Journal of
Fertility Sterility and Reproductive Health

Research Article Open Access

Attenuating Effects of Housefly Larvae Extract in Triton-Induced Reproductive and Ovarian Disorders in Female Rats

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Received Date: January 09, 2024 **Accepted Date:** February 09, 2024 **Published Date:** February 12, 2024

Citation: Reda ElMazoudy (2024) Attenuating Effects of Housefly Larvae Extract in Triton-Induced Reproductive and Ovarian Disorders in Female Rats. J Fertil Steril Reprod Health 1: 1-23.

Abstract

Undoubtedly, numerous bioactive constituents, phytochemicals, and extracts were validated for their health beneficial effects to alleviate complications of Triton-related hypercholesterolemia focusing entirely on hypocholesterolemia, however, ovarian tumours and reproductive disorders, the major risk factors of hypercholesterolemia, was ignored. So, the aim is elaborated to investigate the larva extract of housefly combined with Triton as a preliminary study. The female rats were induced intraperitoneally by 200 mg/kgbw Triton for hypercholesterolemia. The hypercholesterolemic females gave 200mg/kg larval extract orally for improvement activity. In addition to non-lipidemic control and extract-treated group (n = 10/30days). All lipid profile markers, total cholesterol 33.77%, triglycerides 24.95%, and low-density lipoprotein 46.34%, very low-density lipoprotein 59.73%, were significantly decreased in Triton with extract group except the levels of high-density lipoprotein were increased 25%. Besides, the larval extract significantly enhances the depleted ovarian catalase, superoxide dismutase, glutathione peroxidase, glutathione activities and lowers the malondialdehyde levels. The anti-inflammatory effect was enhanced by lowering xanthine oxidase and nitric oxide levels. A corresponding alleviation in ovarian cytotoxicity was noticed in the suppression of lactate dehydrogenase, α-amylase, and myeloperoxidase concentrations. Levels of maternal and embryo-fetal toxicities and fertility were improved in the group of the larval extract with Triton. Concisely, the extract of housefly larvae could attenuate the reproductive and ovarian disorders via retrieving the activities of associated parameters, and that extract can potentially be a promising candidate for human therapeutic development.

Keywords: Housefly larva; Inflammation; Triton; Fertility; Implantation; Ovary

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Introduction

Reproductive and ovarian disorders have been proven to be a major closely socioeconomic complication related to hypercholesterolemia (Artham, *et al.*, 2008) [1]. Alterations in the metabolism of lipids and lipoprotein profile are regarded as the main risk factors for the induction and development of these diseases (Shi, *et al.*, 2000) [2].

Cumulative evidence reported that long-term high concentrations of cholesterolemia can cause damage to human and animal health, impairing the normal physiological functions (Liu, *et al.*, 2019) [3]. Of note, reproductive toxicity of hyperlipidemia can cause ovarian damage (Wang, *et al.*, 2019) [4]. Chronic hypercholesterolemia mediated physiological alteration and metabolic distortion leads to organ dysfunction, massive germ cell degeneration, cancer progression and other pathophysiological consequences (Giri, *et al.*, 2018) [5]. Moreover, it can affect the blood-follicle barrier (Siu and Cheng, 2012) [6], destroy the ovarian tissue structure, reduce the quality and quantity of ova (El-Sayyad, *et al.*, 2018) [7].

Ovarian dysfunction affected by lipid abnormalities including low levels of high-density lipoprotein cholesterol (HDL-C), high triglyceride (TG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) and significantly higher lipoprotein concentrations (Tsouma, *et al.*, 2014) [8]. An animal study found ovarian alterations that suggest a potential impact of hyperlipidemia on the hormonal profile (Patel and Shah, 2018) [9]. Hyperlipidemia exerts a clear effect on oocyte quality and early embryo growth that is triggered by lipotoxicity-induced endoplasmic reticulum stress, mitochondrial dysfunction and apoptosis (Broughton and Moley 2017) [10].

Hypercholesterolemia induced oxidative stress by disruption of prooxidant/antioxidant balance in the blood and ovarian tissues and dramatically reduced catalase (CAT) and superoxide dismutase (SOD) activities (Borzoei, *et al.*, 2018) [11]. As a consequence, hyperlipidemia induced follicular atresia, apoptosis, impaired steroidogenesis (Serke, *et al.*, 2012) [12]. Hyperlipidemia plays a vital role in the pathogenesis of ovarian histology and subsequently increased oxidative stress-inducing an inflammatory environment. Furthermore, oxidative stress is believed to contribute to infertility by interfering with fundamental processes involved in reproduction, including gametogenesis, folliculogenesis, fertilization, implantation, and placentation (Agarwal, *et al.*, 2005) [13]. Previous studies have demonstrated that hypercholesterolemic rats showed a significant reduction in the antioxidant activities of CAT, and SOD, in addition to an increased number of atretic follicles and relatively decreased antral follicles (Cordier, *et al.*, 2013) [14]. In the same vein, a study by Lin, *et al.* (2017) [15] reported a decreased number of small follicular populations in the ovaries of hypercholesterolemic dams (El-Mansi, *et al.*, 2019) [16].

Regarding pathophysiological markers of malignant ovarian tumour, several gynaecological studies showed that the elevation of serum lactate dehydrogenase (LDH) reflects tissue injury in different inflammatory and severe malignancies including ovarian tumours (Gonzalez-Billalabeitia, *et al.*, 2009) [17].

The chemical constituents of housefly larvae extract indicate a high protein content (Hussein, *et al.*, 2017) [18]. It is believed that the housefly larvae represent a probable and productive source of proteins in food and feed (Van, 2013; Zhao, *et al.*, 2017) [19,20]. Many studies have reported bio-components including proteins, saccharides, vitamins detected in housefly larvae extract (Feng, *et al.*, 2010) [21]. Due to bioactivity, housefly larvae extract is extensively used as an antioxidant, antibacterial, and antitumor in multiple biotherapies to cure various ailments such as chronic wounds, osteomyelitis, and purulent inflammation (Ai, *et al.*, 2012) [22]. It has been detected different proteins, including catalase proteins, anti-bacteria, peptide, and cuticle proteins in housefly larvae extract, which participates in the anti-oxidative activity and is capable to scavenge reactive oxygen radicals (Ai, *et al.*, 2013; Mei, *et al.*, 2018) [23,24]. Previously, *in vitro*, and *in vivo*, housefly larvae extract exhibited cytotoxic activities which indicate the extract may contain antitumor peptides and may have therapeutic effects (Chernysh, *et al.*, 2002) [25].

Moreover, the larval extract has been reported to be associated with a variety of health therapy including hepatoprotective in hepatic damage induced by carbon tetrachloride in rats (Wang, *et al.*, 2007) [26], antihepatitic B virus in a cell line (Lu, *et al.*, 2014) [27], hyperlipidemia (Ai et al., 2008) [28], antitumor (Cao, *et al.*, 2010) [29], antifungal (Fu, *et al.*, 2009) [30], and anti-inflammatory (Chu, *et al.*, 2011) [31], in addition to their capacity to ameliorate immunomodulatory proficiency (Cao, *et al.*, 2012) [32]. Recent research has revealed that housefly larvae powder treatment increase protein activities of CAT, SOD, have a protective effect in memory deterioration and may inhibit oxidative stress-induced damage (He, *et al.*, 2019) [33].

In summary, based on the above considerations, the present work aimed to investigate the impacts of Triton-induced

hypercholesterolemia in altering the ovarian structure and function as well as the susceptibility of reproductive efficiency and fertility. In addition to illustrating the chemical constituents of housefly larval extract; highlighting its ameliorative role in the restoration of the ovarian activity and attenuation of cellular damage.

Materials and Methods

Chemicals

The non-ionic detergent Triton WR-1339 (TRT, tyloxapol, polymeric p iso-octyl polyoxyethylene phenol) is used as a base of the animal model of hyperlipidemia (Lee, *et al.*, 2005) [34]. It was purchased from Sigma Chemicals Pharmaceuticals Co, Mumbai. Triton was dissolved in sterile saline solution (pH 7.4) and a dose of 400 mg/kgBW (2.5 ml/kg) intraperitoneally injected (Oh, *et al.*, 2006) [35].

Collection, sampling, identification and rearing of housefly larvae

For a collection of Dipterous larvae, glass beakers containing fermented vegetables and kitchen wastes left in the botanical garden of Faculty of Science, Moharram Bek, Alexandria University, Egypt. For sampling, the larvae of housefly maggots *(Musca domestica* Linnaeus (Insecta: Diptera: Muscidae) were selected among the gathered dipterous larvae and other crawling species with regular forceps (Ahmed, *et al.*, 2011) [36]. The larvae of housefly maggots were mainly identified by the shape of the posterior spiracles of the third larval instar (Oldroyd and Smith, 1973) [37]. The spiracles are slightly elevated and spiracular openings are sinuous slits that were surrounded by an oval black border (Sanchez-Arroyo and Capinera, 2015) [38]. Thereafter, the larvae were maintained for rearing in glass beakers containing chicken mash covered with a thin mesh.

Extract preparation

For preparing larval extract (LPE), 200 gm of frozen third instar housefly maggots were homogenized in a glass beaker using a homogenizer with a PT300 tip, apply four short pulses (5 sec at 3500 rpm) separated by 10 seconds of incubation on ice to avoid warming the crude homogenate. Thereafter, the homogenate was transferred to a motor-driven glass/Teflon beaker, kept on ice and 10 full strokes (800 rpm) were applied to the sample. Then the obtained larval homogenate was filtered through a layer of nylon mesh. The residue material was transferred again to the motor-driven glass/Teflon beaker with 1 ml distilled water per g of the original weight of maggots, and five full strokes (800 rpm) were applied and then filtered again through the nylon mesh (Wang, *et al.*, 2007) [26]. Thereafter, the filtrate was further centrifuged at 10000g for 10 mints (IEC Micromax, Microcentrifuge). Finally, the supernatant of the larval protein extract was kept in Eppendorf tubes at -20°C until needed (Chu, *et al.*, 2011) [31].

Chemical analysis of larval extract

The experimental detection of the chemical composition of crude housefly larva extract was performed using high-performance liquid chromatography by previous procedures of Mei, *et al.* (2018) [24]. Briefly, the whole larval extract was loaded for further purification by the RP-HPLC equipment. 300µl was submitted to separation then eluted under reversed-phase-high performance liquid chromatography (RP-HPLC) using a DL-Cl8 column (75 µm x 150 mm, 3 µm, 1.5µl India) at 25°C. The mobile phase was composed of 0.1% formic acid-water (A) and 0.1% mixture of formic acid and acetonitrile (B). The detection wavelength of elusion at 255 nm and the injection volume was 10 µL. The chromatographic gradient was performed at 130min. After lyophilization, the fractions were dissolved in 100 µl of ultrapure water.

Animal selection and care

Ninety-five-day-old female Wistar albino rats (155–160g) was obtained from the animal house of Research Medical Institute (RMI), Alexandria University, Alexandria, Egypt. Before the experimentation, the animals were acclimatized for two weeks in a thermoneutral controlled laboratory room (25±2°C) with a 12h:12h photoperiod (light/dark) cycle and 45%–50% humidity. Under these conditions, females were freely kept on the diet of animal sterilized commercial pellets (chaw) and water *ad libitum*. All experiments were carried out under the Guidelines for care and use of animals, approved by the Local Ethics Committee of Faculty of Medicine, Alexandria University. For experimental and comfort acclimation and avoiding potential environmental stressors that may lead to stress and distress, rats were maintained according to the guidelines of The Guide for the Care and Use of Laboratory Animals (NRC, 1996; 2003b; 2008) [39-41].

Induction of acute hyperlipidemia

Experimentally, to induce the model of acute hyperlipidemia, a single 400 mg/kg Triton WR-1339 (TRT) was injected intraperitoneally to 12 hours-fasted female rats dissolved in 2.5 ml/kg sterile saline solution (0.9 %, pH 7.4). After Forty-eight hours of Triton injection, levels of serum total cholesterol and triglycerides was estimated. Rats with elevated lipid profile levels were considered hyperlipidemic and assigned in the present research. The pain alleviation strategies taken into considerations to minimize pain, distress and anaesthesia to the animals may include appropriate animal handling and restraint, minimization of tissue trauma during dissection or use of minimally invasive surgical techniques used of general and local anaesthetics. In addition to the application of the "Three Rs" provide the underlying principle to the ethical care and use of laboratory animals: refinement of experimental procedures to reduce or eliminate pain and distress, reduction in the number of animals being used, replacement of animals with other reliable models by using alternative methodologies. We must justify the procedures following current institutional regulations and policies of the animal care and use committee.

Groups planning

24 hours before experimentation, female rats fasted overnight but kept on drinking water *ad libitum*. All scheduled experiments were carried out in the morning (8.00-9.00 am) to maintain uniformity with the experiments. The larval extract was dissolved in saline solution and administered orally by gavage using a gastric tube at the dose of 200 mg/kg/day (He*, et al.*, 2019) [33]. Female rats were divided into experimental groups (10 female/each) as shown in the following design (synopsis):

Plan I (24-h design)

Group I: (GCS) saline-treated control females**,** administered 2.5 ml/kgBW saline.

Group II: (TRT) hyperlipidemic females, orally given 400 mg/kgBW Triton.

Plan II (daily for 30-days design)

Group I: (GCS) saline-treated control females**,** administered 2.5 ml/kgBW saline.

Group II: (TRT) hyperlipidemic females, orally given 400 mg/kgBW Triton.

Group III: (LPE): larval protein extract females, oral received 200 mg/kgBW extract.

Group IV: (TRT+LPE) 400 mg/kgBW Triton coadminstrated with 200 mg/kgBW extract.

Mortality, behaviours and body weights

Mortality and signs of physical and neurological behaviour were monitored and recorded during the experimentation period. Changes in body weight (if any) for each female was determined before the administration of test substances and at least weekly thereafter.

Blood collection

After the duration of the two experimental plans, blood samples were collected directly from the ventricle of the heart of overnight-fasted rats and then females were euthanized. Thereafter, serum was separated by centrifugation at 4500 rpm, for 15min, for various biochemical experiments (Chacko*, et al.*, 2013) [42]. The collected serum was stored at -20 °C until used.

Analysis of serum lipid profile

Total cholesterol (Zak's, 1977) [43], triglycerides (Rice, 1970), and high-density lipoprotein (HDL-C) (Varley*, et al.*, 1980) [44] were quantified enzymatically using commercially available kits (Pars Azmoon kit, Iran, Tehran) on an automatic analyzer (Abbott, model Alcyon 300, USA). Low-density lipoprotein (LDL-C) was determined by Friedewald*, et al.* (1972) [45] formula while very-low-density lipoprotein (VLDL) by Henry, *et al.* (1998) [46].

Estimation of ovarian oxidative stress activities

From each female, the right ovary was excised out, weighed, and homogenized in phosphate buffer saline (PBS, pH=7.4; 0.1M) to 1:5 W/V final preparation. The homogenate was centrifuged at 2400 rpm at 4 °C for 15 min. Thereafter, the supernatant was immediately stored at -20 °C for assay of antioxidant enzyme activities according to the following procedures. Lipid peroxidation (Malondialdehyde, MDA) was measured spectrophotometrically at 532 nm by the production of thiobar-

bituric acid reactive substance (TBARS), essentially according to the technique of Ohkawa, *et al.* (1979) [47] (nmol MDA/mg protein). Superoxide dismutase (SOD) activity was estimated by a spectrophotometric method (Marklund and Marklund, 1974) [48]. The intensity of chromogen in butanol was measured at 520 nm. Catalase activity was quantified by measuring the decomposition of hydrogen peroxide (H_2O_2). The absorbance was read at 570 nm (Aebi, 1983) [49]. Reduced glutathione (GSH) was determined by the method of Ellman (1959) [50]. 10% of trichloroacetic acid (TCA) was added to the homogenate and centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5, 50-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm. The activity of Glutathione-Px was assayed by the method of Paglia and Valentine (1967) [51] through the conversion of the oxidized glutathione (GSSG) into the glutathione with Low-density associated oxidation of NADPH to NADP. The absorbance was measured at 340 nm.

Determination of protein content

For correction of enzymatic activities results, protein content for each ovarian sample was measured by Lowry, *et al.* (1951) [52] Folin phenol reagent (technique). using bovine serum albumin as standard.

Lactate dehydrogenase activity

According to the manufacturer's instructions, serum lactate dehydrogenase (LDH) activity was assayed as a marker of coronary atherosclerosis and ovarian tumour.

Serum α-Amylase activity assay

The principal steps of the α-amylase assay are that α-amylase break down by hydrolysis of the kit substrate, p-nitrow phenol maltoheptaoside (PNP-maltoheptaoside) to smaller fragments that release a chromogenic substrate and finally to glucose and PNP which then can be recorded at 405 nm.

Measurement of XO level activity

Spectrophotometrically, xanthine oxidase (XO) activity was evaluated at 292 nm under aerobic oxidation of xanthine at 25 °C by quantifying the rate of formation of uric acid using commercial (xanthine oxidase Kit, Abcam, ab102522). Briefly, in wells containing a mixture of 2.5 ml of 0.20 M phosphate buffer (PH 7.5), 0.60 µl of 0.3 mM xanthine, 10µl of the xanthine oxidase, and incubated (pH 7.5, 37 °C/20 minutes). Thereafter, the activity of mixture-enzymatic reaction was monitored by the spectrophotometric at a referenced absorbance of the formation of uric acid (U/mg protein).

Ovarian myeloperoxidase assay

Myeloperoxidase (MPO) detection was performed according to the protocol of the commercial kit (Abcam, ab105136). 100 µl of the supernatant was added to 20µl of phosphate buffer (10 mmol/L, pH 6.0) and 10 µl of o-dianisidine hydrochloride (1.6mmol/L, pH 6.0) containing 0.0005% (w/v) H_20_2. At 460 nm, the change in absorbance was monitored on a UV–vis spectrophotometer and expressed as (mmol/mg tissue) (Bradley, *et al.*, 1982) [53].

Ovarian nitric oxide activity

By using the spectrophotometric protocol of Feelisch and Noack (1987) [54], which provides an accurate, and convenient measure of total nitrate/nitrite in the ovarian tissue. The first step converts nitrate to nitrite by using nitrate reductase. The second step uses Griess Reagent to convert nitrite to a deep purple azo compound. The amount of the azo chromophore accurately reflects nitric oxide amount at the absorption difference of wavelength (401 and 421 nm at 37 °C) between the reading of blank and samples.

Parameters of female reproductive performance

After the treatment period for 30 days, one treated female rat in the estrus phase was cohoused individually with one proven fertile male rat for two weeks. The vaginal plug was checked daily for each female for confirmation of copulation (ElMazoudy, *et al.*, 2011) [55].

Mating, copulation, and gestation index

The mating index was judged by detection of a vaginal plug and was considered as zero-day of gestation (GD0). Females with no evidence of mating (no vaginal plug) were recorded sterile. Thereafter, copulated females were separated in an individual cage. Pregnancy was evident by the presence/absence of parturition and/or by examining implants at necropsy. The mating, copulation and gestation indices were calculated.

Evaluation of fetal outcomes

On GD19, the pregnant females were intraperitoneally euthanized (90mg/kg ketamine and 10mg/kg xylazine) and sacrificed. Ovaries were dissected out and then corpora lutea were counted. From exposed uterine horns, the number of implants, resorptions, dead and live fetuses was determined per uterus (Salewski, 1964) [56]. Thereafter, the pre-and post-implantation loss percentage was estimated. Besides, external malformations were examined. The fetal sex ratio and the crown-rump length was also recorded.

Ovarian histopathology

Small pieces of the ovarian tissues (approximately 1.0 cm^2) were fixed overnight in 10 % neutral formalin solution, and they were passed through the usual recognized routine method of tissue processing (dehydration, clearing and embedding in paraffin wax). Serial microtome sections (5µm) thick were cut and finally stained by usual haematoxylin-eosin (H&E) and/or trichrome Mallory's stain (Bancroft and Gamble, 2008) [57].

Statistical analysis

One way-ANOVA performed by using the software package of SPSS (version 13.0 for Windows). The statistical result in each figure displays a comparison of the mean differences between the groups using the Tukey-Kramer test. Data are expressed as the mean ± standard deviation (SD). Differences were considered significant when $P \leq 0.05$. The differences that were considered significant between the Triton-treated group and controls in the acute experiment was evaluated by Student's t-tests at $P \leq 0.05$.

Results

Chemical composition of larval extract and maternal toxicity

The chemical composition of housefly larva extract was calculated on a 100% dry matter basis (Table 1). All treated female rats showed no observable maternal toxicity response, drastic changes in general health (behavioural changes) or vaginal bleeding. The survival rate was also normal with no death occurring on experimental days (0-30). Meanwhile, most animals were still ready to engage in a physically energetic pursuit.

Maternal and organ weights

By the completion 30th day of the experiment, body weight gain was significantly higher in the TRT group compared with control and LPE groups, whereas no significant difference between LPE and the control (GCS) group ($P \leq 0.05$) (Figure 1). The concomitant larval protein extract with Triton (TRT+-LEP) displayed a significant decrease in body weight by 40.13% compared with the TRT group, however a significant increase by 68.84% compared with the control (GCS) group which was approaching the GCS group ($P \leq 0.05$) (Figure 1). On the other hand, the mean ovarian weight was significantly increased in the Triton-treated group compared with the control and larval protein extract group (39.7, 32.3%, respectively) whereas no statistical significance between LPE and GCS groups. Interestingly, the ovarian weight of females in the TRT+LPE group significantly decreased compared with TRT and GCS groups by 37.12% and 16.81%, respectively ($P \leq 0.05$) (Figure 1).

Table 1: Chemical composition of housefly larva extract.

All values are calculated on a 100% dry matter basis

% Composition	Chemical composition
27.17	Dry Matter
55.21	Crude Protein
23.88	Crude fat
8.74	Crude Fiber
7.16	Crude ash
5.01	Nitrogen-free extract
43.09	Essential amino acids
56.91	Nonessential amino acids
40.60	Saturated fatty acid
59.39	Unsaturated fatty acid

Data are shown as means values

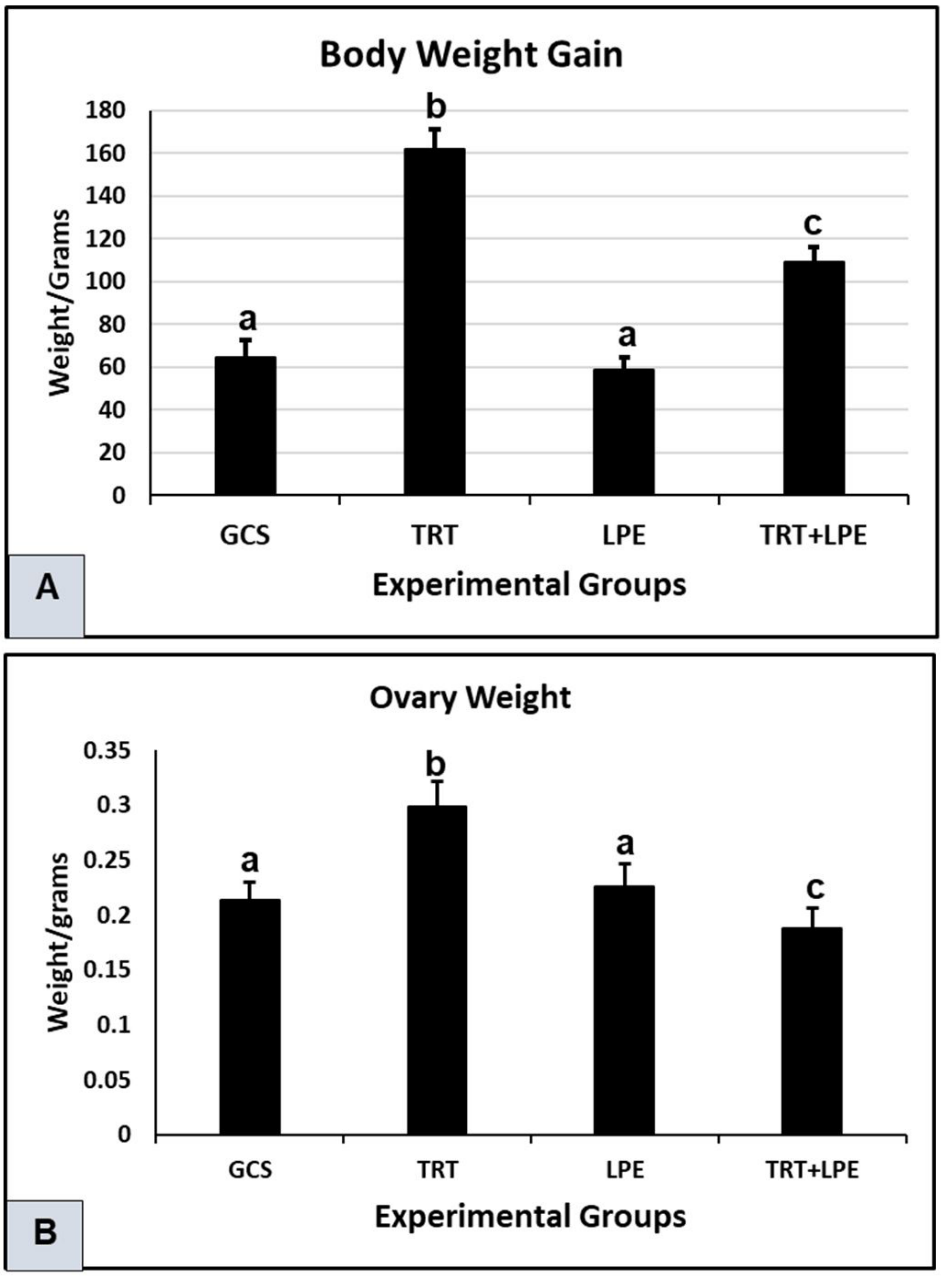

Figure 1: Effects of Triton and/or combination with larval extract on A: body weight gain and B: ovary weight after 30 days of oral administration

Data are shown as means ± SD, N= 10 rats/group.

Mean values with different superscript (a, b, c) were differences at $P \leq 0.05$.

GCS: Positive Control.

Lipid profile

Statistically, 24 hours of hyperlipidemic induction by Triton WR-1339 caused a significant elevation in the measured levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL-c), very low-density lipoprotein (VLDL-c), however a significant decrease in high-density lipoprotein (HDL-c) compared with the control saline ($P \leq 0.05$) (Figure 2).

Status of the serum lipid profile of hyperlipidemic rats after 30 days of oral larval extract (TRT+LPE) exhibited drastic amelioration evident by a significant lowering in TC, TG, LDL-c, and VLDL-c by 33.77%, 24.95%, 46.34%, 59.73%, respectively compared with the hyperlipidemic Triton group (TRT) ($P \leq 0.05$) (Figure 2). Whereas the levels of high-density lipoprotein were increased by 25% relative to the hyperlipidemic (TRT) group. The greatest improvement of larval extract attended and well evident in levels of TG and VLDL-c after 30 days of treatment that deem the most critical indicator in hyperlipidemia diseases (Figure 2).

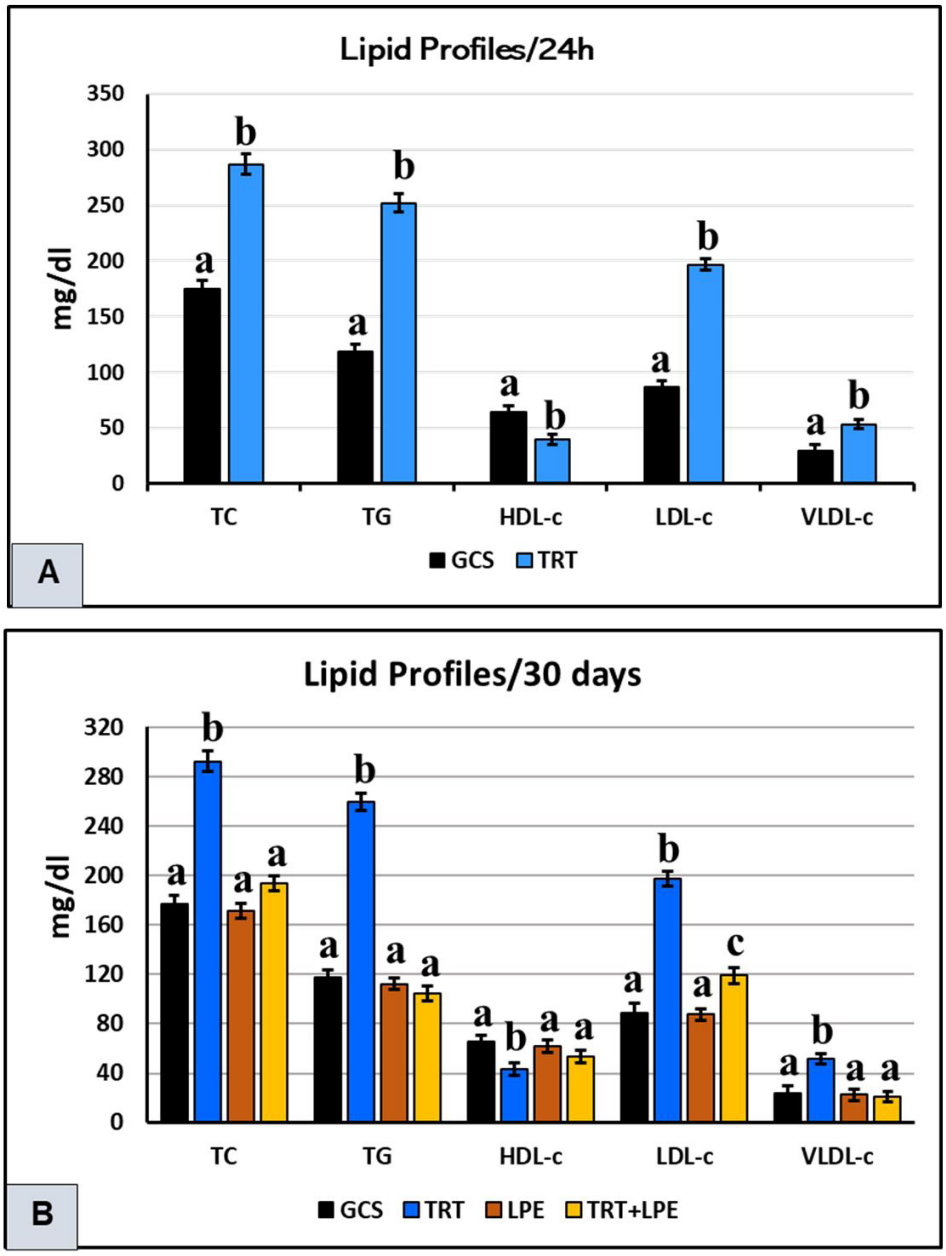

Figure (2): Levels of serum lipoproteins (lipid profile) of Triton (TRT), larval extract (LPE) and their combination (TRT+LPE) of female rat A: after 24 hours and B: after 30 days of oral administration

Data are presented as mean ± SD, N= 10 rats/group.

Mean values with different superscript (a, b) in the same parameter were differences at P≤0.05. GCS: Positive Control

Lactate dehydrogenase

Triton-treated females showed an extremely significant elevation in levels of LDH, a marker of ovarian tumour, compared with the GCS group. However, co-treatment 200 mg/kg larval extract with Triton for 30 days can significantly suppress the increase in LDH marker and come nearer to normalized values of the control group *(P≤0.05)* (Figure 3). In contrast, the normal values of LDH levels were noticed in larval extract-treated females (LPE).

Atherogenic index

There is an outstanding reduction in the atherosclerogenic index (AI) in the TRT+LPE group approaching normal values (*P≤0.05*) (Figure 3).

Ovarian oxidative stress markers

The depleted SOD, CAT, GSH, and GPx activities induced by Triton in hyperlipidemic female rats (TRT) was significantly attenuated in (TRT + LPE) treated group (*P≤0.05*) (Figure

4). Otherwise, oral administration of larval extract with Triton significantly inhibit the increase of ovarian MDA level and succeeded in approaching normal values ($P \leq 0.05$) (Figure 4). Interestingly, the larval extract per se significantly reduced MDA levels and elevated the activities of antioxidant enzymes as well, compared with the control saline group.

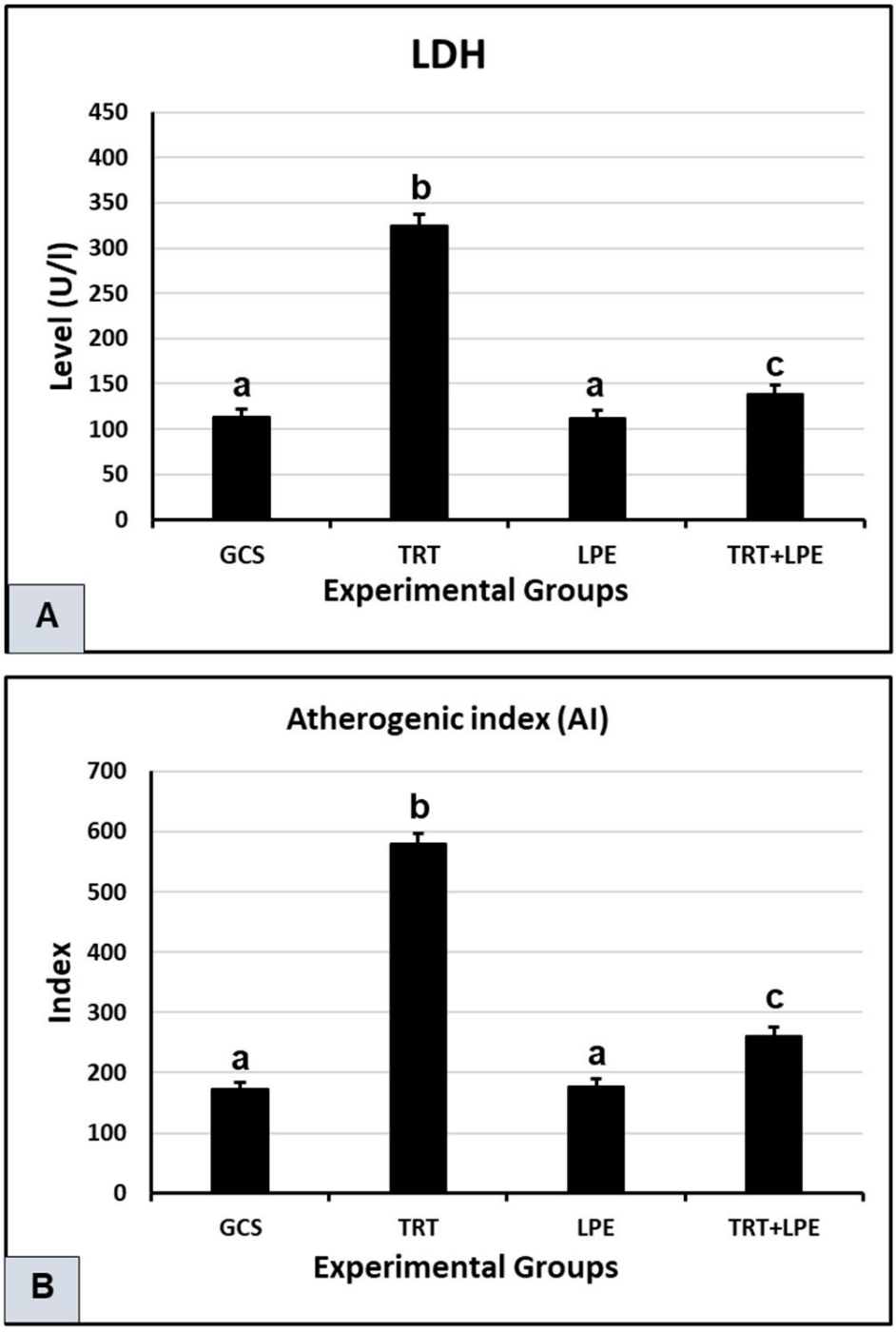

Figure (3): The effect of Triton (TRT), larval extract (LPE) and their combination (TRT+LPE) on level of A: serum lactate dehydrogenase (LDH) and B: atherogenic index (AI) of female rats compared to the controls

Data are shown as means ± SD, N= 10 rats/group.

Mean values with different superscript (a, b) were differences at $P \leq 0.05$.

GCS: Positive Control

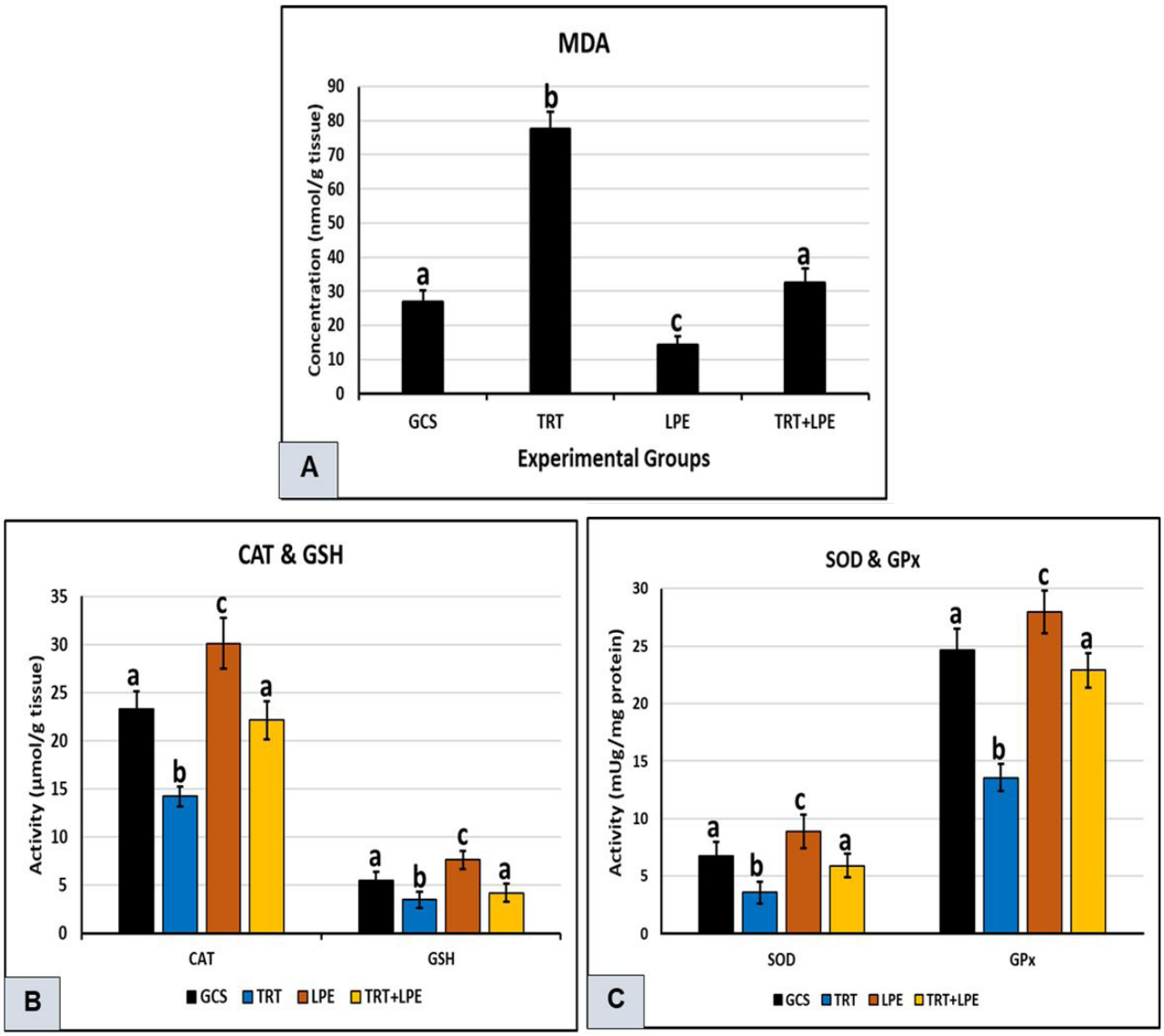

Figure (4): The effect of Triton (TRT), larval protein extract (LPE) and their combination (TRT+LPE) on ovarian oxidative activities. A: levels of MDA; B: activities of catalase CAT and glutathione GSH; C: superoxide dismutase SOD, and glutathione peroxidase GPx) of female rats. Data are shown as means ± SD, N= 10 rats/group.

Mean values with different superscript (a, b, c) in the same parameter were differences at $P \leq 0.05$.

GCS: Positive Control

α-amylase levels

Levels of serum α-amylase were significantly increased in females treated with Triton (564.33 U/L±10.76), vs control females (279.53U/L±6.17) showing a higher incidence of hyperamylasemia. However, no significant difference in levels of serum α-amylase between the LPE group and the GCS group (273.65U/L±5.41, vs 279.53 U/L±6.17). After amelioration with larval extract, serum α-amylase level approaches normal values (391.17UL ±7.01).

XO, NO and MPO activities

Triton significantly elevated the activities of xanthine oxidase (XO), nitric oxide (NO) and myeloperoxidase (MPO) compared with the control group ($P \leq 0.05$) (Figure 5). On the other hand, larval extract in the LPE+TRT group reversed the increasing trend in activities of XO, NO, and MPO ($P \leq 0.05$) (Figure 5). This indicates that the larval extract had a potent anti-inflammatory effect on XO, NO and MPO activity. This anti-inflammatory property alleviates ovarian inflammation and protects from the spreading of cellular proliferation of ovarian carcinoma.

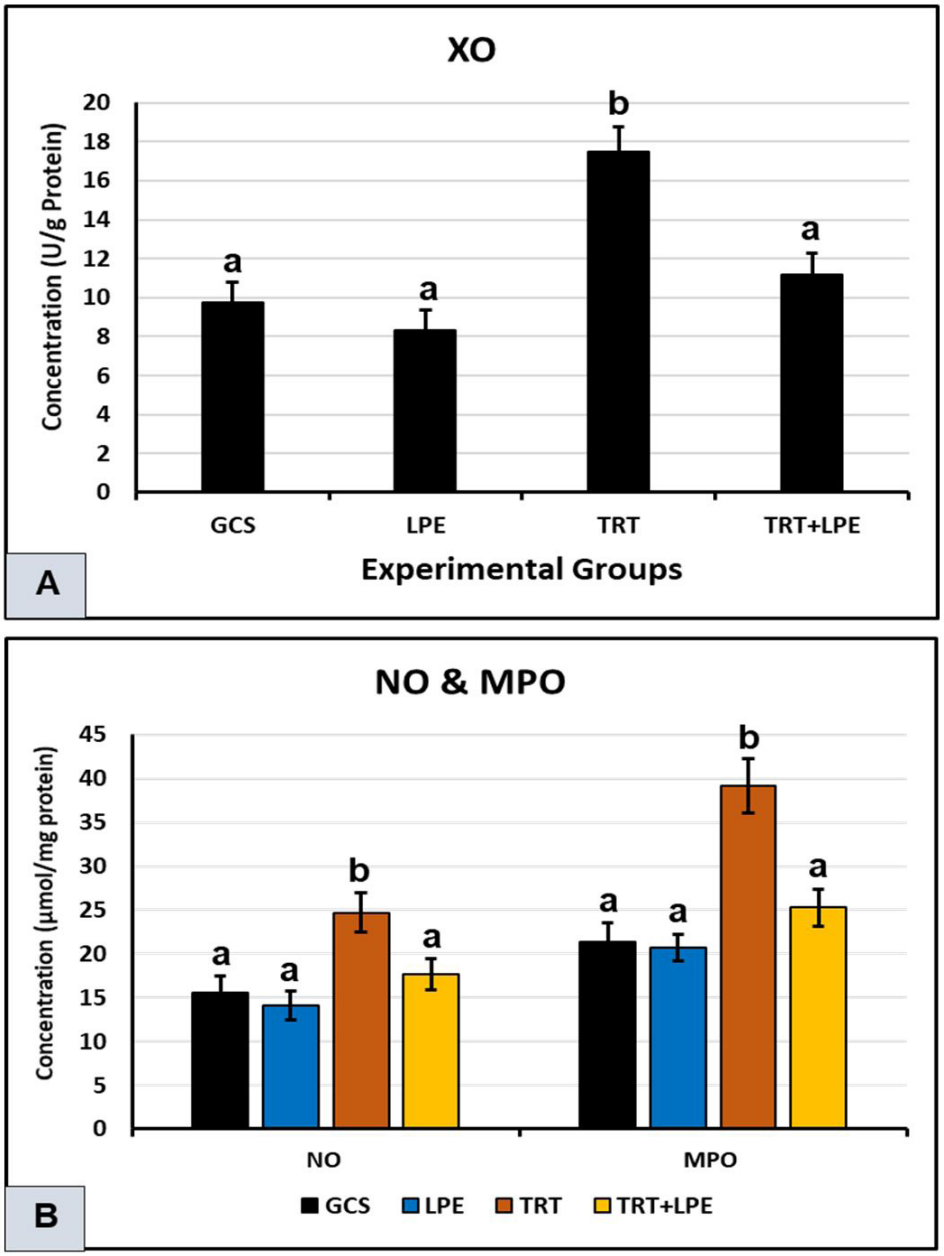

Figure (5): The effect of Triton (TRT), larval protein extract (LPE) and their combination (TRT+LPE) on A: ovarian xanthine oxidase (XO), B: nitric oxide (NO) and myeloperoxidase (MPO) activities of female rats compared to the controls.

Data are shown as means ± SD, N= 10 rats/group.

Mean values with different superscript (a, b) in the same parameter were differences at $P \leq 0.05$.

GCS: Positive Control

Maternal receptivity

All cohabited females in the TRT+LEP group showed 100% copulation and gestation index, compared to 80% and 75%, respectively of the females in the TRT group ($P \leq 0.05$; Table 2). This fundamentally attributed to an increase in the sexual receptivity effect of the extract. There was no adverse effect of the larval extract on copulation and gestation index respective to the control group (Table 2). Interestingly, no preterm delivery and/or abortion were observed in all groups.

Maternal reproductivity

The parameters of maternal reproductive efficiency are shown in Table 2 and Figure 6. The number of implantation sites was significantly decreased in the Triton-treated females with a significantly decreased number of corpus luteum compared to control ($P \leq 0.05$; Table 2; Figure 6). Whereas there were no treatment-related effects on these induced parameters in the TRT+LEP groups compared to control ($P \leq 0.05$; Table 2). These alterations may due to the direct effect of Triton on the repro-

Table 2: Effect of the oral administration of TRT and/or LEP on maternal reproductive potency of females after cohabitation with untreated male rats

Reproductive parameters	Experimental groups			
	GCS	LPE	TRT	TRT+LPE
Copulation index (%)	100	100	80	100
Gestation index (%)	100	100	75	100
Litter size	10	10	6	10
Implants/litter	13.35±0.54[a]	12.95±0.55[a]	9.83±0.65[b]	12.34±0.25[a]
Viable/litter	13.11±0.75[a]	12.07±0.63[a]	8.66±0.62[b]	12.11±0.91[a]
Resorbed/litter	0.24±0.05[a]	0.36±0.02[a]	1.17±0.31[b]	0.21±0.01[a]
Corpus luteum/dam	13.41±0.88[a]	13.01±0.43[a]	10.10±0.61[b]	12.47±0.78[a]
Fertility potential %	99.6 ±4.05[a]	99.5±3.96[a]	97.3±3.16[a]	98.9±3.78[a]
Pre-implantation loss %	0.45±0.65[a]	0.46±0.77[a]	2.67±1.01[b]	1.04±0.36[c]
Post-implantation loss %	1.79±0.55[a]	2.89±0.86[a]	11.90±1.01[b]	1.70±0.47[a]
Fetal weight (g)	5.24 ± 1.07[a]	5.31 ±0.94[a]	7.43 ±1.82[b]	4.99 ±0.79[a]
Crown-Rump Length (CRL) (mm)	4.55 ±0.95[a]	4.28 ±0.89[a]	3.10 ±0.98[b]	4.53 ±0.83[a]
Male: Female	64:60	67:63	32:34	62:60

Data are shown as means ± SD, N= 10 rats/group.

Mean values with different superscript (a, b,) in the same row were significant differences at $P \leq 0.05$.

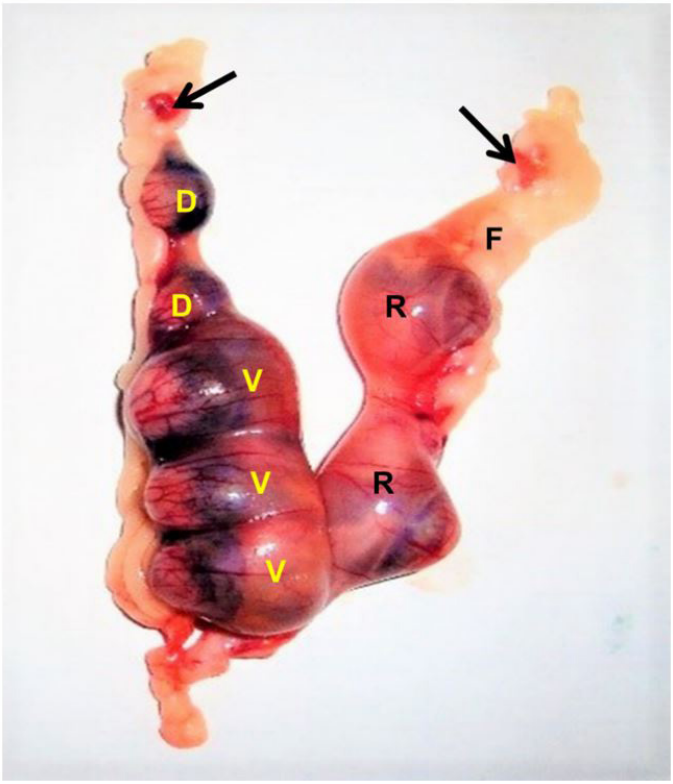

Figure (6): Gravid uterine horns with rat fetuses from hypercholesterolemic female rats treated by TRT (gestation day 19, GD19). Ovary (arrow) embedded in fatty tissue (F), viable fetuses (V), dead fetuses (D). 20X.

ductive capacity of the female. The number of viable conceptuses was significantly decreased in TRT and the number of resorbing conceptuses was significantly increased vs control females ($P \leq 0.05$; Table 2; Figure 6). Interestingly, there are no effects on the index of fertility potential of females, albeit the receptivity and capacity of females were decreased. Moreover, in the TRT group, there was a significant increase in both pre-implantation and post-implantation loss comparable to other groups, which mainly due to an increase in fetal resorptions and/or to a lethal effect of Triton ($P \leq 0.05$; Table 2). These findings commensurate with the mean number of viable fetuses for each pregnant female in the Triton group which was significantly decreased compared to the other groups. On the other hand, there were no effects of larval extract-related on post- and pre-implantation loss or the number of fetal viabilities per pregnant rat compared with the control group.

Reproductive outcomes and fetal findings

Related adverse effects on fetal crown-rump length (CRL) and weight were observed in the Triton group compared to control which were significantly decreased ($P \leq 0.05$; Table 2; Figure 7). Normal, well-formed fetuses and no malformations were observed in TRT+LEP and LEP group compared to the control group.

Ovarian histopathology

The findings of ovarian histopathology in the Triton group exhibited marked alterations related to ovarian carcinoma and tumour definite by fibrinoid necrosis in the interstitial region (N, Figure 8A). Distinct changes were noticed in group TRT, such as follicular cyst (F: Figure 8A) and epithelial cyst (E: Figure 8B) and autophagic vacuoles containing cell debris forming residual bodies of lipofuscin pigment granules in brown atrophy (L: Figure 8A). Further, ovarian tissue of Triton-treated females was replaced by hyperplasia of Sertoli cells (S: Figure 8B), degenerated oocytes (O: Figure 8B). Otherwise, co-administration of 200 mg/kg LEP with TRT for 30 days alleviated ovarian histopathology in comparison with the hyperlipidemic group (TRT). The females of this group displayed moderate changes in the ovarian interstitial (T: Figure 8C), corpus luteum (C: Figure 8C), and Graafian follicles (G: Figure 8C).

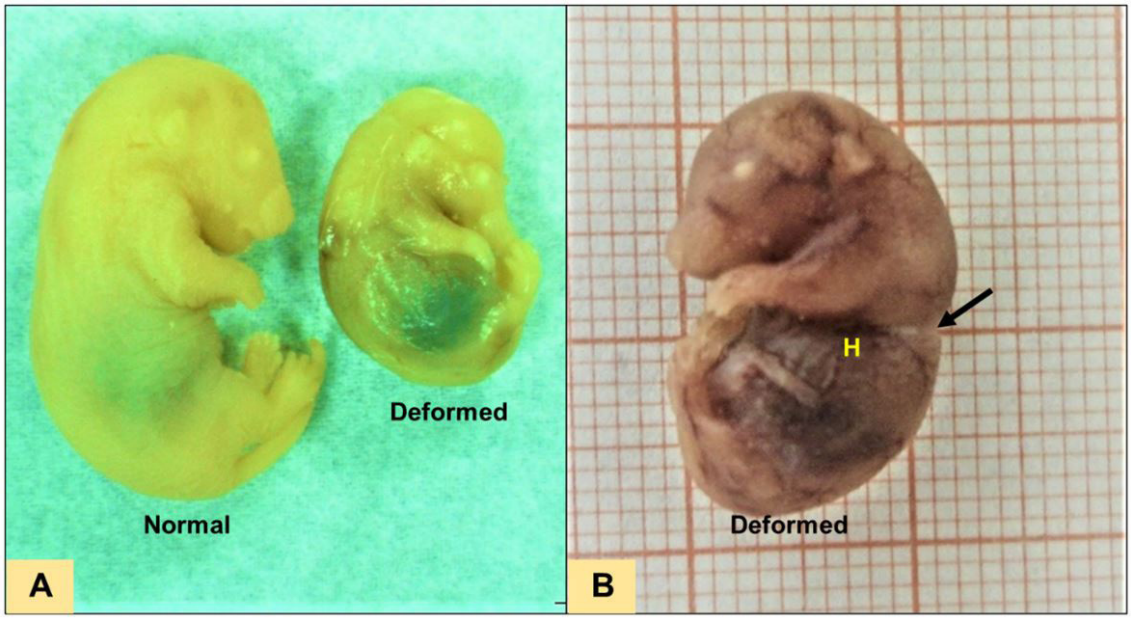

Figure (7): Morphological anomalies in rat fetuses from dams treated with 200mg/bw TRT. (A) stunt pup (Deformed), vs normal (control) on left. (B) subcutaneous hemorrhage fetus (H), crown-rump defect (arrow). 20X.

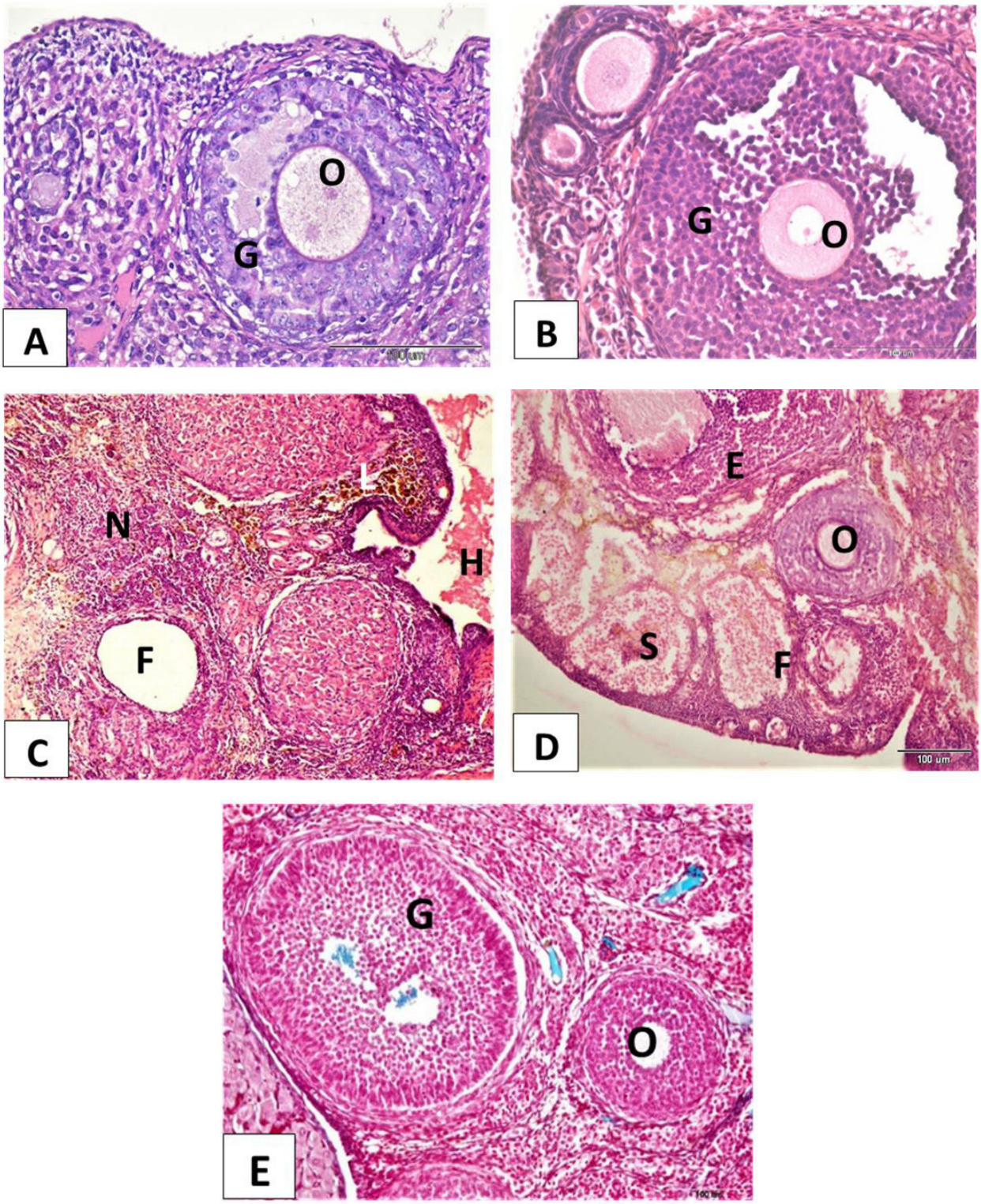

Figure (8): Histopathology of ovarian tissue from the female rats treated with Triton and/or larval protein extract. A: control group; B, extract group; C and D, Triton group; E, TRT+LPE group. Notice: Graafian follicles in different size (G), normal corpus luteum (C), ovarian stroma (T), fibrinoid necrosis in the interstitial regions (N), follicular cyst (F), epithelial cyst (E), autophagic vacuoles containing cell debris to form residual bodies of lipofuscin pigment granules in brown atrophy (L). Ovarian tissue was replaced by hyperplasia of Sertoli cells (S), degenerated oocytes (O), Inflammatory hemorrhage (H). Bar =100µ. Masson's Trichrome

Discussion

Comprehensively, this is the first investigation to predict the role of housefly larval extract on hyperlipidemia-induced reproductive and ovarian disorders and evaluate the associated influencing factors. The results indicate that larval extract significantly restored weight gain and ovary weight in female mice which may be regarded as being caused by the reduction mechanism of food intake induced by d-galactose that exists in larval extract (Wu, *et al.*, 2017) [58]. The mechanism of larval extract potency to achieve lowering of TC, TG, and LDL-c levels in hypercholesterolemic females or to reach hypolipidemia is not entirely understood but may attribute to stimulation of the activity of hepatic lipase (Cedó, *et al.*, 2017) [59] and/or hepatic LDL receptors (Yoo, *et al.*, 2008) [60]. As reported by other studies, the hypolipidemic action can also be due to alterations of enzymatic pathways involved in cholesterol metabolism (Mbikay, *et al.*, 2014) [61]. The diminished triglyceride induced by larval extract represents an important anti-atherogenic factor in the prevention of atherosclerosis risk and coronary artery-related disorders through regulation of cholesterol esterification and maintaining lipoprotein metabolism (Kong, *et al.*, 2014) [62]. According to Roberfroid (2000) [63], it is possible to explain the decreased triglyceride and cholesterol (hypocholesterolemia) may due to the presence of antibacterial peptides in Triton which possesses bifidogenic property causing an increase of Lactobacillus and Bifidobacterium in the cecum of females through regulation of serum lipid metabolism. Further, the increase in Lactobacillus and Bifidobacteria, in turn, selectively increase acetic acid and propionic acid (Short-Chain Fatty Acid) which induce metabolic modification of hepatic tissues (Park and Park, 2014) [64].

Since VLDL-c concentration related to the predominant changes in harmful LDL-c, so, as confirmed by (Sanchez, *et al.*, 2010) [65], hypolipidemia may be interpreted through promoting LDL-c decomposition and simultaneously then sterol clearance and empowering of LDL-c receptor. Further, it may be related to the suppression of the hepatic HMG-CoA reductase activity (bifidogenic effect in larval extract) which important for the biosynthesis of cholesterol (Rogi, *et al.*, 2011) [66] resulting from downregulation of hepatic SREBP-1α and SREBP-2 gene expression which maintain cholesterol metabolism (Rotllan and Fernandez-Hernando, 2012) [67]. Lowering blood LDL-c may also be in line with increase LDL catabolism through regulation of liver LDL receptor or decrease apolipoprotein B production, which represents an essential component of LDL and VLDL (Cha, *et al.*, 2016) [68].

Binding the peptides of larval protein with bile acid (Higaki, *et al.*, 2006) [69], suppression of the cholesterol production and absorption by fermentation products of Lactobacillus and Bifidobacteria with the recycling of bile salt (a metabolite of cholesterol) (An, *et al.*, 2011) [70] and increased excretion of cholesterol (Nishimura, *et al.*, 2009) [71], all together might participate to the decrease of cholesterol levels in the female rats. Also, the ability of residual peptides and undigested proteins to bind with bile acids suppress the solubility of micellular cholesterol and inhibit their absorption through the small intestinal epithelium resulting in their increase in excrement. By this mechanism, the cholesterol-lowering capacity of larval extract maybe, partly attributable to the weak digestibility of larval protein, the contents of amino acids in protein extract and the faecal elimination of TC (Um, *et al.*, 2013) [72]. In this regard, previous reports assumed that the strength of the blood cholesterol-lowering effect may be correlated to the existence of high content of cysteine in the larval protein extract (Kashima, *et al.*, 2014) [73]. It is fully understood that the up-regulation of hepatic CAT, SOD1, p-AMPK, and decrease of PPARc expression by larval extract may be implicated in the amelioration of the hypercholesterolemia parameters through reduction the lipid accumulation (Mei, *et al.*, 2018) [24]. Lipid-lowering findings could be also expected due to the downregulation of gene expression of the transcription factor, Sterol regulatory element-binding transcription factor 1 (SREBF1), in fat depots suggesting this gene is a target for some larval extract constituents (Al-Hasani and Joost, 2005) [74].

Evidence showed that hypercholesterolemia generates high levels of reactive free radical species that are involved in ovarian dysfunction and the development of reproductive complications (Kowluru and Mishra 2015) [75]. Retrieving of antioxidant SOD, CAT, GPX activities in LPE +TRT treated group may because of the existence of the antibacterial peptides and heat shock protein (HSP70) in addition to the abundance of cuticle protein contents that rich in 70% of actin such as chitosan and chitin that acting as strong antioxidant, however, further studies should clarify the precise mechanism (Ai, *et al.* 2013) [23]. It has been reported that, *in vivo* and *in vitro*, larval protein extract exerts its antioxidant effect through reinforcing the scavenging ability of SOD (Zhu, *et al.* 2013) [76]. Recently, Mouithys-Mickalad, *et al.* (2020) [77] indicated the capacity of amino acid compositions of proteinaceous materials exists in the larval extract to scavenge free radicals via the hydrogen atom transfer and/or single electron transfer mechanism (Esfandi, *et al.*,2019) [78].

Besides, the hydrophobic, and amphiphilic nature of amino acids could reinforce the radical scavenging activity of extract through the single hydrogen (electron) atom transfer mechanism (Zou, *et al.*, 2016) [79]. So, the antioxidant effects of larval extract may be accountable for diminishing the cytotoxicity induced by oxidative damage status participating in the protection of ovarian tissue from histological disorders (Mai, *et al.*, 2010) [80].

Elevation in serum levels of lactate dehydrogenase (LDH) indicates membranous damage and seepage of this enzyme into the bloodstream mainly due to TRT-induced oxidative stress (Kwon and Ha, 2014) [81]. This seepage causes ovarian dysgerminoma (Pressley, *et al.*, 1992) [82] with atherosclerosis under cases of ischemia leading to numerous spread necrotic lesions in the ovary (Chazov, *et al.*, 1969) [83]. Also, the increased serum LDH activity might be interpreted through the up-regulation of LDH gene expression and corresponding isoenzymes (Jaiswal, *et al.*, 2018) [84]. Although the precise mechanism of TRT action on the LDH activities in rat ovary is still unknown, it seems that TRT exerts an indirect negative effect on the Kreb's cycle mediated by allosteric suppression of pyruvate dehydrogenase complex leading to an accumulation of excess of unused acetyl-S-CoA (Jaiswal, *et al.*, 2018) [84]. In another mechanism, those elevations of LDH activities were normalized by the co-administration of the larval extract with Triton suggesting that larval extract possibly acted through neutralizing the production of free radicals during hypercholesterolemia (Leekha, *et al.*, 2019) [85]. Also, such improvement in LDH activity may probably be attributed to the ability of chitosan for fusion and sealing of membrane phospholipid integrity and permeability by forming phospholipid aggregates and repairing the cracks in phospholipid layers (Cho, *et al.*, 2010) [86].

Many reports have concluded that serum amylase significantly increases in ovarian adenocarcinoma with an unclear mechanism. In 1994, Seyama, *et al.* [87] stated that the amylase type 1 (AMY1) gene was highly expressed in adenocarcinomas (Seyama, *et al.*, 1994) [87]. Others also reported that the amylase type 2 (AMY2) gene was associated with ovarian carcinoma due to the transformation of neoplastic cells (Watanabe, *et al.*, 2010; Kawakita, *et al.*, 2012) [88,89]. As a growth promoter, non-nutritional polysaccharide dietary supplement, and lower digestibility by mammalian enzymes, taken together, support the role of reinforcing chitosan to an improvement enzyme activity of amylase (Xu, *et al.*, 2018) [90].

It was found that xanthine oxidase increases the flux of free radicals and oxidative stress especially superoxide anions that capable of elevation of lipid peroxidation (Marsillach, *et al.*, 2010) [91]. On the other hand, Triton produces a generation of reactive oxygen species (ROS) and increases the activity of xanthine oxidase and the levels of nitric oxide (NO) resulting in possibly cellular damage (Valko, *et al.*, 2007) [92]. As a result, the xanthine oxidase-mediated superoxide anions could scavenge free nitric oxide to yield a potent oxidant factor, peroxynitrite, causing further injury (Baskol, *et al.*, 2012) [93]. Therefore, increased activities of XO, NO, MPO together with the noticed positive relationship between them, may indicate that female rats with ovarian injury are exposed to oxidative stress in addition to elevated lipid peroxidation, which may lead to increased ovarian carcinoma.

Indeed, oxidative stress is the main motif that succeeds in inducing the vast diversity of diseases. On the other hand, several reports showed that the housefly larvae extracts contain potent multifunctional bioactive compounds and peptide fractions possessing antibacterial, antioxidant, and antitumor activities (Ai, *et al.*, 2012; Sun, *et al.*, 2014; Zhang, *et al.*, 2016) [22,94, 95]. It had been reported that the antioxidant activity may be due to a higher proportion of hydrophobic, positively charged, and aromatic amino acids contained in the larval extract (Rao, *et al.*, 2012; Chi, *et al.*, 2015) [96,97].

Wu (2010) suggested functional amino acids found in housefly larvae may participate in regulating the metabolic pathways to enhance the reproductive potency of the animal. The deficiency of these amino acids deteriorates protein synthesis and the homeostatic environment Wu (2010) [98].

Essentially, the tumour inhibitory effects of the larval extract could be related to the high chelating ability of chitosan and its direct cytotoxic effect on the tumour cells (Senevirathne, *et al.*, 2006; Hou, *et al.*, 2007) [99,100]. Furthermore, the chelating ability and donor property of electron couples (oxygen and nitrogen) can explain the essential role of chitosan in the antioxidative mechanism (Marianti, *et al.*, 2017) [101].

In the present findings, it is evident that the elevation of myeloperoxidase and NO levels, as well as the activity of XO, were reduced by co-administration of the larval extract with Triton. Chitosan confers an anti-inflammatory effect for the larval extract through a reduction in the regulation of YKL-40, a biomarker glycoprotein of inflammation, by binding with it

resulting in improvement of angiogenesis (Gudmundsdottir, *et al.*, 2015) [102] and enhancement of cellular proliferation (Einarsson, *et al.*, 2013) [103]. On the other hand, Marmouzi, *et al.* (2019) [104] confirmed that the chitosan derivatives suppress inflammatory mediators.

Again, oxidative stress-induced inflammatory agents play a significant role in numerous diseases such as ovarian tud mour development (Azuma, *et al.*, 2015) [105]. Besides, hypercholesterolemia linked to different alterations in the status of inflammatory biomarkers (Kwaifa, *et al.*, 2020) [106].

The development of malignant tumours, polycystic ovary and reproductive disorders is closely correlated with disturbed activities of molecular inflammatory agents (XO, NO, MPO). For example, nitric oxide plays a crucial role as a modulator molecule in female reproduction, involving fertilization, oocyte maturation, folliculogenesis, and implantation to become actively cytotoxic in higher levels (Valko, *et al.*, 2007) [92] . Further, nitric oxide-mediated oxidative stress can be responsible for the endothelial vascular dysfunction noticed in histopathology (Baskol, *et al.*, 2012) [93]. Collectively, these results allude that the Triton-induced reproductive and ovarian disorders could be restored by the antioxidant, anti-inflammatory and anti-tumour bioactive components of larval extract per se.

Conclusion

In the literature, the available information on the effects of the protein-rich extract of housefly larva on reproductive and ovarian disorders in hypercholesterolemic female rats was elusive. Henceforth, antioxidant, anti-inflammatory as well as antitumor activities can render the extract a promising candidate to offer potent multifarious protection against Triton-induced damage. Besides, the multiple components of the extract are also important to be worthy of attention in investigating the mechanism and expanding the application range of their wide use in nutraceuticals, biomedicine, and pharmacy as a potential therapy. These findings indicate that the larval extract is good hypolipidemic activity. The amelioration may be due to the efficiency of the larval extract on modulation and relieving the depletion of scavenger activities of cellular antioxidants caused by Triton. These results also prove that the larval extract considered a source of relief leading to improvement of oxidative activities. Special emphasis, these findings suggest that larval extracts able to ameliorate the diagnostic markers of ovarian tumour. The present toxicological findings revealed that larval extract was safe for oral use in rats even at the dose of 200 mg/kgbw.

Disclosure of financial and competing interest

The authors have no financial participation with any entity or organization with a financial benefit or financial conflict concerned with the materials or subject matter for this manuscript. Also, no assistance was utilized in writing and the production of the present manuscript.

Funding

There is no personal Funding or supported grant from any Foundation.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Authors' contributions

Reda ElMazoudy, A. Attia, L.Alsamad conceived and designed the study. L. Alsamad, A. Attia performed the experiments and collected the data. Reda ElMazoudy wrote the primary manuscript. A. Attia provided their expertise and reviewed the manuscript.

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