



Organ Toxicity and Estrogen Like Effects of Cuminum Cyminum.L Seed Essential Oil: A Hormonal, Histopathological and Immunohistochemical Study in Female Mice

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Abstract: Background: Cumin (*Cuminum cyminum* L.) seeds possess many nutritional values and a wide range of dietary and therapeutic applications. Hypothesis: Long-term administration of cumin caused toxic effects and infertility in male rodents but its hormonal effects in females have been remained unclear. Purpose: We aimed in the present study to determine the acute and repeated dose toxicity of cumin essential oil with investigating the biochemical, hormonal and histopathological effects in female mice. Study Design and Methods: After providing the essential oil and analyzing by GC_MS analysis, toxicity assessments performed by OECD 425 and 407 guidelines. We used Chemiluminescence Immunoassays and immunohistochemical assessments for determination of serum levels and tissue levels of steroids. Results: Acute test didn't show any sign of toxicity in doses up to 2000 mg/kg but in repeated dose test, hepatotoxic and nephrotoxic effects were recorded by biochemical and histopathological evidence. Significant weight gains in all dose groups ($p < 0.001$) which were exposed to cumin essential oil showed its obesogenic effects but significant weight gains in the uterus ($p = 0.001$), mild edema in endometrium, moderate edema in perimeter layers of animals which were exposed to low and medium cumin doses, as well as its hyperplastic effects in endometrial epithelium of high dose group suggested the endocrine disrupting effects which was confirmed by marked estradiol elevation and antiprogesterone properties in all dose groups.

List of Abbreviations

GC/MS analysis: Gas chromatography/Mass spectrometry; OECD: Organization for Economic Co-operation and Development; IAUPS: Islamic Azad University, Pharmaceutical Sciences Branch; CCAC: Canadian Council on Animal Care; NIH; National Institutes of Health

Keywords: *Cuminum cimum. L;* Essential oil, Endocrine disruptors; Estradiol; Progesterone; toxicity

1. INTRODUCTION

Cumin (*Cuminum cyminum* L.) seeds are active reservoirs of numerous bioactive compounds with considerable nutritional values, various health benefits and a wide range of dietary and therapeutic applications(1) .It is also a globally popular flavor for many cuisines, particularly in India, Middle East countries, Mediterranean region, China, South Asia, Northern Africa and Latin American countries (2) because this small and aromatic herb of the family of Apiaceae enhances the appetite, taste perception, digestion, vision, strength and lactation(3). Nowadays this plant has been suggested for the treatment of cardio metabolic diseases (4), obesity(5), fever, diarrhea, vomiting, abdominal distension, edema and puerperal disorders (6).

Cumin seeds contain around 5% volatile oil including Cymene, terpenoids, and Cumin aldehyde which have been identified as the major volatile components of cumin seed in combination with other related

aldehydes(7). In 1992the anti-carcinogenic activity of Cumin aldehyde was suggested and it was introduced as a potent inhibitor of both squamous cell carcinoma and hepatomas(8) .Cumin essential oil has been approved for several pharmacological activities, such as antibacterial , antifungal and antioxidant effects ,anti-nociceptive and anti-inflammatory effects , anti-obesity (9) ,anti-diabetic(10) ,antihyperlipidemic , hepatoprotective (11) and cough suppressant (12)properties in herbal medicine, .Although in rodent models , Cuminum cyminum essential oil showed antiepileptic, antidiarrheal(13) and antihypertensive (14) effects, its long-term administration caused toxic effects in male mice and according to a recent study(15), Cuminum cyminum extract reduced the testosterone levels, spermatogenesis and caused infertility in male rats (16). We aimed in the presentstudy to recognize the constitutes of *Cuminum cyminum* seed's essential oil and to investigate its possible toxic effects in acute and repeated dose models as well as its endocrine effects in female mice.

2. MATERIALS & METHODS

2.1.Plant Material

Cuminum cyminum samples were prepared locally from herbal dealers in Tehran and seeds were compared with their receptive standards in the herbarium that were in the Department of Pharmacognosy, Islamic Azad University, and Pharmaceutical Sciences Branch. The samples were stored in the standard condition in Pharmaceutical Sciences Research Center for the preparation of essential oil and other studies.

2.2.Essential Oil

Cumin (400 g) was powdered and submitted to hydro distillation in a Clevenger-type apparatus for 3 hours. At the end of distillation, the essential oil was collected, dried with anhydrous Na₂SO₄, measured, and transferred to clean glass vial and kept at a temperature of -18°C for further analyses.Essential oil sample analysis was performed on a Hp-6890 gas chromatograph (GC) equipped with a FID and a DB-5 capillary column, 30 m × 0.25 mm, 0.25 µm film thickness at a temperature of 60 °C increased by 4°C/min up to 240 °C. The carrier gas was N₂ at a flow of 2.0 ml/min; injector port and detector temperature were 250°C and 300°C, respectively. The operating conditions were the same as those described above but the carrier gas was He. Mass spectra were taken at 70 eV. Scan mass range was from 40-400 m/z at a sampling rate of 1.0 scan/s. Quantitative data were obtained from the electronic integration of the FID peak areas. The components of the sample were identified by their retention time, retention indices, relative to C₉-C₂₈ n-alkanes, computer matching with the WILEY275.L library and as well as by comparison of their mass spectra with data already available in the literature. The percentage of composition of the identified compounds was computed from the GC peaks areas without any correction factors and was calculated relatively. The result of the oil analysis is the average of three replicates (17).

2.3.Experimental Animals and Housing Conditions

Studies were carried out using female mice (6-8 weeks old, 25-30 g), obtained from Animal house of Tehran University of Medical Sciences, Iran. Mice were kept in cages under standard condition (temperature 25±2°C) with 12 hours light/dark cycle. They were provided with standard pellet diet and free access to drinking water ad libitum. The animals were acclimatized to the environment for a week before the commencement of the experiment. The cage cleaning schedule, air filtration and recirculation, health checks and facility maintenance were carried out in accordance with the IAUPS Standard Operating Procedures, and such activities were recorded in the animal room records. Animals were housed and maintained according to the Ministry of Health of Iran for the Care and Use of Laboratory Animals, CCAC Guidelines for Care and Use of Experimental Animals and IAUPS Standard Operating Procedures. An investigation using experimental animals contained a statement confirming the adherence of the research to the Principles of Laboratory Animal Care published by NIH (Committee for the Update of Guide for the Care and Use of Laboratory Animals, 2010) and approved by the ethical committee of IAUPS.

2.4.Acute Oral Toxicity Test

In this study, single oral doses of cumin essential oil (2000 mg/kg) were administered by oral gavages to 6 female mice after the randomized division of animals to two groups(3 animals in each group).Group one received the essential oil with the olive oil as the solvent and group two received this essential oil with distilled water. Mice were observed for mortality and any sign of toxicity for 14

hours and also signs of toxicity and weight changes were investigated during 14 daily follow-up protocol. This study was done according to OECD 425 toxicity assessment guideline (18), (19).

2.5. Repeated Dose Oral Toxicity Study

Twenty-five mice were randomly divided into 5 groups (5 mice in each group; 25,250, 500, 1000 mg/kg and control). All the groups were administered orally from the essential oil on the basis of their body weights once daily for 6 days per week over a period of 14 days for 250,500,1000mg/kg dose groups and 28 days for 25mg/kg dose group. Control groups received equal volumes of distilled water daily. This study was done according to OECD 407 toxicity assessment guideline (20).

2.6. Clinical observations

Clinical signs (Lack of desire to drink water, Lack of desire to eat, lack of response to environmental stimuli, no treatment about the surface of body, failure to clean the body surface, color changes of body hair, body hair loss, drowsiness, stool color change, change of urine color, bringing up the tail, abnormal gait, ataxic gait, change of eyes and skin color, salivation, tearing, ness eyes and death) and weight changes were recorded once daily. Water and food consumption were measured during all periods of each study.

2.7. Histopathological Studies

All animals in each dose groups of 25,250,500,1000 mg/kg were considered for histopathological studies which mean liver, lung, heart, kidney and uterus were removed from the animals at the midpoints for 250,500,1000 mg/kg groups and at the endpoint for 25 mg/kg group. Organ weights were recorded and compared in each group with related control, then the intact organs were fixed in 10% buffered formalin and dehydrated in graded series of alcohol, cleared in xylene and embedded in paraffin wax. Multiple sections from each block were prepared at 5 micrometers and stained with hematoxylin and eosin (H&E).

2.8. Hormonal Studies

Blood samples were collected according to standard protocol and the plasma was separated by coldcentrifuge and collected in micro tubes. Estradiol and progesterone hormones levels were measured at all dose groups at the midpoint (250,500,1000mg/kg) and endpoint(25 mg/kg) of study and compared with control by Chemiluminescence Immunoassays(CLIA) method using Cobas E411 from Roche company with a serial number of 15D3-16 in a collaborating private laboratory.

2.9. Immunohistochemical Studies

As previously described (21) dewaxed and rehydrated tissue sections were subjected to antigen retrieval using microwave oven and boiling citrate buffer (pH =6.0). Endogenous peroxidase activity and nonspecific binding sites were blocked by incubating sections in 0.3% hydrogen peroxide in methanol for 30 min. and 3% BSA for 60 min., respectively. Sections were then incubated 30 minutes at Room Temperature with Estrogen Receptor (ER,Clone 105,Dakocytomation), and Progesterone Receptor (PgR ,Clone 636 , Dakocytomation) , that recognize the nuclear and cytoplasmic expression of human and rodent proteins in uterus tissues. The results were visualized using the envision system (Dakocytomation) based on the manufacturer's instruction with necessary modifications. Sections were also counterstained with Meyer's haematoxyline. In each series, a section in which incubation with the primary antibody was omitted used as negative control. The ideal staining conditions were established in our preliminary experiments. Staining was considered negative only after careful examination of the entire tissue section.

2.10. Statistical analysis

In this analysis, treatment and control groups were compared with each other. When variances were not significantly different data were analyzed by one-way analysis of variance (ANOVA) and the Student's t-test. Values were expressed as means \pm SD. The level of significance was set at $p < 0.05$. All statistical methods were performed by SPSS 21.

3. RESULTS

3.1. GC/MS Analysis

The hydro distillation of cumin gave pale yellow essential oil with pleasant odor. Table 1 shows the list of compounds whose GC/MS concentration is not less than 0.1% of total peak concentration.

Twenty-three components were identified in the seeds essential oil which represented about 94.5% of the total composition. The components of the essential oil were identified as 1-phenyl-1-butanol (40.8%), cuminal (28%) and 1-Isopropylidene-3-n-butyl-2-cyclobutene (9.9%).

Table1:GC-MS Analyses of C. Cyminum Fruits Essential Oil.

Compound	KI ^b	KI ^c	% (fruits)
β-Pinene	981	979	2
α-Phellandrene	1001	1003	0.3
o-Cymene	1021	1026	1.5
Limonene	1030	1029	0.2
γ-Terpinene	1058	1060	4
trans-Dihydrocarvone	1206	1201	0.3
Cuminal	1238	1242	28
trans-p-menth-2-ene-7-ol	1257	1254	0.5
1-Isopropylidene-3-n-butyl-2-cyclobutene	1271	-	9.9
1-Phenyl-1-butanol	1297	-	40.8
p-Mentha-1,4-diene-7-ol	1311	1306	0.7
trans-Caryophyllene	1421	1418	0.2
trans-β-Farnesene	1455	1458	0.3
epi-Bicyclosesquiphellandrene	1477	1480	0.2
α-Curcumene	1486	1483	0.4
Zingiberene	1501	1495	0.2
Elemol	1553	1547	0.2
Caryophyllene oxide	1581	1583	0.1
Carotol	1591	1594	0.5
Dill apiol	1625	1621	0.4
β-Eudesmol	1653	1649	0.2
7-epi-Amiteol	1660	1656	2.5
β-Tumerone	1672	1669	1.1
Total			94.5

^aCompounds listed in order of elution.

BKI (Kovats index) measured relative to n-alkanes (C9-C30) on the non-polar DB-5 column under condition listed in the experimental section.

CKI, (Kovats index) from literature

3.2. Acute Oral Toxicity Studies

Recording clinical signs, no death and no sign of toxicity were recorded in the first 24 hours of administrations using two different solvents. Normal physical activities were recorded in all animals during the next 14 days follow-up period.

3.3. Repeated Dose Toxicity Study

3.3.1. Survival and Clinical Signs

Animals survived healthy in dose groups of 250,500 and1000 mg/kg for 14 days period of study in water as a solvent group. Normal physical activities were recorded in all animals during this period of study.

3.3.2. Total Body Weight

As explained in Table 2, significant weight gains were detected in all high and medium dose treatment groups when compared with control despite using equal levels of water and food during this 14-day study.

Table2: Changes in Water and Food Consumption and Total Body Weight after 14 and 28 Days Oral Administration of Cseo li Four Dose Groups Presented by Mean (Sd)

Variables	Control	25mg/kg	p-value	250mg/kg	p-value	500 mg/kg	p-value	100 mg/kg	p-value
Weigh	24.31	28.79	<0.000	30.14	<0.000	29.91	<0.0001**	29.35	0.001*

Organ Toxicity and Estrogen Like Effects of Cuminum Cyminum.L Seed Essential Oil: A Hormonal, Histopathological and Immunohistochemical Study in Female Mice

t(g)	(3.11)	(2.78)	1***	(1.44)	1***	(1.76)	*	(1.05)	**
Water(ml)	190.38 (78.83)	210.69 (97.85)	0.725	209.53 (134.88)	0.725	223.07 (77.04)	0.673	236.76 (191.45)	0.469
Food(g)	67.23 (36.51)	74.26 (35.11)	0.627	77.26 (46.11)	0.627	66.68 (47.45)	0.740	53.63 (33.77)	0.295

Ranges of p-values were explained under following conditions:

*: 0.05>p-value>0.01

** : 0.01>p-value>0.001

***: 0.001>p-value

3.3.3. Necropsy Studies

After careful considerations, absolute and relative weights of heart, kidney, liver, ovaries, lungs and uterus were recorded at the midpoint of study. The absolute and relative weights of most of the organs remained unchanged from corresponding control groups and no gross changes were observed except the weight of uterus which was increased significantly (p=0.001) as described in Table 3.

Table3: Organ Weights in Different Dose Groups

Variables(g)	25mg/kg	250mg/kg	500mg/kg	1000mg/kg	control	p-value
liver	1.20 (0.141)	1.20 (0.141)	1.20 (0.13)	1.13 (0.098)	1.06 (0.165)	0.456
Lung	0.210 (0.00)	0.210 (0.00)	0.200 (0.11)	0.19 (0.2)	0.176 (0.011)	0.203
Kidney	0.245 (0.007)	0.245 (0.007)	0.260 (0.15)	0.235 (0.007)	0.238 (0.008)	0.806
Uterus	0.083 (0.004)	0.085 (0.007)	0.070 (0.12)	0.075 (0.007)	0.038 (0.013)	0.001**
heart	0.195 (0.006)	0.205 (0.007)	0.190 (0.16)	0.190 (0.014)	0.190 (0.015)	1.00

*: 0.05>p-value>0.01

** : 0.01>p-value>0.001

***: 0.001>p-value

3.4. Histopathological Studies

Pathological studies were performed at day 14 on all resected organs of 1000,500,250 mg/kg doses and at day 28 for the dose of 25 mg/kg.

Organ effects of Cuminum Cyminum essential oil in high and medium dose groups (x40)

3.4.1. Liver

In hepatic tissues of high dose group (1000mg/kg) necrosis of hepatocytes were seen. In medium doses (500 and 250) mg/kg, focal infiltration of mononuclear cells and hydropic degeneration of hepatocytes were detected (Fig 1A, 1B).

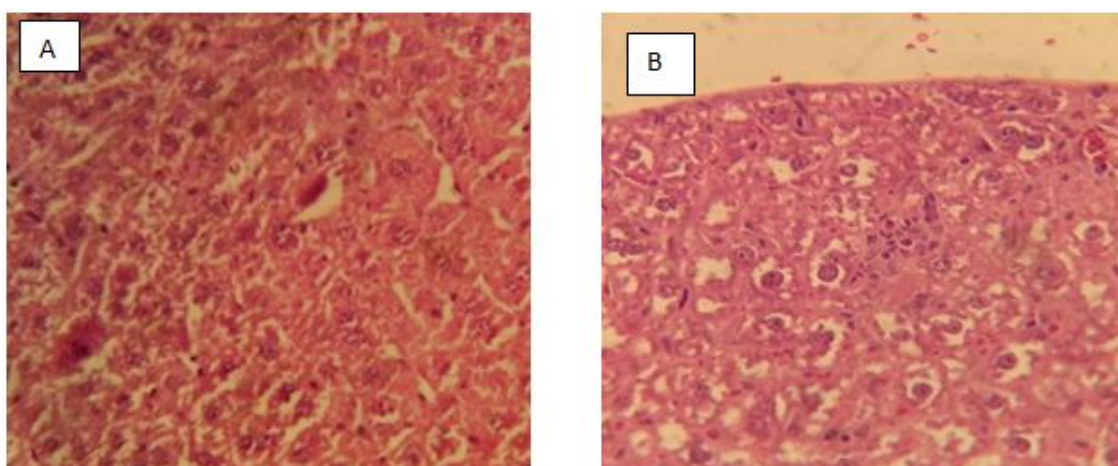


Fig1. A: Hepatic Tissue With Necrosis of Hepatocytes (1000mg/Kg), B: Hepatic Tissue, with Focal Infiltration of Mononuclear Cells (250mg/Kg)

3.4.2. Lungs

In pulmonary tissues of high dose group (1000mg/kg) accumulation of mononuclear cells in the alveolar duct and mild congestion were detected. Pulmonary tissues of lower doses remained normal (Fig 1C).

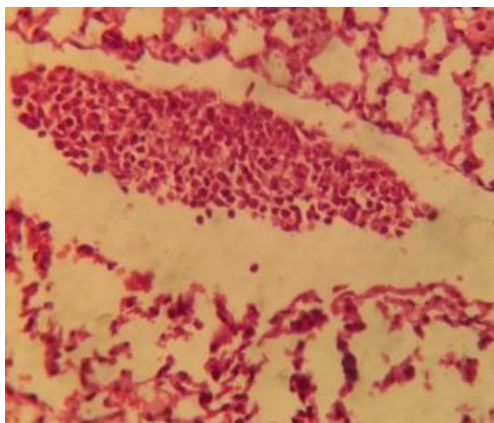


Fig1C. Accumulation of Mononuclear Cells in the Alveolar Ducts (1000 mg/kg)

3.4.3. Kidneys

Mild degeneration of proximal tubules and renal tissue with epithelial necrosis in proximal tubule was recognized in high dose group. Hydropic degeneration of proximal tubules in 500 mg/kg and multifocal mononuclear cells infiltration in 250mg/kg dose groups were identified too (Fig 1D).

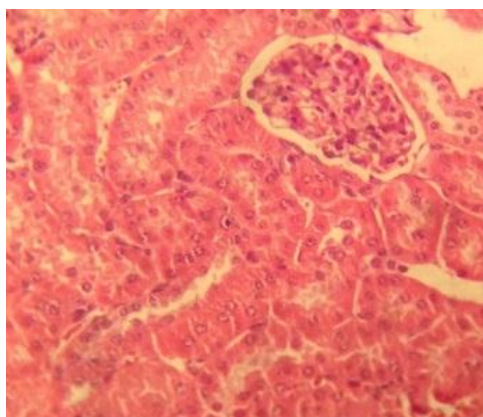


Fig1D. Epithelial Necrosis in Proximal Tubules of Right Kidney (1000mg/Kg)

3.4.4. Hearts

Normal structures were seen in the hearts of animals in different dose groups (Fig 2A).

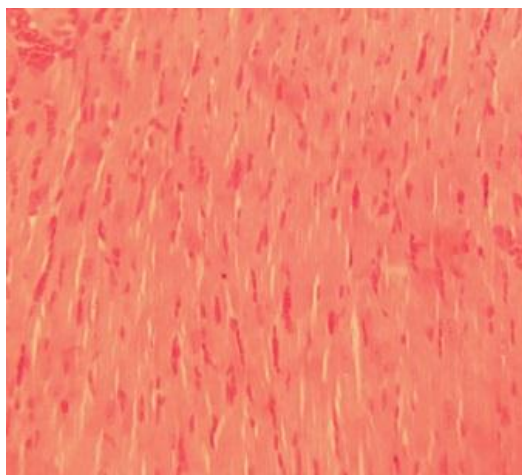


Fig2A. Cardiac (1000 Mg/Kg) with Normal Structures in All Dose Groups after 14 and 28 Days Oral Administration of CSEO, A: Cardiac Tissue 1000 Mg/Kg

3.4.5. Ovary

Normal structures were seen in the ovaries of animals in different dose groups (Fig 2B).

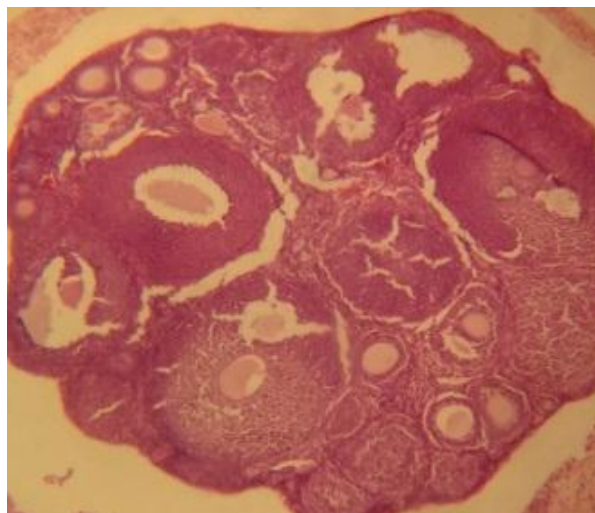


Fig2B. Ovarian Tissues (1000 Mg/Kg) with Normal Structures in All Dose Groups after 14 and 28 Days Oral Administration of CSEO

3.4.6. Uterus

Although mild edema in endometrium and moderate edema in perimeter layer of low and medium doses observed, respectively, the surface of endometrial epithelium was hyperplastic in high dose group (Fig 3 A, B).

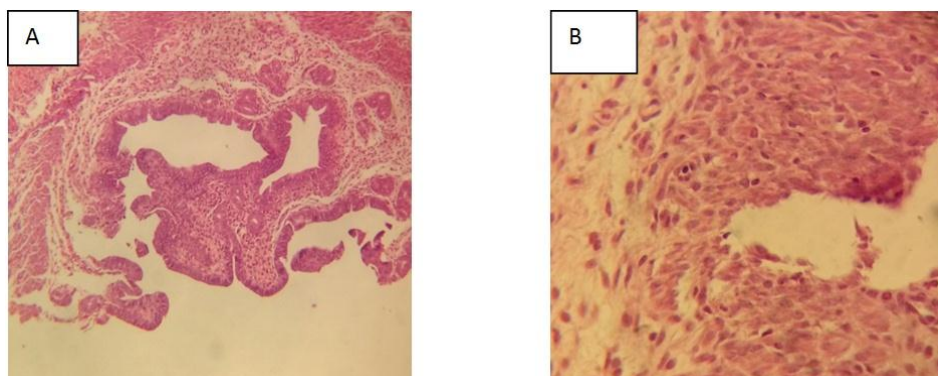


Fig3. Toxic Effects of Cuminum Cyminum Essential Oil on the Uterus of Female Mics (X40)

A. Uterus Tissue with Normal Structure Showed Moderate Edema in Endometrial Stroma and the Surface of Endometrial Epithelium was Hyperplastic.

B. in All Lower Doses Moderate Edema in Uterus Perimeter Layer was seen.

3.5. Hormonal Studies

3.5.1. Steroid Hormones:

Our studies showed a significant increase in estradiol levels ($p < 0.001$) in all doses except 25mg/kg and a significant decrease in progesterone levels in all dose groups ($p = 0.001$) in comparison to control group (Table 4).

Table4. Progesterone and Estradiol Hormone Levels in Control, 25,250,500 and 1000mg/Kg Dose Groups.

Variables	Control	25mg/kg	250mg/kg	500mg/kg	1000mg/kg	p-value
Progesterone (µg/ml) Mean (SD)	3.10 (0.10)	1.50 (0.40)	1.40 (0.70)	3.0 (0.01)	2.90 (0.42)	<0.001**
Estradiol (ng/ml) Mean (SD)	17.2(7.26)	16.20 (4.8)	34.58 (9.71)	94.6.0 (41.24)	46.14 (25.28)	0.001*

3.6. Immunohistochemistry

Nuclear expression of PgR was found in the ovaries and uterus of low dose groups without any significant difference with control although estrogen receptor was highly overexpressed in all uterus

slides of low dose (25mg/kg) group compared with negative control (Fig 4 A, B). The same pattern was found in the ovaries of low dose group when compared with negative control (Fig 4C, D).

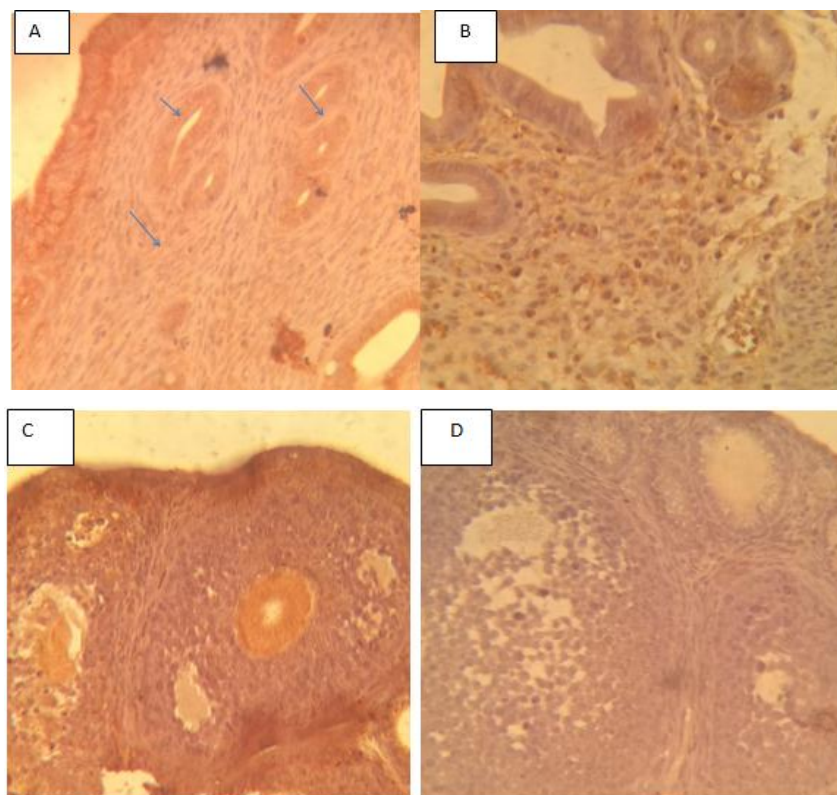


Fig4. Immunohistochemical Expression of Progesterone Receptor (PR) in Uterus and Ovary of Low Dose (25mg/Kg) and High Dose (500 Mg/Kg) Group of Animals

- A. Endometrial overexpression of PR in 25 mg/kg (x 250)
- B. Overexpression of PR in endometrial tissue of 500 mg/kg (x400)
- C. Mild ovarian expression of PR in in 25 mg/kg (x 400)
- D. Negative expression of PR in ovarian tissue of 500 mg/kg group(x400)

4. DISCUSSION

There are numerous natural products and medicinal plants that are reported to disrupt the normal functioning of the endocrine system. They may produce adverse health effects in hormone-responsive target tissues and other organs in both humans and animals (22). For centuries, as a folk medicine throughout the world, recent studies showed its potent hormonal effects with contraceptive efficacy (16). Cumin derived products are frequently used in over-the-counter pharmaceuticals, weight loss, and food products for a variety of health benefits as described before. Due to existing initial data on cumin endocrine effects, we have proposed this study to provide sufficient information on toxicity and hormonal effects of cumin essential oil as a routine dietary agent with considerable nutritional values, suggested weight loss properties and beneficial effects on insulin metabolism compared with orlistat120 and placebo (5).

After providing the essential oil and analyzing using Gas Chromatographic _Mass Spectral (GC_MS) analysis, standardization and toxicity assessments in acute and repeated dose models were performed. Although this essential oil showed hepatotoxic and nephrotoxic effects at high and medium doses, it was considered safe in acute and repeated dose (28 days study) in doses lower than 25 mg/kg from clinical, biochemical and histopathological viewpoints. On the other hand, hormonal studies showed another picture from this routine dietary factor according to the following observations:

All dose groups showed significant weight gains despite using equal ranges of food and water comparable with control. Although recent studies showed the clinical benefits of its powder (22) and methanolic extracts (5) in two clinical settings for weight loss program, our study in female mice

showed opposite effects. Significant weight gains in all dose groups ($p < 0.001$) which were exposed to cumin essential oil by oral administrations even after a short-term (28 days) repeated dose study showed its obesogenic effects in female animals clearly. It seems that abnormal weight gain in the present study is mainly due to an unbalanced energy intake and expenditure, which could be a consequence of disrupting endocrine functions of this agent.

Although the organ weights remained unchanged in all dose groups (table 3), we detected significant organ weight gains in the uterus of female mice. Mild edema in endometrium and moderate edema in perimeter layers of animals which were exposed to low and medium cumin doses, as well as hyperplastic effects in endometrial epithelium of high dose group (Fig 1), confirm the endocrine disruptive effects of cumin essential oil as previous studies in rat revealed the association between early-life exposure to endocrine disruptors and overall size of uterine fibroids (23), (24).

C. cyminum essential oil at dose levels of 250,500 and 1000 mg/ day was orally administered to female mice for 14 days caused marked elevation in the body weight, uterus and estradiol levels ($p < 0.001$) whereas the progesterone levels were decreased significantly in all dose groups including 25 mg/kg after 28 days study ($p = 0.001$). Phytoestrogen properties of cumin extract has been previously described (25) and it was identified useful in treating postmenopausal osteoporosis and estrogen-related disorders in bilaterally ovariectomized rat as an osteoprotective product comparable with estradiol (26). This study suggests the phytoestrogenic effects of cumin essential oil which should be confirmed by later studies using specific models. On the other side, another study showed a marked reduction in sperm density in the cauda epididymis and testes and sperm motility in the cauda epididymis as well as fertility reduction, significant decreases in the number of testicular cells, secondary spermatocytes and round spermatids (27) with its endocrine disruptive effects. The same effects should be checked on cumin essential oil in male animals in the future

5. CONCLUSION

Cumin essential oil caused estrogen-like effects in female mice in doses upper than 25 mg/kg but more studies in lower doses could be beneficial for its future therapeutic application as a safe phytoestrogen.

Oral exposure to cumin oils is widespread; however, little was known regarding the acute and long-term effects of such exposure with especial emphasis to its hormonal properties. Since there is no academic study on cumin essential oil toxic effects, we conclude the safety and lack of toxicity of this extract for short-term (28 days) use in doses up to 25 mg/kg. Present clinical, biochemical and histopathological evidence suggesting that cumin essential oil may possess endocrine disrupting activity first surfaced with several pieces of evidence in present work. Hence, it is necessary to establish the scientific basis for the therapeutic actions of this folk medicine as it may serve as the source for the development of more effective drugs for its estrogenic and ant progestin activities. More studies on its hormonal effects in males as well as its phytoestrogen effects in doses lower than 25 mg/kg could be beneficial for its future applications in health and food products.

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