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Metiram fungicide-induced histomorphological and biochemical changes in rat ovaries: Attenuation by *Nigella sativa* oil

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Abstract

Metiram is a fungicide used against fungal diseases of field crops. *Nigella sativa* L. (Black seed) is widely distributed herb with wide therapeutic uses. This work aims to study the effect of N. sativa oil on ovarian toxicity induced by metiram fungicide in albino rats. Treating animals with metiram caused many histopathological alterations. The number of ovarian follicles decreased and most of them degenerated which accompanied by increase of atretic follicles and increase of collagen fibers. Histochemical results revealed a decrease in carbohydrates content. Metiram significantly decreased the levels of both LH, FSH and estradiol. It also caused an increase in lipid peroxidation and decreased the ovarian antioxidant enzymes, SOD, CAT. This study revealed The ameliorative effect of *N.sativa* oil against metiram -induced ovarian toxicity through significant increase in number of healthy follicles with an decrease of the atretic follicles and collagen fibers. Morover, *N.sativa* treatment led to a significant increase in FSH, LH and estradiol levels. A decrease in lipid peroxidation and increase in antioxidant enzymes were recorded. These results give new insight on beneficial effect *N. sativa* oil against female gonadal toxicity and this effect may be attributed to its antioxidant activity.

Keywords: *Antioxidant enzymes, N. sativa oil, Ovarian tissue; Rat.*

1 Introduction

Fungicides are a group of pesticides applied against wide range of fungal diseases of field crops, fruits, nuts and ornamentals. Although fungicides, proved useful, it does not excluded from expressing its hazardous actions in mammalian animals and human. Intoxication with these chemicals constitutes a major problem, especially because this could be happened by ingestion of contaminated crops like fruits and vegetables. Metiram (polyram) is a non-systemically acting fungicide of dithiocarbamate group. It is used to prevent crop damage in the field and to protect harvested crops from deterioration in storage or transport (Charls et al., 2000). Exposure to metiram was accompanied by adverse effects. Sortwell et al., (1977) reported follicular hyperplasia in thyroid of female rhesus monkeys treated with metiram. Kornuta et al., (1996) reported that metiram is one of the pesticides which showed genotoxic effect. Treating rabbits with high-dose level of metiram was resulted in minimal to moderate exfoliation and ulcerative dermatitis in the skin (Ullmann et al., 1987). Whalen et al., (2003) showed that the fungicides (maneb, metiram, and ziram) have cytotoxic effects onboth T and NK lymphocyte function. Metiram was found to induce histopathological as well as biochemical alterations in the liver of albino mice (Sakr et al., 2009). Sakr and Badawy (2011) reported that metiram-inhibited spermatogenesis and induced apoptosis in albino mice.

Many traditional plants products are in use today due to their therapeutic potential .*Nigella sativa* L. is an annual herb belongs to Family Ranunculaceae. It grows in Mediterranean countries and it is one of the native plants that are widely distributed in Egypt. Black seeds are ascribed to have many medicinal properties in traditional medicine. It is effective against cough, bronchitis, asthma, chronic headache, migraine, dizziness, chest congestion, dysmenorrheal, obesity, diabetes, paralysis, hemiplegia, back pain, infection, inflammation, rheumatism, hypertension, and gastrointestinal problems such as dyspepsia, flatulence, dysentery, and diarrhea (Datta et al.

2012). The plant extracts as well as seed oil have antimicrobial (Hanafy and Hatem,1991),anti-malarial (Abdulelahet al.2007), anti-inflammatory (Salem,2005), hypoglycemic (El-Dakhakhny et al.2002) and anti-cancerous effects (Raval et al.2010).Moreover, black seeds possess anti-nephrotoxicity (Yaman. and Balikci, 2010) and anti-hepatotoxicity (Farraget al.2007) in experimental animals. Tasawar et al.(2011)reported that black seed (tested on 80 subjects) is effective to change the lipid profile significantly in a way which is beneficial to heart. Treatment of the animals with *N. sativa* improved both genotoxicity and ultrastructural changes induced by CCl_4 (Abou-Gabal et al. 2007). Parhizkar et al. (2011) reported that *N. sativa* possesses estrogenic function which can be helpful in managing menopausal symptoms as an alternative for hormone replacement therapy. Although there are many investigations on the impact of *N. sativa* on various tissues, its effect in metiram-induced ovarian toxicity has not been examined yet. In the present work we evaluated the effects of N. sativa oil on serum antioxidant enzyme activities, lipid peroxidation and histological changes in ovarian tissue exposed to metiram in a rat model. Also we evaluated the changes in FSH, LH and estradiol levels following the treatment of *N. sativa* oil.

2 Materials and Methods

Fifty five Female Wistar rats weighting 155 ± 10 g were used in this work. They were purchased from the breeding center of experimental animals at Helwan University, Helwan, Egypt. The animals were kept in the laboratory under constant temperature (24±2 °C) throughout the experimental work. They were maintained on a standard rodent pellets and water was available ad libitum. Maintenance of animals and experimental procedures was approved by the animal ethical committee in accordance with the guide for care and use of laboratory animals of Menoufia University, Egypt. Animals were divided into 4 groups:

Group I. These animals (10 rats) served as controls.

Group II. Animals in this group (15 rats) were orally given 0.2ml/100 g b.w N. sativa oil 3 days/week for 4 weeks (Kamarzaman et al.2012). *N. sativa* oil was obtained from Cairo Pharmaceutical and Chemical Industries Co., Egypt.

Group III. Animals of this group (15 rats) were orally given 1/10LD50 (284 mg/kg b.w) of metiram3 days/week for 4 weeks dissolved in distilled water (Sakr and Badawy, 2011). This fungicide was obtained from SHOURA Chemical Company, Cairo, Egypt. It consists of 80% active ingredients [zinc ammoniate ethylenebis (dithiocarbamate)-poly (ethylenethiuram disulfide) and 20% inert ingredients.

Group VI. Animals in this group (15 rats) were given the same dose of metiram given to animals of group III followed by *N. sativa* oil (0.2ml/100 g) 3 days weekly for 4 weeks.

Histological, histochemical and quantitative study

Animals from treated and control groups weighted and sacrificed by cervical decapitation after 4 weeks of treatment. Then they were dissected, their ovaries were removed weighted and fixed in 10 % formalin. After fixation, specimens were dehydrated in an ascending series of alcohol, cleared in two changes of xylene and embedded in molten paraffin. Sections of 5 microns thickness were cut using rotary microtome and mounted on clean slides. For histopathological examination, sections were stained with Ehrlich's haematoxylin and counterstained with eosin. Sections of ovaries of control and experimental animals were examined histologically and used for quantitative analysis. All serial sections of the ovary were counted for various stages of development of follicles as described by Bolon et al.(1997). Follicles were classified into small (mean diameter < 20 μm), medium (mean diameter 20–70 μm) and large follicles (mean diameter>70 μm). Atretic follicles were classified into small and large. Masson trichrom method was used for staining collagen. Mean area% of collagen fiber content was measured in the Masson's trichrome-stained sections at a magnification of × 100 for each specimen using the color detect menu. This was done in 10 microscopic fields for each rat and their mean was obtained. Periodic acid Schiff's reaction was used for demonstration of total carbohydrates (Kiernan, 1981).

Hormonal study

For hormonal determination, blood sample was obtained from the inferior vena cava and then centrifuged. Sera were stored at -20 OC until assayed for the biochemical parameters. FSH, LH and estradiol were quantitatively determined in sera by enzyme immunoassay kit (Medix Biotech Inc., USA).

Ovarian oxidative stress

For determination of antioxidant enzymes, ovaries were removed and homogenized in potassium phosphate buffer solution (50 mM, pH 7.5) using a Potter-Elvehiem homogenizer to give a 10% homogenate. Homogenates were centrifuged at 1500 g for 10 min at 4°C; supernatant was recovered, placed on ice, and immediately used for the determination of MDA, CAT and SOD. The activity of superoxide dismutase (SOD) was determined according to the method of Nishikimi et al. (1972). The principal of this method depends on the ability of

SOD to inhibit the power of phenazine methosulphate-mediated to reduce the nitroblue tetrazolium. The catalase (CAT) activity was assayed according to the method of Aebi (1984). Catalase activity was determined from the rate of decomposition of H 2O2. Malondialdehyde MDA, as a marker for lipid peroxidation was measured colorimetrically in ovarian homogenate according to the method of Ohkawa et al. (1979).

Statistical analysis

The results were expressed as mean ± SD of different groups. The differences between the mean values were evaluated by ANOVA followed by Student's "t" test using Minitab 12 computer program (Minitab Inc., State Collage, P.A).

3 Results

Change in body and ovary weights

Exposure of albino rats to metriam led to a significant decrease in the body weight of animals compared to the control ($P<0.0001$). A significant increase in body weight was observed in animals treated with metriam + *N. stativa*. Animals given *N. stativa* alone did not show differences in body weight compared to the controls. Similarly, ovary weights decreased in animals treated with metriam and increased in those treated with metriam + *N. stativa* (Table 1).

Histological observations

Examination of ovarian sections of the control group (group I) showed the normal histological structure of the ovary. The surface of the ovary was covered by a single layer of flat to cuboidal cells (covering epithelium). Follicles in different stages of development were detected. Primary oocyte was surrounded by a single layer of squamous epithelial cells and multilaminar primary follicles were surrounded by a few layers of granulosa cells, were detected. Secondary follicles were also observed and each was formed of several layers of granulosa cells with multiple fluid filled spaces. Several corpora lutea were formed of large pale-staining vacuolated granulosa-lutein and theca lutein cells, enclosing many capillaries in between. Atretic follicles and mature graafian follicle were observed (Fig. 1).

Table 1. Change in body and ovary weight in mg in different animal groups

Treatment group	Body weight(g)	Ovary weight(mg)
Control	155.3 ±3.2	31±7.6
N. sativa	156±2.05	31.3±5.2
Metiram	135.5 29.1*	21.3±17. 1*
Metrium+*N. sativa*	153.5 5.1	30.6±9.19

(*). Significant at P<0.05 compared with control group

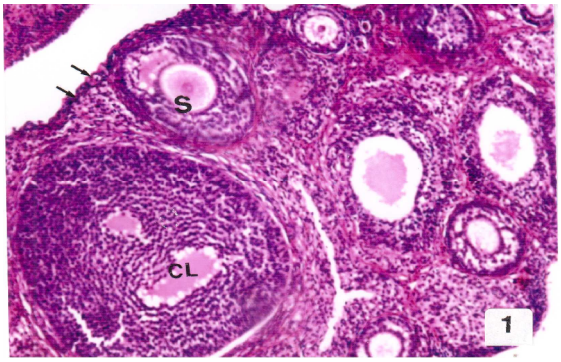

Fig.1. A photomicrograph of a section in the ovary of a control rat showing different types of follicles. Covering epithelium (arrows), CL: corpus luteum, S: secondary oocyte, (H&E X 400).

Ovaries of rats given *N. sativa* oil showed normal histological structure. Examination of sections of ovary of rats treated with metiram showed many histopathological alterations. The follicles were degenerated and showed hyalinization of the surrounding granulosa cells, dislodged oocytes, and widening of the spaces between the oocytes and their granulosa cells (Fig.2). The cells of corpus lutea were degenerated and appeared with pyknotic nuclei. Numerous atretic follicles in different stages of development were detected (Fig.3). Most of the blood vessels were enlarged and congested (Fig.4).

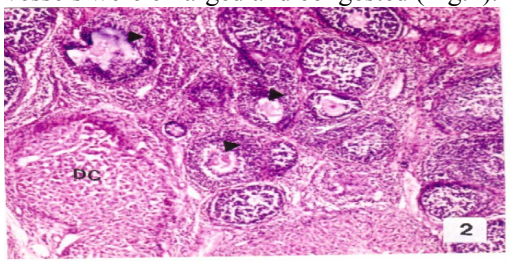

Fig.2. A photomicrograph of a section in the ovary of a rat treated with metiram showing degenerated follicles (arrow heads),DC: degenerated corpus leuteum,(H&E X400).

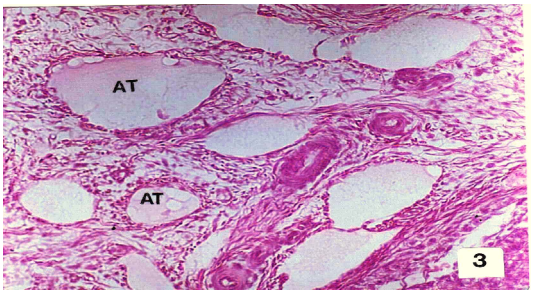

Fig.3. A photomicrograph of a section in the ovary of a rat treated with metiram showing atretic follicles with different sizes (AT),(H&E X400).

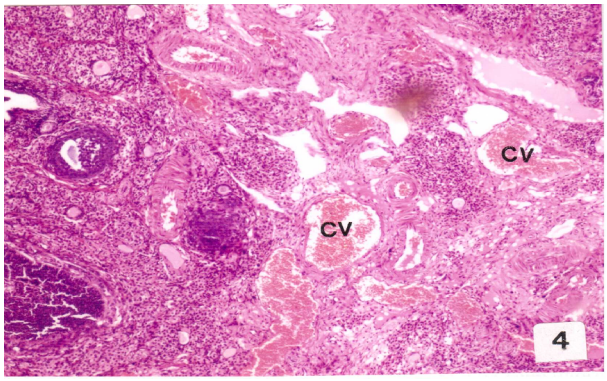

Fig.4. A photomicrograph of a section in the ovary of a rat treated with metiram showing congested blood vessels (CV), (H&EX400).

Ovary of animals treated with metiram and *N.sativa* showed an improvement in the histological appearance. Several normal follicles and corpora lutea with few of congested blood vessels was observed (Fig.5). Stroma of control rats and those given *N.sativa* showed a few collagen fibers (Fig.6), while animals treated with metiram showed an increase in collagen fibers in the stroma and around the blood vessels (Fig.7). Animals given metiram and *N.sativa* showed a reduction in collagen fibers compared with metiram-treated animals (Fig.8).

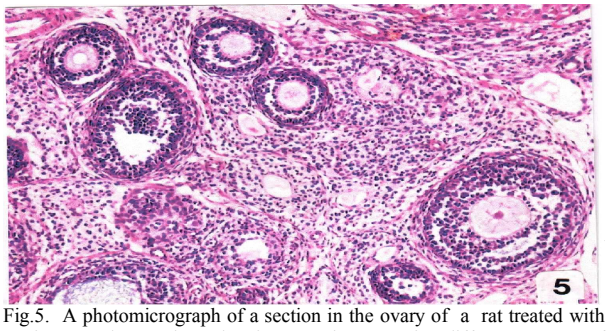

Fig.5. A photomicrograph of a section in the ovary of a rat treated with metiram and N.sativa showing an increase in different types of follicles,(H&E X400).

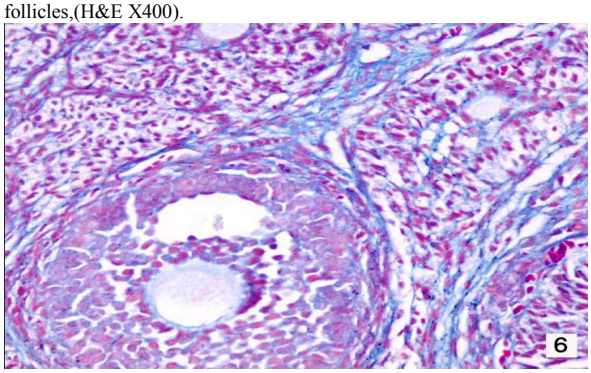

Fig.6. Section in ovary of a control rat showing few collagen in the stroma (Masson trichrom, X400).

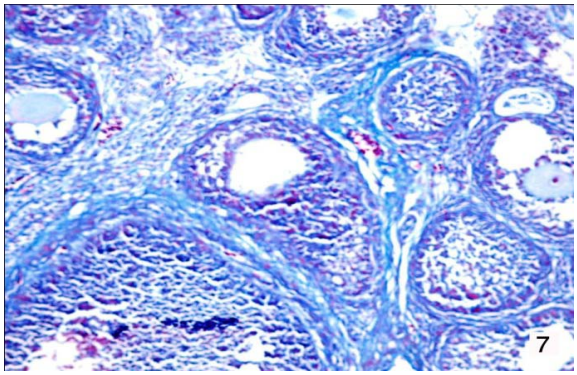

Fig.7. Section in ovary of metiram-treated rat showing increase of collagen (Masson trichrom,X 400)

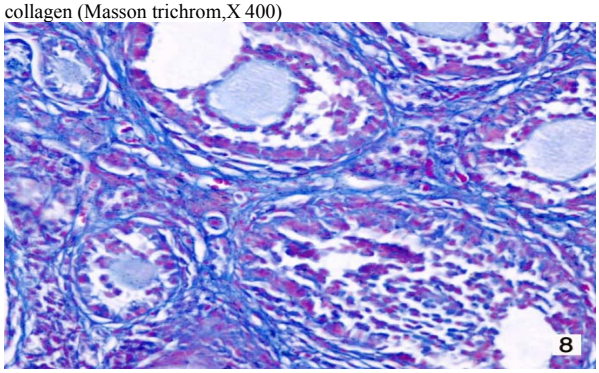

Fig.8. Section in ovary of metiram+ N.sativa treated rat showing decrease of collagen (Masson trichrom, X400).

Quntitative results

Data in table (2) showed that treating rats with metiram for 4 weeks caused significant decrease ($P<0.05$) in the number of medium, large and small follicles compared with control groups. The number of follicles was significantly increased after treating with metiram and *N.sativa*. On the other hand, the number of small and large atretic follicles increased significantly in rats treated with metiram (table 3).The number of atretic follicles decreased in animals treated with metiram and *N.sativa*. Data in table (4) showed the percentage of collagen content in different animals group. An increase in collagen content was recorded in rats treated with metiram compared with control and *N.sativa*-treated groups. On the other hand, the percentage of collagen content reduced in animals given metiram and *N.sativa*.

Table2. Effect of different treatments on the number of ovarian follicles

Treatment	No. of rats	Number of follicles		
		small	medium	large
Control	5	250 ± 11	51± 4	9± 1
N.sativa	5	248 ± 9	49± 5	8± 2
Metiram	5	171 ±5*	22± 3*	3 ±2*
Metiram+ N.sativa	5	222±8	42±3	7±1

(*). Significant at P<0.05 compared with control group

Table3. Effect of different treatments on the number of atretic follicles

Treatment	No. of rats	Number of atretic follicles		
		small	medium	Total number
Control	5	9 ± 2	3± 1	12± 1.4
N.sativa	5	10 ± 1	4± 1	14± 1.5
Metiram	5	18 ±2*	7± 2*	25 ±2.1*
Metiram + N. sativa	5	11 ± 2	4±1	15± 1.5

(*). Significant at P<0.05 compared with control group

Table 4. Mean area% of collagen fiber content in different animal groups

Treatment	Mean ± SD
Control	5.5 ± 0.4
N.sativa	5.8±0.6
Metiram	19.6±1.3*
Metiram + N. sativa	8.6±2.2

(*). Significant at P<0.05 compared with control group

Histochemical Observations

Examination of ovary of control rats or rats treated with N.sativa revealed that the germinal epithelial cells and the stromal cells showed a strong PAS-ositive reaction. The ovum of primary, secondary and Graafian follicles also showed a strong reactivity (Fig.9).Sections in ovaries of animals treated with metiram showed a marked decrease of PAS positive materials in the different parts of the ovary as compared with ovary of both control and *N.sativa* treated rats (Fig.10). On the other hand, rats treated with metiram and *N. sativa* showed an increase in the total carbohydrate contents of the stromal cells, the ovum of primary, secondary and Graafian follicles compared with those of metiram treated animals (Fig.11).

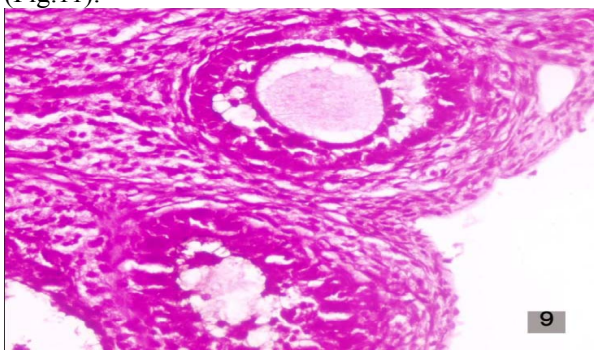

Fig.9. Section in ovary of a control rat showing increase amount of carbohydrates (PAS X 400).

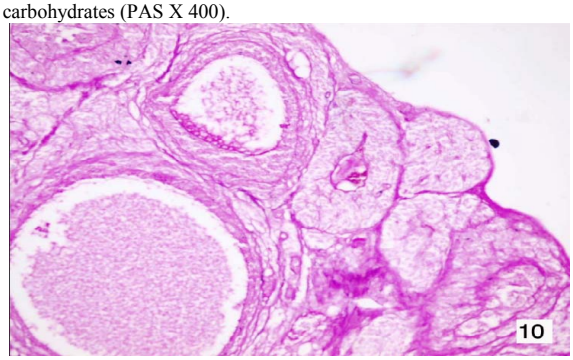

Fig.10. Section in ovary of metiram-treated rat showing decrease of carbohydrates (PASX 400).

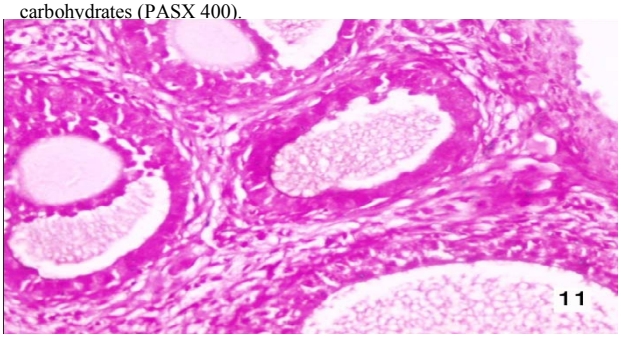

Fig.11. Section in ovary of metiram+ N.sativa treated rat showing restoration of carbohydrates (PAS,400)

Hormonal results

Treating animals with metiram revealed significant decrease in LH level compared with control group. Animals treated with metiram and *N.sativa* showed significant decrease in LH level when compared with metiram treated rats (Fig. 12). Similarly, a significant decrease in FSH was recorded in sera of animals treated with metiram while animals treated with metiram and N.sativa showed significant increase when compared with metiram treated rats (Fig.13). A significant decrease in estradiol level was observed in sera of animals treated with metiram and significant increase was recorded in sera of animals treated with metiram and *N.sativa* (Fig.14). Treating animals with *N.sativa* showed non-significant difference in levels of FSH, LH and estradiol when compared with control group.

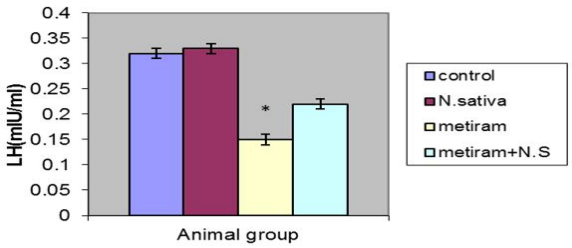

Fig. 12. Change in LH in different animal groups, (*). Significant at P<0.05 compared with control group.

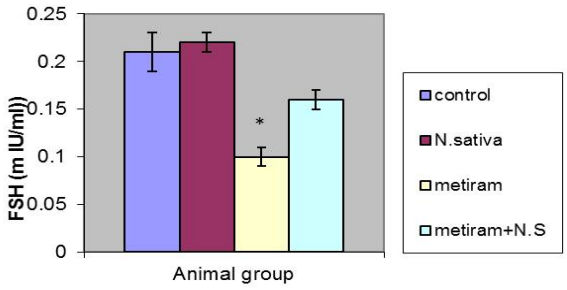

Fig. 13. Change in FSH in different animal groups, (*). Significant at P<0.05 compared with control group.

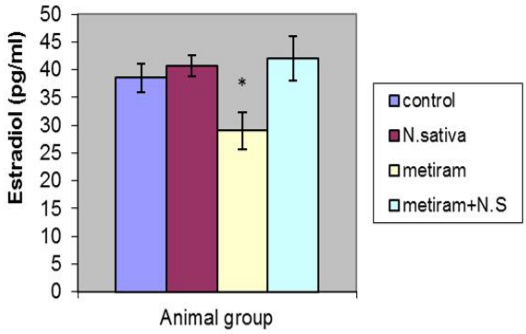

Fig. 14. Change in estradiol in different animal groups. (*). Significant at P<0.05 compared with control group.

Lipid peroxidation and antioxidant enzymes

Data in table (5) showed that metiram treatment exhibited a significant increase in the level of MDA (the marker of lipid peroxidation). Significant decrease in the activities of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), was observed in female rats administered with metiram. On the other hand, rats treated with metiram and *N.sativa* showed a decrease in MDA and an increase in the antioxidant status (SOD, CAT).

Table 5.Effect of different treatments on MDA, SOD and CAT in ovarian tissue of rats

Treatment	MDA(nmol/ mg protein)	SOD(U/mg protein)	CAT(U/mg protein)
Control	15.5±1.5	220±18.6	530±20.4
N.sativa	16.3±1.8	222±20.5	541±19.5
Metiram	29.4±2.2*	165±10.6	258±21.5*
Metiram + N. sativa	20.8±1.3	200±12.5	410±16.5

(*). Significant at P<0.05 compared with control group.

4 Discussion

In the present study, many histopathological changes were seen in the ovary of albino rats after treatment with metiram. The number of ovarian follicles decreased and most of them degenerated which accompanied by increased of atretic follicles. In agreement with these results, Sakr et al. (2011) reported that topsin fungicide caused histopathological alterations in the ovary and decreased the number of follicles with an increase of the atretic follicles. They added that topsin significantly decreased serum levels of both LH and FSH and increased estradiol. Treating rats with mancozeb fungicide was found to induce a significant decrease in the number of healthy follicles with concomitant increase in the number of atretic follicles. The histologic observation of the ovary revealed the presence of less number of corpora lutea and the size of the ovary was also reduced (Bolivar and Kaliwal, 2001). Administration of edifenphos, an organophosphate fungicide, to hemicastrated albino rats caused a decrease of ovarian weight and decreased the number and duration of different phases of the estrous cycle (Nanda and Kaliwal, 2003). Shibayama et al. (2009) reported that atrazine herbicide led to histopathological findings such as decrease in the numbers of corpora lutea, increase in larg -sized atretic follicles and swelling of the luteal cells. The authors suggested that atrazine had an ovulatory effect through suppression of the luteinizing hormone surge. Goldman et al.(1997) reported that the fungicide sodium dimethyldithiocarbamate caused a dose-related suppression of oocyte release in rats and this involves two separate mechanisms, one attributable to an alteration in ovarian hormonal feedback to the brain (or pituitary), inhibiting the LH surge, and the other associated with a direct, as yet undetermined, effect on local preovulatory events within the ovary. Armenti et al. (2008) recorded that exposure to organochlorine pesticide, methoxychlor, resulted in reduced ovulation and fertility and premature aging, possibly by altering ovarian gene expression and folliculogenesis. The authors added that methoxychlor reduced serum progesterone, increased luteinizing hormone and down-regulated Cytochrome P450side-chain cleavage.

A decrease in amount of carbohydrates was recorded in ovary of rats treated with metiram. Similarly, Sakr and Badawy (2011) observed depletion of glycogen in testicular tissue of rats given metiram. El-Sherbiny et al.(2010) reported an alteration in some enzymatic

activity that had led to metabolic degradation and inhibited the carbohydrate synthesis in the ovarian follicles of rats exposed to some insecticides. An increase in lipid peroxidation marker , MDA, and a decrease in the antioxidant enzymes ,SOD and CAT, was recorded in ovarian tissue of rats treated with metiram. These results strongly suggested that metiram has the capability to induce oxidative damage. These results are consistent with the previous study of Sakr et al. (2011) who observed an increase in lipid peroxidation and decrease of antioxidant enzymes in rats treated with the fungicide, topsin.

In the present study, the decrease in healthy follicles with concomitant increase in atretic follicles in rats exposed to metiram was interrupted by some authors. Sarkar et al. (2000) reported that this effect may due to inhibition of acetyl cholinesterase which alters the pituitary as it was observed in the rats treated with pesticide quinolphos. Another possibility may be due to affecting catecholamine neurotransmitter metabolism by inhibiting the GnRH release through the inhibition of DβH (Przewlocka et al. 1975). Pasqualine et al. (1990) reported that the reproductive toxicity of 12-dimethyl (a) anthranene may be due to the direct effect on gonads that may impair reproductive function by direct insult to the cell population within the gonads resulting in the impairment of the feedback mechanism to the hypothalamus and pituitary. In the present work, the ovarian toxicity may be due to the oxidative stress generated by metiram.

The significant decrease in hormonal level (LH, FSH and estradiol) observed in metiram treated rat may explained by two explanations. The first one is hypovascularity associated with congested blood vessels. In this concern, Löseke and Spanel-Borowski (1996) correlated hypoovulation to insufficiently developed microvascular bed in over stimulated ovaries. The second one is stimulation of progesterone antagonists production that reduced preovulatory P4-production in the ovary and down PR expression through pituitary - hypothalamus axis (Donath et al., 2000).

Results obtained in this work revealed the potential protective role of *N.sativa* against ovarian toxicity of metiram. Animals given N.sativa and metiram showed an increase in the number of follicles with decrease of atretic ones together with increase in FSH, LH and estradiol. Similar to these results, Kamarzaman et al.(2014) reported that cyclophosphamide-treatment caused a significant reduction in the mean number of normal primary and secondary follicles, mean ovarian diameters and increased vacuolation with irregular distribution of granulosa cells. They added that the numbers of normal and ovarian diameters were significantly increased in mice given N. sativa and cyclophosphamide as opposed to effects seen in cyclophosphamide-alone. Bayir et al.(2012) reported that the administration of N. sativa was effective in reversing tissue damage induced by ischemia and/or ischemia/reperfusion in rat ovaries. Arak and Assi (2011) reported that adult rats exposed to lead acetate caused a significant reduction in FSH and LH and the treatment of N. sativa seed extract caused significant elevation in these hormones. They added that this elevation may be due to the active constituents of N. sativa which stimulate hypothalamus or pituitary glands to release and secrete gonadotropin hormones.

Attentions have been paid to the protective effects of natural antioxidant against xenobioticas - induced toxicities, especially when free radical generations are involved. The present results showed that *N.sative* caused decrease of lipid peroxidation and improved the antioxidant status in ovary of rats treated with metiram. The antioxidant activity of *N.sativa* was studied by many investigators. Burits and Bucer (2000) tested the possible antioxidant activity of the essential oil seeds, *N.sativa*. They have showed that thymoquinone and the components carvacrol, t-anethole and4-terpineol demonstrated respectable radical scavenging property.Thymoquinone is the active compound of the essential oil with anti-oxidative effect that works as a scavenger of various radical oxygen species including superoxide radical anion and hydroxyl radicals through different mechanisms (Badary et al., 2002).

In vitro studies have shown that extracts of *N. sativa* seeds protect erythrocytes against lipid peroxidation, degradation, loss of deformability and increased osmotic fragility caused by hydrogen peroxide H2O2 (Suboh et al.2004). Meziti et al. (2012) reported that *N. sativa* seeds have a considerable antioxidant activity in vitro and in vivo.The antioxidant activities of the methanolic extract and the fixed oil are confirmed by an *in vivo* assay in mice, the daily oral administration of methanolic extract and fixed oil during 21 days, resulted in a significant enhancement of the blood total antioxidant capacity. Kushawa (2014) reported that *N.sativa* oil is effective in

protecting rats against acetaminophen induced hepatotoxicity via increased resistance to oxidative stress and by reverse cellular damage. Awadalla (2012) reported that co-administration of *N. sativa* oil prevented cisplatin induced reproductive toxicity in male rats and decreased testicular lipid peroxidation. *N. sativa* fixed and essential oils significantly ameliorate free radicals and improve antioxidant capacity in diabetic rats (Sultan et al.2014).The ameliorative effect of *N.sativa* oil against metiram-induced ovarian toxicity observed in the this study, seems to be due to the richness of this oil with antioxidant compounds.

5 References

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