



## Inhibitor Properties of Rue (*Ruta graveolens* L.) on Spermatogenesis in Guppy *Poecilia reticulata* Peters, 1859

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**Abstract:** This study was conducted to investigate the effect of Rue (RE), a medicinal herb having anti-androgenic characteristic on the sexuality of guppy males. In total, 120 males were selected randomly, acclimated and divided equally into four groups that received the RE ethanolic extract at 0, 1, 10 and 100 mg.kg<sup>-1</sup>, respectively for 30 days. The findings revealed that 100 mg.kg<sup>-1</sup> had significantly lower whole-body testosterone levels than control. Furthermore, males administered RE at all doses had a significantly lower gonopodial index than the control group. Whereas, administration of RE at 10 and 100 mg.kg<sup>-1</sup> causes a lowering in sperm counts than the control group. Regarding with histopathological examination, the testes of control males were well-differentiated with an increase in spermatocyte proportion at all spermatogenesis stages with a significant proportion of cystic structures containing spermatozoa. On the other hand, RE-fed animals showed a dose-dependent decrease in spermatocyte and an increase in spermatocytes' number of ducts, indicating the role of RE in reducing the development of guppy's spermatogenesis. In conclusion, Rue can impact the sexual parameters of adult guppy males by confirming the anti-androgenic properties of RE.

**Keywords:** Gonad histology, Gonopodial index, *Poecilia reticulata*, *Ruta graveolens*, Testosterone.

### Introduction

Recently, there are an increasing emphasis on the pharmacological potential of compounds derived from plants. Interestingly, there are very limited data available for the Rue (*Ruta graveolens* L.) toxicity and safety. Rue belongs to a family known as Rutaceae under the Sapindales order. This medicinal plant is

originally native to the Mediterranean region and is introduced to several global areas due to its medicinal and cultural values (Miguel, 2003). The plant contains different classes of phytochemical compounds including cholesterols, glycosides, furoquinolines, tannins, saponins, flavonoids, terpenoids,

volatile substances, coumarins and acridone alkaloids (Kuzovkina *et al.*, 2004; Asgarpanah & Khoshkam, 2012). These compounds explained a broad range of biological activities such as anti-cancer properties (Pathak *et al.*, 2003; Preethi *et al.*, 2006), contraceptive (Maurya *et al.*, 2004), analgesic and hypotensive (Chiu & Fung, 1997), fungicide activity (Oliva *et al.*, 2003; Meepagala *et al.*, 2005), anti-inflammatory (Raghav *et al.*, 2006) and antimicrobial (Ivanova *et al.*, 2005). These compounds are now more common in the diets of fish aquaculture because were used as growth and immunostimulant agents, sex reversal compounds (Kadhim *et al.*, 2023) and antipathogenic (Yilmaz *et al.*, 2015).

Rue can also interfere with reproductive system function and decrease fertility, probably via changes in sex hormone concentration and morphology of reproductive organs (Khouri & El-Akawi, 2005; Sailani & Moeini, 2007). Oral administration of RE in male albino rats suppressed the testosterone (T) levels, decreased sperm count and motility, as well as reduced sexual and aggressive behaviors (Khouri & El-Akawi, 2005). This raises concerns regarding its toxicity in non-target organisms. Nonetheless, the environmental concentration of Rue has not been investigated and the effects of the plant on aquatic animals are poorly understood.

Regarding to the fish, the adverse effects of RE on steroid hormone, impaired reproductive performance and sexual behavior have been reported in the waterborne exposed Zebrafish (*Danio rerio*), as well as male Siamese fighting fish (*Betta splendens*) injected with 300  $\mu\text{g}\cdot\text{g}^{-1}$  of RE (Forsatkar *et al.*, 2016; 2018).

However, sensitivity to endocrine-disrupting compounds (EDCs) could differ across species and routes of exposure (Manibusan & Touart, 2017) and there is little

information available on how the RE effects on the reproductive system in fish.

The Guppy *Poecilia reticulata* Peters, 1859 is a viviparous ornamental fish that belongs to the Poeciliidae (Froese & Pauly, 2024). The male guppy has distinct physical features, including a broad caudal fin, bright body coloration and a modified anal fin used for fertilization (Houde, 1997). Sexually mature male guppies also exhibit specific reproductive behaviors, such as 'display' and 'gonopodium thrust,' to ensure successful copulation (Tian *et al.*, 2017). Because of these discriminable endpoints and short reproductive cycle, the guppy has been widely used as a model for reproductive toxicology and environment pollution (Baatrup & Junge, 2001; Bayley *et al.*, 2002; Kinnberg & Toft, 2003; Li *et al.*, 2019).

Therefore, this study was conducted to estimate an impact of different ethanolic extracts of *R. graveolens* on sexual characteristics of adult male guppy (*P. reticulata*) including sperm count, gonopodial index and spermatogenesis development for 30-days. The testosterone concentration was also determined in the male-fed RE to further confirmation of anti- androgenic impact of plant.

## Materials & Methods

### Preparation of Rue's extract (RE)

Fresh aerial parts of *R. graveolens* were harvested from the garden of medicinal plants located in the College of Pharmacy, University of Karbala. For seven days, leaves were left to be dried at 40°C and then granted. Following the instructions of Forsatkar *et al.* (2018), the extract was prepared by dissolving the 50 g of powder into 500 mL of 70% ethanol and stirring for two days. The ethanol extract was filtered and lyophilized and 4.6 g of powder

was kept at 24°C in a darkened clean jar until further use.

### **Fish**

Sexually mature guppies of  $116 \pm 4$  mg weight were purchased from an ornamental fish hatchery in Kufa, Iraq. Under 12 h light and 12 h dark cycles, the experimental animals were acclimated to laboratory conditions in 135 L glass tanks containing aerated and filtered water at a temperature of  $26 \pm 1^\circ\text{C}$  for two weeks, during which a commercial flake food (Mahiran Co, Iran) was given. The host taxonomy was followed by Rodriguez (1997).

### **Dietary exposure to extract**

The experimental diets were prepared by supplementing of commercial diet (Flake Food, Mahiran Co., Iran) with 0, 1, 10 and 100 mg.kg<sup>-1</sup> of Rue extract (RE), (Kinnberg & Toft, 2003). The male guppy (n = 120), were randomly distributed into four triplicate groups (n = 30) in 50L tanks and were fed daily with 100 mg of experimental diets for 30 days. Assuming an equal consumption of food per fish, these values result in average doses of 0, 3.3, 33, or 330 µg of RE per gram of fish.day<sup>-1</sup>. During the feeding period, water temperature (°C), pH, dissolved oxygen (mg.l<sup>-1</sup>), total hardness (mg.L<sup>-1</sup> as CaCO<sub>3</sub>) were  $26 \pm 1$ ,  $7.8 \pm 0.2$ ,  $7 \pm 0.5$  and  $180 \pm 20$ , respectively.

### **Evaluation of testosterone concentration**

Steroids were extracted to determine the concentration of testosterone in each animal based on Forsatkar *et al.* (2018). From each replication (n = 9), three animals were selected, anesthetized by clove powder solution at 100 ppm, weighed and manually homogenized at 1:4 volume of the phosphate-buffered saline (PBS) for 5 min on ice. The homogenized samples were centrifuged (2000 rpm at 4°C for 4 min), the supernatant was aspirated, transferred to glass tubes containing

5 mL diethyl ether, vortexed for 1 min, frozen at -20°C for 2 h and the ether layer was transferred into new tubes. The diethyl ether was then evaporated off under airflow, yielding a lipid layer containing the steroids. The extract was stored at -20 °C and then the testosterone concentrations were estimated using a commercial ELISA kit based on the manufacturer protocol (Pishtaz Teb Diagnostics, Iran).

### **Gonopodium index and sperm count**

Post experimental period, nine fish from each group (3 per replicate) were randomly selected to determine the gonopodium index and sperm count as described by Bayley *et al.* (2002). Briefly, the sampled males were lightly anesthetized by clove powder (50 ppm) and photographed under the microscope using a digital camera (Epson Perfection V600). Digital images were analyzed to determine the gonopodial index (length of gonopodium /standard length of the fish) using Image-J software.

After photographing, the sperm count in the male guppy was performed by stripping the fish belly toward the gonopodium and ejaculate was evacuated in a glass plate. The guppy ejaculates were collected and transferred to 100 µL 175 m MKCl solution to aid separation. Then, the suspensions were placed on a Neubauer Chamber hemocytometer to count sperm cells by light microscope (Olympus, Japan).

### **Gonad histopathology**

At the end of the experiment, three fish from each group (n = 9) were selected and killed with an overdose of clove powder solution at 500 ppm, the testicular tissues removed and fixed in 10% formalin after aseptic dissection. The tissues then were dehydrated and embedded in paraffin. Then, 5 µm sections were haematoxylin and eosin stained

(Hewitson & Darby, 2010) and subjected to light microscope examination (Olympus, Japan).

### Statistical analysis

Kolmogorov-Smirnov test and one-way ANOVA in the SSPS 23 software (Chicago, IL, USA) were used to analyze the collected data at  $P < 0.05$ . Values were represented as mean  $\pm$  SD (Gharban & Yousif, 2021).

## Results

### Testosterone levels

No mortality was observed during the study, clinical observations have not shown a significant sign for a systemic toxicity in the fish (Fig. 1A). Testes were normal in the both, control and treatment groups (Fig. 1B). After 30 days, the testosterone concentrations in male guppy fed dietary RE revealed that 100 mg.kg<sup>-1</sup> (929.4 pg.g<sup>-1</sup>) had a significant reduction in the whole-body testosterone levels than control (1826.5 pg.g<sup>-1</sup>), while insignificant variations were observed between other treated and control groups (1 mg.kg<sup>-1</sup> concentration explains 1897.3 pg.g<sup>-1</sup> and the 10 mg.kg<sup>-1</sup> concentration explains 1679.8 pg.g<sup>-1</sup>) (Figure 2A). ( $P \geq 0.05$ ).

### Gonopodium index and sperm count

Gonopodial index was significantly lower in guppy males fed dietary RE at all doses used compared with the control group (Figure 2). Moreover, the ejaculate of control males contained an average of sperm cells reaching 3.62 million cells; while males fed 10 and 100 mg.kg<sup>-1</sup> RE showed a significant lowering in their values, with a decrease of 14% and 19% corresponding to 3.11 and 2.93 million sperm cells, respectively (Fig. 2C). However, no significant difference was found in the sperm cells of males at the lowest dose group (1 mg.kg<sup>-1</sup>) in comparison to those of control males ( $P \geq 0.05$ ).

### Histopathological results

The gonads in control group shows normal histological texture without any significant occupied lesion can be seen in the tissue. However, the 1 mg RE concentration shows mild pathological changes in the gonad including mild degenerative changes in the spermatogonia and spermatozoa (Fig. 4). In contrast, the 10 mg.kg<sup>-1</sup> of BW. Treated of RE explained severe pathological changes in the gonads including necrosis of spermatogonia and spermatozoa (Fig. 5). Interestingly, treatment with 100 mg.kg<sup>-1</sup> B.W. of RE showed highly damage in the gonads including severe necrosis in the spermatozoa and spermatogenesis (Fig. 6).



**Fig. (1):** Gross pathological examination of male guppies after 30 days of feeding dietary Rue (*R. graveolens*) extract revealed no external (A) or testis (B) gross lesions.



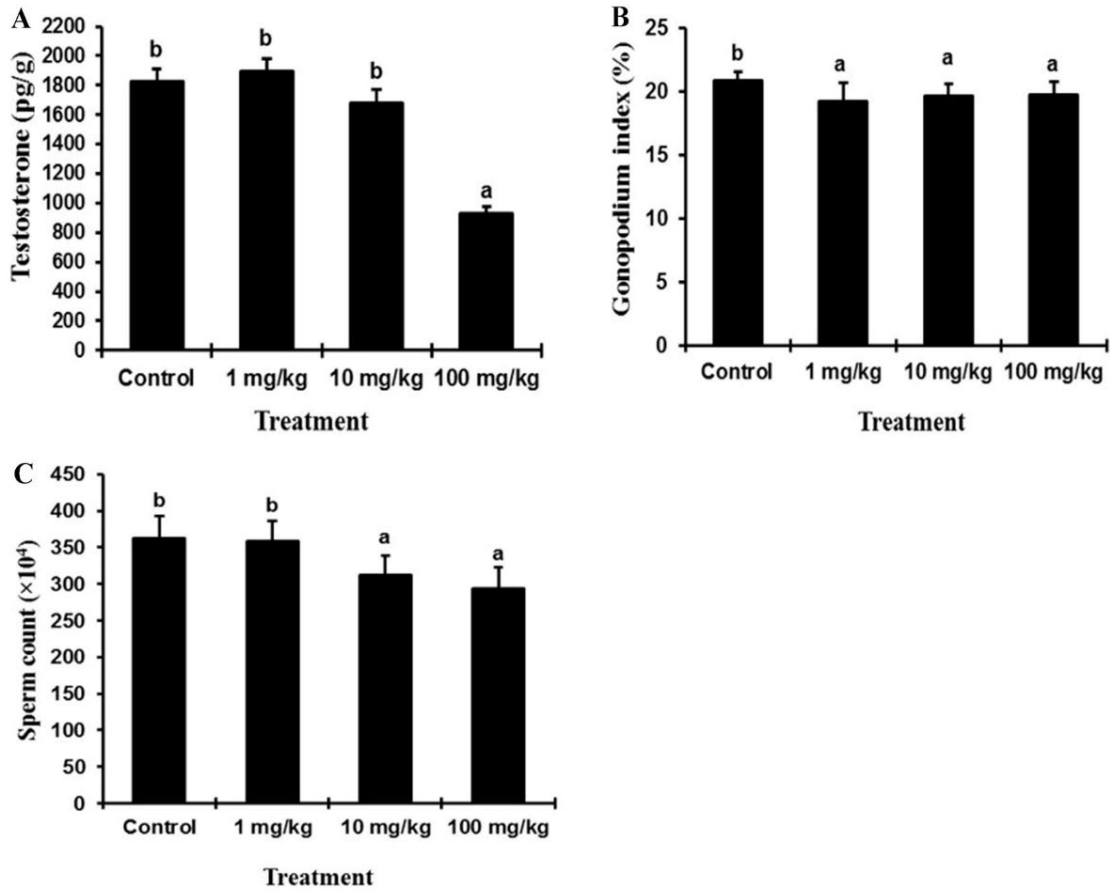


Fig. (2): Testosterone (T) level (A), gonopodium index (B), sperm count (C), in the male guppies fed with 0, 1, 10 and 100 mg.kg<sup>-1</sup> of dietary Rue (*R. graveolens*) extract (RE) for 30 days. Different letters refer to significant variation ( $p < 0.05$ ). pg.g<sup>-1</sup> = pekoe gram/ gram.

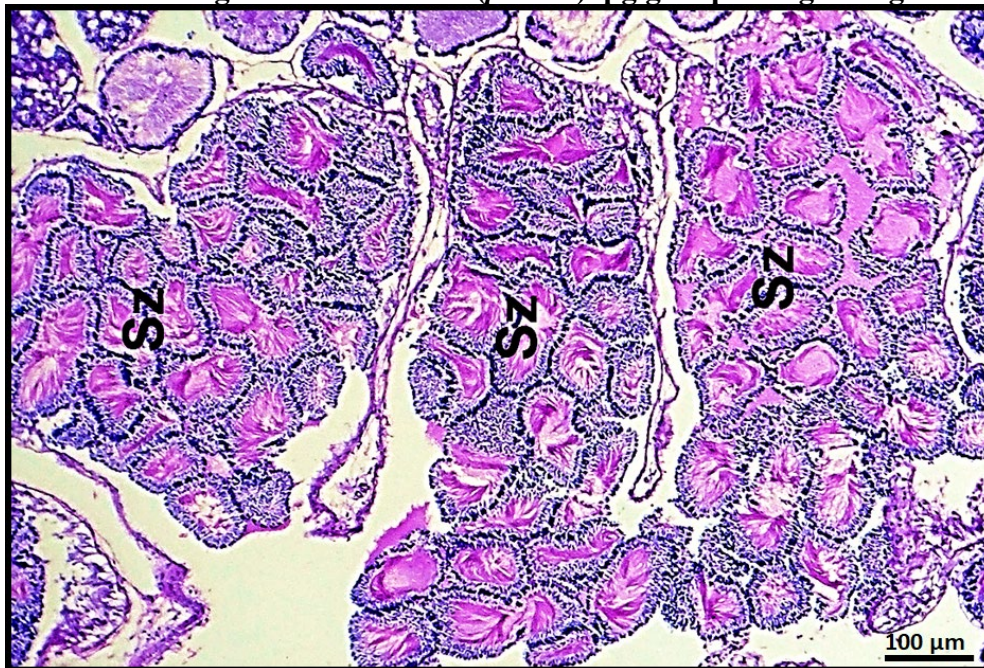


Fig. (3): Control group. The histological section of the gonads in the fish of control group shows no significant occupied lesion (SOL) can be seen in the gonads. The section shows normal texture of spermatogonia, spermatogenesis processes and sperm formation. The tissue is stained with H and E stain and the section is captured using a light microscope and digital camera. Sz: spermatozeugmata.



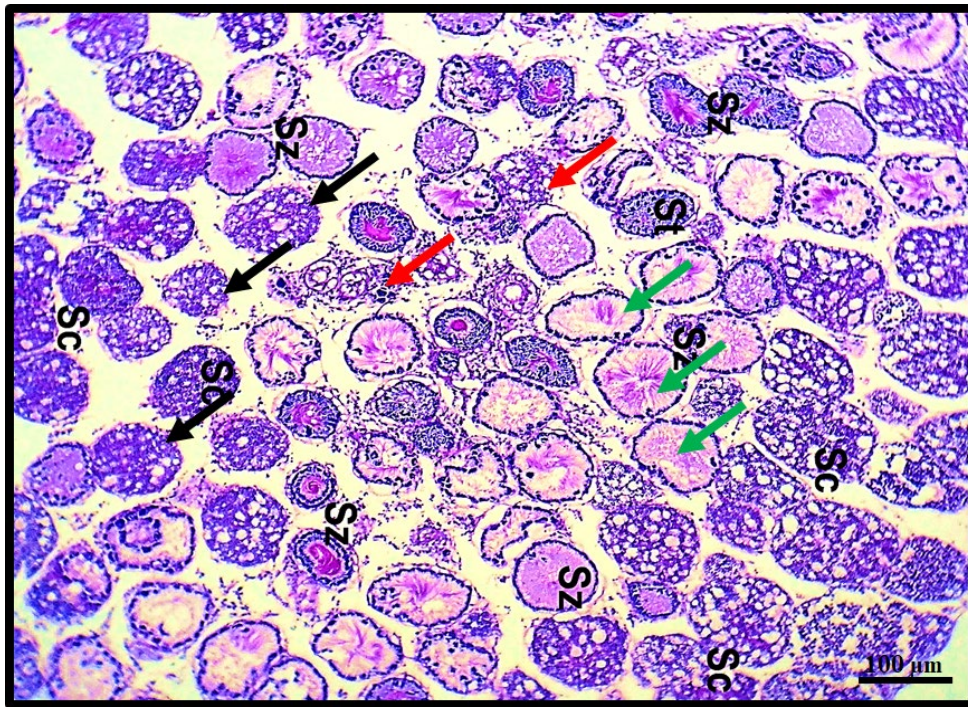


Fig. (4): The histopathological changes in the gonads of Guppy *Poecilia reticulata* in the group that was treated with  $1 \text{ mg.kg}^{-1}$  *Ruta graveolens*. The section shows mild degenerative changes in the gonads especially in the spermatogonia (black arrows) and spermatozoa (red arrows), while the section shows normal seminiferous tubules without any significant occupied lesion (SOL) can be seen in the tissue (normal spermatogenesis, green arrows). The tissue is stained with H&E stain and the section is captured using a digital camera and light microscope. Sc: spermatocytes; St: spermatids; Sz: spermatozeugmata.

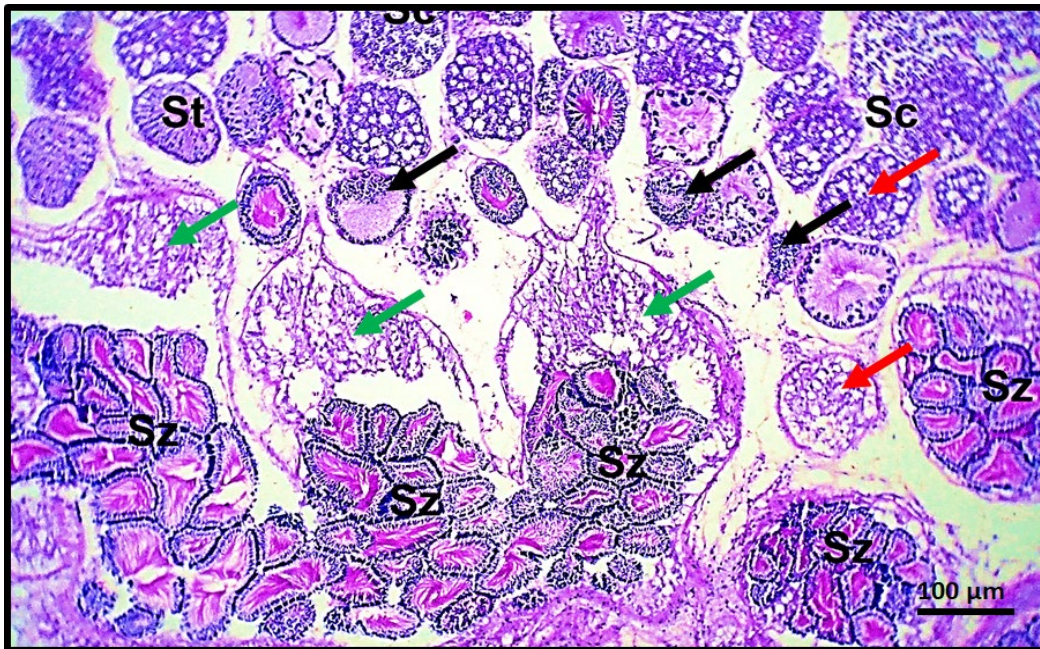


Fig. (5): The histopathological changes in the gonads of Guppy (*Poecilia reticulata*) in the group that was treated with (*Ruta graveolens*  $10 \text{ mg.kg}^{-1}$ ). The section shows severe damage in the gonads especially in the spermatogonia (black arrows) and spermatozoa (red arrows), the section shows severe necrotic changes can be seen in the tissue (seminiferous tubules, green arrows). The tissue is stained with H&E stain and the section is captured using a digital camera and light microscope. Sc: spermatocytes; St: spermatids; Sz: spermatozeugmata.



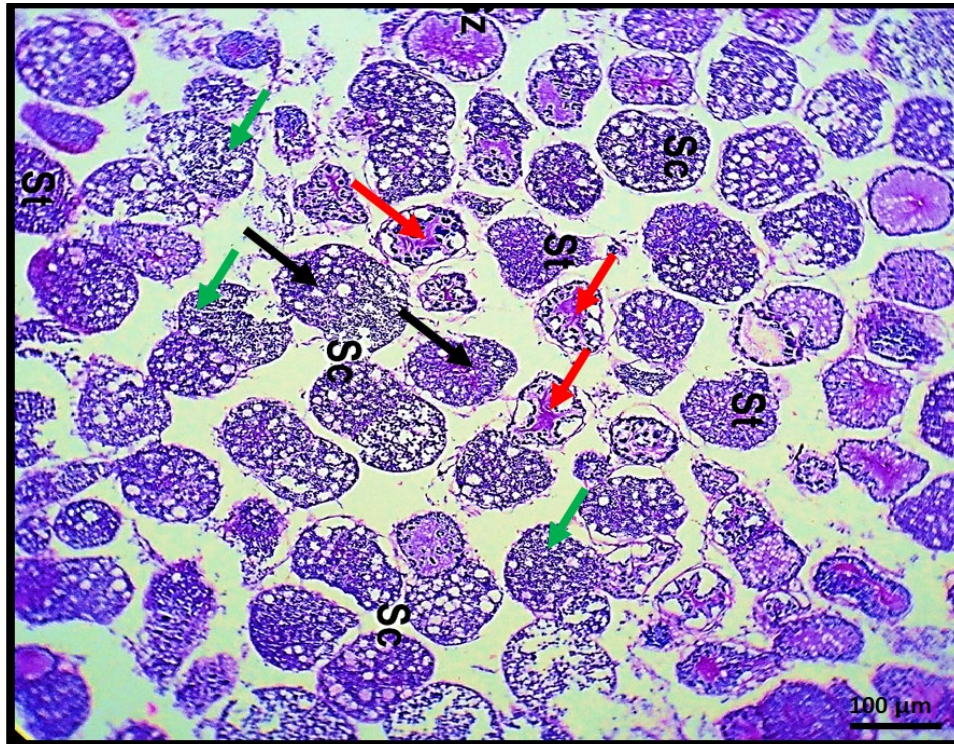


Fig. (6): The histopathological changes in the gonads of Guppy (*Poecilia reticulata*) in the group that treated with *Ruta graveolens* 100 mg.kg<sup>-1</sup>. The section shows highly severe damage in the gonads especially in the spermatogonia (black arrows) and spermatozoa (red arrows), the section shows severe necrotic changes can be seen in the tissue (seminiferous tubules, green arrows). The tissue is stained with H&E stain and the section is captured using a digital camera and light microscope. Sc: spermatocytes; St: spermatids; Sz: spermatozeugmata.

## Discussion

Various chemicals in aquatic environments with endocrine-disrupting potency can affect aquatic organisms (Blair *et al.*, 2013). Researchers revealed that endocrine disruption by chemical substances can impact sexual characteristics in fish (Matthiessen *et al.*, 2018); however, they are mainly focused on pesticides and there is limited data regarding pharmaceutical products.

The guppy is an ideal species to investigate the reproductive toxicology and have been widely employed to assess endocrine-disrupted compounds such as tributyltin and bisphenol A (Haubruge *et al.*, 2000), vinclozolin fungicide (Baatrup & Junge, 2001)

and 2, 2'-Dithiobis-pyridine antifouling biocides (Li *et al.*, 2019).

The results of the present study have shown that dietary supplementation with a medicinal plant, RE significantly decreased testosterone level, sperm count and gonopodium index, as well as inhibited spermatogenesis development of the male guppy which further confirms the anti-androgenic potency of the plant consistent with previous reports in rats and fish (Khoury & El-Akawi, 2005; Halvaei *et al.*, 2012; Forsatkar *et al.*, 2018).

Our result revealed that RE adversely affected testosterone levels in the male guppy, agreeing with the study of Forsatkar *et al.* (2018) that demonstrated observable decreases in concentration of testosterone in males of *D.*

*rerio* that exposes to sub-lethal levels of Rue extract, as well as similar to findings recorded post exposing for other anti-androgenic endocrine disruptor chemicals in fish (Baatrup & Junge, 2001; Crago & Klaper, 2012; Ahmadvand *et al.*, 2016).

Previous studies have shown that *R. graveolens* extract significantly decreased the number of Leydig cells, as well as testosterone concentration in male rats (Khouri & El-Akawi, 2005; Sailani & Moeini, 2007; Walker, 2009; Halvaei *et al.*, 2012).

On the other hands, increased testosterone aromatization into 17 $\beta$ -estradiol has been shown in many studies investigating anti-androgenic endocrine-disrupting chemicals in fish (Jensen *et al.*, 2004; Cheshenko *et al.*, 2008). Therefore, decreasing testosterone levels of males fed RE may be associated with aromatization and/or decreased endogenous androgen production due to the reduced Leydig cells, as well as altering the androgen signaling pathways (Walker, 2009).

A significant lowering in the number of sperm cells was detected in ejaculates of guppy males fed with RE when compared with the male in control group. In addition, results from histological examinations of the testes of treated fish showed dose-dependent decreases in spermatogonia, in parallel with increasing of spermatocytes in ducts, proposing that RE can inhibit the development of spermatogenesis. These results align with mammalian studies where reduced sperm count and changes in testicular structure were reported in the male Rats fed with ethanol extract of *R. graveolens* (Khouri & El-Akawi, 2005; Sailani & Moeini, 2007), as well as identical to results observed post exposing for other anti-androgenic endocrine disruptors in guppy males (Baatrup & Junge, 2001; Bayley *et al.*, 2002; Kinnberg & Toft, 2003). Similar responses were also found in zebrafish (*D.*

*rerio*) exposed to sub-lethal levels of Rue extract (Forsatkar *et al.*, 2018), as well as in the male siamese fighting fish (*B. splendens*) that was injected with 300  $\mu\text{g}\cdot\text{g}^{-1}$  of the plant extract (Forsatkar *et al.*, 2016).

Androgen signaling via androgen receptors (ARs) localized in the Leydig, Sertoli, peritubular cells regulates the proliferation cells regulates proliferation and differentiation of the male germs, as in the absence of the T or AR spermatogenesis does not proceed beyond the meiosis stage (Ikeuchi *et al.*, 2001; De Gendt *et al.*, 2004). Therefore, it can be suggested that the impaired spermatogenesis of male guppy-fed RE may be a result of altering the androgen signaling pathways and T reduction.

Moreover, meiosis arrest can occur through T and/or Insulin-like growth factor 1 (IGF-1) via extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) signaling in T-independent pathways via down-regulation of *boule* gene expression (Ahmadvand *et al.*, 2016), involving in male meiosis and nervous system (Hoopfer *et al.*, 2008).

Therefore, spermatogenic failure in this study, as well as abnormal behaviors reported in fish exposed to *R. graveolens* (Forsatkar *et al.*, 2016; 2018), may be associated with *boule* dysfunction, as well.

The males fed with RE had a lower gonopodium index than the control fish agreeing with the previous studies reporting the decreased gonopodium index in male poeciliid exposed to anti-androgenic endocrine disruptors (Toft & Baatrup, 2001; Bayley *et al.*, 2002), which may be explained as a general effect of the plant on growth and secondary sex characteristics (Bayley *et al.*, 2002).



## Conclusions

This study revealed the effects of alcoholic extracts of a medicinal plant, *R. graveolens* on spermatogenesis and sexual characteristics in male guppy. The results further confirmed the anti-androgenic potential of *R. graveolens*, as well suggest *P. reticulata* as a suitable species for bio-monitoring of endocrine disruptors in aquatic environments. Nonetheless, further researches are necessary to investigate the mechanism of action and immunotoxic effects of the plant in fish.

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## Contributions of authors

**A.M.K.**, Suggestion the proposal of the article, Laboratory techniques, wrote the manuscript.

**A.A. A.**, Suggestion the proposal of the article, Laboratory techniques, wrote and revised the manuscript.

**S.M.A.**, Statistical analysis, revised the manuscript.

**I.S.A. A.**, Laboratory techniques.

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## Conflict of interest

There is no conflict of interest between the authors

## Ethical approval

All ethical guidelines related to Fish and care issued by national and international organizations were implemented in this report.

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المميزات التثبيطية لعشبة الشذاب (*Ruta graveolens* L.) على عملية تخليق النطف في أسماك الكوبي *Poecilia reticulata* Peters, 1859

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**المستخلص:** أجريت هذه الدراسة لمعرفة تأثير عشبة الشذاب (RE)، وهي عشبة طبية ذات خصائص مضادة للأندروجين، على الحياة الجنسية لذكور أسماك الجوبي. وتم اختيار 120 ذكراً من الأسماك المذكورة للتجربة حيث تم اقلمتها في الوسط التجريبي وتوزيعها عشوائياً وبأعداد متساوية على أربع مجموعات تلقت المستخلص الإيثانولي للعشبة عند 0 و 1 و 10 و 100 ملغم. كغم<sup>-1</sup> من عشبة RE على التوالي لمدة 30 يوماً. كشفت النتائج الدراسة أن 100 ملغم. كغم<sup>-1</sup> من العشبة كان لديهم انخفاض مستويات هرمون التستوستيرون في الجسم بالكامل بشكل ملحوظ عن السيطرة. وعلاوة على ذلك، الذكور المعطاة عشبة RE في جميع الجرعات أظهرت انخفاض ملحوظ في قيمة الدليل التناسلي (gonopodial index) مقارنة بمجموعة السيطرة. بينما، أظهر إعطاء عشبة ال RE بجرعة 10 و 100 ملغم. كغم<sup>-1</sup> إلى انخفاض عدد الحيوانات المنوية مقارنة بمجموعة السيطرة. أما بالنسبة للفحص النسيجي فقد كانت خصى ذكور السيطرة متباينة بشكل جيد في زيادة نسبة الحيوانات المنوية في جميع مراحل تكوين الحيوانات المنوية بشكل كبير وكذلك نسبة الهياكل الكيسية التي تحتوي على الحيوانات المنوية. من ناحية أخرى، كانت الحيوانات التي تم إعادة تغذيتها من جديد قد أظهرت انخفاضاً في الحيوانات المنوية وزيادة في الخلايا المنوية وعدد القنوات اعتماداً على جرعة المادة العشبية وذلك يدل على دور عشبة ال RE في الحد من تطور أسماك الجوبي وتكوين الحيوانات المنوية. استنتجت الدراسة الى انه هناك تأثير واضح لعشبة ال RE على المعايير الجنسية لذكور أسماك الجوبي البالغة وتأكيد الخصائص المضادة للأندروجين التي تمتلكها العشبة.

**الكلمات المفتاحية:** انسجة الغدد التناسلية، الدليل التناسلي، *Poecilia reticulata*، عشبة الشذاب، هرمون التستوستيرون.