Journal of Genetic and Environmental Resources Conservation, 2015, 3(2):138-144.
www.jgerc.com

Physiological and histological effects of alcoholic and aqueous extract of *Nigella sativa* seed on fertility potential in male mice

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Abstract

The aim of this study is to investigate the effect of alcoholic and aqueous extracts of *Nigella sativa* seeds on the reproductive values, some sexual hormones level and histological change of treated and normal male mice at one spermatogenesis cycle (five weeks). Sixty albino Swiss male mice were used as animal model, they divided into six groups equally, and 1st group served as control group, the 2nd group was treated orally with 150 mg/kg. B.w. of aqueous extract of *Nigella sativa* seeds, the 3rd group treated with alcoholic extract 150 mg/kg. B.w. The 4th group was treated with 100 mg\Kg of CCl_4 and the 5th group treated with CCl_4 (100 mg\Kg) and aqueous extract (150 mg/kg. B.w), finally 6th group treated with CCl_4 (100 mg\Kg) and alcoholic extract (150 mg/kg. B.w). The parameters were determined, sperm characteristic (sperm concentration, motility, viability and abnormal sperm) levels of testosterone, FSH and LH hormones and histopathological study. The results of the present study showed that the treatment with CCl_4 caused a significant decrease ($P>0.05$) in the sperm concentration, motility and increase the dead sperm and abnormalities. The results showed significant ($P< 0.05$) increases in sperm concentration, motility and decrease in dead and abnormal sperm in groups treated with aqueous and alcoholic extract of *Nigella sativa*. The animals treated with aqueous and alcoholic extract combination with CCl_4 showed improvement in sperm concentration and motility compared to group treated with CCl_4. The FSH, LH and testosterone significant ($P< 0.05$) increased in treated groups with aqueous and alcoholic extract of plant compared to group treated with CCl_4 and improvement in group treated with aqueous and alcoholic extract combination with CCl_4. The histopathological sections of testis of treated mice with CCl_4 showed sever changes in testicular architecture. This was attributed to the cytotoxic activity of CCl_4. CCl_4 exposure induced histopathological changes in the testis including morphological alterations of the seminiferous tubules. Seeds extract have positive fertility and histopathological effects on the testis of mice.

Key words: *Nigella sativa*, Testicular functions, Tetra carbone chloride, Histopathology, Mice.

Introduction

Nigella sativa is a species plant belonging to the family Ranunculceae. It is cultivated in several countries in the Mediterranean region and Asia. The seed were used in the orient as condiment or flavoring and also in traditional medicines application (Rchid *et al*., 2004). It has been shown that *N. sativa* has bronchodilator, antibacterial, hypertensive, antioxidant, anti-tumor and-diabetic properties. The aqueous extract exhibited an inhibitory effect on nitric oxide production in macrophage of mice and was validate for the treatment of rheumatism. The oil of *N.sativa* was a potent analgesic and anti-inflammatory drug in rats (Hajhashemi *et al*., 2004).

A wide majority of medicine plants possess pharmological principles, which has rendered them useful as curatives foe numerous ailments according to the World Health Organization (WHO) reports, 70-80% of the world population confide in traditional medicine for primary health care (WHO, 2002). Plants and derivatives played a key role in world health and long been known to possess biological activity. In addition, plants have a long folklore of use in aiding fertility, including fertility enhancing properties and aphrodisiacal qualities (D'Cruz *et al*., 2010). *N. sativa* seeds has been used in traditional medicine as a neural theraby for promotes females menstruation, carminative, laxative. Animals studies have been shown that extract of *N.sativa* seeds have many therapeutic effects such as gastro protective, anti-tumor anti-anxiety, anti-inflammatory and anti-oxidant (Kanter *et al*., 2005).

The seeds of N. sativa as many different chemical components, including mucilage, crud fiber,

reducing sugar, resin, alkaloids, flavonoids, organic acids, sterols, tannins, saponins and protein. In, Addition, it has a high content of unsaturated fatty acids, especially Linoleic acid (55%) Oleic acid (23.4%) and palmitic acid (12.5%). It is know that biological activity of N. sativa seeds is attributed to its essential oil component. The main compounds contained Thymoquinone (30-48%), P-mycine (7-15), Csrvacrol (6-12%), 4-terpinol (2-7%), T-anethol (1-4%) and Sesquiterpens (1-8%) (Gilani *et al*., 2004). Thymoquinone and its derivatives are the most putative pharmacologically active constitute of *N.sativa* (Padhye *et al*., 2008). (Samir, 2007) Shown that administration of *N. sativa* oil to hyperlipidemic rats improved their reproductive efficiency and produced additional protection against hyperlipidemic induced reduction infertility. Also, (Mukhalad *et al*., 2009) concluded that the aqueous extracts of *N. sativa* have increased spermatogenesis of male rats. In addition, (El-Tahomi *et al*., 2010) shown that the inclusion of a mixture of equal quantities from radish rocket and black cumin (Nigella) meals. The expense of approximately 50% soybean meal protein improved the sperm characteristics and reduced free radicals in the seminal plasma. Therefore, regardless to value of plant used in traditional medicine for drug discovery of fertility. This study was concluded to examine the effect of alcoholic and aqueous extracts of Nigella seeds on fertility potential, gonadotropin hormones and testosterone in male mice.

CCl_4 are widely used as model compound to induce hepatotoxicity and elucidate its mechanisms of action following exposure to these compounds (Kim *et al*., 2010). Effects such as fatty degeneration, fibrosis, hepatocellular apoptosis and carcinogenicity have been associated with CCl_4 toxicity.

Impairing crucial cellular processes such as lipid metabolism with the potential outcome of fatty degeneration while the reaction between CCl_4 and DNA is thought of function as initiator of tissue cancer. This radical can also react with oxygen to form the trichloromethyl peroxy CCl_3O_2 radical a highly reactive species. This compound initiates the chain reaction of lipid peroxidation (Weber *et al*., 2003).

CCl_4 binds to cytochrome P450 reductase. The enzyme substrate complex then loses a chloride ion and a free radical (CCl_3.) intermediate is generated which reacts with oxygen or takes a hydrogen from a donor to yield a secondary radical or reacts with lipids or proteins(Augusti *et al*., 2006). The lipid radicals thus formed add on molecular oxygen to generate lipid peroxyl radicals, which steals the hydrogen atoms from other lipid molecules and the process of lipid peroxidation propagates. Trichloromethyl (CCl_3.) radical even reacts with reduced glutathione (GSH) and causes various pathological and toxicological manifestations (Sultana *et al*., 2005). CCl_4 increases intercellular Ca^{2+} concentration and, releasing harmful cytokines that leads to the death of the tissue and oxidative stress. Although modern medicine has made tremendous advancements, effective drugs that offer protection from liver damage, stimulate liver function or help to regenerate liver cells are still not available (Chattopadhyay, 2003).

Materials and Methods

Aqueous extract: Fifty gram of Nigella sativa seed powder were put in flask, then 500 ml of distilled water were added in percentage 1:5w.v. During extraction the mixture was shacked for three hours, the suspension was filtered by Whatman filter paper and filtrates concentrated by using rotary evaporator. The crud extract was stored at 4C (Harborne, 1977).

Alcoholic extract: Fifty grams of seed plant were extrication with absolute methanol under continuous stirring for 8hr. at room temperature; filtrate extract was concentrated by rotary evaporator (Harborne, 1977).

Experimental Design: Sixty healthy albino male mice with average body weight (25-28) gm, were used for this study. They were obtained from animal house of the Biotechnology research center \ Al-Nahriane University. Mature male mice were kept in an air-conditioned room at 25±2 °C with light\dark period of 14/10hrs. Male mice were divided into six groups each one involved ten animals. Aqueous and alcoholic extract of *Nigella sativa* in a dose of 150 mg\kg Bw\day were orally administrated to the male of group one and two respectively for five weeks. The third group was orally administrated with 100 mg\kg Bw\day of CCl_4. While the fourth and fifth groups were orally administrated aqueous and alcoholic extract of *Nigella sativa* in a dose of 150 mg\kg Bw\day combination with100 mg\kg Bw\day of CCl_4 (The plant extract and CCl_4 were given orally by stomach tube as a dose of 0.1 ml\day for five weeks). The sixth group allowed drinking distilled water only as control group. The epididymis was put in small Petri dish containing RPMI-1640 medium, organ was minced into tiny pieces with micro surgical scissors until getting homogenized solution ,which contain the sperm suspension then subjected to sperm characteristics, including microscopic examination to record the concentration of sperm(sperm\ml), sperm motility, abnormal sperm morphology and

sperm viability. The portion of blood samples were collected and allowed to coagulate at room temperature, the blood centrifuged at 3000 r.p.m for 10 min, and clear non-haemolysed supernatant serum were quickly removed and stored at -20°C for measurement of FSH, LH and testosterone (aBio merieux Italia S.P a vidia Campigliano, 58 50015-poin tAEMACF,Italia,mini-VIDAS.

Evaluation of sperm parameter: Semen sample were collected from the caudal epididymis, and the samples were analyzed immediately after collection. A drop of sperm suspension was placed on slid and covered with cover slip. Concentration of sperm (sperm\ml) was calculated from the mean number of sperm in five high powers microscopically field. This number was multiplied by a factor of one million(x 106 sperm\ml). The sperm suspension 50µl was placed over slid and covered by slid cover, using light microscope, several field were examined to estimate the percentage of individual motility of sperm. A total of 100 sperm from each mouse were examined for morphological change and viability. Dead\a Live ratio was determined using 1% Eosin and 5% Nigrosin in 3% sodium citrate dehydrate solution (Raji *et al*., 2003, Kisa *et al*., 2004).

Histopathological Examination: Testes were dissected out and fixed 10% neutral formalin solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grads of alcohol. These specimen were cleared in xylene, embedded in paraffin, sectioned at 4-6 microns thickness and stained with Hematoxylen and Eosin, then examined microscopically (Persnell and Schreebman, 1997).

Collection of blood: At the end of the experimental period, blood was drawn from the animals by heart puncture. Separated serum were used to determine testosterone, FSH, LH After collection of blood samples.

Testosterone, FSH, LH assay: Bio merieux Italia S.P. a vidia campigliano, 58 50015-point A EMA (F1) Italia miniVIDAS. Was used for the hormonal assay. In testosterone, FSH and LH tests the assay principle combines an enzyme immune assay sandwich method with a final fluorescent detection (ELFA).

Results and Discussion

Effect of aqueous and alcoholic extract of *Nigella sativa* in this study was presented in Table (1), the result showed a significant (P<0.05) increase in sperm concentration in animals treated with aqueous and alcoholic extract (150 mg\kg WB\day), (44.56±5.56, 43.65±6.11) respectively compared to animals which treated with 100mg\kg Bw\day. of CCl_4 (28.65 ± 7.54) and animals treated with aqueous and alcoholic extract of plant combination with CCl_4 (36.41±7.06, 35.02±5.24) respectively. The results of statistical analysis indicated a significant (P<0.05) increase in sperm motility in all groups compared with animals treated with CCl_4.Table -1. It has been found that the CCl_4 exposed animals were shown in table- 1 increase in dead and abnormal sperm (29.32±3.30, 25.32±3.62) respectively significantly differed from those which treated with aqueous and alcoholic extract combination with CCl_4. Also the result showed a significant increase in the level of FSH, LH and Testosterone in animals which treated with aqueous and alcoholic extract (150 mg\kg Bw\day) compared with animals treated with 80mg\kgBw\day of CCl_4, while the results showed improvement in the level of FSH, LH and Testosterone in the animals which treated with aqueous and alcoholic extract (150 mg\kg Bw\day) combination with 100mg\kg B.W\ of CCl_4 compared with animals treated 100mg\kgBw\day of CCl_4 (Table 2).

The histopathological study of testes of mice treated with CCl_4 showed decrease diameter of seminiferous tubules and Leydig cells and increase interstitial space compared to control group notes normal diameter of semineferous tubules and Leydig cells and testes of mice treated with alcoholic extract combination with CCl_4. CCl_4 exposure induced histopathological changes in the testis including morphological alterations of the seminiferous tubules, and degeneration of spermatogenic cell.

Reactive oxygen species (ROS) belong to class of free radicals are highly reactive oxidizing agent. Production of ROS in various tissues such as testis is common event; however, the increases in its synthesis stimulate the DNA damage and oxidation of cells (Strzezek *et al*., 2012). The sperm membrane contains a high amount of unsaturated fatty acids, peroxidation could lead to the damage of lipid matrix structure in sperm membrane and could be associated with impaired motility (Aiten *et al*., 2013).

In addition, ROS are highly reactive molecules that can react with fatty acids and proteins. The oxidation of these molecules can produce disturbance in permeability of cell membrane. Sperm are susceptible peroxidative damage due to existence of unsaturated fatty acid which is responsible for regulation of sperm maturation, sperm lipid peroxidation could destroy the lipid structure of the sperm membrane, and it could inhibit spermatogenesis (Kumanov *et al*., 2006).

Table (1): Effect of aqueous and alcoholic extract of *Nigella sativa* and CCl_4 on sperm parameters in albino male mice.

Groups	Sperm concentration 10^6 sperm\ml	Motility %	Viability %	Morphologically abnormal %
Control	41.72± 7.43 A	84.05± 11.33 A	10.084± 3.54 A	8.26± 1.40 A
Aqueous extract	44.56± 5.56 A	87.12± 8.24 A	10.13± 1.62 A	6.36± 1.53 A
Alcoholic extract	43.65± 6.11 A	85.23± 7.25 A	11.19± 2.66 A	7.435± 1.80 A
CCl_4	28.65± 7.54 B	60.63± 5.33 B	29.32± 3.30 B	25.32± 3.62 B
Aqueous extract + CCl_4	36.41± 7.06 C	80.67± 7.94 C	17.27± 2.62 C	15.32± 2.60 C
Alcoholic extract + CCl_4	35.02± 5.24 C	79.71± 9.08 C	18.22 ± 1.64 C	14.04± 1.33 C

Values are means ± standard error.
Different letters refer to significant differences ($p<0.05$) compared between columns groups.

Table (2): Effect of aqueous and alcoholic extract of *Nigella sativa* and CCl_4 on FSH, LH and testosterone in albino male mice

Groups	FSH mIU/ml (means±SE)	LH mIU/ml (means±SE)	Testosterone ng/ml (means±SE)
Control	1.58± 0.03 A	1.7 0± 0.06 A	1. 76± 0.08 A
Aqueous extract	2. 23± 0.03 A	1. 90. ± 0.07 A	1. 98± 0.09 A
Alcoholic extract	1.90.± 0.07 A	1.85 ± 0.10 A	1. 85± 0.06 A
CCl_4	0.93± 0.03 B	0.91± 0.04 B	0.85 ± 0.06 B
Aqueous extract+CCl_4	1. 39± 0.07 A	1.5 6± 0.03 A	1.2 0 ± 0.05 C
Alcoholic extract +CCl_4	1.30 ± 0.05 A	1.4 1 ± 0.29 A	1.32 ± 0.05 C

Values are means ± standard error.
Different letters refer to significant differences ($p<0.05$) compared between columns groups.

The generation of small amount of free radicals play role in biological junction. Oxidative stress can produce interrelated derangements of cellular metabolism, including increase in intracellular free calcium, alteration of protein and nucleic structure, damage to membrane ion transport and permeability and destruction of the cell by lipid peroxidation (Szymonic-Lesiuk *et al*., 2003).

Removal of hydrogen atom from unsaturated fatty acids by free radicals generates Carbone – centered lipid radicals .These lipid radicals add molecular oxygen to form lipid peroxyl radicals. The toxicity of CCl_4 probably depends on formation of the trichloromethyl radicals (CCl_3) which in the presence of oxygen interacts with it to form the toxic trichloromethyl peroxyl radicals CCl_3 O_2 (Behar-Cohen *et al*., 1996).

Damage of tissue induced by generated ROS has been proposed to be contributing factor in a variety of human diseases including male infertility. The high production of ROS and their oxidative stress can be initiated by a variety of factors, including exposure to CCl_4, such as acetaminophen and carbon tetrachloride CCl_4 increase in oxygen reactive radical production lipid peroxidation in different tissue. Lipid peroxidation is an important factor that may induce morphological changes .Sperm are particularly susceptible to oxygen

radicals induced damage because their membranes contain large amount of fatty acids. ROS mediated damage in the sperm membrane, may defective sperm function (Sanchez et al., 2006).

This could be an outcome of loss of membrane permeability and increase oxidative stress as suggested earlier it is well understood that CCl_4 converted to trichloromethyl CCl_3 free radicals which initiate a chain of reaction by abstracting hydrogen ion from polysaturated fatty acids (PUFA) and result in increased production of thiobarbituric acid reactive substance, the major products of lipid per oxidation. in fact, peroxidation of lipids, particularly those containing PUFA can change the biological membrane and sometime result in severe cell damage (He *et al*., 2006; Lee *et al*., 2007).

CCl_4 has been demonstrated to cause acute necrosis and apoptotic injury and impairment function. The mechanism of CCl_4 involves oxidative damage by metabolism of CCl_4 to CCl_3. which ultimately results in cell death with accumulation of lipid peroxidation and intracellular calcium ions and triggers secondary damage (Kovalovich *et al*., 2001).

The cause of testosterone hormone increase may be due to the effect of black seed on the enzymes which effect on the metabolism and steroid secretion in the testis. The increase in sperm concentration was due in part to the increase in testosterone and FSH level in testicular tissue, since these two hormones were responsible for spermatogenesis in semineferous tubules, while testosterone is responsible for epididymal function in maturation of sperm. The black seed contain alkaloids and phenols which stimulate the secretion of FSH and testosterone (Mclachlan *et al*., 2002).

The testes, epididymis and other reproductive organs are structurally and physiologically dependent upon the testosterone and other androgen. Testosterone stimulate growth and secretary activity of the reproductive organs a significant increase of these hormones increase the number of germinal cells of testis. LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli and cells of the seminiferous tubules and indirectly stimulates spermatogenesis via testosterone (El-Tahomi *et al*., 2010)

The extract increased sperm count, these parameters in mammals are regulated by the LH, and FSH binds with receptors in the Sertoli cells and stimulates spermatogenesis.

At previous study shown increased the number of Leydig cells .it is possible that testis semineferous tubules induced directly by the *N. sativa* extract or indirectly by testosterone and stimulated sperm counts (Singh *et al*., 1995).

N. sativa oil is an effective free radicals scavenger showing antioxidant activity and protection against the damage caused by free radicals. Therefor the oil is useful in hyperlipidemia induced free radicals. *N. Sativa* oil protects some tissues from oxidative stress and lipid peroxidation (Ozugurlu *et al*., 2005).

The *Nigella sativa* seed oil treatment led to increase in LH and FSH levels which may be due to the direct effect on hypothalamus which in turn increases Gonadotropic releasing hormone (GnRH), furthermore fatty acids can stimulate GnRH-dependent pathway that promoter changes in gonads function the increase in testosterone level in treated groups may be due to the effects of *Nigella sativa* oil to stimulate the activity of 17B-hydroxysteroid dehydrogenises the most important key enzyme in the testosterone synthesis pathway (Gromadzka *et al*., 2002).

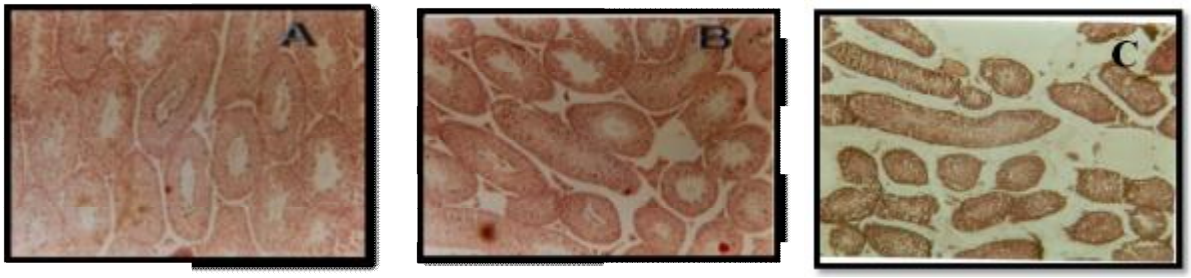

Figure (1): A- Section in testes of mice (control group), showing normal structure of seminiferous tubules and Leydig cells stain 10X (H and E). B- Section in testes of mice (treated extract and CCl_4), showing normal structure of seminiferous tubules and Leydig cells decrease interstitial space (I.S.) stain 10X(H and E). C- Section in testes of mice treated withCCl_4, showing degeneration of seminiferous tubules and Leydig cells and increase interstitial space. 10X (H&E) Stain.

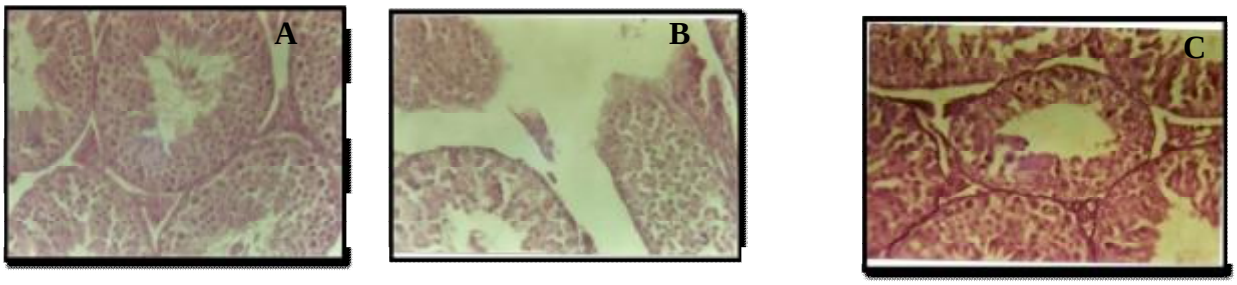

Figure (2): A: Section in testes of mice (control group), showing normal structure of seminiferous tubules and Leydig cells and interstitial space (I.S.). 40X (H&E) stain. B- Histological section of treated testes with CCl_4 show increase interstitial space and degeneration of seminiferous tubules (ST). 40X E&H stain. C- Histological section of treated testes with plant extract and CCl_4 show normal diameter of seminiferous tubules and decrease interstitial space. 40X E&H stain.

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