil) suppress TNF that induced lipid peroxidation and has antigenotoxicity against chemotherapy.[56,57] In addition, it was found other two protective substances in fennel oil (*d*-limonene and β-myrcene) that proved their chemoprevention effect.[19] Fennel contains little amounts of polyacetylene in non- polar extracts that have cytotoxicity against lymphoplastic cell lines.[58] Moreover, fennel oil may be succeeded in inhibition of DNA damage induced by etoposide due to their antioxidant propriety. This due to high contents of fennel oil of polyphenols and flavonoids.[59]

In the present study, etoposide induced structurally chromosomal aberrations in bone marrow cells (somatic cells) that may be due to their rapid division and their high contents of top II that was considered the main target of etoposide. Cleavage complexes are important for top II to perform its function. Lowering the cleavage complex concentration (lowering the enzyme level) leads to cell failure to make chromosomal segregation so mitotic failure.[60,61,62] Etoposide has the ability to induce

chromosomal aberrations in normal and malignant cells, fragmentation of chromatin, sister chromatin exchange and micronuclei.[63,47] Etoposide administration accompanied with secondary tumors as secondary myeloid leukemia specially in childhood cancer.[64] In agreement with the present study, it was found that etoposide induced chromosomal aberrations in mice bone marrow at doses (5, 10, 15, and 20 mg/kg b. wt.).[65] In contrast, other observations confirmed that etoposide failed to induce chromosomal aberrations in mice bone marrow at doses (10, 15 mg/kg b. wt.) and caused chromosomal aberrations at higher dose (20 mg/kg b. wt.) in female mice[66] and male mice.[67] This difference yield from changing the harvest time. Similarly, it was observed that etoposide clastogenisity was concentration and time dependent.[68] Determination the dose is very important in medicine even if using medicinal plant. The over dose of medicinal plants don't cure the illness only but it can cause damage itself. In the present study, fennel oil low dose (0.5 mg/kg b. wt.) was effective dose and showed normal structural chromosomes near to control group. While the high dose of fennel oil showed slight toxicity on structural chromosomal aberrations which higher than the control. This may be due to estragole (one of the components of fennel oil) in fennel oil which consider a good alkylating reagent that alkylate DNA molecule and form a covalent bond with DNA bases so, it may be consider carcinogen.[69] It was found that the high doses of pure estragol was genotoxic dose. Moreover, it was found that anethole and flavonoids present in fennel have a protective role which reclaim estragol effects in low does.[70] Fennel has a protective effect on chromosomes of bone marrow of mice (dose dependent effect).[71] Fennel seeds and anethole able to reduce the chromosomal aberrations induced in mice bone marrow.[72] Anethole enhance coffee antigenotoxicity.[73]

In the present study, mitotic index of etoposide-treated rats showed a significant decrease compared with control this may be due to etoposide arrest cells division and prevent metaphase in somatic cells. Proliferating bone marrow cells are very sensitive and affected by chemotherapy. This is due to the S-phase dependent effect of these drugs.[63,74] Accumulated DNA breaks caused by etoposide prevent the cell to inter the mitotic phase in cell division. Inhibition of top II function slows down cell cycle progress at S phase and cause cells to be arrested at the G_2 phase which delay entry in to mitosis then cause the cell death.[75,76,16]

Fennel oil succeeded to restore mitotic index values to normal range. Fennel oil high dose showed more obvious improvement in combination with etoposide than the low dose this may be due to antioxidant propriety of fennel oil.[77]

The results of mitotic index of testis indicated that germ cells were more sensitive to etoposide than somatic cells and showed a significant inhibition in the percentage of mitotic index in testis when compared with control. Chemotherapeutic agent can easily reach to the proliferating epithelium of seminiferous tubules and harm the later stages of differentiated germ cells which are very sensitive to cytotoxic agents.[78] Etoposide affected mitotic index of testis cells may be due to testis cells contain high amounts of topoisomerase II that is required for spermatogenesis this make testis cells good target for etoposide.[79] Etoposide caused the death of proliferating cells including the male germ cells and increase the number of apoptotic germ cells during adulthood and pre-puberty.[33] Etoposide induce chromatid breaks 4 hours after administration in dividing mouse spermatogonia.[67] Structural aberrations and aneuploidy were observed in germ cells of mice treated with etoposide and induced heritable chromosomal abnormalities in male germ cells at clinical doses that yield abnormal reproductive outcomes.[80] More studies proved that etoposide make a significant mutagenicity in primary spermatocytes which normally undergo meiosis.[81]

In the present study, fennel oil low and high doses showed non-significant differences on mitotic index values in testis compared with control group. A marked increase in mitotic index values was observed in testis of animals treated with etoposide with either doses of fennel oil (low or high). Fennel extract elongated the germ cell division of rats and increase metaphase stage of testis cells.[82] Fennel seed and anethole inhibited chromosomal aberrations in germ cells of mice.[72]

Fennel oil contain other compounds as limonene, afrol, α pinene, camphene β-pinene, β-myrcene, α- phellandren, P-cymene, 3-carnene, camphor and cis-anethole, volatile oils, anisic acid, anisic aldehyde, salts Na, K, P, coumarine and vitamins A, C, α-terpineol which have biological functions and give fennel oil its protective effect.[18,77]

The present study showed that etoposide cause elevation in sperm head abnormalities which restored to normal level after treatment with each dose used of fennel oil. Etoposide statistically increase sperm head abnormalities may be due to free radicals species released from etoposide administration. Similarly, it was reported that sperm number and motility affected by free radicals as reactive oxygen species and increasing lipid peroxidation measured by MDA level.[83] In agreement with the present study it was confirmed that etoposide in combination with other drugs induced toxicity to germ cells which impair sperm count, motility and morphology due to the oxidative stress induced.[84] Etoposide administration resulted chromosomally abnormal sperms.[80,67] Animals treated with fennel oil showed a degree of productivity may be due to antioxidants substances in fennel oil that improve the activity of sperm defense antioxidant system. Similarly to the finding.[35,85] In contrast, it was found that high doses of fennel affected sperm's motility and fertility

decreased.[86] Oral administration of fennel oil inhibited sperm abnormalities caused by cyclophosphamide.[31] More studies confirmed that fennel extract increase fertility which consider a novel medicine for treatment infertility. It increase sex hormone in female mice and improve oxidative status in serum.[87] Fennel seed and pure anethole were used for improvement sperm head abnormalities.[72]

In the present study, animals treated with etoposide showed increase in protein level this is may be due to disorders in protein metabolism[88] or due to evaluation of serum enzymes.[20] Etoposide increase total protein in rat kidney.[89] In agreement with the current study, it was proved that total protein revealed to its normal values in rats treated with fennel oil.[22,20] Similarly, co-administration of fennel oil with dill oil maintained the normal levels of total protein.[90]

CONCLUSION

The present study concluded that fennel oil has a protective potential against etoposide-induced genocytotoxicity in male albino rats.

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