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Original article

Origanum majorana essential oil improves the rat's sexual behavior and testicular oxidative damage induced by imidacloprid via modulating the steroidogenesis pathways

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ABSTRACT

The neonicotinoid insecticide imidacloprid has been linked to significant reproductive damage in mammals. *Origanum majorana* essential oil (OME) is a natural herbal product used in the management of many diseases due to its strong antioxidant effects. The oil was hydrodistilled from *O. Majorana* and analyzed using GC/MS then its possible protective mechanisms against IMI-induced reprotoxicity in male rats were investigated. 28-adult male Wistar rats were divided into 4 groups as follows: group (1) control group, group (2) OME, group (3) IMI, and group (4) IMI + OME. The treatments were applied daily via oral gavage for 60 days. Remarkable abnormalities in both territorial aggressive and sexual behaviors were observed in IMI-treated rats with a significant elevation of serum FSH and LH as well as altered testicular redox status. Along with inhibition of the testicular expression of StAR and aromatase genes and serum total testosterone in addition to abnormal sperm count, viability, motility, and morphology. Histopathological examination showed severe degeneration and necrosis in both germ cells and Leydig cells with atrophy in most of the seminiferous tubules. Co-administration of OME with IMI notably improved all the above-mentioned studied parameters, and restored rats' spermatogenesis, sexual behavior, and favorably modulates the levels of both testosterone and gonadotropic hormones via its potent antioxidant effect. These findings support the use of OME as a fertility enhancer and suggest that it could be used to manage pesticide-induced male infertility.

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Abbreviations: CAT, Catalase; EF, Ejaculation frequency; EL, Ejaculation latency; FSH, Follicle stimulating hormone; GCA-S'cytes, Germ cell arrest at spermatocytes; GSH, Reduced glutathione; HPT, Hypothalamic-pituitary-testicular axis; IF, Intromission frequency; IL, Intromission latency; IMI, Imidacloprid; LH, Luteinizing hormone; LPO, Lipid peroxidation; MDA, Malondialdehyde; MF, Mount frequency; ML, Mount latency; OME, *Origanum majorana* essential oil; PEI, Post-ejaculatory interval; ROS, Reactive oxygen species; SC, Sertoli cells; S'gonia, Spermatogonia; StAR, Steroidogenic acute regulatory protein; TCA, Trichloroacetic acid.

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1. Introduction

Neonicotinoid insecticides are extensively utilized to protect the harvest and domesticated animals against the wide scope of pests (Matsuda et al., 2001, Tomizawa and Casida, 2005). The United States Environmental Protection Agency (US-EPA) enlisted the imidacloprid pesticide (IMI) [1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine] in "Group E" carcinogen, which implies that there is no proof of cancer-causing nature in people (Duzguner and Erdogan, 2012). Even though IMI is somewhat harmful to people and domesticated animals, it might in any case affect some organs like the brain, heart, kidney, testes and gastrointestinal organs causing severe disorders (Abbassy et al., 2014). Several studies reported the toxic impacts of IMI in

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mammals, including mutagenic (Bal et al., 2012), teratogenic (Hafez et al., 2016), and hepatorenal (Hassanen et al., 2022) and immunotoxic impacts (Subapriya et al., 2005, Weisburger, 2001). Some recent studies revealed that prolonged exposure of rats to IMI can cause testicular damage, hormonal imbalance, and spermogram alteration manifested by a reduction in sperm count and motility, and elevation in sperm mortality and abnormality, all of which could prompt infertility in male rats (Bataneh and Nusier, 2006, Hassan et al., 2019, Khouri and El-Akawi, 2005). (See Fig. 1).

Oxidative stress is considered the primary conceivable component of IMI-initiated poisonousness in the cell especially in the male reproductive system due to its high polyunsaturated fatty acids content making it more vulnerable to oxidative damage (Zowail et al., 2019). ROS can modify oxidant/antioxidant balance by expanding lipid peroxidation (LPO) and exhausting both enzymatic and non-enzymatic antioxidants in the cell which trigger cells and DNA damage (Abbassy et al., 2014, Mansour and Mossa, 2009).

Recently, significant consideration has been committed to therapeutic plants especially those enriched in polyphenols, for the most part, flavonoids and phenolic acids, which show cell reinforcement properties, hydrogen-giving and metal-chelating limits as potential chemopreventive specialists (Khalaf et al., 2020). The phenolic compounds have affirmed defensive impacts against oxidative stress damage induced by numerous poisons through scavenging ROS and upgrading host cell antioxidants (Subapriya et al., 2005, Weisburger, 2001). Marjoram (*Origanum majorana*) belongs to the mint family (Lamiaceae) which is a common aromatic medicinal herb (Gurib-Fakim, 2006). *Origanum majorana* essential oil has clear restorative properties like antioxidant, antimutagenic, and anti-inflammatory effects (Mossa and Nawwar, 2011). This plant is disseminated throughout Asia, Europe and North America and is regularly known as Wild marjoram or wintersweet (Zowail et al., 2019). It is broadly utilized in the conventional medication as a diuretic and antispasmodic alongside other therapeutic uses, such as energizer, expectorant, antibacterial, anticancer, anti-inflammatory, antioxidant and purgative (Lemhadri et al., 2004).

The widespread application of IMI in pest control results in its environmental bioaccumulation; thus, humans and animals' expo-

sure has become a reality. On the other hand, the risk associated with environmental pollutants could be lessened with the use of natural plant-based agents. However, the interaction of *Origanum majorana* with IMI has not been referred to yet. Therefore, this study aimed to investigate the possible protective effect of *Origanum majorana* essential oil (OME) against IMI-induced reproductive dysfunction and infertility in male rats by measuring the changes in the sexual behavior, and hormonal analysis. Additionally, we study the testicular redox status, histopathological alteration, and gene expression.

2. Materials and methods

2.1. Chemicals

Anhydrous sodium sulfate, ethanol, chloroform, and sodium citrate were purchased from Sigma Chemical Co., Germany. Commercial imidacloprid (70% WP, (NE)-N-[1-[(6-chloropyridin-3-yl)methyl]imidazolidine-2-ylidene]nitroamide, C₉H₁₀ClN₅O₂) was obtained from Kafr El-Zayat Pesticides & Chemicals Company (Kafr El-Zayat, Gharbia, Egypt). We dissolved 3 mg IMI in 1 mL deionized distilled water to form solution with a concentration of 0.3% (W/V) and we prepared it freshly by the required concentration of the active ingredient (45 mg/kg bwt).

2.2. Preparation and identification of *Origanum majorana* essential oil

2.2.1. Plant material and essential oil extraction

Origanum majorana L. fresh aerial parts (leaves, stems, and flowers) were collected from the Experimental Station of Medicinal Plants, Faculty of Pharmacy, Cairo University, Giza, Egypt, in May 2020. The plant was identified and authenticated at the herbarium of the Faculty of Science, Cairo University. The aerial parts were cut into small pieces and hydrodistilled using Clevenger apparatus to obtain the essential oil. The distillation continued for 5 h till no more condensing oil was noticed, then, the distilled oil was dried over anhydrous sodium sulfate and stored in a freezer at -20°C until analyzed and further used for biological activity evaluation (Mossa and Nawwar, 2011).

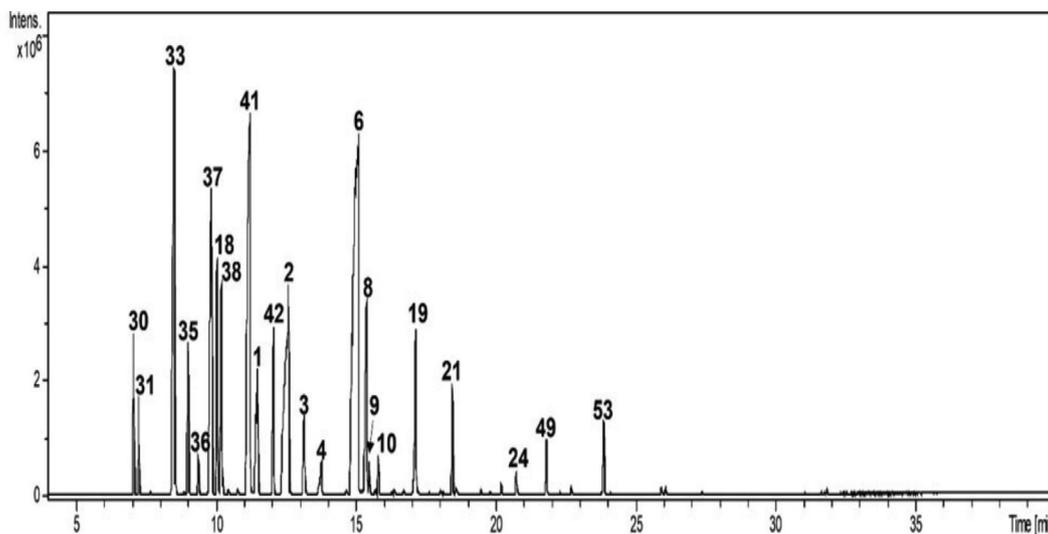


Fig. 1. GC-MS representative chromatogram of volatile oil of aerial parts of *Origanum majorana* L. herb. The corresponding compound names for volatile peaks follow that listed in Table 1. Notes: 1, *cis*-Sabinene hydrate; 2, β -Linalool; 3, *cis*-2-*p*-Menth-1-ol; 4, *trans*-*p*-2-Menth-1-ol; 6, 4-Terpineol (Terpinen-4-ol); 8, α -Terpineol; 9, *trans*-*p*-Menth-1-en-3-ol (*trans*-Piperitol); 10, *cis*-*p*-Menth-1-en-3-ol; 18, *m*-Cymene; 19, Linalyl acetate; 21, 4-Terpineol acetate; 24, Geranyl acetate; 30, α -Thujene; 31, α -Pinene; 33, Sabinene; 35, β -Myrcene; 36, α -Phellandrene; 37, α -Terpinene; 38, Limonene; 41, γ -Terpinene; 42, Terpinolene; 49, Caryophyllene; 53, Elixene.

The quality of *origanum Majorana* essential oil (OME) was assessed by the yield and physical analysis such as color and odor. Specific gravity was measured at 20 °C, refractive index using Abbe digital refractometer (Leica, Depew, New York) at 25 °C and solubility in ethanol. The oil (1 µL) was diluted in 1 mL chloroform for GC–MS analysis, and applied for compounds limits determination (Issa et al., 2020b).

2.2.2. GC/MS analysis of the essential oil

GC–MS analysis was performed on a Shimadzu GCMS-QP2010 (Kyoto, Japan) equipped with Rtx-5MS fused bonded column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) (Restek, USA) equipped with a split-splitless injector. The injector temperature was set at 250 °C. The subsequent gradient temperature program was used for volatiles analysis. The initial column temperature was held at 45 °C for 2 min and ramped at a rate of 5 °C/min to 300 °C, and then kept constant at 300 °C for 5 min. Helium was used as the carrier gas with a flow rate of 1.41 mL/min. Blank runs were made during samples analyses. The quadrupole mass spectrometer was operated in EI mode at 70 eV with the interface temp. at 280 °C and ion source temp at 200 °C. A scan range was set at m/z 40–500. Diluted samples (1% v/v) were injected with split mode (split ratio 1: 15). Three replicates were analyzed for the essential oil sample.

2.3. Animals and experimental protocol

Twenty-eight adult male albino Wistar rats (170 ± 20 g) were attained from the Department of Veterinary Hygiene and Management's Animal House, Faculty of Veterinary Medicine, Cairo University, Egypt. Animals were kept in plastic cages and standard commercial pelleted feed and water were provided *ad libitum*. They were closely inspected for health conditions and acclimatized for two weeks before the beginning of the experiment. All the procedures and the experimental design were authorized and accepted by the institutional animal care and use committee of Cairo University (IACUC), Animal Use Protocol (AUP Vet Cu 12102021360).

Rats were randomly allocated into 4 groups (n = 7). Animals were given imidacloprid (IMI) and/or *origanum oil* (OME) daily via oral gavage for 60 days. Group (1) received 10% Tween 80 in normal saline and was kept as a control group. Group (2) received 200 mg/kg bwt OME. Group (3) received 45 mg/kg bwt IMI. Group (4) received co-treatment of IMI (45 mg/kg bwt) and 200 mg/kg bwt OME. OME was weighed in a clean empty beaker and then suspended in saline with the aid of 10 % Tween 80 to give a constant final volume of administered doses (5 mL/kg) (Issa et al., 2020a). The dose of imidacloprid represented 1/10 the LD50 and was selected according to previous study (Hassan et al., 2019), as well as the dose of OME (Pimple et al., 2012).

2.4. Behavioral measurements

2.4.1. Territorial aggressive behavior test

Rat's aggressive behavior was tested in a rectangle observation cage with measurements of 45 cm in length, 27 cm in width, and 40 cm in height. A stud male rat was kept alone for 10 days in the testing cage and considered the resident. Then the tested male (intruder) of no previous contact was placed with the resident in the test arena and confronted with the resident for 5 min test period. All the testing sessions were recorded, and the aggressive behavior was analyzed using behavioral tracking software (Solomon coder, www.solomoncoder.com). The following aggression parameters were reported: lateralization, boxing bouts, fights with

the stud male, as well as ventral presenting posture (supine posture) (El-Iethy et al., 2011, Khouri and El-Akawi, 2005).

2.4.2. Sexual behavior test

Male rat's sexual behavior was measured by using a stimulus untreated female (sexually receptive female). Then the male rat was placed alone in the mating cage measuring (45 cm length × 27 cm breadth × 40 cm height) for 5 min for habituation before introducing to the receptive female. Male copulatory behavior was video recorded during a 15 min session, and the sexual activity was analyzed using behavioral tracking software (Solomon coder, www.solomoncoder.com). The following parameters were monitored; mount latency (ML), intromission latency (IL), ejaculation latency (EL), mount frequency (MF), intromission frequency (IF), ejaculation frequency (EF) i.e., mating potential, post-ejaculatory interval (PEI), as well as the intromission ratio, which was estimated using the formula (IF/ (IF + MF) (Bataineh and Nusier, 2006, Heijkoop et al., 2018). The latency of any rat that failed to mount, intromit, or ejaculate was recorded as 15 min for statistical testing.

2.5. Sampling

After behavioral assessments, blood samples were collected from all rats and serum samples were obtained after centrifugation at 3500 Xg for 15 min which was then preserved at – 20 °C till used for hormonal analysis. Afterward, Rats were euthanized by the cervical dislocation and immediately clear semen samples were collected from the tail of the epididymis and vasa differentia to perform the semen analysis. Additionally, testicular samples were collected and divided into two parts, the first part was preserved at 10% neutral buffered formalin for histopathology, while the second part was maintained at – 80 °C for oxidative stress assessment and molecular studies.

2.6. Hormonal analysis

Serum testosterone levels were measured using an ELISA kit (Diagnostic Biochem., Canada, CAN-TE-250). While, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations were measured using a rat-specific ELISA kit following the instructions of the manufacturer (Sun Long Biotech Co., LTD, Catalog # [SL0286Ra](#), Catalog # [SL1093Ra](#)).

2.7. Semen analysis

The collected semen samples were freshly examined for sperm cell concentration, sperm motility, sperm viability, and sperm abnormalities by the method of Bearden and Fuquay (Bearden and Fuquay, 1997). Briefly, a drop of 2.9% sodium citrate solution was added to the sperm sample on a warm slide and then examined under the microscope to determine the percentage of the motile spermatozoa in the field (0–80%). Afterward, smears of sperm samples were stained with eosin 1% and nigrosine 5% to evaluate the live/dead ratio (viability percentage) and morphological changes. Regarding sperm cells concentration, the sperm samples were diluted and stained with 2% eosin solution using an RBCs pipette, and the sperms were similarly counted by hemocytometer to RBCs.

2.8. Oxidative stress evaluation

Testicular tissues were homogenized using a 5 mL cold buffer of 50 mM potassium phosphate, pH 7.5 per gram tissue for MDA Assay, while for GSH assay 1 mM EDTA was added. On the other

hand, 1 mL/L Triton X-100 was added to the previous solution for CAT activity assay. Finally, tissue homogenates were centrifuged at 4000 rpm for 15 min at 4 °C and the supernatants were separated for the redox state evaluation. Lipid peroxidation (MDA) level, GSH content and CAT activity were measured by using the test kits (Biodiagnostic Co., Egypt, CAT. No. MD 25 29, GR 25 11, CA 25 17).

2.9. Pathological studies

2.9.1. Histopathological examinations

Formalin-fixed testicular and epididymal samples were treated through application of ascending concentrations of alcohol for dehydration and cleared by xylol, then sliced at 4.5 µm to obtain paraffin sections stained by H&E for histopathological examinations using Olympus light microscope (Bancroft and Gamble, 2008).

2.9.2. Testicular lesion scoring systems

For quantitative microscopic evaluation of the testicular lesions, Johnsen (Johnsen, 1970) and McLachlan et al. (McLachlan et al., 2007) scoring systems were applied. Concerning Johnsen scoring, a ten-point scoring system was assessed to quantify the spermatogenesis based on counting the different germ cells and Sertoli cells lining each seminiferous tubule for a total of 100 tubules. Afterward, the number of tubules was multiplied by the score then the result was summed across different scores and divided by the number of evaluated tubules to calculate Johnsen score mean. The criteria formulated by Johnsen were summarized as the following: (10) normal spermatogenesis and numerous sperm cells with normal organized germinal epithelium, (9) many sperm cells with disorganized germinal epithelium, (8) few sperm cells, (7) no sperm cells with numerous spermatids, (6) no sperm cells with few spermatids, (5) neither sperm cells nor spermatids present but numerous spermatocytes found, (4) few spermatocytes, (3) only spermatogonia, (2) sertoli cells only, (1) neither germ nor sertoli cells were present.

McLachlan scoring system that summarized into the Copenhagen index was calculated via the evaluation of 20 seminiferous tubules and the results were expressed as a compound index of four digits based on the histopathological pictures of different stages of spermatogenic cells within the tubules. The first digit described the general pattern of the testis as the following: (1) adult, (2) immature, and (3) neoplasia. The second digit described the most predominant histological pattern of seminiferous tubules as the following: (1) homogenous (uniformity of histological pattern) with normal spermatogenesis, (2) homogenous with reduced spermatid count, (3) homogenous with germ cell arrest at spermatocytes (GCA-S'cytes), (4) homogenous with only spermatogonia (S'gonia) and Sertoli cells (SC), (5) homogenous with Sertoli cells only (SCO), (6) heterogeneous (mixed histological pattern) and predominantly 1 or 2, (7) heterogeneous and predominantly 3, (8) heterogeneous and predominantly 4, (9) heterogeneous and predominantly 5, (0) other. The third digit represented the next important histological pattern like the following: (1) normal spermatogenesis, (2) reduced spermatid count, (3) GCA-S'cytes, (4)

only S'gonia + SC, (5) SCO, (6) hyalinized tubules, (7) undifferentiated tubules, (8) hyalinized and undifferentiated tubules with microliths, (9) hyalinized and undifferentiated tubules with Leydig cell nodules, (0) other. The fourth digit described other abnormalities as the following: (1) inflammatory cells infiltration, (2) fibrosis, (3) Leydig cell hyperplasia, (4) Leydig cell degenerations or aplasia, (5) microliths, (6) granuloma, (7) 1 + 3, (8) 3 + 5, (9) undifferentiated tubules, (0) no abnormalities.

2.9.3. Histomorphometric analysis

It was performed using ImageJ software for the measurement of the seminiferous tubular diameters, germ cell layer thickness, and the mean percentage of empty seminiferous tubules about the total tubular numbers. Twelve circular seminiferous tubules per testicular section were randomly examined at low magnification (×100) in five sections representing five rats per group according to the method described previously by Azouz and Hassanen (Azouz and Hassanen, 2020).

2.10. Quantitative real-time PCR for *stAR* and *aromatase* genes

Isolation of the total testicular tissue RNA was done using Easy-RNA™ Cell/Tissue RNA Mini Kit (Biovision#K1337). The synthesis of first-strand cDNA was performed using SuperScript Reverse Transcriptase (Thermo Scientific) following the manufacturer's instructions. Quantitative PCR was done using Power Track™ SYBR Green Master Mix Applied Biosystems™ on an ABI Prism Step OnePlus Real-Time PCR System (Applied Biosystems) following the manufacturer's instructions. The primer sets of our candidate genes were collected in the Table 1. The Target mRNA expression was normalized to ACTB.

2.11. Genotoxicity study

2.11.1. DNA laddering assay

DNA fragmentation assay was employed as follows: A (200 mg) of the testicular tissue was mechanically homogenized using a hypotonic lysis buffer. The tissue homogenates were then centrifuged, and the pellets encompassed the total intact DNA (P) while the supernatants (S) included the fragmented DNA. Both P and S fractions were separated and each of them was treated with 0.5 mL of 25% trichloroacetic acid (TCA) then incubated at 4 °C for 12 hrs. DNA was collected by centrifugation followed by the addition of 80 µl of TCA (5%) to each sample and incubated at 90 °C for 20 min. Then, we added 1 mL diphenylamine reagent to each sample and all tubes were incubated overnight at room temperature. The optical density (OD) was measured at 600 nm. We determined the DNA laddering percentage according to the following equation: % DNA fragmentation = [S/ (S + P)] X 100 (Hassanen et al., 2021).

2.11.2. Genomic DNA agarose gel electrophoresis

The agarose gel electrophoresis was performed for the extracted genomic DNA using the DNeasy kit (Qiagen). The extracted DNA was electrophoresed using 2% agarose gel containing ethidium bromide.

Table 1

The primer sets of the studied genes.

	Sense	Antisense	Amplicon	Accession no
StAR	TGGCTGCCAAAGACCATCAT	TGGTGGGCAGTCCTTAACAC	241	NM_031558.3
Aromatase	TGACGTCAGTACAACCTCGG	CAAGTCCACGACAGCTGAT	235	NM_017085.2
ACTB	CCGCGAGTACAACCTTCTTG	CAGTTGGTGACAATGCCGTG	297	NM_031144.3

Abbreviations: StAR, Steroidogenic acute regulatory protein; ACTB, beta actin housekeeping gene.

Table 2Effect of the imidacloprid (IMI) and/or *Origanum majorana* essential oil (OME) on some hormonal assay.

Groups Parameters	Testosterone (ng/mL)	FSH (ng/mL)	LH (IU/L)
Control	6.50 ± 0.21 ^a	7.02 ± 0.14 ^a	1.24 ± 0.10 ^a
OME	6.53 ± 0.26 ^a	6.65 ± 0.23 ^a	1.21 ± 0.11 ^a
IMI	3.53 ± 0.19 ^b	10.58 ± 0.68 ^b	2.46 ± 0.15 ^b
IMI + OME	4.83 ± 0.30 ^c	8.32 ± 0.24 ^c	1.60 ± 0.11 ^c

Data are expressed as mean ± SEM. (n = 7 rats/ group). Values with different letters within the same column are significantly different at P ≤ 0.05.

2.12. Statistical analysis

Statistical analysis was performed using SPSS (V25) including the calculations of Means ± standard deviation (SD) using one-way analysis of variance (ANOVA) followed by a posthoc test. Statistical significance was set at the P < 0.05 level.

3. Results

3.1. Yield and physical constants

The yield of essential oil obtained from *Origanum majorana* L. reached 1.1 % (V/W) on fresh weight basis, which is following the usual yield. The color of the oil was light yellow to orange. The odor was thyme green herbal spicy as determined by olfactory expertise. The oil was soluble in ethanol. Specific gravity at 25 °C ranged from 0.9350 to 0.9430 and refractive index at 20 °C ranged from 1.5000 to 1.5300.

3.2. GC–MS peaks identification in *Origanum majorana* essential oil

We aimed in this study to assess the volatiles constituents in *Origanum majorana* L. essential oil (OME) grown in Egypt during the flowering season. The identified compounds and their relative

proportions are listed according to their order of elution on Rtx-5MS fused bonded column in supplementary materials (Table S1).

GC/MS analysis of *Origanum majorana* oil results in the identification of 55 volatile components categorized in 8 different classes viz. alcohols, aromatics, esters, ketones, monoterpene hydrocarbons, oxides, phenol/ethers and sesquiterpene hydrocarbons. Alcohols, monoterpene hydrocarbons, esters and sesquiterpene hydrocarbons were the major volatile classes in OME. Several metabolites were reported as representative components of OME, including *cis*-sabinene hydrate, β -linalool, 4-terpineol, α -terpineol belonging mainly to alcohols, in addition to sabinene, α -terpinene, limonene, γ -terpinene, terpinolene belonging to monoterpene hydrocarbons.

Alcohols constituted the most abundant class in OME accounting for 47.19 %. Linalool (peak 2) was the major detected volatile component that reached 17.76% while 4-terpineol (peak 6) was detected at ca 13.54%. *cis*-Sabinene hydrate, α -Terpineol, *cis*-*p*-2-Menthen-1-ol, *trans*-*p*-2-Menthen-1-ol were found next to linalool and 4-terpineol in OME at ca. 5.89, 4.74, 2.38 and 1.15% of the total volatiles composition, respectively.

Monoterpene hydrocarbons comprised the second most abundant volatiles class in OME amounting for 39.55% of its total volatile composition. γ -Terpinene (peak 41) was the major detected monoterpene hydrocarbon and the second most abundant volatile component in OME accounting to ca. 14.26% of its total essential oil composition. Followed α -terpinene, sabinene, terpinolene, limonene, β -myrcene and α -thujene (peaks 37, 33, 42, 38, 35 and 30) amounting for ca. 8.21, 5.62, 3.32, 3.25, 1.90 and 1.34 of the total volatile components in OME, respectively. Further minor monoterpene hydrocarbons were detected in lesser amounts.

Esters were the third dominant volatile class in OME (6.2%), represented by linalyl acetate as the major ester (3.64%, peak 19), followed by 4-Terpineol acetate (1.96%, peak 21), geranyl acetate (0.31%, peak 24) and neryl acetate (0.71%, peak 23). Other esters found were bornyl acetate and α -terpinyl acetate. Sesquiterpene hydrocarbons constituted the fourth abundant volatiles class in

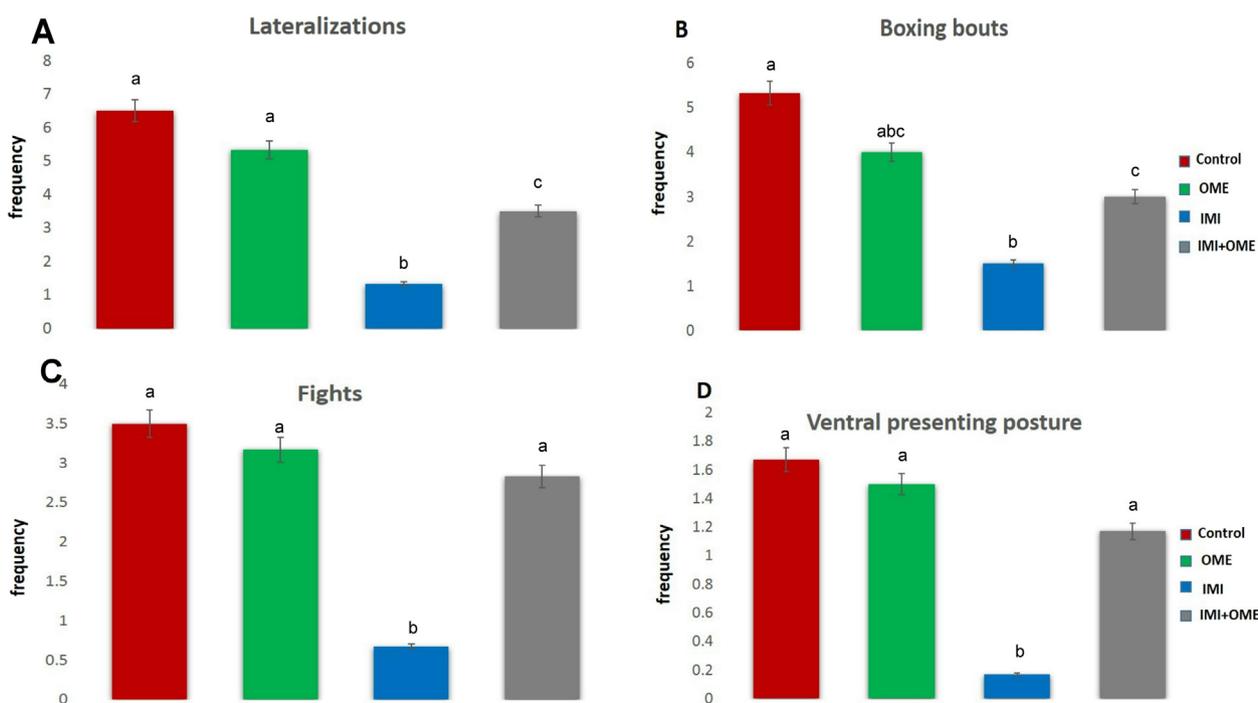


Fig. 2. Bar charts representing the effect of imidacloprid (IMI) and/or *Origanum majorana* essential oil (OME) on rat's aggression behavior. (A) Lateralization frequency, (B) boxing bouts frequency, (C) fights frequency, (D) ventral presenting posture frequency. Data are presented as mean ± SEM (n = 7). Values with different letters were significantly different at P ≤ 0.05.

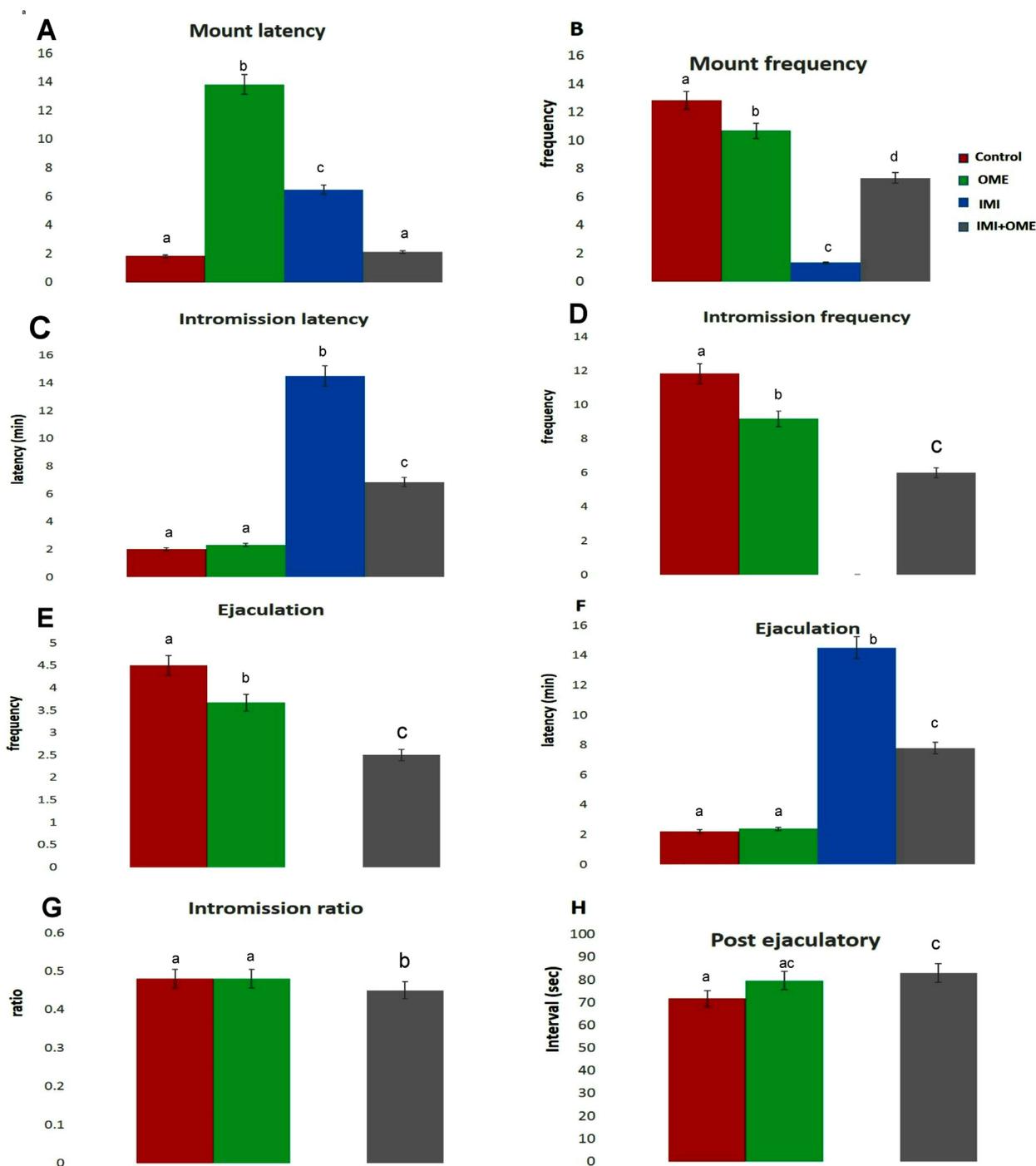


Fig. 3. Bar charts representing the effect of imidacloprid (IMI) and/or Origanum majorana essential oil (OME) on rat's sexual behavior. (A) Mount latency, (B) mount frequency, (C) intromission latency, (D) intromission frequency, (E) intromission ratio, (F) ejaculation latency, (G) ejaculation frequency, (H) post ejaculatory interval. Data are presented as mean ± SEM (n = 7). Values with different letters were significantly different at P ≤ 0.05.

Table 3
Effect of the imidacloprid (IMI) and/or Origanum majorana essential oil (OME) on the testicular redox status.

Parameters Groups	MDA (nmol/g)	GSH (mg/g)	CAT (U/g)
Control	31.9 ± 0.17 ^a	38.5 ± 2.1 ^a	9.2 ± 0.67 ^a
OME	29.1 ± 0.17 ^{ac}	41 ± 1.27 ^{ac}	10.1 ± 0.58 ^{ac}
IMI	57.4 ± 0.16 ^b	22.6 ± 0.96 ^b	4.6 ± 0.48 ^b
IMI + OME	46.3 ± 0.12 ^d	30 ± 1.29 ^d	6.58 ± 0.67 ^d

Data are expressed as mean ± SEM. (n = 7 rat/ group). Values with different letters within the same column are significantly different at P ≤ 0.05.

OME amounting to 4.42%. Caryophyllene was the major sesquiterpene hydrocarbon detected in OME accounting for ca. 2.35%, (peak 49), followed by elixene (1.78%, peak 53), in addition to other minor sesquiterpene hydrocarbons.

m-Cymene (peak 18) was the only aromatic detected in OME amounting for 1.6% of its total volatile content. Three oxides only were detected in OME amounting to about 0.76%, namely, eucalyptol (1,8-Cineole), Caryophyllene oxide, Ledene oxide-(II) at concentrations 0.39, 0.27 and 0.1% (peaks 44, 45 and 46), respectively of the total volatile content of OME. Ketones constituted about 0.26

of the volatiles of OME. It contained five minor constituents: *trans*-dihydrocarvone, pulegone, carvone, *trans*- β -ionone and hexahydrofarnesyl acetone. Phenol/ethers comprised the minimal group of volatiles in OME with carvacrol being the only constituent at ca. 0.06%.

3.3. Behavioral measurements

3.3.1. Territorial aggressive behavior

A notable profound lessening effect was observed for all measurements of territorial aggression including boxing bouts, fights, ventral presenting posture and lateralization in IMI-exposed rats. Administration of OME along with IMI caused a remarkable recovery in all territorial aggressive parameters compared to IMI-treated rats. In addition, OME significantly advanced the territorial aggressive parameters to the level of the control group (Fig. 2).

3.3.2. Sexual behavior test

Exposure to IMI caused a significant increase in mount, intromission and ejaculation latencies compared to their counterparts in the control group. In addition, there was a notable reduction in frequencies of mounts, intromission, and ejaculation. Furthermore, IMI exposure significantly prolonged the post-ejaculatory intervals and decrease the intromission ratio. Co-administration of IMI with OME significantly alleviated all IMI-caused changes in sexual behavior of male rats. The alleviation was comparable with the level noted in the control group (Fig. 3).

3.4. Hormonal analysis

Data illustrated in Table 2. revealed that IMI administration at a dose 45 mg/kg bwt for 60 days led to significant inhibition of serum testosterone level and significant elevation of serum gonadotropins (FSH and LH) when compared to the control group at ($p < 0.05$). On the other hand, co-administration of OME (200 mg/kg bwt) with IMI significantly modulates serum testosterone, FSH, and LH concentrations, when compared to IMI, exposed group at ($p < 0.05$).

Table 4

Effect of the imidacloprid (IMI) and/or Origanum majorana essential oil (OME) on the sperm's motility and concentration.

Parameters	Motility (%)	Concentration ($\times 10^7$ /epididymis)
Control	68 \pm 2 ^a	12.86 \pm 0.70 ^a
OME	71 \pm 2 ^a	13.50 \pm 0.72 ^a
IMI	49 \pm 5 ^b	8.60 \pm 0.60 ^b
IMI + OME	67 \pm 2 ^a	9.50 \pm 0.51 ^{ab}

Data are expressed as mean \pm SEM (n = 7 rats/ group). Values with different letters within the same column are significantly different at $P \leq 0.05$.

Table 5

Effect of the imidacloprid (IMI) and/or Origanum majorana essential oil (OME) on the sperm cell's viability and morphological abnormalities.

Parameters Groups	Live Sperms (%)	Morphological abnormalities			
		Head Abnormalities		Tail Abnormalities	
		Deformed Head (%)	Detached Head (%)	Curved Tail (%)	Coiled Tail (%)
Control	89.40 \pm 0.81 ^b	2.40 \pm 0.24 ^a	1.20 \pm 0.37 ^a	2.10 \pm 0.37 ^a	2.40 \pm 0.20 ^a
OME	90.00 \pm 0.71 ^b	1.20 \pm 0.37 ^a	1.00 \pm 0.32 ^a	2.00 \pm 0.32 ^a	1.60 \pm 0.24 ^a
IMI	68.20 \pm 1.71 ^a	11.80 \pm 0.37 ^c	2.90 \pm 0.55 ^a	11.60 \pm 0.51 ^c	14.60 \pm 0.51 ^c
IMI + OME	82.20 \pm 1.59 ^b	5.60 \pm 0.40 ^b	2.30 \pm 0.37 ^a	5.00 \pm 0.55 ^b	6.20 \pm 0.37 ^b

Data are expressed as mean \pm SEM (n = 7 rats/ group). Values with different letters within the same column are significantly different at $P \leq 0.05$ in an ascending order.

3.5. Oxidative stress evaluations

Imidacloprid receiving group showed significant elevation in MDA levels and reduction in both GSH content and CAT activity in testicular tissues in comparison with the control group. Otherwise, coadministration of OME with IMI improved testicular redox state via reduction of MDA levels and enhancement of GSH content and CAT activity compared to IMI group but still high in contrast to the control group. Moreover, the OME receiving group showed significant improvement in the oxidant/antioxidant balance compared with the control group (Table. 3).

3.6. Semen analysis

There was a significant reduction in sperm motility, concentration, and viability with marked elevation in sperm abnormalities in IMI receiving group compared with other groups. On the other side, OME showed significant improvement in the aforementioned sperm parameters compared with the IMI group and showed some similarities with the control group (Tables. 4Table 5.).

3.7. Pathological studies

3.7.1. Histopathological examination

Testicular sections obtained from rats in the control group and those receiving OME showed homogenous seminiferous tubules with organized germinal epithelium and Sertoli cells as well as normal spermatogenesis (Fig. 4a). Interstitial tissue showed normal Leydig cell count, blood vessels and a thin layer of fibrous tissue. On the other hand, testicular sections obtained from IMI receiving rats showed heterogenous seminiferous tubules with various pathological alterations. Most of the seminiferous tubules showed germinal epithelial degeneration with intraluminal necrotic cell debris while others were contracted and appeared as sacs lined by Sertoli cells only or lined by spermatogonia and Sertoli cells (Fig. 4b). Germinal epithelium showed several degenerative changes in the form of spermatogenic cytoplasmic vacuolization, tubular vacuolation, undifferentiated germ cells, germ cells exfoliation, and reduced various germ cells count (Fig. 4c). Most sections showed degeneration and partial loss of Leydig cells (Fig. 4d) accompanied by Leydig cell hyperplasia in some parts. Interstitial tissue showed congestion, edema, and mild inflammatory cells infiltration in some sections (Fig. 4e). Tunica albuginea showed congestion and mild inflammatory cells infiltration (Fig. 4f). *Origanum* oil markedly improved the pathological pictures and the testis appeared with normal histological structure. The seminiferous tubules are nearly homogenous with organized germ cell layers separated by interstitial Leydig cells and fine connective tissue layers containing blood vessels (Fig. 4g-h).

3.7.2. Testicular microscopic lesion scoring

Imidacloprid receiving group showed the lowest mean Johnson score and highest Copenhagen index compared with other groups.

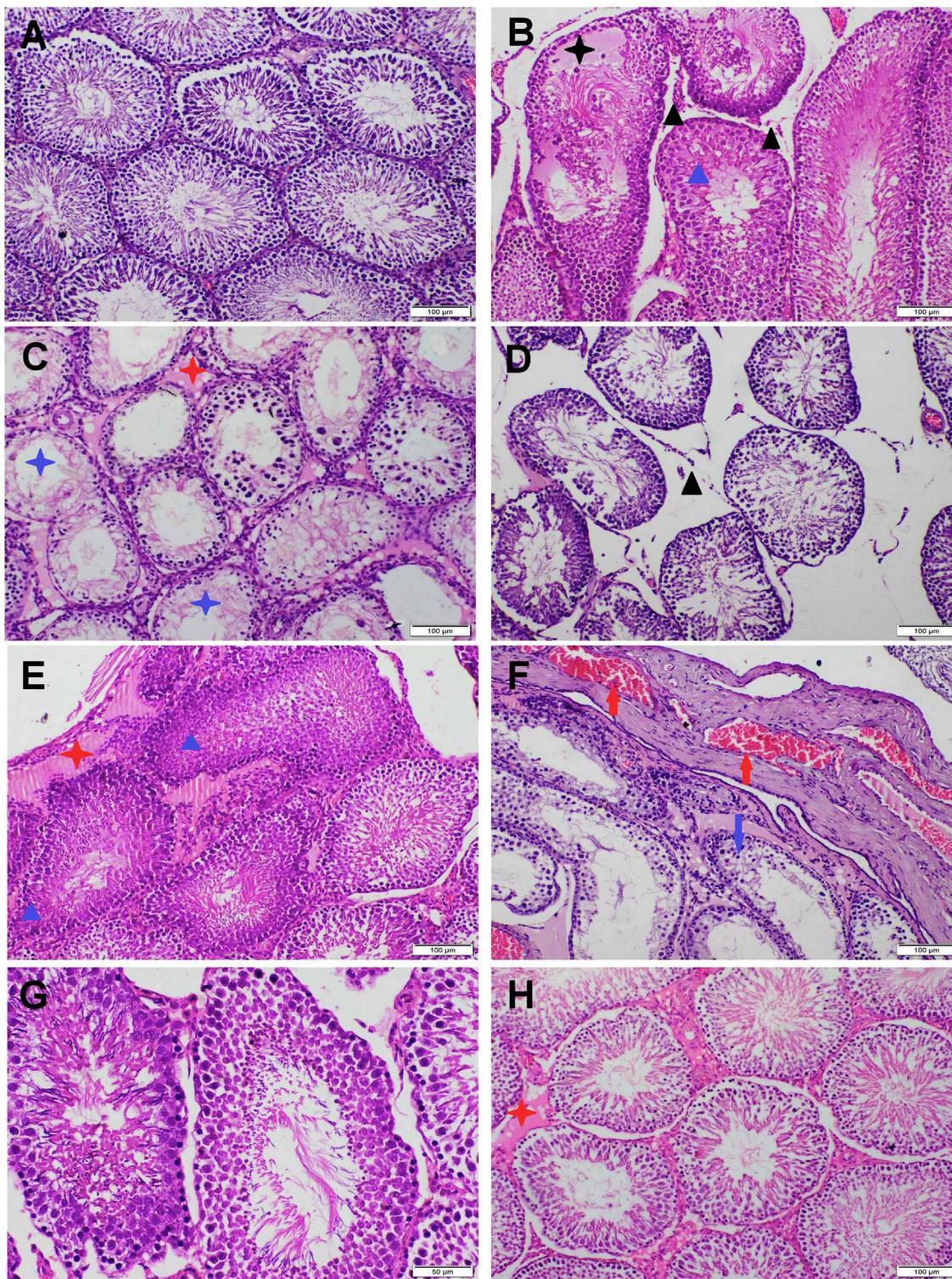


Fig. 4. Photomicrograph of testicular tissue sections stained by H&E representing; (A) Control group showing homogenous seminiferous tubules with normal histological architecture. (B-F) IMI receiving group showing; degeneration of Leydig cells (black arrow head), Vacuolization (blue arrows) and necrosis of germ cells (blue arrow head), Proteinous material in the tubular lumen (black star), Empty seminiferous tubules (blue stars), Interstitial edema (red stars), Vascular congestions (red arrows). (G-H) IMI + OME receiving group showing homogenous seminiferous tubules with normal histological structure except for interstitial edema (red star).

On the other hand, coadministration of OME with IMI could improve both Johnson scoring and Copenhagen index when compared with IMI group still higher than those of the control group. In addition, there was no significant alteration in both scoring values between OME receiving group and the control group (Table 6).

3.7.3. Histomorphometric analysis

Imidacloprid receiving group showed a significant decrease in germ cell layer thickness and seminiferous tubular diameter with increasing in the diameter of the seminiferous lumen and mean % area of empty seminiferous tubules compared with the control group. On the other hand, the entire histomorphometric measure-

Table 6
Johnsen and McLachlan scoring system in different groups.

Groups Scoring	Control	OME	IMD	IMI + OME
10	90	94	0	0
9	10	6	0	10
8	0	0	5	20
7	0	0	12	5
6	0	0	5	60
5	0	0	10	5
4	0	0	15	0
3	0	0	20	0
2	0	0	30	0
1	0	0	0	0
Mean Johnson score	9.9	9.9	3.9	6.7
Copenhagen index	1.1.1.0	1.1.1.0	1.7.4.4	1.6.2.0

Abbreviations: IMI, imidacloprid; OME, Origanum majorana essential oil.

Table 7
Histomorphometric analysis of the seminiferous tubules in different groups.

	Control	OME	IMI	IMI + OME
STD (um)	193.6 ± 3.64 ^a	195.6 ± 5.04 ^a	166 ± 8.12 ^b	190 ± 10.61 ^a
SLD (um)	7.6 ± 1.45 ^a	7.4 ± 1.17 ^a	58.6 ± 2.93 ^c	12.8 ± 1.85 ^b
GCT (um)	68.2 ± 0.58 ^a	67.4 ± 0.97 ^a	18 ± 3.41 ^b	66.2 ± 2.52 ^a
EST%	0 ± 0.0	0 ± 0.0	35.9 ± 3.15 ^b	7.8 ± 1.22 ^a

Data are expressed as mean ± SEM (n = 12 ST/section in 5 rats/ group). Values with different letters within the same column are significantly different at P ≤ 0.05 in an ascending order.

Abbreviations: IMI, imidacloprid; OME, Origanum majorana essential oil, STD, seminiferous tubular diameter; SLD, seminiferous luminal diameter; GCT, germ cell layer thickness; EST%, percentage of empty seminiferous tubules related to the total tubular number.

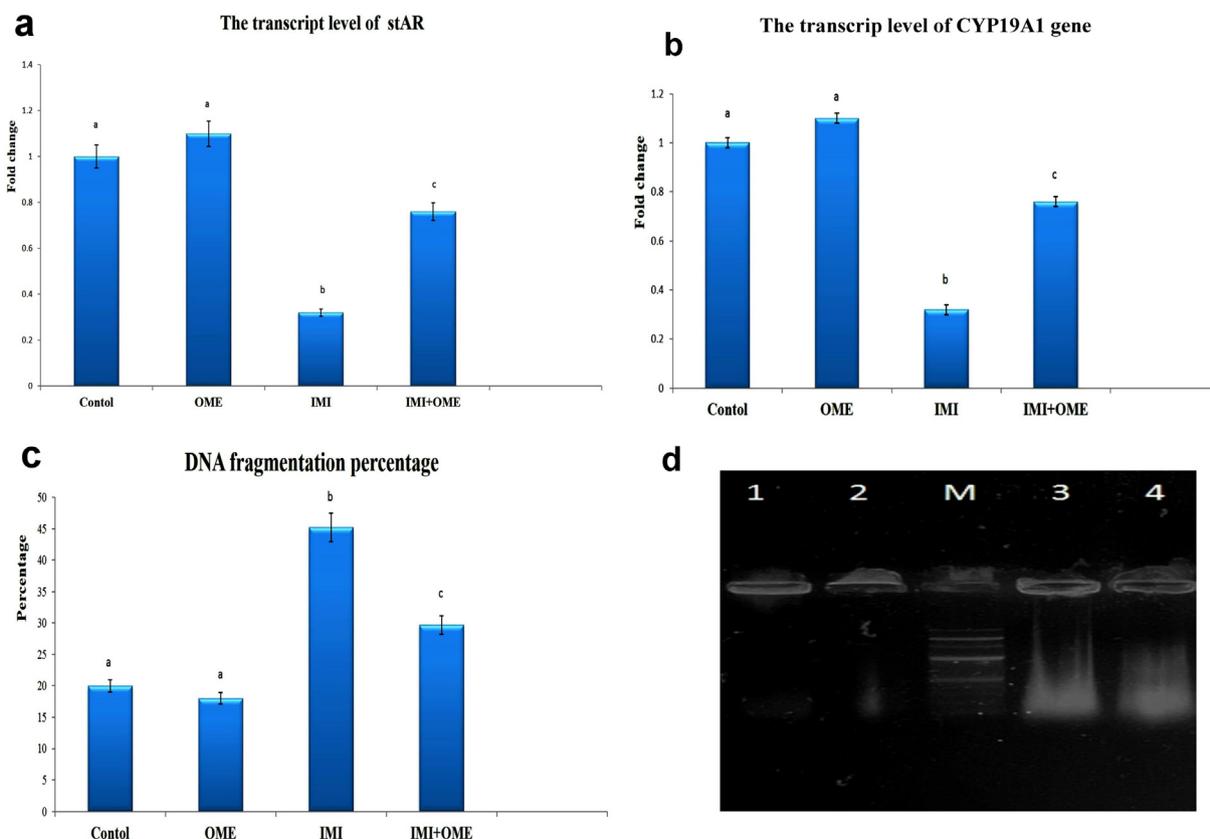


Fig. 5. Bar charts representing, (A) transcript levels of StAR, (B) transcript levels of aromatase, (C) DNA laddering percentage, (D) DNA fragmentation pattern shown on agarose gel electrophoresis (2%). Lane 1: Control; lane 2: OME; lane 3: IMI; lane 4: IMI + OME. M: 100 DNA ladder. Data are presented as mean ± SEM (n = 7). Values with different letters were significantly different at P ≤ 0.05.

ments were markedly improved in the group receiving OME with IMI. Moreover, no significant difference was detected in the aforementioned analysis between the control group and those receiving OME (Table. 7).

3.8. Quantitative real-time PCR for *stAR* and *aromatase* genes

There was a significant down-regulation in the transcript levels of both *stAR* and *aromatase* genes in testicular tissue homogenates obtained from IMI receiving group compared with other groups. Co-treatment of OME with IMI upregulated the transcript levels of the aforementioned genes compared with IMI group (Fig. 5A&B).

3.9. DNA laddering assay

Imidacloprid receiving group showed the highest DNA laddering percentage in the testicular tissue (45%) compared with other groups. On the other hand, co-treatment of OME with IMI significantly reduced the DNA laddering percentage to 29%, which is still high when compared with the control group (Fig. 5C). The same results were confirmed by the electrophoretic mobility of the DNA fragments on the agarose gel. The DNA laddering was obvious in testis obtained from IMI receiving group compared with a control group and showed a moderate decline in the group receiving OME + IMI but still prominent when compared to the control group (Fig. 5D).

4. Discussion

Pesticides are considered the major endocrine disrupters because of their deleterious effects on the hypothalamic-pituitary-gonadal pathway (Sweeney et al., 2000). In addition, recent surveillance studies recorded male infertility and testicular damage among farmers which may be related to pesticides exposure. So, it is very important to discover new strategies to reduce pesticide-induced reproductive toxicity. The current study is designed to investigate the different mechanisms of IMI-induced male infertility as well as to discover the mechanistic role and possible protective effect of OME against IMI-induced male reproductive toxicity.

The results of the present study confirmed that IMI caused testicular oxidative stress damage manifested by increasing MDA levels with decreasing GSH and CAT activity in testicular tissue homogenates and this agreed with several previous studies (Najafi et al., 2010). Several studies confirmed that oxidative stress is an important mechanism of IMI-induced reproductive toxicity via increasing ROS production with depletion of enzymatic and nonenzymatic antioxidants. This is related to the fact that testes enriched with polyunsaturated fatty acids make them more vulnerable to lipid peroxidation and oxidative stress (Bal et al., 2012, Hafez et al., 2016). Free radicals generation exert direct toxic effects on both cell and mitochondrial membrane increasing mitochondrial permeability and enhancing the release of calcium and cytochromes (Hassanen et al., 2019). Increasing cytosolic calcium levels activates several enzymes causing protein degradation, lipid peroxidation, DNA fragmentation, and finally cell damage (Morsy et al., 2021). Moreover, the release of cytochromes as cytochrome *c* activates caspase cascades and initiates the process of programmed cell death (Hassanen et al., 2020). In addition, mitochondrial dysfunction causes the disturbance in ATP production and Na/K/ATPase pump and induced cellular degenerative changes and finally necrosis (Morgan et al., 2021). All of these data suggest our histopathological findings that showed germinal epithelium degeneration and necrosis with seminiferous tubular atrophy. Testicular oxidative stress damage induced by IMI impaired male fer-

tility manifested by germ cell loss leading to a reduction of sperm concentration and motility, as well as increasing in sperm abnormalities suggesting abnormal spermatogenesis related to testicular dysfunction and disturbance in the androgenic and gonadotropic hormones.

Serum testosterone, FSH, and LH have crucial roles in the spermatogenesis mechanism and their strict balance is essential for normal testicular function (Holdcraft and Braun, 2004). In the current study, IMI caused a significant reduction in serum testosterone with marked elevation in FSH and LH concentrations and this is in agreement with several previous studies and attributed to testicular oxidative stress induction (Mikolić and Brčić Karačonji, 2018, Saber et al., 2021). Furthermore, recent research conducted by Yuan et al. (Yuan et al., 2020) found that IMI interferes with androgen receptors and alters the expression of genes involved in the steroidogenesis pathway in male mice. Also, IMI-induced oxidative stress and extensive degeneration in Leydig cells were confirmed by histopathological examination, therefore it can disrupt testosterone secretion (Arıcan et al., 2020). On the other hand, testosterone biosynthesis is regulated by pituitary gonadotrophic hormones (FSH and LH). LH enhances the Leydig cells for testosterone synthesis, while FSH promotes Sertoli cells for the release of androgen-binding protein, which is an essential component for balanced testosterone required by germ cells during the spermatogenesis process (Ruwanpura et al., 2010). The raised serum levels of LH and FSH could be related to hypothalamic-pituitary-testicular (HPT) axis negative feedback control predisposed by decreased testosterone levels (Saber et al., 2021). These findings suggested that IMI interferes with the normal HPT axis and alters the steroidogenesis pathway as well as spermatogenesis in male rats.

Furthermore, the decrease in testosterone levels induced by IMI suggested our results showing a significant reduction in all territorial aggression parameters in IMI receiving group. In rodents, the testosterone hormone is responsible for regulating male aggression and there is significant evidence indicating that aggression rises in parallel with testosterone levels (Batrinos, 2012, Simpson, 2001). Aggression has also been observed to be influenced by the opposite effect, particularly olfactory characteristics (Guillot and Chapouthier, 1996). As a result, the resident male's tendency to attack could be explained by differential olfactory detection of the intruder as a stranger via various processing cues offered by the opponent. As testosterone has been linked to smell coding, this could explain why the resident's olfactory signals were less intense, leading to less identification of the imidacloprid-exposed intruder and, as a result, less defensive and aggressive behavior. The current study revealed that IMI harmed the sexual performance of adult male rats indicated by prolonged time to first mount, intromission, and ejaculation, with a reduction in total frequencies of these parameters. These results were consistent with previous research and attributed to the reduction in testosterone production because of oxidative stress-induced testicular damages (Lonare et al., 2016, Tappert et al., 2017). The current findings showed that IMI provoked spermatogonia apoptosis showed to high DNA fragmentation. Moreover, IMI inhibits the steroidogenic pathway that occurs in testes through downregulation of the relative mRNA levels of the *Aromatase* and *StAR* genes. Some reports recorded that IMI reduced testosterone synthesis by downregulating the major genes in steroidogenesis (Mohamed et al., 2017). *Aromatase* is the key enzyme involved in gonadal steroidogenesis as it irreversibly converts androgen into estrogen. While, the *StAR* gene is essential for the acute regulation of steroidogenesis (Abdelhamid et al., 2020). The deficiency of *aromatase* and/or *StAR* is directly correlated to impairment of the spermatogenesis and development of the germ cell. The resulted infertility is featured by oligospermia, seminal DNA fragmentation and germ cell arrest

(Yuan et al., 2020). The transcript of both genes is absolutely associated with sperm quality and adversely correlated with oxidative stress (Saber et al., 2021).

Conversely, coadministration of OME with IMI significantly decreased MDA levels and increased GSH and CAT activity in testicular tissue suggesting its potent antioxidant effects that were reflected on the histopathological pictures of testis and showed normal histological structure. However, the co-administration with OME significantly decreased the apoptotic DNA fragmentation and upregulated the transcript of the studied genes and modulated the reprotoxic effect of the IMI. These beneficial effects are attributed to the high phenolic and flavonoids composition of the OME (Mohamed, 2018). In addition, the antioxidant activity of OME could explain its relieving impact on reduced aggressive parameters observed with imidacloprid-treated rats and improved all-male sex behavior parameters. It also could decrease the serum LH and FSH concentrations and alleviate the serum testosterone level that consequently enhanced the spermatogenesis in the current study manifested by increasing sperm concentration, vitality, and motility. This beneficial modulatory role of OME could be attributed to its direct effect on the central nervous system, gonadal tissues, and the HPT axis (Abbasi-Maleki et al., 2020, El-Ashmawy et al., 2005).

The antioxidant and anti-apoptotic effect of marjoram essential oil was reported by El-Wakf et al. (El-Wakf et al., 2015) as it protects against testicular tissue apoptosis in obese male rats. *O. Majorana* and other plants related to the Lamiaceae family's redox regulatory role have been widely examined (Couladis et al., 2003, El-Wakf et al., 2015, El-Ashmawy et al., 2005). *Origanum* oil and its ethanolic extracts exhibited antioxidant potential like alpha-tocopherol (Couladis et al., 2003). Furthermore, the reactive oxygen species (ROS) quenching power of essential oil, alcoholic and aqueous extracts from *O. Majorana* has been previously investigated (Couladis et al., 2003, dos Santos Dantas, 2019). We speculated that the protective effect of *Origanum* spp. on DNA could be due to the enhancement of the antioxidant defense mechanism and free radicals scavenging that results in the reduction of lipid peroxidation accumulation, activation of antioxidative enzymes that quench ROS and thereby inhibition of tissue peroxidative damage (Arami et al., 2013).

5. Conclusion

Imidacloprid reprotoxic effect exhibited in adult male rats via exertion of oxidative stress in the testicular tissue, disruption of testosterone synthesis, and dysregulation of the key genes implicated in testicular steroidogenesis pathways accompanied by histopathological alterations. Notably, concurrent administration of OME alleviates the testicular toxicity induced by IMI through restoring hormonal balance and spermatogenesis, antioxidant status, up-regulation of the relative-mRNA expression level of steroidogenesis and consequently minimizing the impairment of the histological structure of testicular tissue.

6. Ethics approval and consent to participate

All Institutional and National Guidelines for the care and use of animals were followed.

7. Availability of data and materials

All data are available on request.

8. Authors' contributions

S.M performed the experimental study and measured the animal's sexual behavioral changes. M.Y.I responsible for preparation and characterization of *Origanum majorana* oil. N.H.H performed the hormonal analysis and carried out data analysis. A.M.H performed the oxidative stress evaluations and semen analysis, M.A.I carried out the quantitative RT-PCR of the examined genes and DNA laddering assay. E.I.H conceived the study, designed the experiment, reviewed all the results, drafted the manuscript, and performed the pathological studies. All authors read, revised, and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jsps.2022.06.016>.

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