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Original article:

Subchronic study of sperm morphology, genotoxic and mutagenic effect of *Lepidium sativum* seeds aqueous extract *in vivo* "*Lepidium sativum* induced genotoxicity and mutagenicity"

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Abstract

Lepidium sativum (LS) is an annual fast-growing herb that is now cultivated worldwide. It has been used for a long time as a traditional way in curing various diseases due to its different pharmacological properties. However, excess use of alternative medicine without medical supervision could lead to reverse toxic effect. Therefore, the present study aimed to evaluate the cytotoxic, genotoxic and mutagenic effect of oral administration of excess LS seeds aqueous extract *in vivo*. Animals were segregated into six groups; negative control, LS (200mg/kg), LS (400mg/kg), and LS (800mg/kg) daily administration for two consecutive weeks and LS (200mg/kg), and LS (400mg/kg) day by day administration for three consecutive weeks. Results show that high doses of LS, especially for daily schedule, increases mortality rate, leads to animal weakness, hair loss and abdominal bloating. In addition, LS administration distorts colon, liver, kidney and testis tissue in a dose dependent manner. Also, daily treatment increases cytotoxicity and genotoxicity of bone marrow cells detected by micronucleus assay and increases sperm abnormalities. Surprisingly, the present study records point of mutation in exon 5 of P53 gene in colon and liver tissue using SSCP assay. In conclusion, LS seeds extract is toxic in high dose administration for daily treatment and should be used in a therapeutic dose with no excess under medical supervision.

Keywords: *Lepidium sativum*; subchronic; genotoxicity; cytotoxicity; mutagenicity

1-Introduction

Lepidium sativum L. (LS, Garden cress) is an annual fast-growing herb, locally known as "El-Rshad", and it is native to Egypt country, west of Asia, and now it is cultivated worldwide. In the past, LS have been traditionally used in curing of various diseases due its different pharmacological properties (**Tiwari and Kulmi, 2004**).

It was reported that LS seeds have different nutritional and medicinal properties, such as a galactagogue, a diuretic, an alternative tonic, relieving minor pain and skin inflammation, natural aphrodisiac, a rubefacient, relieving gas in the alimentary tract, stimulate menstrual flow **(Nadkarni and Nadkarni, 1954)**, also seeds can cure bronchitis and asthma **(Kloos, 1976)**. The aqueous extract of LS seeds has a hypoglycaemic potential in both normal and diabetic rats without having any effect on the secretion of insulin **(Eddouks et al., 2005)**. Also, LS seeds were used for bacterial and fungal infections treatment, and rapid bone fracture healing **(Al-Yahya et al., 1994)**. Their seeds paste was applied to relieve rheumatic joints pain and swelling, might be useful in treatment of hiccup, dysentery, diarrhea and skin disease that is linked to blood impurities **(Gupta et al., 2010)**.

Early phytochemical studies of LS have confirmed the presence of flavonoids, coumarins, triterpenes, sterols Sulphur glycosides, and different imidazole alkaloids **(Radwan et al., 2007)**, such as lepidine, semilepidine and glucosinolates **(Gill and MacLeod, 1980)**. LS seeds contain different volatile components including benzyl isothiocyanate and benzyl cyanide as the main ones. In addition, they contain unsaponifiable matter including β-sitosterol and α-tocopherol. LS seeds contain mucilages, in which they can give different monosaccharides such as glucose, galactose, mannose, arabinose, xylose and various uronic acids by hydrolysis **(Divekar et al., 2010)**.

Many research articles report the safety and various pharmacological potential of LS seeds extract, however, in our per knowledge there are very few studies about their toxicity. In which, mice administrated LS seeds extract in drinking water at a dose of l00 mg/kg/body weight per day for a period of 90 days in a chronic study leads to a non-significant (25%) mortality of the animals in comparison to control group (15%) **(Al-Yahya et al., 1994)**. In another study, dietary LS seeds for rats 10% (w/w) were toxic but not fatal, while higher dietary dose 50% (w/w) for 6 weeks was lethal, decreased growth rate and caused entero-hepato-nephrotoxicity **(Adam, 1999)**. In addition, **Nath et al. (1992)** report teratogenic and anti-ovulatory effect of high dose of LS seeds on rats and their extracted oil cause skin irritation or mild inflammation and

should be used in a little quantity **(Pullaiah, 2006)**. Therefore, the aim of our present study is to focus on the histopathological, cytotoxic, genotoxic and mutagenic effect of oral administration of aqueous extract of LS seeds at different concentrations and different treatment schedule on male albino mice.

2-Materials & Methods

2.1 Animals

Forty male albino mice (*Mus musculus*; 8-10 weeks old; 26-30g, body weight) were used as experimental animals. They were obtained from King Fahad Center for Medical Research (animal house), Jeddah, KSA and were fed dry balanced meal for experimental animals provided by the General Organization for Grain Silos and Flour Mills in Jeddah, with a constant source of water. All experimental procedures and animal maintenance were conducted in accordance with the accepted standards of animal care per cage (Council of Europe, European Convention for the Protection of Vertebrate Animals 2006). We have followed the European Community Directive (86/609/EEC) and National Rules on Animal Care.

2.2 Preparation of *Lepidium sativum* seeds aqueous extract and animal grouping

Lepidium sativum (LS) seeds were purchased from herbal shop in Al Taif governate. The herbalist mentioned that seeds were grown in Al-Qaseem region of Kingdom of Saudi Arabia (KSA). The aqueous seeds extraction of LS was prepared according to **Mahassni and Khudauardi (2017)** and mice were segregated into 6 groups (7 mice per each except control), negative control (untreated, 5 mice), LS orally administrated groups *via* oral gavage daily for two consecutive weeks LS (200mg/kg) D, LS (400mg/kg) D and LS (800mg/kg) D and mice administrated day by day for three consecutive weeks LS (200mg/kg) D/D, and LS (400mg/kg) D/D.

2.3 Survival rate and abnormal morphological determination

Number of died animals were recorded throughout the whole time of the experiment and then used to calculate the survival rate/percentage. Also, any change of the external morphology of survival animals was recorded.

2.4 Mouse sacrifice and tissue harvesting

All survival animals were sacrificed by cervical dislocation at the end of their treatment, dissected and then colon, liver, kidney, testes, epididymis and bone marrow tissues were collected for further examination.

2.5 Histopathological evaluation

Colon, liver, kidney and testis tissues were traditionally sectioned in 5 µm thick paraffin sections and then stained by hematoxylin and eosin dyes (H&E) for detection of any histopathological alternations **(Ogino et al., 2006)**.

2.6 Sperm abnormalities

The cauda epididymides were exited from mice and cut into small pieces in 2 ml saline, then sperm suspension was smeared on a slide, air-dried, methanol fixed (10 min) and finally stained with 1% eosin (5 mins) (**Watanabe and Endo, 1991**). Thousand sperm cells were counted per animal and then the percentage of abnormal sperms in total was calculated.

The sperm abnormality rate = the abnormal sperm number ÷ 1000×100.

2.7 SSCP-PCR amplification

PCR product of exon 5 fragment of P53 gene was amplified using forward primer**:** 5'-TCTCTTCCAGTACTCTCCTC-3'; reverse primer: 5'-AGGCGGTGTTGAGGGCTTAC-3' from nuclear DNA through a succession of incubation steps at different temperatures: initial denaturation at 94°C for 5 min, DNA double stranded denaturation at 94°C for 30 s, primer annealing at 58°C for 60 sec, and primer extension at 72°C for 1 min, for 30 cycles and then final extension at 72°C for 10 min for complete amplification. PCR product was denatured by using formamide dye (95% formamide, 4 M urea, 0.1% bromophenol blue, 0.1% Xylene cyanol FF and 0.5µl 15% Ficoll) at 94ºC for 5 min and then was kept on ice for 10 mins **(Dai et al., 2005)**. The denaturated PCR products were separated into single strand DNA through 9% polyacrylamide gel electrophoresis (acrylamide: bisacrylamide =19:1v/v), then gel stained in 100ml of 1×TBE and ethidium bromide and finally DNA bands were visualized under transilluminator.

2.8 Micronucleus assay

Bone marrow smear was prepared according to the method described by **Schmid**, **(1975)** and then slides were stained with two different dyes, May-Grünwald and Giemsa, to increase contrast between different cells and micronuclei. From each mouse, 1000 polychromatic cells (PCEs) were examined for micronucleus (MN). Also, the ratio between PCEs to normochromatic cells (NCEs) in total 100 cell per each mouse was recorded to assess bone marrow geno- and cytotoxicity.

2.9 Statistical analysis

Data were expressed as the mean ± standard deviation (M±SD) and statistical analysis was performed using student t-test and one-way ANOVA to test the significance difference between groups using GraphPad software (GraphPad, 2017) ®.

3. Results

3.1 Survival rate and morphological change

Oral administration of LS seeds aqueous extract day by day for low (200mg/kg) and high (400mg/kg) doses doesn't cause any record of animal death for 3 consecutive weeks and therefore the survival rate is 100% as the control untreated mice. However, daily oral

administration of LS seeds extract causes mice death that is related to dose concentration, in which the highest dose 800mg/kg shows the highest mortality rate and in turn decreased survival rate in comparison to untreated control mice **(Figure 1)**. Due to lower survival rate for the daily administration of LS seeds extract, we scheduled their treatment for 2 consecutive weeks only. **Figure 2** shows that daily oral administration for different LS seeds extract doses leads to weakness and loss of body weight, hair loss **(A)**, abdominal bloating **(B)** that is due to stomach and ileum bloating **(C)**, and inflammation of the pericardium (pericarditis, **D**).

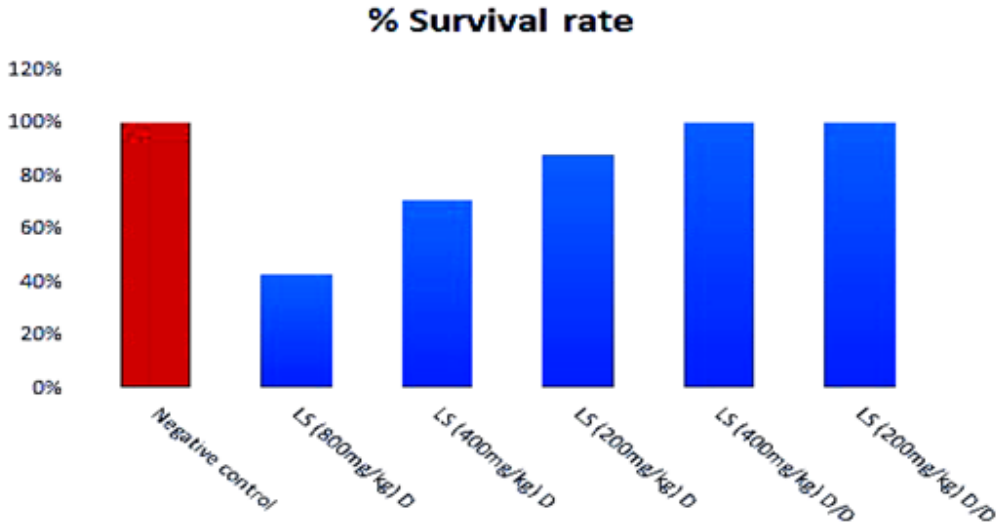

Figure 1: Effect of LS different concentrations on the percentage of survival rate of treated animals.

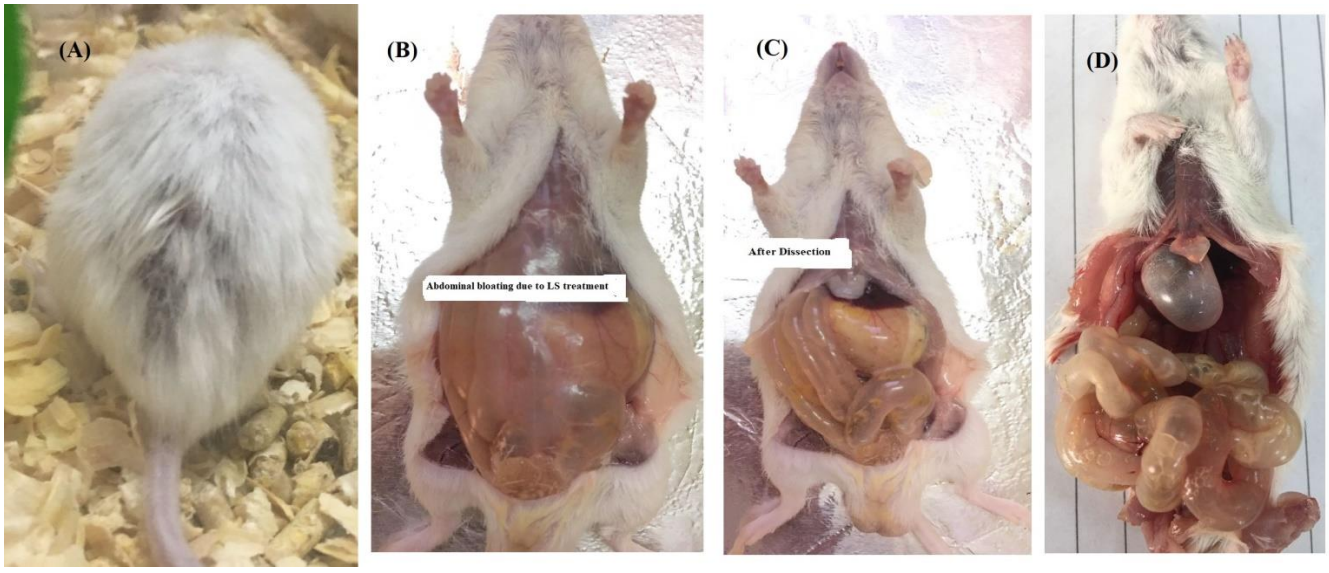

Figure 2: *Lepidium sativum* daily treatment for different concentrations leads to hair loss (A), abdominal bloating (B), ileum and stomach bloating (C) and pericarditis (D).

3.2 Histological examination

Different sections were done from colon, liver, kidney and testis tissues to evaluate the histopathological effect of different LS seeds extract doses especially at the daily treatment (200mg/kg, 400mg/kg and 800mg/kg) because they cause a higher toxic effect than day by day treatment. By microscopic examination of different tissue sections, we report damage in their architectures due to LS administration, that is increased in a dose dependent manner and daily treatment shows more severity. In colon tissue as shown in **Figure 3**, daily treatment of LS seeds extract leads to infiltration of inflammatory cells in the mucosal and submucosal regions, few hyperchromatic nuclei, crypts with dilated lumen and edema. There is an accumulation of infiltrated cell in the submucosal region that forms inflammatory nodules as a sign of colitis that related to the highest dose.

Liver tissue was distorted due to daily administration of different LS seeds extract doses that appears as a slight leucocytic infiltration, cytoplasmic degeneration of hepatocytes, polyploid hepatocytes with one large nucleus or more than one nucleus and appearance of apoptotic cells that are contracted in size with hypereosinophilic cytoplasm that might be surrounded by Kupffer cells. Moreover, there are necrotic (degenerated) cells that appear swollen with nuclear fragmentation or karyolysis **(Figure 4)**. In addition, **Figure 5** shows that LS seeds extract especially for 800mg/kg in a daily treatment leads to cell necrosis, infiltration of leucocytes and congestion of uriniferous tubules of kidney tissue.

Testis tissue sections of mice treated groups, especially with LS (400mg/kg) and LS (800mg/kg), show different features of damage. This appears as cell necrosis, atrophy and vacuolization of spermatocytes in seminiferous tubules, loss of Leydig cells and others present with irregular shapes, damage appeared more sever at 800mg/kg dose. Numerous seminiferous tubules appeared with a depletion of germinal epithelium, loss of spermatids and spermatozoa, absence of Sertoli cells, distortion of basement membrane with spermatogenic imperfection as shown in **Figure 6**.

3.3 Sperm abnormalities

In turn, the present study evaluates that oral administration of LS at different doses in different scheduled time significantly increased sperm abnormalities in comparison to negative control group, except for LS (200mg/kg) day/day administration **(Figure 7)**. Different sperm abnormalities were recorded as hookless and amorphous head; looped neck and midpiece, and sperms stickiness. It is clear that; sperm abnormalities decreased by decreasing dose and day by day have mild effect than daily administration.

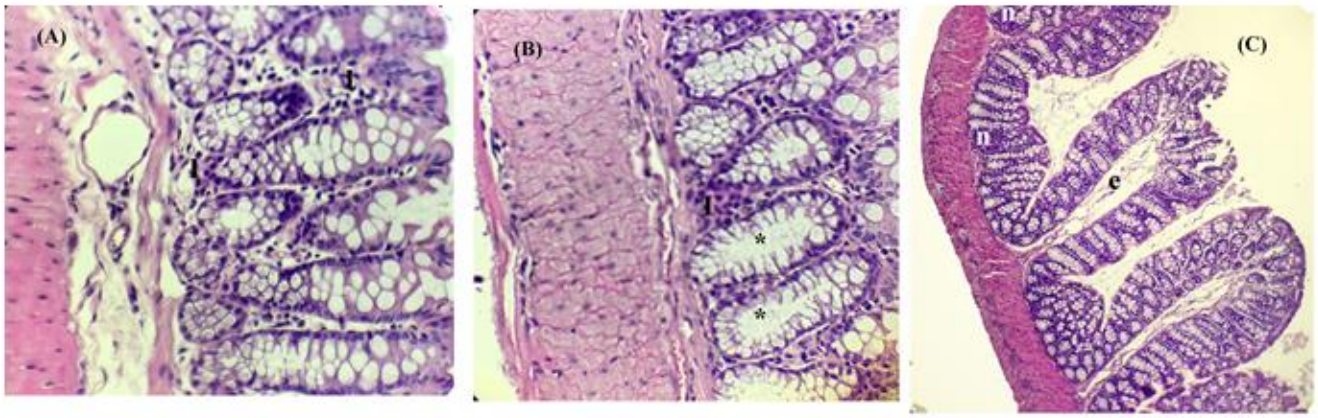

Figure 3: Photomicrograph of colon section of daily treatment of LS (200mg/kg) (A), LS (400mg/kg) (B) and LS (800mg/kg) mice groups (C) showing crypts with dilated lumen (*), inflammatory infiltrate (I), edema (e), hyperchromatic nuclei and inflammatory cells nodule (n). (H&E with magnification 400x for A & B, 100x for C).

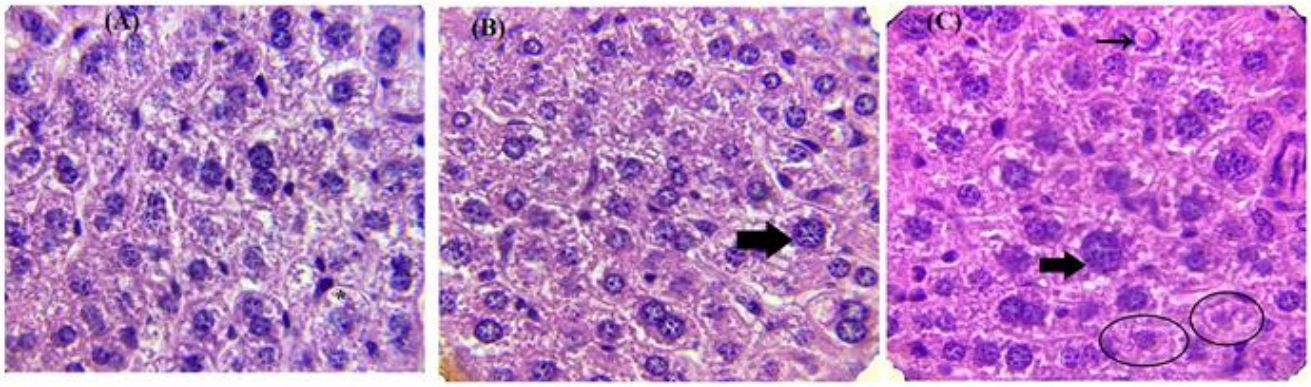

Figure 4: Toxic potential of daily treatment of LS (200mg/kg) (A), LS (400mg/kg) (B) and LS (800mg/kg) (C) in liver tissues, showing cytoplasmic degeneration (*), polyploid hepatocytes (large arrow), apoptotic cell (small arrow) and necrotic cells (black circle) (H&E with magnification 1000x).

3.4 PCR-SSCP

Daily administration of LS seeds aqueous extract leads to point mutation in exon 5 of P53 gene in both liver and colon tissues. We report only one animal per each daily treatment as shown in **Figure 8**, in which there is a band shift pattern at 400mg/kg dose and 800mg/kg in liver tissue (gel A, lanes 4 and 5, respectively), while in gel (B) lane 2 shows point mutation in colon tissue due to LS (200mg/kg) daily administration.

3.5 Micronucleus assay and PCEs/NCEs ratio

All LS oral administrated groups (daily and day by day) show bone marrow cells genotoxicity that appears by induction of micronucleated

polychromatic erythrocytes (MnPCEs) that is significant in comparison to negative control group. In which oral daily administration of LS (800mg/kg) have the greatest toxicity, however, 200mg/kg (D/D) group shows the least genotoxicity as shown in **Figure 9**. In addition to LS genotoxicity, daily and D/D oral administration increases cytotoxicity of bone marrow cells due to significant decrease of the PCEs/NCEs ratio in comparison to negative control group as shown in **Figure 10**. In confirmation to other results, LS (800mg/kg) daily administration shows the greatest toxicity, while 200mg/kg in day by day administration shows the least cytotoxicity.

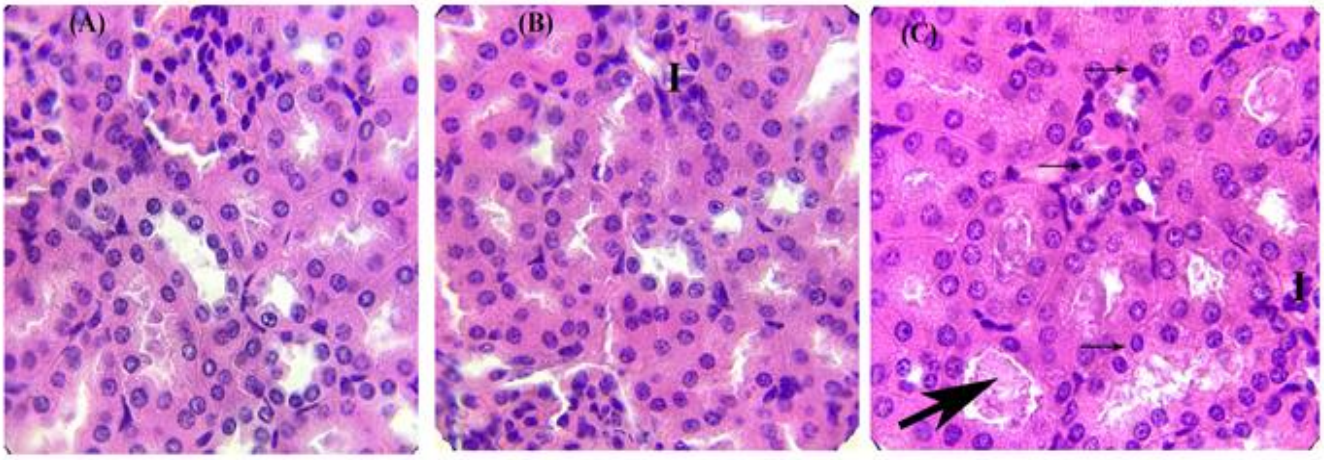

Figure 5: Daily treatment of LS (200mg/kg) (A), LS (400mg/kg) (B) and LS (800mg/kg) (C) damages kidney tissue that shows cell infiltration (I), necrosis (small arrow) and congestion (large arrow) (H&E with magnification 1000x).

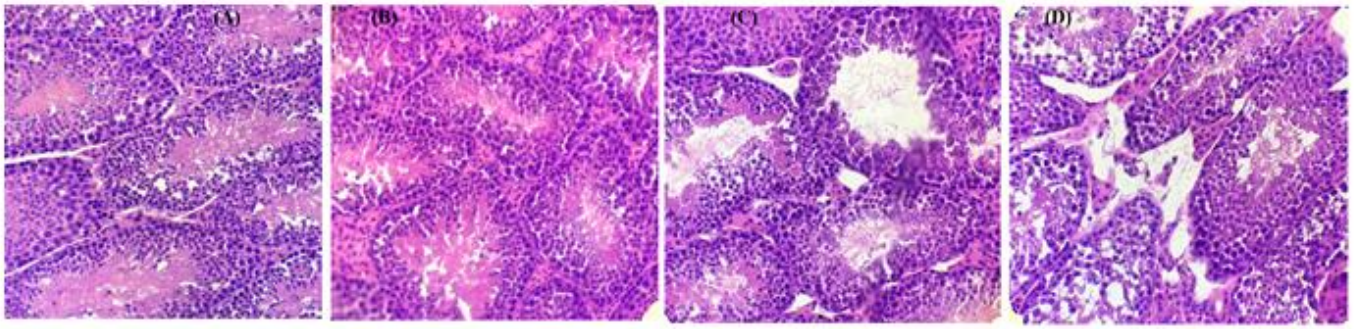

Figure 6: Photomicrograph of testes section of negative control (untreated, A), daily treatment of LS (200mg/kg) (B), LS (400mg/kg) (C) and LS (800mg/kg) mice groups (D) showing damage of tissue in treated groups (H&E with magnification 400x).

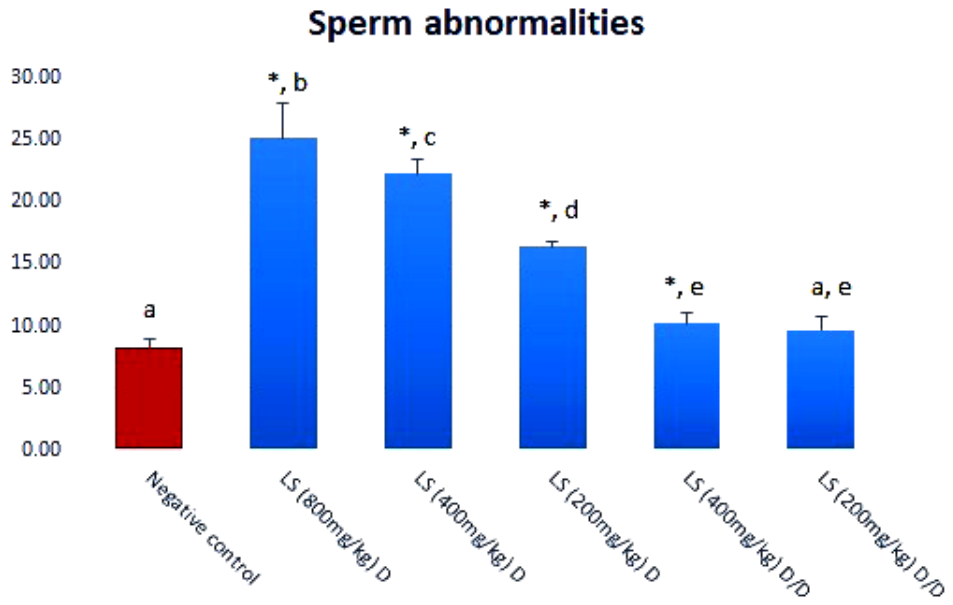

Figure 7: LS treatment at different concentrations increases the percentage of sperm abnormalities at P > 0.05. * Statistically compared with negative control group. Same small letters considered to be statistically non-significant, while different small letters mean statistically significant between different groups.

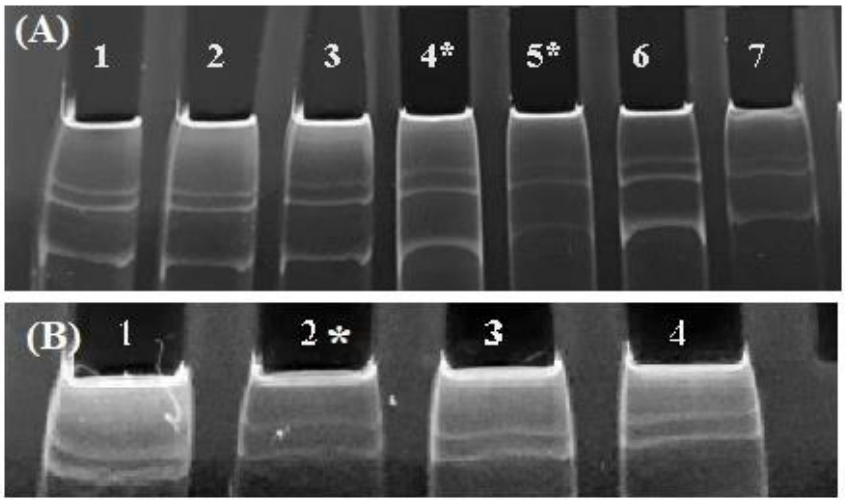

Figure 8: Representative 9% polyacrylamide gel showing polymerase chain reaction-single strand conformation polymorphism for p53 exon 5 (A) in liver and (B) colon tissues. Lane 1 represents negative control group in both gels; in (A) Lanes 2–7 represent different LS treated groups, in which lanes 4 and 5 show bands shift pattern, they represent LS (400mg/kg) and 5 represents LS (800mg/kg) daily treatment, respectively, while in gel (B) lane 2 show band shift pattern that represents LS (200mg/kg) daily treatment.

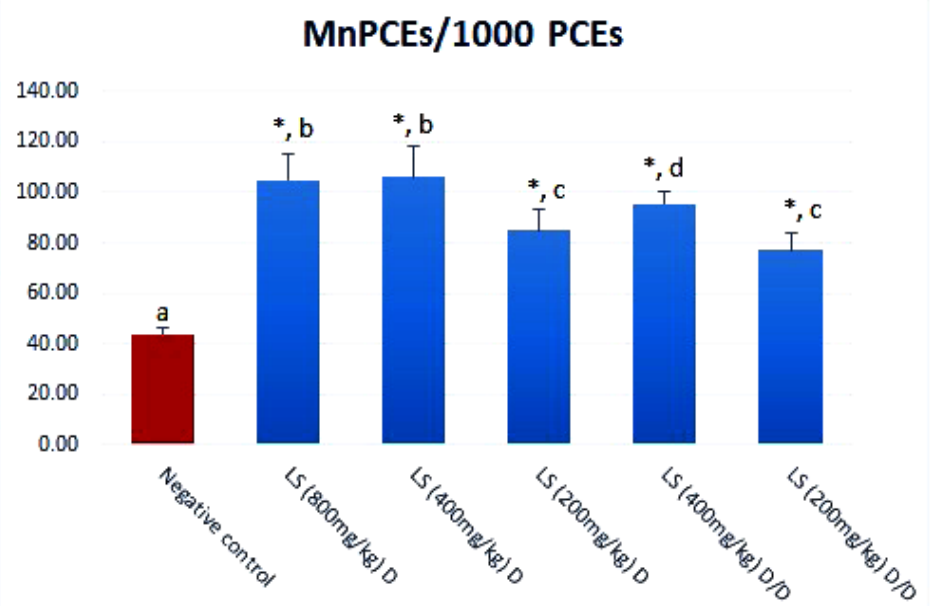

Figure 9: Effect of *LS* seeds aqueous extract at different concentrations on MnPCEs/1000 PCEs at P > 0.05 in bone marrow cells. * Statistically compared with negative control group. Same small letters considered to be statistically non-significant, while different small letters mean statistically significant between different groups.

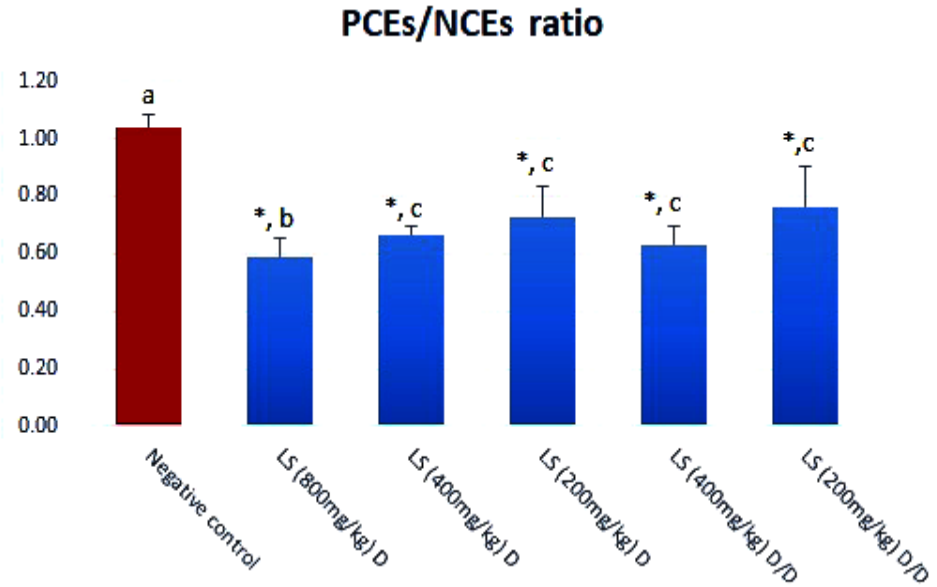

Figure 10: Different concentrations of *Lepidium sativum* seeds extract decreases PCEs/NCEs ratio in mice bone marrow cells at P > 0.05. * Statistically compared with negative control group. Same small letters considered to be statistically non-significant, while different small letters mean statistically significant between different groups.

4. Discussion

Lepidium sativum seeds were used in a traditional therapy without caring of high doses side effects and loss of any medical supervision. Few research studies reported possible side effects of excess use of LS seeds. The present study revealed the genotoxic, cytotoxic and mutagenic effects of LS seeds aqueous extract of high doses, especially daily administration, however the least dose (200mg/kg day/day) could be used as a safe dose in this schedule. Extract of LS administration, especially daily high doses, leads to abdominal bloating that is agreed with previous study that reports excess use of LS leads to indigestion **(Pullaiah, 2006)** and inhibit peristaltic movement of intestine **(Manohar et al., 2009)**.

The present study records weakness of animals that received high dose daily, loss of their body weight, hair loss and increase in morbidity rate that might be attributed to disturbance of different tissue functions, such as liver and kidney, and anemia that have been estimated before by **Adam (1999)**. They found that by feeding Wistar albino rats with LS seeds at 2%(w/w) was not toxic, however 10%(w/w) was toxic but not lethal, but higher dose 50%(w/w) for 6 weeks was entro-hepato-nephrotoxic, decrease growth rate and in turn leads to animal death. Toxicity was accompanied with anemia and leukopenia that is related to change in serum AST, ALT, total protein, cholesterol and urea **(Adam, 1999)**.

LS seeds contain high content of benzyl isothiocyanates that affects the colon tissue as a result of high doses administration in a daily schedule. That was confirmed before **(Kassie et al., 1999)**, in which exposing rats to high dose of benzyl isothiocyanates (200 mg/kg b.wt) induces DNA damage in colon and gastric mucosa cells that is detected by SCGE assay. Moreover, benzyl isothiocyanates caused chromosomal aberrations and sister chromatid exchanges in Chinese hamster ovary cells that mimics the ionizing radiation and radiomimetic chemicals **(Musk et al., 1995)**. There is an assumption that benzyl isothiocyanates high doses genotoxicity might be attributed to free radicals that is evaluated by TBARS assays, which reports their induction of lipid peroxidation **(Kassie et al., 1999)**. Also, DNA damage according to this stress induces bone marrow cells toxicity.

Previous study reports mild toxicity of LS (2 mg/ml) aqueous seeds extract oral administration on liver tissue that appears as dilatation of central veins and focal endothelial degeneration of hepatic sinusoids. However, LS (4 mg/ml and 8 mg/kg) higher doses exerts higher toxicity represented by more central veins congestion with more vascular hemorrhage, infiltration of leucocytes, and appearance necrotic

hepatocytes and small pyknotic nuclei as a hallmark of apoptosis **(Bafeel and Ali, 2009)**. They conclude that liver damage in the form of apoptosis and necrosis returns to the high dose of isothiocyanates that are present in LS seeds extract **(Burow et al., 2007)**. **Okulicz et al. (2008)** report gluconasturtiin and phenethyl isothiocyanates of LS caused increase in liver cholesterol and triglyceride content with a decrease in glycogen content that leads to hepatocytes vacuolization. In our per knowledge, it is the first study to report the mutagenic potential of LS daily doses on colon and liver tissues that appears as a point of mutation due to their high toxicity.

In addition, daily oral administration of high doses LS distorts testicular tissue and in turn increases sperm abnormalities. **Kamani et al. (2017)** found that daily oral consumption of high dose 400 mg/kg of LS seeds ethanol extract decreased the weight of epididymis and they conclude that LS hyper dose has reverse effect on genital gonads **(Meikle et al., 1996)**. LS seeds consist of several fatty acids, in which α-Linolenic acid (34%) was the major, then oleic (22%) and linoleic (11.8%) **(Diwakar et al, 2010)**. High concentrations of oleic acid significantly reduced testosterone production, cholesterol concentration of testis's Leydig cells and the activity of cholesterol esterase enzyme in the cytosol and mitochondria. In turn, they correlate between the usage of high doses of essential fatty acids and reverse effect on genital gonads **(Meikle et al., 1996)**.

5. Conclusion

LS seeds aqueous extract at high doses for daily oral administration exerts cytotoxic, genotoxic and mutagenic effect on different tissues *in vivo*. Therefore, LS seeds extract must be used in a known therapeutic dose under medical supervision due to their toxicity if used in high doses. Further study must be done to clarify the mechanism of LS genotoxicity and mutagenicity.

6. Conflict of interest

All authors declared no conflict of interest.

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