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Response Against Ethinyl Estradiol and Levonorgestrel of *Daphnia Magna*: Immobilization and Oxidative Stress Assessment

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KEYWORDS

Steroids; Ethinyl estradiol; Levonorgestrel; *Daphnia magna*; Immobilization; Oxidative stress

ABSTRACT:

Ethinyl estradiol (EE2) and levonorgestrel (LNG) are synthetic steroids commonly found in contraceptive pills that induce behavioral, physiological and biochemical changes in both human and animal models. The toxic effects of EE2 and LNG are well known, but no reports about their combination exist. Hence, this investigation aims to assess individual and combined effects at low relevant environmental concentrations in a 1:5 ratio similar to that found in most combined birth control pills on a freshwater microcrustacean, *Daphnia magna.* The acute toxicity of EE2, LNG and their mixture was examined after 24 h and 48 h on daphnia juveniles through the immobilization test, which subsequently enabled the median inhibitory concentration (IC_{50}) to be determined. Additionally, the effects of oxidative stress were examined in *D. magna* adults over a 48-hour exposure. *D. magna* immobility was concentration-dependent, decreasing for EE2 and increasing for LNG, while the mixture inhibited motility to a greater extent. In daphnia adults, oxidative stress responses showed a significant decrease in GSH contents and CAT activity, along with an increase in the activity of GST and MDA levels in treated groups compared to the control ($p < 0.05$). These findings suggest that EE2 and LNG alter the antioxidant system, notably in combination, due to generating reactive oxygen species (ROS) in *D. magna*.

1. Introduction

The amount of emerging chemicals currently present in the environment, individually or in mixtures, is still being determined (Zhanyun Wang et al., 2020). Among these compounds, steroid-synthetic hormones from human and veterinary pharmaceuticals are widely used in aquatic ecosystems worldwide (Weizel et al., 2018); they are all biologically active and, thus, hypothetically, can impact exposed non-target species (Runnalls et al., 2010). Ethinyl estradiol (EE2) and levonorgestrel (LNG) are the most commonly used in oral contraceptives. They are synthetic versions of natural hormones found in the female body (Gunnarsson et al., 2019) due to their ability to mimic natural and endogenous hormones at minute concentrations (Dzieweczynski and Hebert, 2013) by interfering with the hormonal homeostatic processes necessary for the growth and development of different tissues (Sharma and Chadha, 2021).

EE2 and LNG are commonly found in aquatic surfaces down to the ng/L range and are excreted particularly by pregnant women (Laurenson et al., 2014). Both compounds are environmentally persistent and can bioaccumulate and biomagnify in aquatic organisms due to their high hydrophobic character, lipophilicity, and resistance to degradation (Olivera and Luengo, 2019). The concentration of EE2 in fresh water and rivers can range from 1 to 22 ng/L with a predicted half-life between 2-81 days under aerobic conditions, which is not

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degraded but decreases from 1–0.62 µg/g very slowly (Adeel et al., 2017). However, LNG has been detected in municipal sewage treatment plants, surface water, and groundwater at concentrations ranging from 11 to 79 ng/L (Steinbach et al., 2023), showing resistance to degradation even under natural energy radiation, which reduces only 80% of this substance (Narváez et al., 2019).

These compounds act as natural hormones; thus, they do not have a dose-response effect on invertebrates (Vandenberg et al., 2012). In this regard, (Luna et al., 2015) showed that exposure to EE2 decreased the number of daphnids produced per female in *D. magna* at minute concentrations (0.1 and 1.0 µg/L). Similarly, LNG can seriously affect embryonic development and reproductive functions at low concentrations (Hua et al., 2015). However, high concentrations of steroids can alter behavior, feminize male fish thoroughly, and decrease reproduction (Aris et al., 2014).

Much research has focused on a single pollutant effect; however, steroid mixtures and mechanisms are critical ecotoxicological concerns (Aris et al., 2014). Most pollutants are present independently at concentrations too low to harm the ecosystem, whereas some do so when combined with other substances (Carvalho et al., 2014). Consequently, their prevalence in food and drinking water poses a serious risk to human and animal health (Adeel et al., 2017; Sharma and Chadha, 2021).

Daphnia magna, a zooplanktonic water flea, is a crucial bioindicator organism in ecotoxicology (Svigruha et al., 2021) due to its high sensitivity to various factors, including interactions between stressors and pollutants, in particular, steroid hormones (Luna et al., 2015), which may have an impact on their survival patterns in aquatic environments and require further research (Yisa et al., 2023).

It has been demonstrated that exposure to sex steroids causes modifications in enzymatic and nonenzymatic antioxidants in various aquatic non-target species (Belhaj et al., 2018; Rodrigues et al., 2021), affecting growth, sexual maturity, and reproductive behaviors (Aris et al., 2014). For this reason, oxidative stress biomarkers are used as indicators to assess the state of health of aquatic organisms (Maharajan et al., 2018; Rodrigues et al., 2021; Yisa et al., 2023). Therefore, we used *D. magna* in the current study to assess the individual and combined ecotoxicological effects of EE2 and LNG at environmentally relevant concentrations with a 1:5 ratio, focusing mainly on immobilization and antioxidant activity.

2. Materials and methods

2.1. Reagents and test solutions

The drug hormones used are derived from three mini-dosed oral contraceptives: estrogen-progestin pill: 30 µg/150 µg EE2/LNG; and a progestin-only pill: 30 µg LNG drugs (Pfizer laboratory); and oestrogen alone: 50 µg EE2 (Effik laboratory). Stock solutions of each hormone were prepared by dissolving them in an ISO medium, noting that EE2 ($C_{20}H_{24}O_2$) was dissolved according to the method of (Clubbs and Brooks, 2007) in acetone but modified to 0.1% and LNG ($C_{21}H_{28}O_2$) in 0.1% ethanol (Contardo-Jara et al., 2011). The tested concentrations were obtained by diluting the stock solutions immediately in distilled water before the experiments and were stored at 4 C° in the dark.

2.2. Experimental animals and breeding conditions

Crustaceans were collected from lakes where their waters harbor many types of microcrustaceans; located in the Gulf of Annaba, which is an enormous gulf of the Mediterranean Sea located in Annaba, in the province of El Tarf, 400 km east of Algiers, and were transported rapidly with their natural medium to the laboratory in 7 L aerated tanks.

Once the species were confirmed, *D. magna* was maintained from a culture performed in our laboratory over many generations and was incubated in a growth chamber at a constant temperature of 20 ± 2 °C and light cycle (16 h light, 8 h dark) in an aquarium containing approximately 15 L of dechlorinated tap water medium (total hardness 169.8 mg/L ± 18 of $CaCO_3$ using a photometer; pH = 7.8 ± 0.2), and an air pump provided oxygenation. The medium was renewed twice a week, and normal *Chlorella vulgaris* (5 mg/L) and *Saccharomyces cerevisiae* (2.5 mg/L) were given to *D. magna* in the initial week of growth. Juveniles were extracted daily following the maturation process, while adults were provided with standard Chlorella daily. *D. magna* juveniles less than 24 hours old, sorted from at

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least the third clutch, were used in the acute ecotoxicity tests, and adults were used for the oxidative stress biomarkers, noting that all clones were extracted from the same culture.

2.3. Experimental design

After conducting a series of preliminary tests on *D. magna* with different concentrations of EE2 and LNG ranging from 0.1 to 500 µg/L, we reserved four concentrations for each hormone and three treatment groups were performed for the immobilization test and enzymatic biomarkers:

- **Control group:** a blank control was utilized without the addition of any reagents or solvents (culture media ISO);
- **EE2 Group:** 0.1; 0.5; 5; and 25 µg/L;
- **LNG group:** 0.5; 2.5; 25; and 125 µg/L;
- **EE2/LNG group:** Co1 = 0.1/0.5; Co2 = 0.5/2.5; Co3 = 5/25; and Co4 = 25/125 µg/L.

All the groups involved in the immobilization test were exposed for 24 h and 48 h and only for 48 h for the oxidative stress biomarkers. The chosen concentrations of EE2 referred to one of several reports (Rodrigues et al., 2021; Zheng et al., 2020), where the difference between the concentrations of each hormone is based on doses contained in the majority of combined contraceptive pills with a 1:5 ratio (0.03 µg EE2; 0.15 µg LNG).

2.4 Acute toxicity test

2.4.1 Immobilization test and IC_{50} levels

The acute toxicity test was performed according to the standard protocol for *D. magna* acute test 202 (OECD, 2004), and the IC_{50} of daphnids immobilized at 24 hours and 48 hours was determined. Three biological samples of 20 unfed neonates aged less than 24 h were introduced into 30 mL glass tubes containing 10 mL of ISO medium at varying test concentrations in a darkened area. Immobility was the designated endpoint for the assessment. In particular, daphnids that exhibited an inability to swim were categorized as immobile.

Additionally, those who displayed movement only in their antennae but failed to swim within 15 seconds following gentle agitation were also classified as immobile. The fixed proportion in the control must be less than or equal to 10% for the test to remain valid. Immobilized juveniles were counted visually and confirmed under a light microscope (Leica ATC 2000 Microscope, Wetzlar, Germany) to determine the 50% inhibitory concentration (IC_{50}). The average $IC_{50,}$ which induces the immobilization of 50% of daphnids after 24 h and 48 h of exposure to the tested molecules (IC_{50}/24 h; IC_{50}/48 h), was calculated from the previously tested concentration range by the Probit method (Finney, 1952).

2.5. Homogenate preparation

The enzymatic activities were carried out on fifteen adult individuals (n = 4 per group), collected and rinsed with distilled water thrice, exposed for 48 hours to increasing concentrations of EE2, LNG and EE2/LNG in quadruplicate, and then placed in Eppendorf microtubes and frozen (- 20 °C) for storage. For enzyme analyses, specimens were sonicated in 1.5 ml Eppendorf microtubes with 1 ml phosphate buffer (100 mM), 1 ml EDTA (ethylene diamine tetra-acetic) (0.02 M), 1 ml phosphate buffer (0.1 M, pH 6), and 1 ml Tris-HCl (trisaminomethane-hydrochloride) (50 mM, pH 7.5), for CAT, GSH, GST and MDA, respectively, using an ultrasonic homogenizer, where aliquots were separated for each biomarker. Afterwards, supernatants were centrifuged at 5000 rpm for 10 min at 4 °C.

2.5.1 Oxidative stress and antioxidant biomarkers

CAT activity was determined using the (Claiborne, 1985) method to evaluate absorbance at 240 nm and was monitored every 15 s for 1 min to measure enzyme activity fluctuations. The results were represented as (mol/min/mg of proteins), with a hydrogen peroxide extinction coefficient of 0.04 mM/cm. GSH was measured based on the method of (Weckbecker and Cory, 1988), where a fraction of the supernatant from the homogenization of living organisms in 1 mL of EDTA (0.02 M) was added to 0.2 mL of (ASS) sulfosalicylic acid in an Eppendorf tube. The reaction was started by adding 0.025 mL of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to 0.5 mL of supernatant after agitation and centrifugation at 1000 rpm for 5 min. GSH levels were measured instantly at 412 nm in control and treated daphnid samples, and the results are represented as (mol/mg of protein). As outlined by (Habig et al. 1974), GST activity was

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established by tracking the pairing of GSH with CDNB. After homogenizing the samples in 1 mL of phosphate buffer (0.1 M, pH = 6), the homogenate was centrifuged for 30 minutes at 14000 rpm. The reaction was initiated by mixing 1.2 mL of CDNB (1 mM)/GSH (1 mM) with 0.2 mL of supernatant in a clean quartz cuvette. The production of S-2,4-dinitrophenyl glutathione conjugate was measured by measuring the increase in absorbance at 340 nm after each 1 min interval during a 5 min period. Lipid peroxidation results from ROS-induced oxidation of lipid membranes and was measured by the (Buege and Aust, 1978) method used to quantify MDA levels expressed in (mmol/mg protein).

2.6. Statistical analysis

The acute toxicity was characterized by determining the 50% inhibitory concentration (IC_{50}), and the immobility rates were adjusted using the (Abott 1925) formula' and then converted into Probits, followed by a two-way analysis of variance (ANOVA) used for multiple comparisons between different groups, where $p < 0.05$ was considered significant. Enzymatic assays were conducted in quadruplicate, and the results are shown as the means ± standard errors of the mean (SEM) to assess the correlation between EE2 and/or LNG concentrations and their respective effects. The intergroup variations were evaluated for statistical significance for oxidative stress and antioxidant biomarkers using one-way analysis of variance (ANOVA) and then subjected to Tukey's multiple comparisons test (GraphPad Prism 9). The statistical significance level was established at $p < 0.05$, where values with different letters (a/b/c/d/e) significantly differ. To characterize the response of oxidative stress biomarkers and immobilization using a multivariate approach, a principal component analysis (PCA) coupled with a correlation circle and a Ward dendrogram was performed using XLStat.

3. Results

3.1. Immobilization test and IC_{50} levels

Under current exposure conditions, EE2, LNG, and their combination significantly affected the immobility of daphnids ($p < 0.05$) (Fig. 1a, b, c). The immobility of daphnids had a concentration-dependent pattern, decreasing for EE2 (≤ 5 µg/L) and increasing for LNG (≤ 125 µg/L), but the combination of both hormones stimulated much more of the effect on immobilization; this has been reported only with low concentrations, for example: at 0.1 μg^{-L} of EE2 inhibition was 33.33%, at 0.5 µg/L of ING 20%, but was (40%) when these two concentrations are combined (Co1 = 0.1/0.5 µg/L). However, the maximum hormone exposure concentration caused the most significant immobilization, 100% induced by 25 µg/L EE2 and combined hormones (Co4 = 25/125 µg/L) after 48h of exposure and 93.33% for 25 µg/L LNG. The control groups did not report any inhibition. On the other hand, the effect of EE2 and LNG on immobilization was weaker after only 24 h and was reflected in the IC_{50} values, which were reduced markedly, as described in Table 1 below.

3.2. Oxidative stress and antioxidant biomarkers

3.2.1. effect on catalase (CAT) activity

As shown in (Table 2), the decrease in catalase activity was significant ($p < 0.05$) in all EE2-treated groups. Additionally, in groups exposed to 2.5 and 25 µg/L of LNG, CAT activity was reduced compared to the control and only showed a significant ($p < 0.05$) decrease at 25 µg/L. However, at 125 µg/L, the catalase activity remained almost the same as that of the control. Following treatment with combined hormones, CAT activities were significantly decreased in all groups compared to the control ($p < 0.05$), especially with Co3 = 5/25 µg/L, reaching its lowest activity level.

3.2.2. Effects on reduced glutathione (GSH) content

Compared with the control, the decrease in GSH levels was significant ($p < 0.05$), inversely proportional to concentrations of EE2, and was lower in all groups that received treatment (Table 2). However, the reduction in GSH levels following treatment with LNG was concentration-dependent and notably significant at 25 and 125 µg/L. Our research indicated that the GSH levels declined significantly ($p < 0.05$) in all treated groups treated with combined hormones.

3.2.3. Effects on glutathione-S-transferase (GST) activity

Table 2 shows that following EE2 treatment, all groups revealed significant GST activity rate enhancements ($p < 0.05$) except for 0.5 µg/L. While the detected changes were not concentration dependent, GST

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activity was significantly higher in the 0.1 and 25 µg/L exposed groups compared to the control and the other concentrations. After 48 h of treatment with LNG, the enhancement in GST activity was observed in all groups in a concentration-dependent manner but was significant ($p < 0.05$), starting from 2.5 µg/L compared to the control, and notably elevated at the highest concentration, 125 µg/L. Exposure to the mixture caused a significant increase ($p < 0.05$) in GST activity with all treated groups and reached its highest stimulation level at the lowest combined concentrations of Co1 = 0.1/0.5 and Co2 = 0.5/2.5 µg/L.

3.2.4. Effects on lipid peroxidation levels

After exposure to EE2, all groups showed a significant rise in MDA levels ($p < 0.05$), as shown in Table 2, except for the 5 µg/L group. Nevertheless, there was no concentration-dependence in the observed changes, while compared to the control and the other higher concentrations, the MDA content in the groups exposed to the lowest concentrations of EE2 (0.1 and 0.5 µg/L) was significantly the greatest. Following a 48 h LNG treatment, MDA levels were elevated in all groups in a concentration-dependent manner; however, the rise was significant ($p < 0.05$) starting at 2.5 µg/L compared to the control. MDA content increased significantly ($p < 0.05$) in all treated groups after exposure to the mixed steroids, reaching its peaking stimulation level at the minimal combined concentrations of Co1 = 0.1/0.5 µg/L, and the highest one, Co4 = 25/125 µg/L.

3.3. Principal component analysis (PCA)

We conducted a principal component analysis (PCA) to analyze correlations between variables presented by 4 biomarkers of enzymatic and nonenzymatic oxidative stress and percent immobilization measured in *D. magna* specimens belonging to 5 experimental batches (Control and four combined concentrations of Co = EE2/LNG). This analysis revealed two main gradients among the parameters measured, with a correlation of 83.96%. The first gradient was associated with a strong influence of treatments on GST, MDA and immobilization percentage. The first axis described a correlation of 70.18% of the total variance of these three parameters. The second gradient was linked to the other two biomarkers, CAT and GSH, with a correlation of 13.78% (Fig. 2A).

The PCA biplot reveals two clear pairs of components (Fig. 2B), clearly showing the impact of the mixture exposure at the lowest and highest concentrations (Co1 = 0.1/0.5 µg/L and Co4 25/125 µg/L) on the variables measured (MDA, GST and percentage of immobilization), while the second was associated with the other parameters (CAT and GSH)

Table 1. Values of the half maximal inhibitory concentration (IC_{50}) calculated for 24 h and 48 h EE2 and LNG exposure (n = 3) are grouped in the following table:

Treatment	Time (h)	Regression equation	Correlation coefficient R^2	IC_{50} (μg^{-L})	95% Confidence interval
EE2	24h	$y = 1.288 + 2.374x$	0.977	20.61	-0.925 - 3.500
	48h	$y = -4.062 + 5.987x$	0.977	1.66	-10.357 - 2.232
LNG	24h	$y = 1.4 + 2.139x$	0.993	117.34	0.293 - 2.507
	48h	$y = -0.046 + 3.111x$	0.950	2.23	-5.36 - 5.268

Note: Probit analysis using Excel/Prism determined the accumulated inhibition (%) of *D. magna* neonates treated with different EE2 and LNG concentrations for 24 and 48 h and the IC_{50} with 95% confidence limits. The logarithm of concentration is represented by the letter X in the regression equation, while the inhibition of daphnids is represented by the letter Y.

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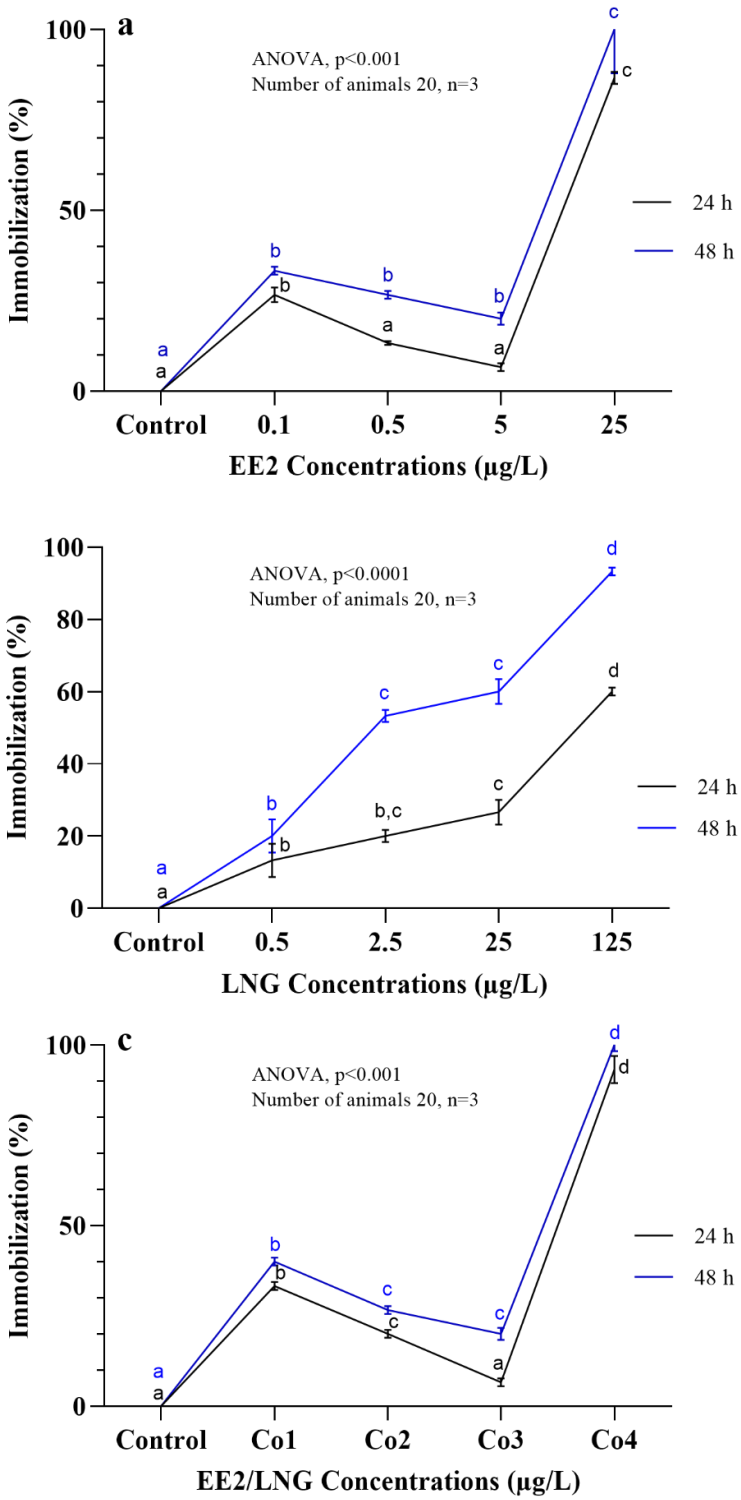

Fig. 1. Immobilization of neonatal *D. magna* exposed to EE2 (**a**), LNG (**b**), and combined EE2/LNG (**c**) after 24 and 48 h of exposure. Concentrations are given as the mean ± SEM (n = 3 per group). Values with different letters (a/b/c/d/e) are significantly different.

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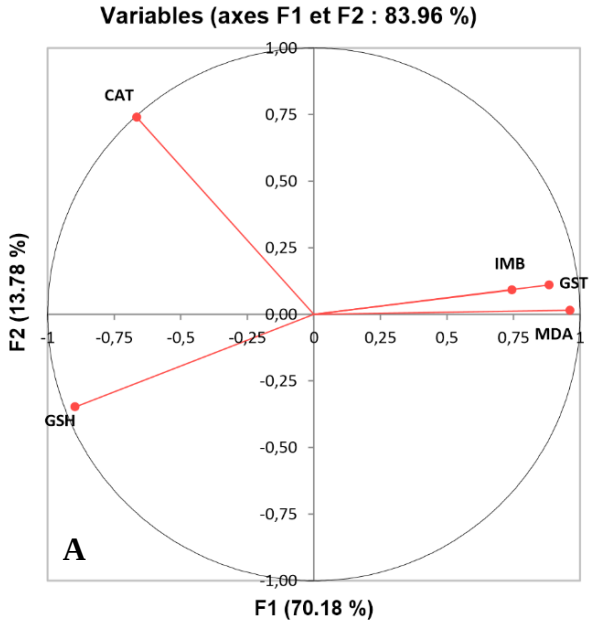
Table 2. Changes in CAT activity, GSH level, GST activity, and MDA amount in adult *D. magna* following 48 h of exposure to increasing concentrations of individual and combined EE2/LNG (Co) hormones. Data are expressed as the means ± SEM (n = 4 per group).

Treatment (μg^{-L})		Parameters			
Groups	**Concentrations**	**CAT**	**GSH**	**GST**	**MDA**
EE2	**Control**	20.70 ± 2.51^{a}	0.96 ± 0.14^{a}	$127.63 \pm 4.25^{a,c}$	5.96 ± 0.14^{a}
	0.1	3.79 ± 1.08^{b}	0.10 ± 0.03^{b}	157.60 ± 8.27^{b}	11.77 ± 0.61^{b}
	0.5	10.51 ± 0.50^{c}	$0.27 \pm 0.17^{b,c}$	$139.20 \pm 2.50^{a,b,c}$	9.60 ± 0.55^{c}
	5	1.95 ± 1.06^{b}	$0.39 \pm 0.15^{b,c}$	135.18 ± 8.00^{c}	7.06 ± 0.43^{a}
	25	4.81 ± 0.64^{b}	0.56 ± 0.11^{c}	156.69 ± 11.74^{b}	8.89 ± 0.53^{c}
LNG	**Control**	$20.70 \pm 2.51^{a,c}$	0.96 ± 0.14^{a}	127.63 ± 4.25^{a}	5.96 ± 0.14^{a}
	0.5	$17.64 \pm 3.10^{a,c}$	$0.74 \pm 0.07^{a,b}$	$149.10 \pm 7.05^{a,b}$	$7.14 \pm 0.47^{a,b}$
	2.5	$12.94 \pm 2.40^{a,b}$	$0.70 \pm 0.13^{a,b}$	159.02 ± 13.78^{b}	$8.30 \pm 0.60^{b,c}$
	25	6.20 ± 3.80^{b}	$0.52 \pm 0.16^{b,c}$	181.87 ± 9.43^{c}	8.85 ± 0.70^{c}
	125	21.93 ± 3.19^{c}	0.22 ± 0.14^{c}	309.89 ± 8.85^{d}	10.65 ± 0.66^{d}
Combined	**Control**	20.70 ± 2.51^{a}	0.96 ± 0.14^{a}	127.63 ± 4.25^{a}	5.96 ± 0.14^{a}
EE2/LNG	**Co1**	12.52 ± 2.24^{b}	0.09 ± 0.04^{b}	370.80 ± 4.15^{b}	11.91 ± 0.25^{b}
	Co2	$7.77 \pm 1.45^{b,c}$	$0.49 \pm 0.03^{c,d}$	335.67 ± 5.59^{c}	9.25 ± 0.29^{c}
	Co3	0.75 ± 0.22^{d}	0.60 ± 0.11^{c}	274.46 ± 5.82^{d}	9.90 ± 0.71^{c}
	Co4	$4.84 \pm 3.79^{d,c}$	$0.30 \pm 0.13^{b,d}$	301.26 ± 5.58^{e}	12.97 ± 0.66^{b}

Note:
EE2 = Ethinyl estradiol; LNG = Levonorgestrel; Co = Combined EE2/LNG; Co1 = 0.1/0.5; Co2 = 0.5/2.5; Co3 = 5/25 and Co4 = 25/125 µg/L. Units: Catalase (µmole H2O2/min/mg protein); GSH (µmol/mg protein); GST (U/mg protein); MDA (mmol/mg protein). Means that do not share the same lowercase letter (a/b/c/d/e) are significantly different ($p < 0.05$), according to one-way ANOVA followed by the multicomparison Tukey test.



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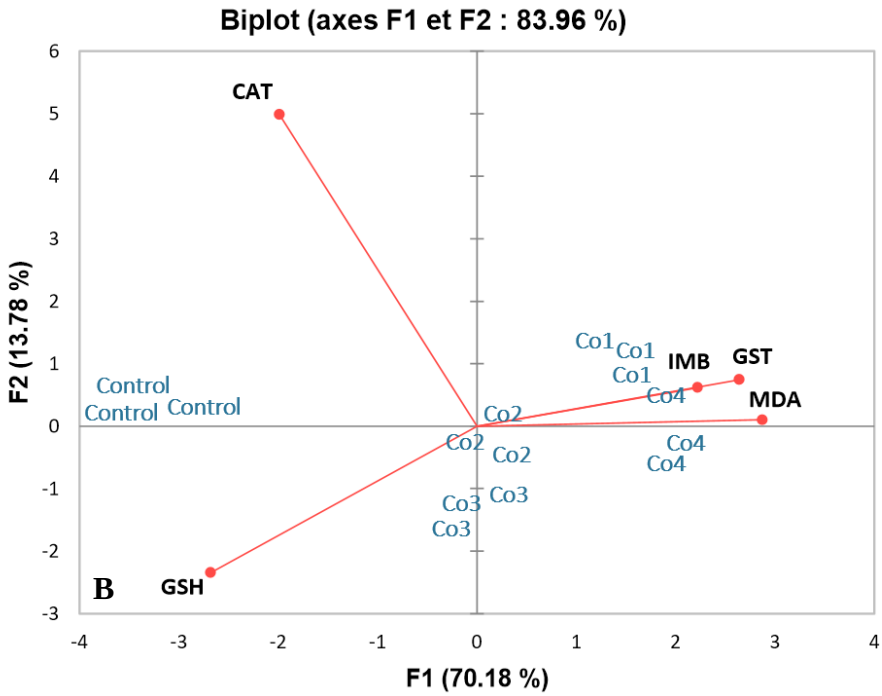

Fig. 2. A Principal Component Analysis of connection viz a viz toxicity parameters and different concentrations of EE2, LNG and EE2/LNG for *D. magna.* The PCA test was carried out on 05 variables of oxidative stress and immobilization rate. **Fig. 2.A.** Factorial plane F1: (70.18%), F2 (13.78%); **Fig. 2.B.** Biplot results, projection of stress oxidative variable and immobilization marker in specimen control and mixture treated group on the first two principal axes. Abbreviations: Co1 = 0.1/0.5; Co2 = 0.5/2.5; Co3 = 5/25; Co4 = 25/125 µg/L; IMB = immobilization.

4. Discussion

There is an ongoing discussion about whether invertebrate species are negatively impacted by residues of natural and manufactured sex steroids (Svigruha et al., 2021). Available scientific data on toxicity and adverse effects show that *D. magna* is sensitive to synthetic steroids (Zheng et al., 2020). The present study was carried out to investigate the potential toxicity of EE2, LNG and their mixture on motility and the antioxidant defense system in *D. magna*.

Aquatic species in their early life stages are more vulnerable to estrogens than adults when exposed acutely; thus, embryonic and young stages have been employed to assess the aquatic ecosystem's biological values (Aris et al., 2014; Liu et al., 2012). In this regard, the acute toxicity of the different concentrations (0.1, 0.5, 5 and 25 µg/L) of EE2 was evaluated on the immobilization of juvenile *D. magna* to estimate the inhibition concentration (IC_{50}) and their 95% confidence intervals at 24 and 48 h of exposure. Our results showed that the immobility percentage of daphnids had a concentration-dependent pattern, decreasing for minute concentrations of EE2 (≤ 5 µg/L); the observed results are similar to those of (Razekenari et al., 2023), who found that the survival rates of juvenile crustacea *Neocaridina davidi* were stimulated significantly by concentrations of 0.02 and 0.2 µg/L; it is plausible that EE2 was metabolized and assimilated as a nutrient rather than a harmful xenobiotic, enabling the cells to keep their specific intracellular homeostatic amounts of xenobiotics in balance (Moumeni et al., 2016). In contrast, juveniles exposed to higher concentrations above 25 µg/L for 24 h were immobile, and all died after 48 h, which can be explained by the inherent difficulty of catching the food, leading to increased mortality (Goto and Hiromi, 2003). In addition, *D. magna* was more sensitive, with an IC_{50} of 20.61 µg/L after 24 h and 1.66 µg/L after 48 h of exposure, which explains why mobility was impacted more over time, compared to (Jaser et al., 2003), who reported that the EC_{50} (24 h) for *Ceriodaphnia reticulata* is approximately 1814 µg/L of EE2.

The present study revealed that the immobility of daphnids had an increasing concentration-dependent

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pattern in exposure to (0.5, 2.5, 25, and 125 µg/L) LNG, and we established that this progestin was less toxic than EE2 since the IC_{50} was increasingly higher, 117.34 and 2.23 µg/L after 24 and 48 h, respectively. Similar results have been obtained in *D. magna* exposed to norethindrone (a contraceptive drug containing a progestin hormone) (Goto and Hiromi, 2003); the authors have explained that immobilization was affected by the reduction in food capture.

However, combining the two steroids was more noxious than their isolated effect, where the lowest concentrations had the most toxicity, and we contend that EE2 has a higher toxicity potential than LNG since the immobilization in exposure to the mixture was in the same manner as EE2; this result was similar to those studied by (García-García et al., 2014), who showed that population growth of *Anuraeopsis fissa* was affected at the lowest concentrations of hormones 31.25/6.25 then 62.5/12.5 µg/L of LNG/EE2 respectively after six days. Recently, some studies have shown that combinations of steroid hormones can suppress the reproduction of various species of aquatic invertebrates (molluscs and crustaceans in particular), even when the concentrations of the individual hormones are too low to have an individual effect (Ojoghoro et al., 2021). Additionally, (Goto and Hiromi, 2003) revealed that while norethindrone (contraceptive progestin) alone did not affect *D. magna* reproduction, EE2 at 0.1 µg/L and a combination of EE2 and norethindrone at 0.006 or 0.094 µg/L drastically reduced the number of offspring.

Antioxidants are the primary defense system that reduces the toxicity related to free radicals (Maharajan et al., 2018). The overproduction of ROS can lead to redox alterations by the disturbance of the activity of enzymes involved in the oxidative stress status (Duan et al., 2022). In the present study, the exposure of *D. magna* adults to individual and combined steroids EE2 and LNG for 48 h caused a disturbance of the redox status, as revealed by the reduction in GSH levels and CAT activity along with increased MDA content and GST activity, as determined by the PCA test.

Lipid peroxidation initiates a chain reaction when free radicals assault lipids, and MDA levels may rise due to lipid degradation (Maharajan et al., 2018). Following 48 h, EE2 and LNG separately or combined exposure induced an increase in MDA levels compared to the control groups in a concentration-dependent manner, decreasing for EE2 and increasing for LNG; the observed increase was associated with a drop in CAT activity and GSH levels. Accordingly, the enhanced MDA levels result from the breakdown of primary hydroperoxides and lipid polymers, which occurs when there is an excessive amount of intracellular H_2O_2 beyond the typical cellular level, which CAT and GST activities were unable to neutralize (Yisa et al., 2023). In this context, (Rodrigues et al., 2021) showed that exposing *D. magna* to EE2 at increasing concentrations ranging from 0.1 to 100 µg/L significantly increased TBARS (thiobarbituric acid reactive substances), leading to greater cytotoxicity. Similarly**,** (Meksem et al., 2007; Shen et al., 2019) confirmed that MDA accumulation in tissues might be related to suppressing antioxidant enzyme function. This conclusion is corroborated by (Mo et al., 2019), who suggested that EE2 causes ROS formation and MDA buildup in *Pelteobagrus fulvidraco* to exhibit harmful effects. Even though it cannot be compared, and due to the lack of information on the effects of the mixture of EE2 and LNG on the antioxidant systems of invertebrates, a study on rodents determined that the mixture considerably increased plasma and kidney MDA levels as well as GSH levels (Olaniyi et al., 2021).

GSH is the most common thiol outside of proteins and is crucial for cellular survival. In our experiment, a reduction in GSH levels was recorded, particularly at 0.1 µg/L EE2, where significant toxicity was exhibited, as evidenced by the excessive production of MDA. The GSH depletion observed may be due to its involvement in neutralizing steroid-induced free radicals and/or its high utilization for conjugation (Faheem and Lone 2018; Shen et al., 2019). However, the significant reduction in the amount of GSH observed in the mixture, particularly with the lowest concentration of Co1 = 0.1/0.5 µg/L, indicates a potentiated synergy between the two molecules, suggesting that their combined effects at low concentrations are increasingly more remarkable than that affected by each of them (Zhu, 2008). Accordingly, a recent study concluded that oxidative stress caused by the synthetic progestin Norgestrel (an active isomer of LNG), at increasing concentrations (0.01 and 1 µg/L) almost in the same range as ours, resulted in histological lesions of the digestive gland of clam *Mactra veneriformis* (Zhao et al., 2023).

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The results of our study demonstrated that catalase activity dropped aggressively in adult *D. magna* after exposure to EE2, LNG and their mixture. In support of our findings, a recent study reported that exposure to 1 µg/L EE2 reduces CAT activity in yellow catfish serum (Mo et al., 2019). For LNG, a hormesis reaction of catalase was observed in zebrafish that resulted from exposure to lower concentrations than higher concentrations (Cardoso et al., 2019). According to (Gasmi et al., 2016; Rodrigues et al., 2021), this suggests the involvement of CAT in the antioxidant defense process in *D. magna*. Consequently, this reduction can be attributed to the inhibition of enzymatic activity by the overproduction of free radicals such as H_2O_2 (Belhaj et al., 2018).

One of the major controlling functions in the cellular redox process and safeguarding tissues against oxidative stress is played by the GST enzyme (Shen et al., 2019), recognized in the elimination of numerous anthropogenic toxicants (residues of active pharmaceutical ingredients) (Svigruha et al., 2021). According to a recent study, increasing GST activities in *D. magna* helped the organism better detoxify psychotropic drugs (Beghoul et al., 2017; Duan et al., 2022). Our findings showed that the GST activity was significantly increased in *D. magna* exposed to EE2, LNG and their mixture compared to the control groups; the observed increase was not concentration dependent, particularly during exposure to low concentrations of EE2 individually or in mixture (Co1 = 0.1/0.5 and Co2 = 0.5/2.5 µg/L), which can be explained by an increase in oxidative stress and lipid peroxidation (Svigruha et al., 2021).

In this sense, (Gasmi et al., 2019; Yisa et al., 2023) suggested that the activity of the GST enzyme was involved in the detoxification of EE2 and LNG, individually or in combination, in the species under study. Similar findings have been recorded in *D. magna* exposed to 0.1, 1, and 10 µg/L EE2 (Rodrigues et al., 2021). Therefore, short-term exposure of *D. magna* to a mixture of four progestogens, including LNG, increased GST gene expression and, consequently, a significant increase in GST activity, indicating that progestins may interact with circuits involved in oxidative stress responses, similar to estrogens (Svigruha et al., 2021). Given that *D. magna* represents a substantial proportion of the feeding regime of secondary consumers such as tadpoles and fish, the analyzed model's results at environmentally relevant concentrations of both hormones indicate their potential threat to the aquatic food chain. Accordingly, pollution profile databases for both hormones must be extended to improve toxicity endpoint predictions and revisions, especially for aquatic environmental risk assessment.

5. Conclusion

The findings of this study demonstrated that exposure to the two steroids EE2 and LNG, even in small amounts, could alter the antioxidant system, thus affecting the motility of *D. magna*, either separately or in combination. This study is instrumental, as it enabled us to assess environmentally relevant concentrations in a ratio inspired by the actual quantities contained in contraceptive pills. Determining the impact of these pharmaceutical ingredients is also an essential strategy for successfully evaluating aquatic environments, considering the hormesis effect that EE2 can have and the synergy that may exist with LNG, subsequently creating potentially toxic effects on non-target aquatic organisms.

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