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GENOTOXICOLOGICAL AND BIOCHEMICAL STUDIES ON *Holothuria atra* FOR ALLEVIATING REACTIVE TEXTILE DYES CONTAINING WASTEWATER IMPACTS IN NILE TILAPIA, *Oreochromis niloticus* (L.)

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Abstract

Anthropogenic organic dyestuffs and their derivatives have been enormously used in various manufactures such as textiles, pharmaceuticals, food, cosmetics and leather. Such environmental chemicals can readily be discharged into the aquatic ecosystem through industrial effluent drainage. Several research studies have indicated that synthetic organic dyes and their derivatives could cause reproductive impairment in aquatic fauna. However, the endocrine-disrupting impacts of such dyestuffs and colorants containing wastewaters have been scarcely explored. Herein, we examined the potential of three different sorts of reactive textile dyes (RTDs), i.e., reactive red 195 (RR195), reactive yellow 18 (RY18), and reactive blue 4 (RB4) to interrupt sex steroids synthesis. Furthermore, our study investigated the biological role of *Holothuria atra* (*H. atra*) in the attenuation of the deleterious effects induced by such organic dyes. For this purpose, tilapia fish juveniles of *Oreochromis niloticus* (*O. niloticus*) were used. Regulatory fluctuations have been observed in three substantial hypothalamic-pituitary-gonadal axis (HPG axis) genes by all tested reactive dyes, i.e., downregulation of *FSHβ*, *LHβ* (sex steroids) and *CYP19A1* (steroidogenic gene) and up-regulation of them in the presence of *H. atra*. Our outputs suggest that some RTDs could act as endocrine disruptors and may consequently severely impact the aquatic environment.

INTRODUCTION

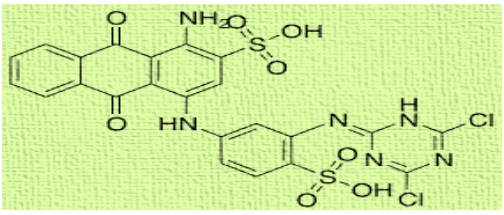
Despite the excessive importance of the textile sector to the Egyptian economy, this industry has many risks to the aquatic ecosystem. This is due to the fact that the textile industry is one of the industries that depend on consuming large quantities of water during fabric dyeing processes [1]. The resulting textile effluents are significant, since they consist of considerable toxic compounds, whether such compounds are organic or inorganic including organic dyes, salts, acids, heavy metals and so on [2]. Relying on the pattern of fabric and dye, the percentage of dye wastage ranges from 5% up to 50% [3]. Therefore, approximately 200 billion liters of coloured effluents are discharged into surface waters worldwide and about 5 million liters into the Egyptian aquatic environment yearly [4].

Since these compounds are characterized by their recalcitrance, persistence and low biodegradability by conventional treatment process, which makes those substances to be more dangerous to aquatic life particularly and to humans in general. There are many types of synthetic organic dyes by the textile industry such as azoic, disperse, vatic, direct, acidic, basic and reactive dyes [5].

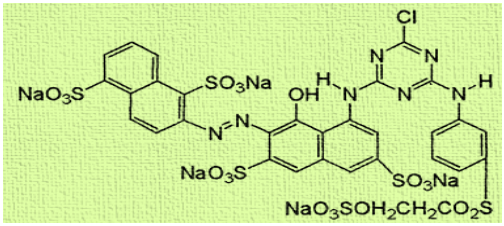
Among these, reactive dyes are one of the most widely applied not only in the textile industry but also in many other industries such as printing, paper, cosmetics,

pharmaceutical, food, etc [6]. The most major patterns of reactive dyes are azo and anthraquinone based dyes [7]. As per previous scientific reports, mutagenic aromatic amines (benzidine, aniline, nitro amino azo benzenes and its derivatives) might be stem from organic azo dyes after biotransformation by intestinal microflora [8,9]. As previously reviewed by [10], anthraquinone-based dyes are more renitent to biodegradation than azoic dyes due to their fused aromatic rings [11].

Reactive blue 4
Molecular weight: 637.43
Molecular formula: $C_{23}H_{14}Cl_2N_6O_8S_2$



Reactive red 195
Molecular weight: 1136.32
Molecular formula: $C_{31}H_{19}Cl\ N_7Na_5O_{19}S_6$



Reactive yellow 18
Molecular weight: 906.12
Molecular formula: $C_{25}H_{16}ClN_9Na_4O_{13}S_4$

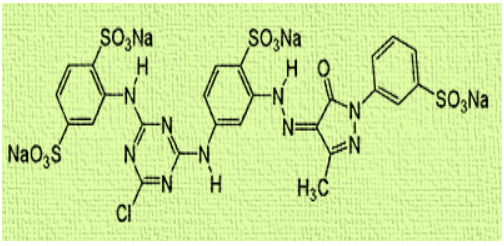


Figure 1. Chemical structure of some reactive textile dyes (RTDs) including Reactive blue 4(RB4), reactive red 195(RR195) and reactive yellow 18 (RY 18).

Anthropogenic textile dyes which act as endocrine-disrupting chemicals (EDCs) might interfere with the reproduction physiology of aquatic vertebrates including fishes [12] throughout their interaction in the hypothalamus-pituitary gonadal-Liver axis (HPGL axis) that coordinate sex steroid hormones synthesis [13]. Moreover, EDCs have been documented to modulate the transcription of enzymes and hormone receptors implicated in sex steroid metabolic pathways [14], leading to a subsidence of populations, productivity and gamete quantity [15] and thus ultimately affecting biodiversity. Many of EDCs are estrogenic, sometimes resulting in feminization and vitellogenin induction in male fish [16,17]. So, as recently stated by [18], the existence of such estrogenic EDCs in the aquatic environment has become an increasingly growing problem.

Generally, various natural products play a crucial role as antioxidants and protective agents against toxic oxidants and multiple health problems, due to its contents of phenolic and polyphenolic substances [19, 20, 21, 22, 23, 24]. Recently, many of studies have pointed out the importance of finding new alternatives as marine organisms based products, particularly Holothurians [25, 26]. Invertebrate sea cucumber *Holothuria atra* is considered one of the most substantial and popular species that are found in the Red Sea on the Egyptian coasts.

H. atra, belonging to Holothuroidea class, is a fertile source of varied bioactive components including saponins glycosides, polysaccharides, minerals, vitamins, peptides, cerebrocides, flavonoids and polyphenolic substances [27,28]. A number of biological and curative functions of the previous scarcely ingredients have been reported by [29]. Consequently, evidences suggested that they act as anti-toxicity, antioxidant, anti-microbial, anti-diabetic, anti-inflammatory, anti-hypertension, anti-coagulant and anti-carcinoma [30, 31].

However, little is known about applying sea cucumbers as protective agents in fish diets. Thus, the objective of the current study was to evaluate endocrine-disrupting, genotoxicity and oxidative stress potentials of selected synthetic reactive dyes for monitoring the various resulting fluctuations in HPG-axis genes and targeted biomarkers in tilapia individuals. In addition, *H. atra* has been applied to study its biological activity against genotoxicity induced by textile dyes. Among the important freshwater fish species,

Nile tilapia is considered as an excellent candidate for biomonitoring and genotoxicological experiments [32,33]. Therefore, tilapia fish have been extremely utilized to study the effects of environmental chemicals on pituitary gene expression [34,35]. The findings of this study will help characterize reactive textile dyes of potential toxicological prominence and stimulate further investigations on sea cucumbers significance against endocrine disruption of environmental chemicals and their derivatives.

MATERIALS AND METHODS

Collection and Preparation of Black Sea Cucumber

Sea cucumber (*H.atra*) samples were caught from the Red Sea (Safaga). The animals were brought to the experimental laboratory in an icebox and then washed by running tap water and cut open. The internal organs were dismissed and washed. The body walls of these animals were dried, milled and then stored at -20ºC until processing.

Fish Samples and Acclimatization

A whole number of 100 distinctly healthy *Oreochromis niloticus* (Nile Tilapia), with average body weight of 100 ± 25 g / fish and 12.8 ± 2.3 cm in length obtained from a local fish farm at Nubaria - National Research Centre (NRC), Egypt. Fish samples were grouped into ten indoor fiberglass aquaria (70 L water capacity). Fish individuals were acclimatized under laboratory conditions for 2 weeks, before the experiment, glass aquaria provided with de-chlorinated water (26.7± 2.1ºC and pH 7.2-8.2) with stabilized aeration. Feeding was done once daily using a pelleted diet (32% protein proportion) at a rate of 1% of the fish's body weight. The water in the glass aquaria was changed once per week to avoid metabolite accumulations. All fish groups received human care in compliance with the guidelines of the Ethical Committee of NRC, 140633, Egypt.

Experimental Design

Aquatic animals were sectioned randomly into one of these groups (n=10 per group): Control group (Non treated water), textile dyes without *H. atra* consist of RR (195) or a mixture of RR (195) + RY (18) + RB (4) with a constant final concentration of 100 mg/L once per week, or mixture of previous RTDs with different doses of powdered *H. atra* including 100, 200 and 300 mg/kg/day. In addition, Zink sulphate ZS (100mg/kg/day) as positive control. Under these conditions, Tilapia fish juveniles lasted for 4 weeks. Through this period, potential deaths were monitored. Commercial dyes were obtained from Textile Industries Division, NRC, Egypt. After the experiment, before dissection, the fish groups were anesthetized, 30 fish juveniles were separated for genotoxic analysis, where gills and liver were isolated. For the remaining fish animals, the liver was segregated and stored at -80ºC for biochemical analysis [36]. Brain samples of all such animals were also removed and stored at -80ºC for gene expression analysis [37] (Table 1).

Table 1. Experimental design of *H. atra* supplemented diet in different concentrations fed to Nile tilapia (*Oreochromis niloticus*) exposed to reactive textile dyes for 30 days.

Groups	Treatment	Dosage (mg)			
1	Negative control group	RR195	MDs	HaP	Zinc sulphate
2	Reactive red 195 (RR195 mg/L)	100	-	-	
3	Mixed reactive dyes (MDs mg/L)	-	100	-	
4	RR(195), mg of HaP/kg diet	100	-	100	
5	RR(195), mg of HaP/kg diet	100	-	200	
6	RR(195), mg of HaP/kg diet	100	-	300	
7	MDs, mg of HaP/kg diet	-	100	100	
8	MDs , mg of HaP/kg diet	-	100	200	
9	MDs , mg of HaP/kg diet	-	100	300	
10	Zinc sulphate				5.00

Micronucleus Assay

The micronucleus test was carried out using smears of gills blood and hepatic cells drawn on clean and dry microscopic slides using fetal calf serum according to [37]. 30 fish samples were analyzed for this test. Nine slides were intended for each group (3 animals per treatment). The slides were dried at room temperature for 24 hours, subsequently, fixed in absolute methanol for 10 min and air-dried at 25ºC. These steps were followed by 10% Giemsa stain solution (v/v) for 10 min. 1000 erothrocytes were examined per each slide using the light microscope. The frequency of micronuclei (%) was recorded at 1000x magnification and calculated for each animal [38].

Oxidative Stress Evaluation in Hepatic Tissues

Glutathione-S-transferase Activity (GST)

GST activity was determined according to [39], quantifying the conjugation of glutathione to the substrate 1-chloro-2,4-dinitro-benzene (CDNB). The measurement was performed at 340 nm for 5 min**.** The enzymatic activity was expressed in nmol of GSH/min/mg protein.

Catalase Activity (CAT)

CAT activity was determined according to [40]. The enzymatic activity was monitored at 510 nm and expressed as $mmol^{-1}$ $H_2O_2/min^{-1}/mg^{-1}$ protein.

Superoxide Dismutase Activity (SOD)

SOD activity was determined according to [41]. The SOD activity was expressed by measuring the increase in absorbance at 560 nm for 5 min at 25ºC. This assay relied on the ability of the enzyme to inhibit PMS-mediated reduction of O_2° to O_2 which then reduced NBT dye. 1.5 U/ assay of the purified SOD produced 80% inhibition. Also, the levels of GST, CAT & SOD were assessed using commercial kits (Bio-diagnostics Co., Cairo, Egypt).

Gene Expression

RNA Isolation and cDNA Synthesis

The RNA was extracted from the brain tissues using a kit produced by Bioer Technology Co., Ltd., China, according to the manufacturer's protocol. The obtained total RNA concentration and purity were measured spectrophotometrically at 260 and 280 nm wavelengths using nanodrop (Jenway, Cole-Parmer Ltd., Staffordshire, UK). Equal amounts of RNA of 0.4µg were reverse transcribed into cDNA in 20 µL reactions containing 1.0µL of RT enzyme mix which consist of (reverse transcriptase enzyme and RNase inhibitor enzyme), 4.0µL of cDNA reaction buffer (5x) which composed of optimized buffer and the final concentration of dNTPs was 0.5mM, 11µL of nuclease-free water and 4.0µL of extracted RNA using (Cosmo cDNA synthesis kit, UK). Reverse transcriptions were conducted in DNA Thermal cycler (Applied Biosystems, USA) at 24ºC for 5 minutes, then incubated for 15 min at 45ºC and terminated at 85ºC for 5 min. Primers were designed for the specific amplification according to [37, 42, 43].

Table 2. List of primers used in qRT-PCR.

Gene	Oligonucleotide sequence	Reference
B-Actin	F 5'-GACCCACACAGTGCCCATCT-3 ' R 5'-TCTCGGCTGTGGTGGTGAA-3 '	[43]
FSHβ	F 5'-GTCGCCCAAAGAACATCAGCCTC-3' R 5'-TGTATCCAGACAAGGTCCCGCAGT-3'	[42]
LHβ	F 5'-AGAATGCTCCTTGCTCTGATGTTG-3 ' R 5'-CAACTCAAAGCCACGGGGTAGGT-3 '	[42]
CYP19a1	F 5'-TCAAACAAAACCCGCACGTG-3 ' R 5'-AAAACTCGGTGCGGTGCATT-3 '	[37]

B-Actin, beta-actin (housekeeping gene); *FSHβ*, follicle-stimulating hormone beta subunit; *LHβ*, Luteinizing hormone subunit beta; *CYP19a1,* aromatase (estrogen synthetase).

Quantitative Real-Time PCR (qRT-PCR)

The primers sequences of three genes: FSHß, LHß and CYP19a1 were shown in (Table 2). Quantitative real-time qRT-PCR (Applied Biosystems step one, USA). qRT-PCR reaction systems 20µL consists of 10µl of Sybr green-2-Go Master mix-High Rox, Canada, 0.6µl of each gene-specific primer, 1.0µl of cDNA (template). ß-actin was used as a reference housekeeping gene. The thermal profile was 95ºC for 30 s, 40 cycles of 95ºC for 5 s, 60ºC for 30 s and 72ºC for 30 s, respectively. The melting curve was obtained to assess the specificity for genes of interest. The threshold cycle (Ct) values were determined for quantitative analysis of gene expression and compared by using the $2^{-\Delta\Delta Ct}$ method. ß-actin was utilized as an internal control for normalization [37, 42].

Statistical Analysis

A randomized complete block design was used for analysis of all data with three replications for each parameter. The treatment means were compared by the least significant difference (L.S.D) test as given by [44, 45] by used Assistant program. The values are expressed as mean ± SD. All statements of significance were based on probability of $P < 0.05$.

RESULTS AND DISCUSSION

Micronuclei (MN) Inducement in Tilapia Gills and Hepatic Cells

The results of this study showed that the exposure of gills erythrocytes in tilapia groups to RR(195) and MDs as positive controls caused a significant increase in micronuclei induction (9.33 and 9.87%, respectively) at ($P<0.05$) in comparison with their respective negative control (0.97%) and different other treatments of *H.atra*. The positive control (ZS) showed a significant increase in MN induction (9.57%). MN percentages have been significantly decreased after treated with various doses of *H.atra* in the presence of RR (195) and MDs, particularly at 200 mg/kg (1.87 and 1.60%, respectively) as shown in (Fig. 2a).

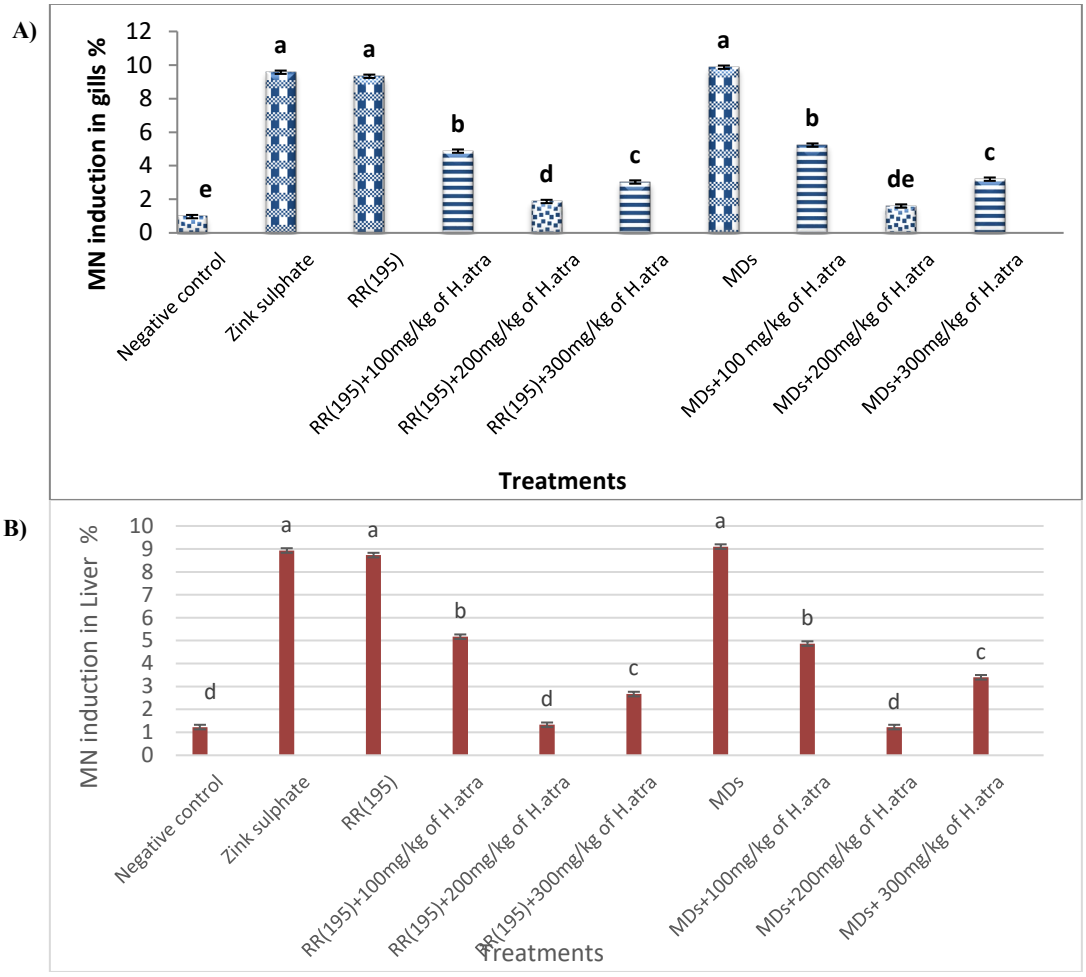


Figure 2. The effect of RR(195), MDs and different doses of *H. atra* on MN induction in both of tilapia gills **A)** and hepatic cells **B)** after 30 days exposure and treatment, the different letters indicate significant differences from each other (ANOVA, LSD test, $p < 0.05$).

Furthermore, our results indicated the exposure of hepatic cells in tilapia groups to RR(195) and MDs after 30 days have caused a significant increase in MN formation 8.73 and 9.10%, respectively at ($P < 0.05$) over their corresponding negative control ((1.23%) and different other treatments of *H. atra*. ZS, a positive control showed a significant increase of MN induction (8.93%). Whilst, MN percentages in tilapia hepatic cells after treating with different concentrations of *H. atra*, particularly at 200 mg/kg were 1.87 and 1.60%, respectively (Fig. 2b).

The obtained outcomes are in line with those reported by [36], they mentioned that *O. niloticus* erythrocytes have displayed an increasing in micronuclei percentage and DNA damage as a result of the exposure to textile effluents at various concentrations.

As per earlier studies elucidated that aquatic pollutants including textile dyes might lead to oxidative damage through disruption in redox state and over-generation of Reactive Oxygen Species (ROS). As a result, the occurrence of DNA damage and genotoxicity [37, 38]. In addition, mitochondrial retrogradation and apoptosis might be excited by ROS [39].

Our results have disclosed the efficient effect of *H. atra* as a potential antioxidant, as it contains several predominant polyphenolic and flavonoid ingredients such as chlorogenic acid, gallic acid, naringenin and catechin, wherein these compounds are characterized by their biological effective role as ROS scavengers by its adjacent hydroxyl groups on aromatic rings [40, 41].

Biochemical Responses of *O.niloticus* by RTDs Eexposure and *H. atra* Treatments

The results of the biochemical profile indicated the induction of a significant increase of GST activity in tilapia hepatic cells ($p < 0.05$) after 30 days of exposure to 100 mg/L of RR (195) and MDs (14.16 and 15.34%, respectively) over negative control. ZS showed also significant increase in GST activity (14.82%). However, the increase in GST activity has been significantly improved after treating with various doses of *H. atra* in the presence of RR (195) and MDs, particularly at 200 mg/kg (4.04 and 5.4%, respectively) as shown in (Fig. 3a).

As well, the activity of CAT increased significantly over negative control ($p < 0.05$) in RR (195) and MDs, ZS as groups of positive controls (66.84, 89.74& 63.37%, respectively). But, the addition of *H. atra* to the previous groups, particularly at 200mg/kg has improved significantly CAT activity (10.64 and 0.06%) over negative control (Fig. 3b).

Likewise, after 30 days of exposure to reactive dyes solely (RR195 and MDs), SOD activity was increased significantly ($p<0.05$) in hepatic cells (745.03 and 675.95%) over negative control. ZS as a positive control exhibited a significant increase in SOD activity (733.05%). Whereas, the increase in SOD activity was ameliorated significantly ($p<0.05$) in RR (195) and MDs groups treated with *H. atra*, especially at 200 mg/kg (24.8 and 7.74%) as shown in (Fig. 3c).

Our findings are in accordance with those obtained from investigation on textile dyes especially azoic dyes triggered oxidative stress through the increase in antioxidant enzyme activity [46,47]. The increase in antioxidases activity might be an indicator of increased toxicity in the existence of RTDs. Whilst, the addition of *H.atra* to the diet has significantly improved the enzyme activity especially at 200 mg/kg.

The efficacious protective role of *H. atra*, likely due to the existence of natural protein, polysaccharides, vitamins, polyphenolic components as ROS scavengers [48,49, 50,51]. As a result, the resulting toxicity by those textile dyes has been decreased. Likewise, other studies have exhibited that both of Fe^{+} chelating vitality and lipid peroxidation have been suppressed by the aqueous and organic extracts of *H. atra* [52,53].

The preceding reports have confirmed the increase in antioxidant enzymes activity including GST, CAT and SOD by textile dyes, probably due to increased production of ROS, implying the ability of antioxidant enzymes to scavenging of radicals generated by oxygen reduction mechanism, illustrating the protective and defensive role of antioxidases versus oxidative stress [47]. On the other hand, the generation of different types of free radicals including H_2O_2, hydroxyl and superoxide radicals by azoic textile dyes, might induce a decrease in antioxidases activity and consequently, reflect a disability in the antioxidant capacity and thus impairment of the cellular defense [54]. However, all the potential scenarios for such an issue relying on the concentration and exposure time to the stressor.

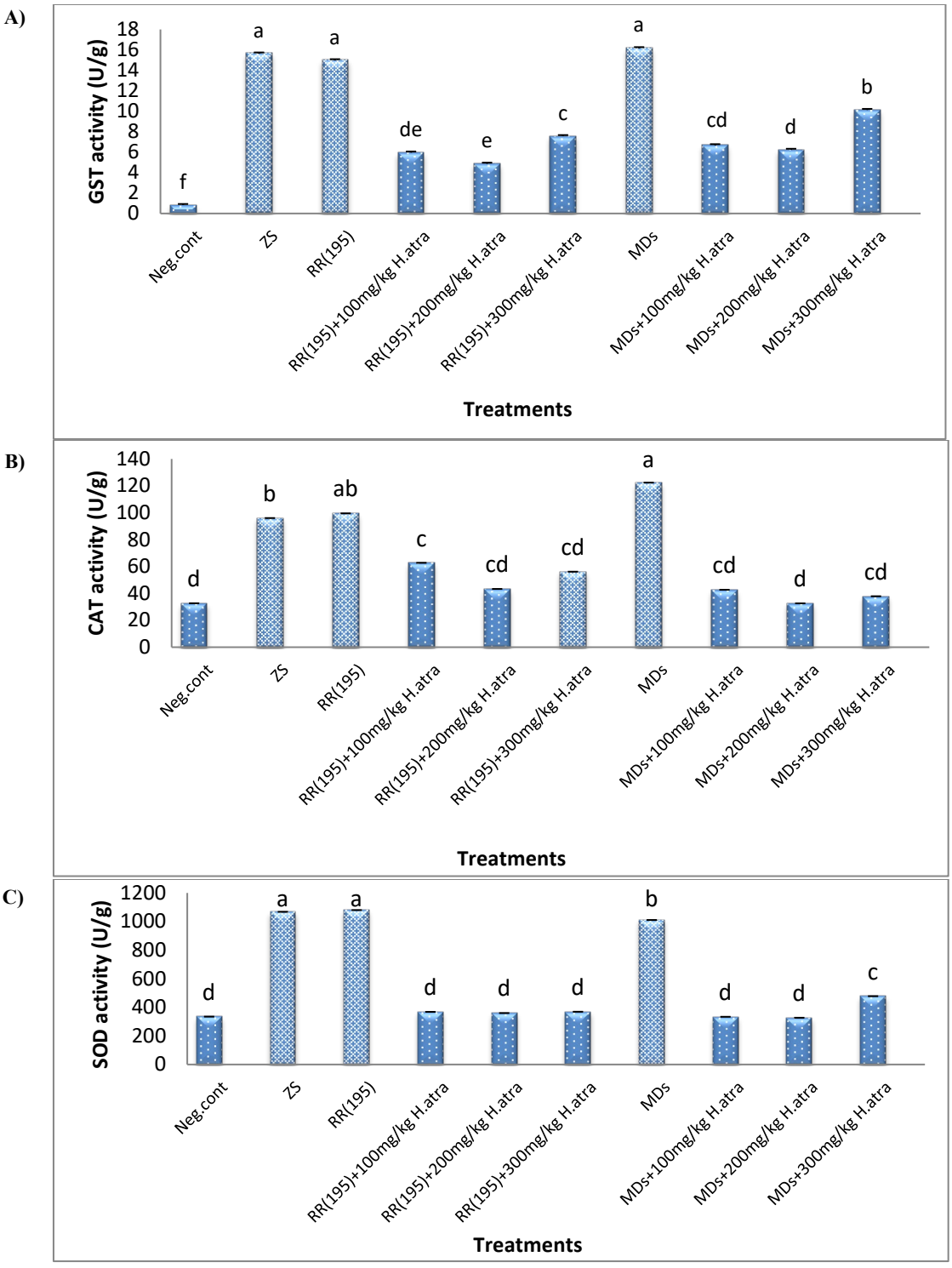


Figure 3. The activity of GST **A)**, CAT **B)** & SOD **C)** after 30 d exposure to RR(195), MDs & different other treatments of *H. atra*. Results as shown as mean ± SD.

mRNA Expression of HPG-axis Genes

The *FSHβ* gene was significantly down-regulated by both RR (195) and MDs in brain cells ($P < 0.05$) over 30 days of exposure. But, after treating with various concentrations of *H. atra*, *FSHβ* was significantly up-regulated over negative control (Fig. 4a). Likewise, our results showed that *LHβ* expression was down-regulated upon exposure to RR (195) and MDs [55]. Whilst, *LHβ* gene was up-regulated after treating with various doses of *H. atra*. However, *LHβ* gene was not altered by MDs exposure [14] (Fig. 4b). After exposure to RR (195) and MDs, *CYP19A1* mRNA was significantly down-regulated. Whereas, *CYP19A1* expression was significantly increased after treating with 100 and 200 mg/kg of *H. atra* (Fig. 4c). All three targeted genes exhibited significant down-regulation by ZS as positive control (Fig. 4a, b, c).

Our results have revealed that the fundamental HPG-axis genes such as *FSHβ*, *LHβ* and *CYP19A1* as a steroidogenic gene have been influenced upon exposure to RR (195) and MDs, implying that inhibition of steroidogenesis pathway, which may lead to decreased levels of sex steroid hormones [56]. The three targeted HPG-axis are playing a key role in steroidogenesis via stimulating of estrogen, vitellogenin synthesis in females and androgen synthesis in males. Therefore, the conversion of cholesterol into sex steroid hormones might be interrupted by those effluent dyes [56,57]. Aromatase encoded by *CYP19A1* gene is responsible for the transformation of androgen to estrogen. Thence, *CYP19A1* up-regulation by various concentrations of *H. atra* may lead to boosting in androgen transmutation to estrogen. As well, the results of this study have indicated an improvement in mRNA expression of both *FSHβ* and *LHβ* by treating with *H. atra* in the presence of RR (195) and MDs, thus amelioration of steroidogenesis and fecundity might be due to its contents of various natural compounds specifically, polyphenolic components and triterpene saponins [58].

Tri-terpene glycosides are playing a paramount role in the proliferation process through boosting of HPG axis capacity and then enhancing the expression level of endocrine related genes, as it may be due to their similar structure to sexual steroids [59, 60]. As per earlier reports [61], the extracted tri-terpene saponins from Asian red ginseng have significantly up-regulated of *FSHR* and *CYP19a1* and thus promoted the development of mice ovarian follicles. However, few researches documented that some sea cucumber extracts have significantly enhanced the oocyte maturation via the increased mRNA expression of *FSHR* in experimental animals [62]. So far, endocrine disruption of textile dyes has not been investigated adequately in aquatic fauna. Bazin et al. [63] have recorded that synthetic textile dyes may have an estrogenic and anti-estrogenic effect as a result of interference with the endocrine system. Therefore, further environmental studies are required to be conducted to understand and confrontation of the ecological problems of RTDs compounds in the aquatic environment.

In conclusion, our findings in tilapia fish demonstrate that induction of disruption in sex steroids synthesis by the three tested reactive textile dyes through fluctuation of some HPG-axis genes, micro-nucleated gills and hepatic cells and elevated levels of some detoxification enzymes in liver cells. However, the different concentrations of *H. atra* have up-regulated mRNA of three HPG axis genes, reduced micro-nucleated cells and ameliorated antioxidant conditions in individuals exposed to RTDs. It is therefore recommended to add *H. atra* as nutritional supplements with fish diet, to mitigate the unfavorable effects of organic textile dyes containing wastewater.

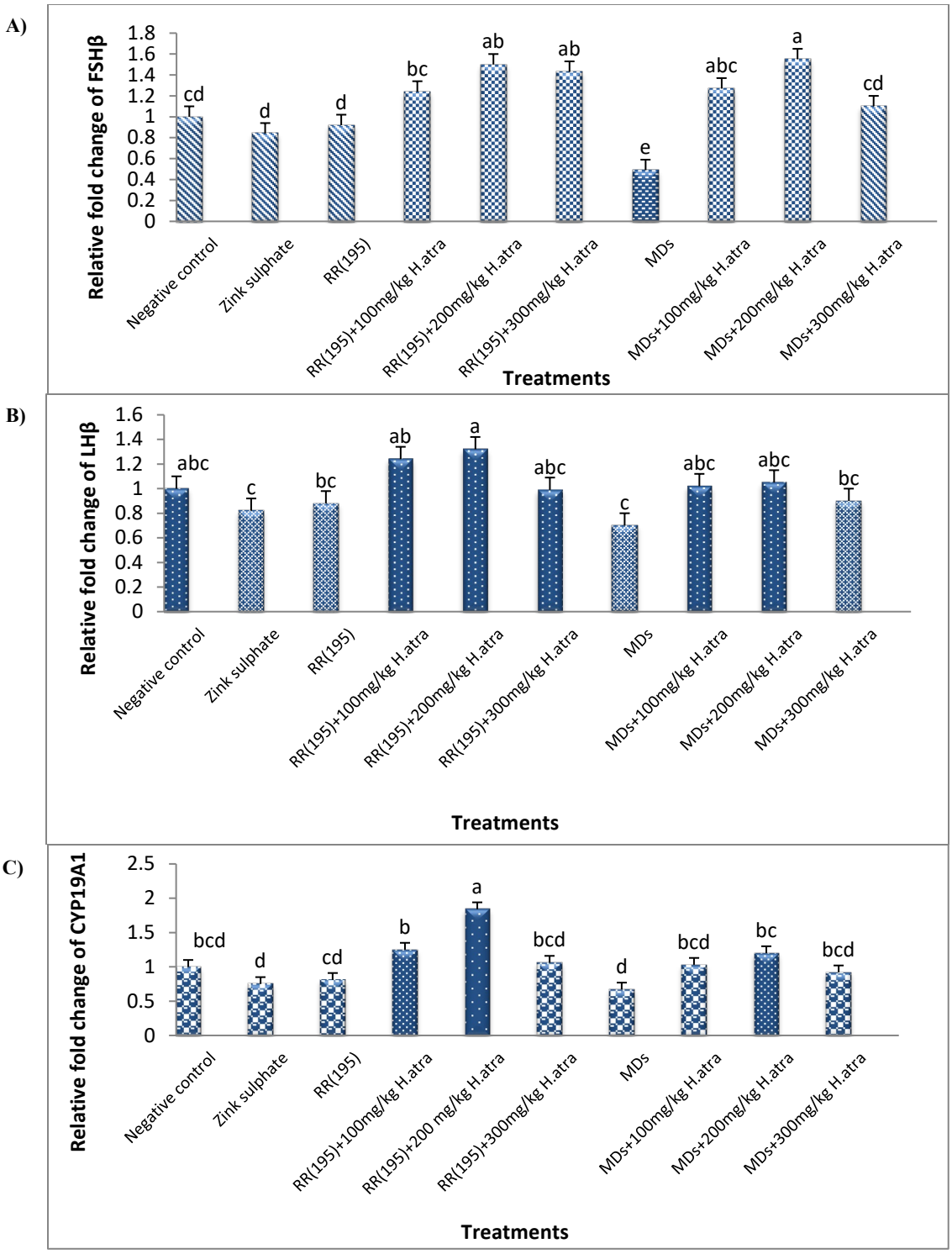


Figure 4. A) FSHβ, **B)** LHβ and **C)** CYP19A1 expressions after 30 d exposure to different treatments of textile dyes and *H. atra*. Results as shown as mean ±SD.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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