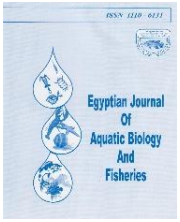
Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(1): 79 – 103 (2024)
www.ejabf.journals.ekb.eg




# Biochemical and Histopathological Impacts of NSAIDs on the Hepatopancreas of the Freshwater Crayfish *Procambarus clarkii*, and Possible Treatment with *Chlorella vulgaris*

**Hanan Ahmed El-Rouby[1], Elham Hassan Ahmed Ali[1], Magdy Tawfik Khalil[2], Asmaa Ali Baioumy Ali[2] ***

[1-] Zoology Department, Faculty of Women for Arts, Science, and Education, Ain Shams University, Egypt.

[2-]Zoology Department, Faculty of Science, Ain Shams University, Egypt.

*** Corresponding author:** Asmaaali86@sci.asu.edu.eg

**ARTICLE INFO**

**Article History:**
Received: Nov.19, 2023
Accepted: Dec. 27, 2023
Online: Jan. 7, 2024

**Keywords**:
*Chlorella vulgaris*,
Hepatopancreas,
Histopathology, NSAIDs,
*Procambarus clarkii*

**ABSTRACT**

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most used pharmaceuticals in the world that introduced into the environment via hospital wastes. These drugs have been detected in many aquatic ecosystems in Egypt, such as the Rosetta Branch of the River Nile, even in drinking water. The objective of this study is to investigate the toxicological effects of ibuprofen (IBP), diclofenac (DCF), and naproxen (NPX) on the freshwater crayfish *Procambarus clarkii* at concentrations that are close to those in its natural environment. The experimental crayfish were exposed for 60 days and divided into 9 groups; including the control, DCF, IBP, and NPX separately or in combination with the three drugs. The used low concentrations were 0.64mg/L, 100µg/L, and 2mg/L, while high ones were 17mg/L, 63mg/L, and 15mg/L for DCF, IBP, and NPX, respectively. The eighth group of crayfish was treated with a mixer of drugs at low doses while the ninth was treated with a previous mixer co-treated with *Chlorella vulgaris* algae. The results showed that lipid peroxidation, nitric oxide contents, glutathione-S-transferase, aspartate transaminase, alanine transaminase, and alkaline phosphatase activities were significantly increased in the hepatopancreas of the crayfish exposed to IBP, DCF, and NPX. On the other hand, total protein, glucose, and calcium contents were significantly decreased after IBP, DCF, and NPX exposure versus the control. Biological treatment with *C. vulgaris* improved marginally these symptoms. Also, obvious histological damage was observed by drug treatments and slight improvement was noticed by adding algae. According to these findings, DCF, IBP, and NPX were to blame for the abnormalities in the biochemical indicators examined and the histological damage at low and high doses. While treatment with *C. vulgaris* adjusted the damage in the mixed low dose although this improvement was not to the level of the control.

## INTRODUCTION

Despite all control programs, pollution has increased in the Nile's main streams and tributaries over the last few decades (Abdel-Satar *et al*., 2017). Water pollution is caused by a variety of factors, such as industrial waste, agricultural drainage waters, oil pollution, heavy metals, and pharmaceutical residues (Parolini, 2020). The presence of pharmaceutical chemicals in the aquatic environment has long been recognized as a problem, and pharmaceutical pollution research has expanded significantly in the last decade (Gómez-Oliván, 2020).

The primary routes for these pharmaceuticals into the environment are hospital wastes, human excretion, the disposal of unused products, and agricultural use (Vulliet and Cren-Olive', 2011; Kumirska, 2020). Human pharmaceuticals are widely used throughout the world depending on the size of the country, and consumption for each pharmaceutical compound that reaches many tons per year (Abdel-Shafy and Mohamed-Mansour, 2013). The most detected prescription classes are nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, beta-adrenergic antagonists (b-blockers), and iodinated X-ray contrast media (Láng and Kohidai, 2012). Many environmental analyses have been conducted in various countries (Togola and Budzinski, 2008; Yu and Chu, 2009; Wang *et al*., 2011).

Fareed *et al*. (2018) found analgesics and antibiotics along the Rosetta Nile River branch in Egypt. Analgesics are the most used self-medication drugs in Egypt and its production and consumption have skyrocketed in recent years, particularly during the Corona period, resulting in their uncontrolled discharge into the environment. Nonsteroidal anti-inflammatory drugs are substances that have anti-inflammatory, analgesic, and antipyretic properties. As a result, they are widely used to alleviate pain in both acute and chronic inflammatory conditions (Gómez-Oliván, 2020).

Diclofenac (DCF), ibuprofen (IBP), and naproxen (NPX) are the most common members of the NSAIDs group of therapeutic agents (Brozinski *et al*., 2013). NSAIDs with similar mechanisms of action have been studied in various species, revealing that exposure to low, environmentally relevant concentrations of DCF, IBU, and NPX can induce significant adverse effects at the molecular, biochemical, and cellular levels, while effects at the individual level (e.g. growth, survival, and reproduction) appear improbable. The consequences may worry for freshwater invertebrates exposed to short- and medium-terms sublethal analgesic doses throughout their lifetimes (Parolini, 2020).

NSAIDs can cause oxidative stress by forming reactive products (Masubuchi *et al*., 2002; Gómez-Oliván *et al*., 2014). Oxidative stress is defined as an imbalance between the body's antioxidant systems and reactive oxygen species (ROS). Normally, metabolic activities inside cells result in the production of ROS, which increases lipid peroxidation (LPO) and total protein content as well as changes in the total activity of diverse antioxidant enzymes (TAA) (Parolini, 2020) and may ultimately damage genetic material. In other studies, DCF exposure at 1000 mg/L significantly affected biomarkers of damage, LPO, and DNA damage in marine mussels (*Mytilus* spp.) at 96h (Schmidt *et al.*, 2011).

The red swamp crayfish (*Procambarus clarkii*) that belongs to *Decapoda*, *Astacidea*, *Cambaridae*, is an invasive species that introduced in the River Nile in the early 1980s. It quickly spread in all aquatic ecosystems including streams, ponds, and marshes; either polluted or clean waters, from the northern Delta to Aswan (Ibrahim and Khalil, 2009). It is one of the most widely cultivated crayfish species for human consumption (Sheir *et al*., 2015). It is a suitable species for use as a bioindicator of invertebrate toxicity in the Nile. *P. clarkii* has been widely used as a model species for the assessment of contaminant effects in the field and is considered a good model organism to study changes induced by pollutants due to the high knowledge of its biological characteristics and its ecologic and economic importance (Yu *et al*., 2018).

To minimize or eliminate risks to human health and the aquatic environment, intensive treatments are currently required to effectively remove analgesics from recycled treated effluent. There are now environmentally friendly methods for treating wastewater from field activities. Because of their ease of cultivation, microalgae such as *Chlorella* sp. are used for this purpose. These microalgae have been widely used to remove heavy metals (Wang *et al*., 2016; Zárate *et al*., 2017), and dyes (Li *et al*., 2011a; Li *et al*., 2011b; Zhou *et al*., 2017; Kazakova *et al.,* 2018). Microalgae, on the other hand, have properties that make them suitable for producing biofuels (Nautiyal *et al*., 2017).

The objective of this study was to investigate the toxicological effects of ibuprofen (IBP), diclofenac (DCF), and naproxen (NPX) on the freshwater crayfish *P. clarkii* and to evaluate the biological treatment of the algae *C. vulgaris* to the physiological and histological effect induced by DCF, IBP, and NPX on hepatopancreas of crayfish *P. clarkii* exposed to sublethal concentrations.

## MATERIALS AND METHODS

### 1. Chemicals (Used Analgesics):

Ibuprofen ($C_{13}H_{18}O_2$) was purchased as a tablet 200mg from Kahira Pharmaceuticals & Chemical Industries Company. Diclofenac ($C_{14}H_{11}Cl_2NO_2$) was purchased as a tablet 100mg from NOVARTIS PHARMA S.A.E. Cairo-Egypt, C.C.-111108. Naproxen ($C_{14}H_{14}O_3$) was purchased as "Momendol" tablets 220mg from Angelini Francesco company (ACRAFSPA), Italy.

### 2. Egyptian Dried Algae:

Dried *Chlorella vulgaris* microalgae were used as a treatment for the mixed low-dose effect. It was obtained from the Botany Department, Faculty of Science, Ain shams university. Purified strains of the green alga *C. vulgaris* Beyerinck, was obtained from Algal Biotechnology Unit, Fertilization Technology Department, National Research Centre, Cairo, Egypt. To obtain the proper inoculum, the isolated *Chlorella* was grown under optimal conditions of BG-II nutrient solution (Stainer *et al.,* 1971). Continuous lighting was provided by day light lamps (5x40w) reflexed from one side to provide approximately 120.e of light intensity. Aeration was accomplished by passing free oil compressed air from the upper hold through a 3mm polyethylene tube and ending with a compact sand distributor. Throughout the incubation period, the room temperature (272$^{o}$C) was recorded. Incubation took place in fully transparent polyethylene bags (75cm length x 5cm diameter x 100 thickness) containing 2.0L of algal broth (El-Sayed and El-Fouly, 2005). When growth reached its peak, the biomass was collected using a cooling centrifuge (RUNNE HEIDBERG model RSV-20) and washed twice. The algal biomass is then oven dried at 60$^{o}$C overnight to produce dried algal biomass.

### 3. Water Samples Collection and Analysis:

Water samples were collected in sterile plastic bottles from 7-sites at the same time in the summer of April 2020 along the Rosetta branch; those drains reserve untreated wastewater from un-served villages located in the domain of the branch water shed from (El-Rahawy, Nekla, Zat-Elcoom, kafer-Hakim, Bortoss, Elmansoruia, and Bani-souf) as shown in Fig. 1. The 7 water samples were taken from the middle of the waterway along the Rosetta

Branch. The samples were frozen in a refrigerator for later analysis by means of HPLC. Each sample volume was 2 liters.

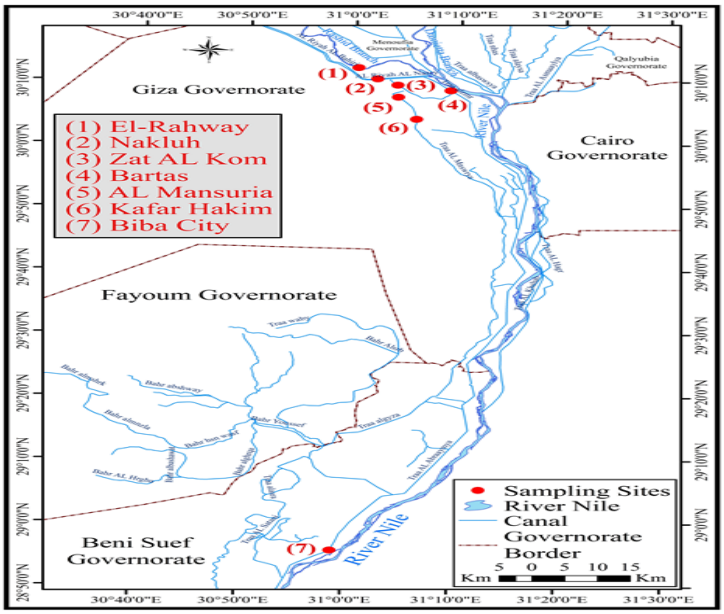



**Fig. 1:** The sampling sites along the River Nile

**Source:** Egyptian General Survey Authority, digital maps of the Arab Republic of Egypt, scales 1: 50,000, 2018. - Google Earth 2023.

## 4. Detection of Selected Analgesics in the Collected Water Samples:

### *4.1. Sample Preparation:*

Prior to high-performance liquid chromatography (HPLC) analysis, the collected water samples were filtered through 0.45 µm syringe filters to remove any particulate matter that could interfere with the HPLC analysis. Then the samples were evaporated using a rotary vacuum (Rodriguez *et al.*, 2014). The concentration factor should be chosen so that the concentration of the target chemical in the sample falls within the range of the calibration curve of the standard. The selected analgesics were determined by two different methods on the HPLC system with a modification of the original methods.

### *4.2. Ibuprofen Detection:*

The development and validation of the assay was performed on an Agilent 1200, column oven, degasser, detector, and a class Empower-2 software. Chromatographic analysis was performed on Agilent XDB (C18) RP Column, 150mm×4.6mm particle size 5µ size column. The flow rate was 1 mL/min, the injection volume was 10 µL, and UV detection was performed at 310 nm. Peak identity was confirmed by both retention time comparison and comparison of spectra obtained from the UV detector was about 4.73 according to the method of Sirisha *et al.* (2014) with modification.

### *4.3. Naproxen, Paracetamol, and Diclofenac Detection:*

Water analysis by HPLC for diclofenac, naproxen, and paracetamol using a selective HPLC-UV detection method at wavelength 220. Waters Symmetry C18 column (3.9 150mm, 5m particle size) with gradient elution of the mobile phase composed of 0.05 M orthophosphoric acid and acetonitrile provided effective chromatographic separation. The gradient elution began with 5% (by volume) acetonitrile, ramped up linearly to 65% in 5min,

and then remained constant until the end of the run. A flow rate of 1.5 mL/min was used to pump the mobile phase. The wavelength detector was set to 220 nm, and drug quantification was done by measuring peak areas. Naproxen, paracetamol, and diclofenac had retention times of about 2.7, 4.2, and 7.4min, respectively. The analytical performance of the HPLC procedure was According to Belal *et al.* (2015). The analgesic standards used in this study were all Sigma HPLC grade.

**5. Experimental Organisms:**

*Procambarus clarkii* samples (n=216) were collected during October 2020 from 2 main sites; the main River Nile at (Nekla) and Abo–Ragwan (El-Giza). Each sample was washed with tap water to remove any adhering contaminants and then transported in an aired box to the laboratory of the animal house in the Women Faculty for Arts, Sciences, and Education, Ain Shams University. Crayfish samples were re-washed again with potable water (individuals ranged from 8 to 10.5 cm in length and 26.8-34.5g weight). At the beginning of the experiment, crayfish were divided into 9groups in triplicate plastic tanks for every group in which one tank contain 8 crayfish, then acclimated in aerated, de-chlorinated fresh water to laboratory conditions for 4 weeks. Exposure was conducted for 60 days in tanks with freshwater (5L/tank) under natural photoperiods. Also, animals were kept under a fixed temperature of 24±2$^{O}$C and 12 hours of a light/dark cycle. Plastic tubes were placed in the tanks to avoid cannibalism and organisms were fed every 48h with extruded sinking fish feed pellets (Skretting, Nutreco Company, Spain), fresh carrot, and zucchini vegetables. Experimental procedures performed according to the Ethical rules for animal protection guidelines of the Faculty of Women for Arts, Sciences, and Education, Ain Shams University.

**6. Experimental Design:**

The experimental design was performed in Women's Faculty and Science Faculty, Ain Shams University for a period of eight weeks. The animals were classified randomly into nine groups in triplicate plastic tanks for every group in which one tank contains 8 crayfish. These groups were: control exposed to dechlorinated tap water, ibuprofen groups treated with low dose (100µg/L) and high dose (63mg/L) according to Trombini *et al.* (2021) and Praskova *et al*. (2011), diclofenac groups treated with low dose (0.64mg/L) and high dose (17mg/L) according to Laurenz *et al*. (2020) and Praskova *et al*. (2011). Naproxen groups treated with low dose (2mg/L) according to water analysis, and high dose (15mg/L) (Li *et al.*, 2016), mixed group treated with low doses of the three drugs and a mixed low dose group co-treated with 0.5g/L of dried *Chlorella* algae (Angulo *et al*., 2018; Al Ketife *et al*., 2020).

**7. Biochemical Studies:**

After completing the experiment, the animals were sacrificed and the hepatopancreas (digestive gland) was dissected out, weighed, and homogenized in 10mmol/L phosphate buffer saline (10% W/V) of 7.4 pH and iced in a refrigerator. The following biomarkers were evaluated:

***7.1. Oxidative Stress Markers:***

Total Antioxidant Activity (TAA) was determined according to the method described by Koracevic *et al.* (2001). The glutathione-S-transferase activity was assayed spectro-photometrically using 1-choloro-2-4 dinitrobenzene (CDNB) and glutathione as described by Habig *et al*. (1974). The final product of lipid peroxidation, Malondialdehyde (MDA) was

measured in the hepatopancreas using the colorimetric method of thiobarbituric acid (TBA) assay according to the method of Draper and Hadley (1990). The malondialdehyde standard solution was prepared by acid hydrolysis of 1,1,3,3 tetra-ethoxy propane (TEP) "Merck" according to Karatepe (2004). The colorimetric determination of nitric oxide was conducted according to Miranda *et al*. (2001) using modified Griess reagent.

***7.2. Enzymes:***

Aspartate amino Transferase (AST) activities were measured in hepatopancreas using kits purchased from Bio Diagnostics Egypt, CAT No: AS1061(45). According to Reitman *et al*. (1957). Alanine Transferase (ALT) activities were measured using the colorimetric Kits method according to Henry *et al*. (1960) purchased from Spectrum Egypt. Alkaline phosphatase (ALP) activities were measured using the colorimetric method according to Belfield and Goldberg (1971) which were purchased from Bio diagnostic, Egypt.

***7.3. Glucose, Total Protein, and Calcium:***

The colorimetric determination of glucose was performed using a kit purchased from Egyptian BIODIAGNOSTIC Company according to Trinder (1969). Total Protein was measured by the colorimetric method according to Doumas (1975), the kit brought from Bio-Diagnostic Co. Egypt. Calcium concentration was evaluated using the Calcium kit (Bio-Diagnostic Co. Egypt) according to the method of Ginder and King (1972).

**8. Histological Studies:**

The hepatopancreas tissue was histologically examined using the method described by Bancroft *et al*. (1996). Following dissection, the tissue was fixed in 10% buffered formalin for 24 hours before being postfixed by washing under running water overnight (6-7hr). With an ascending ethanol series, the tissue was dehydrated (70% for 24hr, 80% for 2hr, 90% for 2hr, 95% for 2hr and 100% for 2hr). It was then cleared in terpineol for three days before being infiltrated in three changes in paraplast inside a 60°C oven and embedded in paraplast. A YD-335 computer microtome was used to cut sections of 5 m thickness (Hunan Kaida Scientific Instrument Comp., Changsha, China). Hematoxylin and eosin (H&E) stained sections were examined under an electric Olympus light microscope (Model CX22RFS1, Tokyo, Japan) and photographed with a Toup Cam microscopic digital camera (S/N:18070500023).

**9. Statistical Analysis:**

Data were represented as means±SE of 6 animals. Statistical analysis was performed by one-way ANOVA. Once a significant F test was obtained, Duncan comparisons were performed to assess the significance of differences among various treatment groups at $p \leq 0.05$. The Statistical Program for social sciences “SPSS” for Windows software, Release 23.0 (SPSS, Chicago, IL) was used.

## RESULTS

**1. Water Samples Analysis:**

The data presented in Table 1 showed that the levels of Ibuprofen were detected in two samples from the 7 investigated samples with 0.073mg /L in El mansuria sample and 0.106 in Kafar-Hakium sample. Whereas the diclofenac concentration was 0.0022, 0.0194, 0.0124, 0.0157 in El-Rahawy, Elmansoruia, Bortoss, and kafer-Hakium samples,

respectively. In contrast, naproxen was detected in all water samples with concentrations 0.0719, 1.9367, 0.2031, 0.1363, 0.4948, 0.6727, and 0.3541 in El-Rahawy, Nakluh, Elmansoruia, Biba, Bortoss, Zat-Alcom, and kafer-Hakium samples, respectively. Paracetamol was detected in Elmansoruia, Biba, Bortoss, Zat-Alcom, and kafer-Hakium samples with concentrations of 0.1624, 0.0152, 0.2272, 0.5065, and 0.2343, respectively. Caffeine was found only in El-Rahawy sample. Naproxen recorded the highest concentration at 1.9367mg/L in Naklih sample, while Caffeine recorded the lowest concentration at 0.0059mg/L in Alrahawy sample.

**Table 1:** The concentrations of different pharmaceutical analgesics in different water samples along the Roseta branch of River Nile (mg/L).

| Place | Alrahaway | Nakluh | Almansuria | Biba city | Bartas | Zat-Alcom | KafarHakium |
|---|---|---|---|---|---|---|---|
| **Ibuprofen** | **ND** | **ND** | **0.073** | **ND** | **ND** | **ND** | **0.106** |
| **Diclofenac** | **0.0022** | **ND** | **0.0194** | **ND** | **0.0124** | **ND** | **0.0157** |
| **Naproxen** | **0.0719** | **1.9367** | **0.2031** | **0.1363** | **0.4948** | **0.6727** | **0.3541** |
| **Paracetamol** | **ND** | **ND** | **0.1624** | **0.0152** | **0.2272** | **0.5065** | **0.2343** |
| **Caffeine** | **0.0059** | **ND** | **ND** | **ND** | **ND** | **ND** | **ND** |

ND= not detected in the sample.

## 2. Biochemical Analysis:

### *2.1. Oxidative Stress Biomarker:*

#### 2.1.1. The Lipid Peroxidation (LPO):

The product of lipid peroxidation (LPO), total malondialdehyde content (MDA) was significantly increased in all the treated groups except the mixed low dose+algae treatment group when compared to the control group at $p<0.05$ as shown in Fig. 2. Whereas the naproxen low and high-dose groups showed a significant increase as compared to all the other treated groups. While the diclofenac-treated groups exhibited a significant increase in LPO content when compared to the ibuprofen-treated group and a significant decrease when compared to the naproxen-treated groups in both low and high doses. Moreover, the mixed low dose and the crayfish group treated with algae showed a significant alleviation in LPO content when compared to the other treated groups.

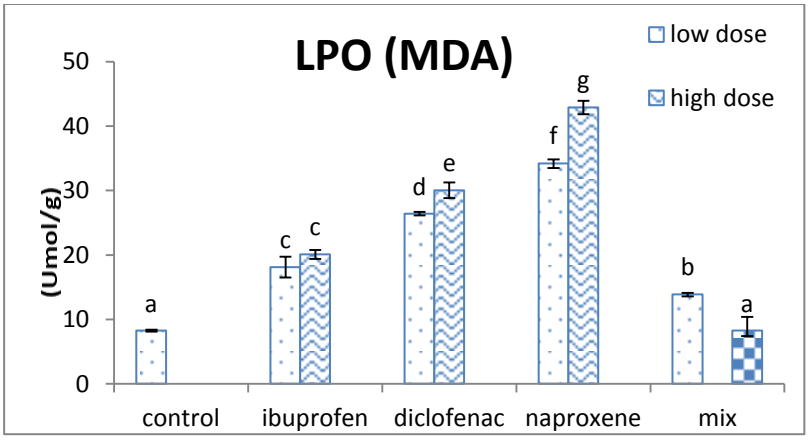



**Fig. 2:** The lipid peroxidation (MDA) in the hepatopancreas of crayfish of different treated groups. Values are represented as means±SE of 6 animals. Significant at $p<0.05$. Groups with the same symbols are not significantly different. The treated group was treated by mixed low doses and treated with algae.

### 2.1.2. The Total Antioxidant Activity (TAA):

Fig. 3 shows that the total antioxidant activity (TAA) in the hepatopancreas was significantly decreased in all the treated groups in comparison with the control group at $p<0.05$. Also, the ibuprofen low dose group exhibited a significant increase in TAA activity when compared to the ibuprofen high dose and the naproxen low dose group. Moreover, the mixed low dose+algae treated group showed a significant increase in TAA activity when compared to the naproxen low and ibuprofen high dose treated groups.

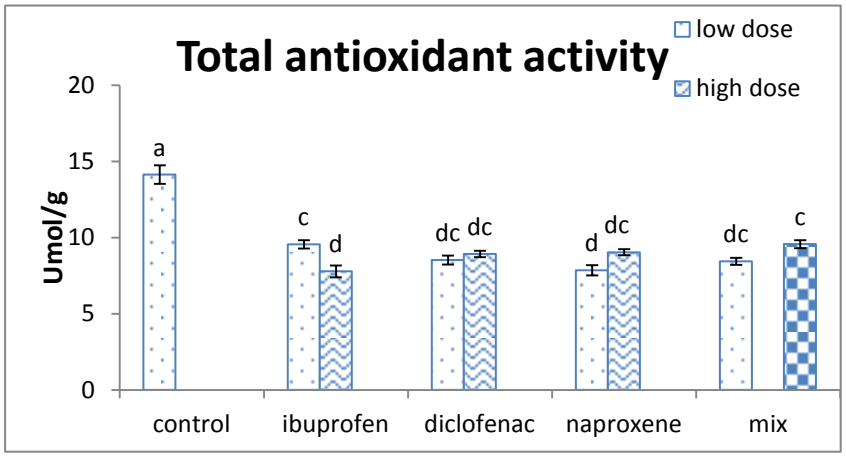



**Fig. 3:** The total antioxidant activity in the hepatopancreas of crayfish of different treated groups. Values are represented as means±SE of 6 animals. Significant at $p<0.05$. Groups with the same symbols are not significantly different. The treated group was treated with low dose mix and treated with algae.

### 2.1.3. The Nitric Oxide (NO):

The data presented in Fig. 4 show that the nitric oxide contents (NO) were significantly increased in all the treated groups in comparison with the control group at $p<0.05$. Also, all the high dose treated groups exhibited a significant increase in NO contents when compared to the other treated groups. Whereas the naproxen low dose and the mix low dose groups showed a significant increase as compared to the ibuprofen low dose group. Moreover, the crayfish group treated with algae showed a significant alleviation in NO content when compared to the other treated groups.

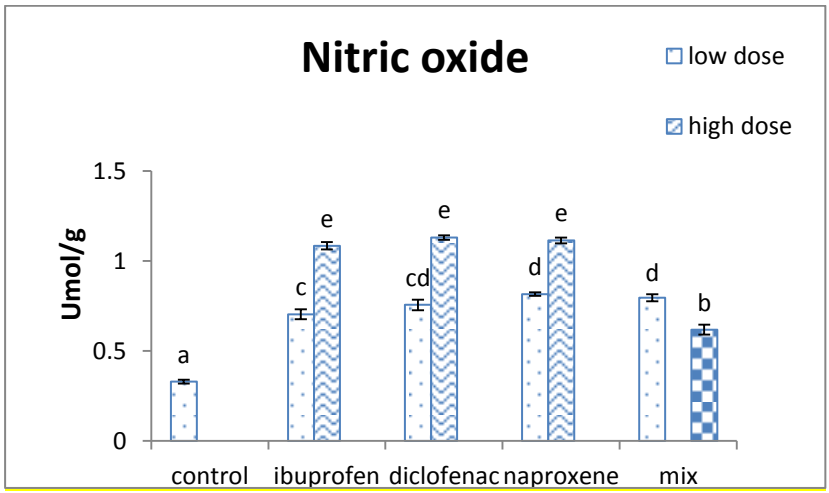



**Fig. 4:** The nitric oxide contents in the hepatopancreas of crayfish of different treated groups. Values are represented as means±SE of 6 animals. Significant at $p<0.05$. Groups with the same

symbols are not significantly different. The treated group was treated by low dose mix and treated with algae.

### 2.1.4. The Glutathione-S- Transferees (GST):

The data depicted in Fig. 5 illustrate that the total glutathione-S-transferees activity was significantly increased in all the treated groups except the ibuprofen low dose and low mix+algae treatment in comparison with the control group at $p<0.05$. While the naproxen high dose treated group exhibited a significant increase in GST activity when compared to all the other treated groups. Whereas the mix low dose group and diclofenac low dose group showed a significant increase in GST activities as compared to the ibuprofen low dose group. Moreover, the crayfish group treated with the mix low+algae showed a significant reduction in GST activity when compared to all the high doses treated groups.

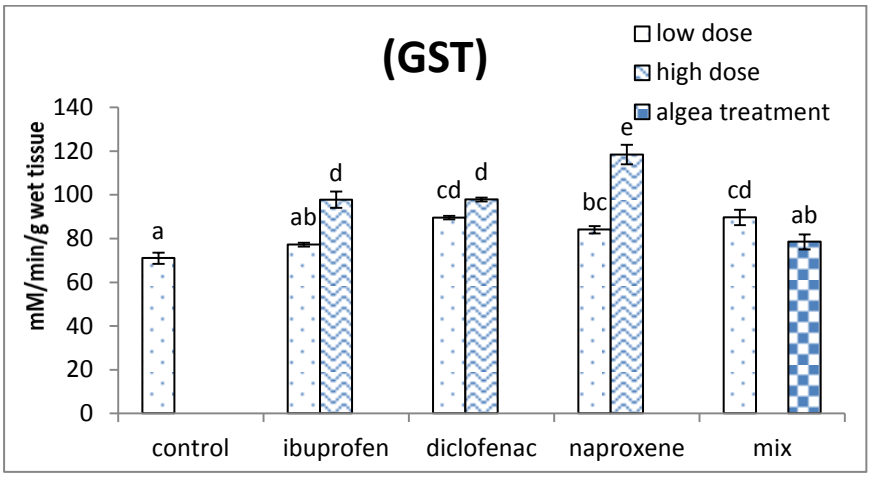



**Fig. 5:** The total glutathione S-transferees (GST) activity in the hepatopancreas of crayfish of different treated groups. Values are represented as means±SE of 6 animals. Significant at $p< 0.05$. Groups with the same symbols are not significantly different. The treated group was treated by low dose mix and treated with algae.

## *2.2. Enzymes (AST, ALT, and ALP):*

The **aspartate amino transferase** (AST) activity was significantly increased in all the treated groups except in the naproxen low dose and the diclofenac high dose treated groups in comparison with the control group at $p<0.05$ as shown in Table 2. While ibuprofen and naproxen high dose groups exhibited a significant increase in AST activity when compared to the diclofenac high dose group. Whereas ibuprofen high dose and the mix low dose group showed a significant increase as compared to the other low dose groups. While the ibuprofen low dose and the low dose diclofenac showed a significant increase in AST activities when compared to the naproxen low dose group. Moreover, the crayfish group treated with the mix low+algae showed a significant elevation in AST activity when compared to the mix low dose treated group. The **alanine transaminase** (ALT) activity was significantly increased in all the treated groups except naproxen low dose and the mix low dose+algae treated groups in comparison with the control group at $p<0.05$. While the ibuprofen high dose group exhibited a significant increase in ALT activity when compared to all the other treated group. Moreover, the crayfish group treated with mix low+algae showed a significant alleviation in ALT activity when compared to the high doses treated groups as shown in Table 2. The total **alkaline phosphatase** (ALP) activity was significantly increased

in all the treated groups in comparison with the control group at $p<0.05$. Whereas the diclofenac low and high-dose groups exhibited a significant increase in ALP activity when compared to all other treated groups. Moreover, the group treated with the mix of low algae showed a significant decrease in ALP activity when compared to all other treated groups (Table 2).

**Table 2:** The aspartate amino transferase (AST, U/g) activity, the alanine transaminase (ALT, U/g) and the alkaline phosphatase (ALP, U/g) in the hepatopancreas of crayfish of different treated groups.

| Group | AST | ALT | ALP |
|---|---|---|---|
| **Control** | $28.50\pm 0.73^{a}$ | $0.17\pm0.00^{a}$ | $3.31\pm0.13^{a}$ |
| **Ibuprofen (low)** | $33.43 \pm 1.40^{b}$ | $0.24\pm0.04^{bcd}$ | $5.63\pm0.20^{d}$ |
| **Ibuprofen (high)** | $47.87\pm 1.27^{c}$ | $0.39\pm0.03^{e}$ | $7.53\pm0.10^{f}$ |
| **Diclofenac (low)** | $35.64 \pm1.52^{b}$ | $0.28\pm0.01^{cd}$ | $8.22\pm0.30^{g}$ |
| **Diclofenac (high)** | $26.67 \pm3.70^{a}$ | $0.30\pm0.02^{d}$ | $10.13\pm0.19^{h}$ |
| **Naproxen (low)** | 26.71 ±1.08a | $0.21\pm0.02^{abc}$ | $5.88\pm0.26^{de}$ |
| **Naproxen (high)** | $53.89 \pm0.90^{d}$ | $0.27\pm0.02^{cd}$ | $6.47\pm0.31^{e}$ |
| **Mixed (low doses)** | $48.72 \pm1.74^{c}$ | $0.25\pm0.02^{bcd}$ | $4.38\pm0.19^{c}$ |
| **Mixed (low+algae)** | $56.88 \pm1.23^{d}$ | $0.18\pm0.03^{ab}$ | $2.63\pm0.10^{b}$ |

Values are represented as means± SE of 6 animals. Significant at $p<0.05$. Groups with the same symbols are not significantly different.

### *2.3. Glucose, Total Protein and Calcium contents:*

A significant decrease was observed in glucose contents in all the treated groups when compared to the control group at $p<0.05$ as shown in Table 3. While mix low dose treated group exhibited a significant decrease in glucose content when compared to the other low dose treated groups. Whereas the ibuprofen low dose group showed a significant increase as compared to the other treated groups except the mix low+algae group. Moreover, the group treated with the mix low+algae showed a significant increase in glucose content when compared to all other treated groups. Table 3 revealed that the total protein contents were significantly decreased in all the treated groups except in diclofenac low, naproxen low and the mix low+algae treatment groups in comparison with the control group at $p<0.05$. Also, the ibuprofen low and high dose groups showed a significant decrease in protein content when compared to diclofenac low dose and the mix low+algae groups. Whereas the mix low+algae and diclofenac low dose showed a significant increase in protein content when compared to the naproxen high and the mix low treated groups. The total calcium contents were significantly decreased in all the treated groups except mix low dose group in comparison with the control group at $p<0.05$ as shown in Table 3. Also, the mix low dose group showed a significant increase in Ca contents when compared to low doses of ibuprofen, naproxen and high dose of diclofenac.

## 3. Histological Studies:

The hepatopancreas of the control crayfish showed a typical glandular tubular shape. It is formed of numerous tubules separated by connective tissues (Fig. 6a). Each tubule has a

central asterisk-like lumen surrounded with a wall formed of epithelial cells. There are four types of cells: absorptive, secretory, fibrillar and embryonic cells. The absorptive cell is the most abundant cell type with a basely located nucleus with apical small vacuoles. The secretory cell has a basely located nucleus and a single large central vacuole. The fibrillar cell is small, darkly stained with a large nucleus. The embryonic cell which are the precursors of all other types of cells, are cuboidal in shape and found in the distal part of hepatic tubules.

**Table 3:** The glucose (mg/g), total proteins (mg/g), and calcium (mg/g) contents in the hepatopancreas of crayfish of different treated groups.

| Group | Glucose | Total Proteins | Calcium |
|---|---|---|---|
| **Control** | $8.75\pm0.30^{a}$ | $0.46\pm0.02^{a}$ | $0.14\pm0.03^{a}$ |
| **Ibuprofen (low)** | $3.31\pm0.08^{c}$ | $0.35\pm0.02^{cd}$ | $0.03\pm0.01^{c}$ |
| **Ibuprofen (high)** | $1.48\pm0.07^{e}$ | $0.32\pm0.02^{d}$ | $0.06\pm0.01^{cb}$ |
| **Diclofenac (low)** | $2.57\pm0.12^{d}$ | $0.48\pm0.03^{a}$ | $0.07\pm0.01^{cb}$ |
| **Diclofenac (high)** | $1.39\pm0.11^{e}$ | $0.38\pm0.02^{bcd}$ | $0.04\pm0.02^{c}$ |
| **Naproxen (low)** | $2.83\pm0.04^{d}$ | $0.42\pm0.02^{abc}$ | $0.03\pm0.01^{c}$ |
| **Naproxen (high)** | $2.55\pm0.09^{d}$ | $0.33\pm0.02^{d}$ | $0.08\pm0.03^{cb}$ |
| **Mixed (low doses)** | $1.41\pm0.07^{e}$ | $0.34\pm0.02^{d}$ | $0.10\pm0.01^{ab}$ |
| **Mixed (low+algae)** | $4.57\pm0.15^{b}$ | $0.45\pm0.03^{ab}$ | $0.06\pm0.01^{cb}$ |

Values are represented as means± SE of 6 animals. Significant at $p<0.05$. Groups with the same symbols are not significantly different.

The hepatopancreas treated with ibuprofen at low dose (100µg/L) showed limited mild histopathological alterations (Fig. 6b). Connective tissue was degenerated and conglomerated in some areas. Although the lumen was asterisk in shape, they were wide. Separation between the tubular wall and tubular cells was distinguished. Numerous absorptive cells degenerated without cell boundaries. It was difficult to distinguish embryonic cells, and if so, it was very few and small. Other types of cells were not affected. After treatment with a high dose of ibuprofen (63mg/L) stain became faint in all sections (Fig. 6c). Sever disintegration was found in all tubular cells. Dilation of tubules make them very narrow to each other without connective tissue in between. It was exceedingly difficult to differentiate between cell types because of severe swelling and degeneration. Presence of deeply stained pyknotic nuclei of epithelial cells. The dose 0.64mg/L of diclofenac exhibited degeneration of connective tissue between the tubules in some areas (Fig. 6d). Conspicuous disruption of some tubules with lysis of their walls was observed. Separation between tubular wall and cells were distinguished. There was a cellular atrophy and lumen shape damage of some tubules. In addition, all cell types were disintegrated. High dose of 17mg/L diclofenac showed a marked and sever deterioration of the tubules (Fig. 6e). Increasing vacuolation, formation of abnormal lumen and some tubules fused together leaving fused large lumen with damaged tubular wall. Cellular atrophy and necrotic cells were found in every section. Marked histopathological alterations were found in hepatopancreatic tubules treated with low dose of naproxen (2mg/L) (Fig. 6f). Tubules were separated with wide spaces with degenerations of connective tissue. Reduced lumen was found in tubules. The disintegration

of the epithelium, and the degeneration and separation of the tubular walls were also distinguished. Signs of damage in hepatopancreas of crayfish were increased using high dose of naproxen (15mg/L) (Fig. 6g). The tissue stained weakly. Tubules and connective tissue were completely degenerated. Complete destruction of tubular cells with vacuolation was observed. Fusion of tubules was investigated leaving irregular mass of damaged cells enclosing central space.

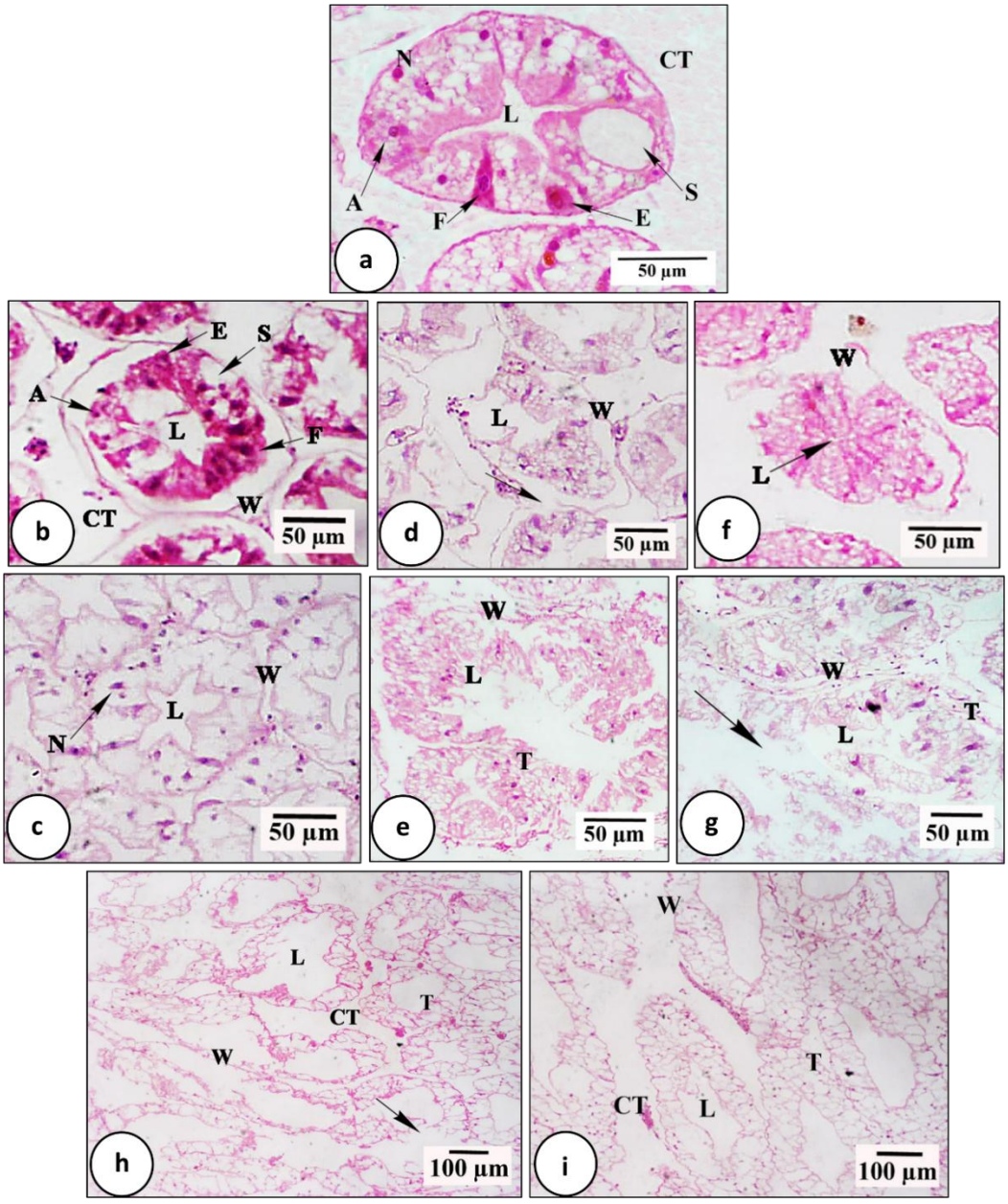



**Fig. 6:** Light micrograph of transverse section of *P. clarkii* hepatopancreas; **a.** General structure. **b**. Treated with low dose of ibuprofen (100µg/L). **c.** Treated with high dose of ibuprofen (63mg/L). **d**. Treated with low dose of diclofenac (0.64mg/L). **e.** Treated with high dose of diclofenac (17mg/L) showing a marked and severe deterioration of the tubules (T) with cellular atrophy and fused of tubular walls (W) with fused large lumen (L). **f**. Treated with low dose of naproxen (2mg/L). **g**. Treated with high dose of naproxen (15mg/L). **h**. Treated with low doses of mixer of ibuprofen, diclofenac and naproxen at 100µg/L, 0.64mg/L and 2mg/L, respectively. **i**. Treated with dried green alga *Chlorella* added to low doses of mixer of ibuprofen, diclofenac and naproxen at 100µg/L, 0.64mg/L and 2mg/L, respectively. CT: Connective tissue; T: Tubule; L: Lumen; W: Tubular wall; A: Absorptive cell; F: Fibrillar cell; S: Secretory cell; E: Embryonic cell; N: Nucleus.

## DISCUSSION

### 1. Water Analysis:

The use of pharmaceutical drugs in Egypt has steadily increased, resulting in the emergence of micropollutants in raw and drinking waters. Pharmaceuticals enter natural waters via hospital wastes, sewage effluent as well as landfill leachates, and their effects on non-target aquatic organisms are unknown (vertebrate and invertebrate). Even at low concentrations (ng/L to g/L), the continuous release of pharmaceutical products into aquatic environments could have long-term negative effects on non-target aquatic organisms (Almeida *et al*., 2020; Parolini, 2020). The most common pharmaceutical class in the environment is nonsteroidal anti-inflammatory drugs (NSAIDs) (Fareed *et al.,* 2018, Parolini, 2020). Ibuprofen (IBP), diclofenac (DCF), and naproxen (NPX) have all been identified and quantified in various bodies of water (Chafi *et al.,* 2022). These findings were consistent with the current findings; the data presented in Table 1 demonstrated the presence of the selected analgesics in the Nile River. Pharmaceuticals are highest at the discharge points of the four drains (AL Rhaway, Nakluh, Zat-Alkom, and Bartas), reflecting the dramatic impact of the four drains on water quality at the Rosetta branch. This could be due to highly contaminated areas or receiving water from specific locations such as hospitals, pharmaceutical plants, farms, and so on. Furthermore, many villages discharge their untreated waste directly into the drain, as well as the number of uncovered villages with sewage systems in their drain domain. Fareed *et al.* (2018) previously reported that 15 pharmaceuticals were traced along the Rosetta branch. The highest concentrations of naproxen, chlorotetracyclin, doxytetracyclin hyclate, penicillin, and oxacillin were 21.189, 20.955, 20.89, 20.09, and 20.029 g/l, respectively, while Oxytetracylin, Tetracyclin, and Ofloxacine were not detected. Wang *et al.* (2010) discovered seven acidic compounds in rivers, including five NSAIDS (salicylic acid, ibuprofen, diclofenac, mefenamic acid, and naproxen) and two blood lipid regulators (clofibric acid and gemfibrozil). Santos *et al.* (2007) found four NSAIDS (diclofenac, ibuprofen, ketoprofen, and naproxen) and a nervous stimulant (caffeine) in influent and effluent samples from four wastewater treatment plants (WWTPs) in Seville. Bagnis *et al.* (2018) and Fekadu *et al.* (2019) also detected concentrations (in the ng/L range) in surface waters.

### 2. Biochemical Analysis:

LPO were significantly increased in all concentrations of DCF, IBP, and NPX alone and with combination exposure in crayfish for 60 days, with a reduction in TAA. This increase could be because CYP produces an oxygenated intermediate - the oxy-cytochrome P450 complex [P450 ($Fe_3$) $O_2$] - during NSAID biotransformation, resulting in the release of superoxide anion ($O_2$) by uncoupling of this reaction (Doi *et al.,* 2002). Because NSAIDs affect the mitochondrion and thus oxidative phosphorylation, increased ROS production, especially of $O_2$, may occur, resulting in an increase in LPO and a decrease in TAA (Salgueiro-Pagadigorria *et al.,* 2004). Cytochrome P450 (CYP) and the CYP2, CYP3, and CYP4 families are known to exist in the invertebrate crayfish (Feyereisen, 2006). The CYP2 subfamily is specifically responsible for the biotransformation of NSAIDs to hydroxylated metabolites. Under normal conditions, the antioxidant defense system is critical in the

neutralization of ROS generated by the redox reactivity of pharmaceuticals. This mechanism is mediated by a cascade of antioxidant enzymes (GPX, SOD, and CAT) that scavenge ROS and convert them into less harmful molecules. The overproduction of ROS due to the prolonged exposure time (60 days) and the metabolism of analgesics in the crayfish hepatopancreas resulted in a significant decrease in TAA when compared to the control.

Furthermore, the current findings are consistent with those reported by Aguirre-Martnez *et al.* (2013), who exposed *Carcinus meanas* (gill, hepatopancreas, muscle, and gonad tissues) to ibuprofen and caffeine (0.1 and 50mg/L) for 28 days. In the same trend, Gómez-Oliván *et al.* (2014) exposed *Daphnia magna* to IBP, DCF, and NPX at concentrations of 2.9 mg/L, 9.7mg/L, and 0.017mg/L, respectively, and found that after 48 hours of exposure, the LPO level increased. Another study found that diclofenac increased LPO in *Hyalella azteca* in a time-dependent manner (Oviedo-Gómez *et al.,* 2010). This rise can be explained by the formation of 4- and 5-hydroxydiclofenac and their subsequent biotransformation to benzoquinones, which increase ROS formation.

When NSAIDs are consumed, they enter the vasculature and acetylate the enzyme COX2 found in the endothelium or circulating leukocytes to produce 15-epi-lipoxin A4, which promotes nitric oxide (NO) synthesis mediated by endothelial (eNOS) and inducible (iNOS) nitric oxide synthase (Paul-Clark *et al.,* 2004). Through a diffusion-limited reaction, when superoxide anion and NO bind, they can form a reactive nitrogen species (peroxynitrite) (Huie and Padmaja, 1993). Without glutathione (GSH), the oxidant peroxynitrite may cause mitochondrial malfunction, leading to permanent damage and a significant decrease in cellular ATP (Jaeschke *et al.,* 2003).

GST activities were significantly increased in the crayfish hepatopancreas after 60 days of exposure to low, high, and mixed low-dose concentrations of ibuprofen, diclofenac, and naproxen. The increase in GST activities may indicate the activation of a biotransformation metabolic pathway mechanism via phase reaction II, as well as the activation of the antioxidant defense system in response to chemical insults (Halliwell & Gutteridge, 2015). The findings of this study agree with the findings of other authors who found an increase in GST activities after IBP exposure to various experimental animals. Nonsteroidal anti-inflammatory drugs (e.g. IBP) are known to disrupt eicosanoid biosynthesis in both invertebrates and vertebrates (via inhibition of the COX pathway and prostaglandins) (Heckmann *et al.,* 2008). A blockage of one of the three membrane-phospholipid arachidonic acid metabolic pathways (along with the lipoxygenase pathway and the cytochrome P450 epoxygenase pathway) may impair several physiological functions, including the immune system, reproduction, and ion transport (Rowley *et al.,* 2005; Heckmann *et al.,* 2008; Ericson *et al.,* 2010)

Previous research (Aguirre-Martnez *et al.,* 2016) found a significant increase in GST activity in hepatopancreas tissues of clams *Ruditapes philippinarum* exposed to the highest concentrations of ibuprofen at 0.1 and 5g/L over 14 days. GSTs have been implicated in the toxic response of aquatic organisms to DCF in numerous studies. Gonzalez-Rey and Bebianno (2014) demonstrated that GSH-DCF conjugation occurs via GSTs isoenzymes in *Mytilus galloprovincialis* marine mussels. Furthermore, Nunes *et al.* (2020) discovered that the ecotoxicological effects of DCF in the marine polychaete *Hediste diversicolor* and the

marine fish *Solea senegalensis* organisms exposed to DCF directly or indirectly showed an increase in GSTs activities in organisms exposed to concentrations like those found in the environment (0.5, 1, and 2g/L). Other studies have found similar changes in GST activity after DCF exposure, such as a study of *Dreissena polymorpha* by Quinn *et al.* (2011). They found that a 96h (acute) exposure to 1 and 1000g/L of DCF significantly increased GST expression when compared to the control group. Thus, the biochemical pathway that leads to the bioactivation of DCF into its reactive metabolites (specifically quinoneimines) appears to correspond to an evolutionary conserved metabolic mechanism. This route, however, ultimately necessitates the conjugation of the formed quinoneimines with reduced glutathione to allow detoxification of these harmful intermediates, a trend that appears to be shared by many species. It is possible that the increase in GSTs activity was due to an increase in detoxification capacity rather than an antioxidant response.

There is no previous study on the effect of naproxen on GST level in invertebrates. So, it is recorded for the first time that GST activity increased in both doses low and high doses of NPX, also, in mixture low dose of three analgesics on hepatopancreas in *P. clarkii*. The effect of naproxen is obvious as NSAIDs effect according to Quinn *et al.* (2011) and Guiloski *et al.* (2017).

The activities of ALP, AST, and ALT in crayfish exposed to the investigated analgesics increased ($p<0.05$) when compared to the control. According to Chen *et al.* (2012), alkaline phosphatase, ALP, catalyses the hydrolysis of phosphate monoesters and is required for protein synthesis. ALP, an enzyme involved in adaptive cellular response to the potential and genotoxicity of pollutants, has also been reported as an important biomarker and could serve as a good indicator of intoxication. The significant increase in ALP activity observed in all treated groups of crayfish may be related to the level of stress the animals were experiencing because of analgesic exposure (xenobiotics). ALT and AST activities are biomarkers of acute hepatic damage and enzymes involved in the conversion of amino acids to keto acids, allowing for the interaction of carbohydrate and protein metabolism during fluctuating energy demands of organisms in various adaptive situations. An increase in transaminase (ALT and AST) activities in crayfish exposed to analgesics, as observed in this study, may indicate analgesic-induced dysfunction. Again, the disruption of energy supply via amino acid metabolism may be to blame for the elevation of these transaminases (Bogé *et al.*, 1992; Lohner *et al.*, 2001).

Proteins are the most abundant organic molecules in living system and play an essential role in the physiology of living organisms by providing information on the general energy mobilization of an animal (Adams *et al.*, 1990). In the present study, there was a significant decrease ($p<0.05$) in the total protein concentration of the hepatopancreas of *P. clakii* in all concentrations used in this study when compared with the control crayfish group. The reason for decreasing total protein concentration is that exposure to different analgesics can alter the oxidative state of cells and thereby increase reactive oxygen species and lead to oxidative stress (Gagné *et al.*, 2006). As proteins are the basis of the structure and function of life, proteins are broken down under stress conditions by organisms to amino acids to meet their metabolic need. A study by Bhattacharya *et al.* (2006) reported a decrease in total protein concentration in *Pilaglobosa* exposed to an untreated tannery effluent. In addition, Trombini *et al.* (2021) showed that the proteomic analysis identified 22 different proteins

whose abundance changed in the hepatopancreas after the mixed pharmaceutical exposure, depending on time and/or dose. Also, the functional analysis of the differentially expressed proteins showed alterations in main cell functions: biotransformation and detoxification of xenobiotics, cytoskeleton homeostasis, carbohydrate metabolism and immune response. the alteration of these specific groups of proteins is probably linked to the ability of cocktail exposure to generate reactive oxygen species (ROS) in the cell and the subsequent onset of an antioxidant response (Gonzalez-Rey and Bebianno, 2011; Bartoskova *et al.,* 2013; Nunes *et al.,* 2018). Also, the increase in the ALT, AST, and ALP activities in the present work could explain this decrease in protein contents.

Crayfish exposed to IBP, DCF, and NPX for 60 days showed a significant reduction in glucose levels due to oxidative stress and increased glucose metabolism. Glucose, also known as dextrose, is a type of carbohydrate known as a simple sugar, or monosaccharide. It provides energy for cell function. A previous study found that pollutant exposure increased glucose metabolism in crustaceans like *Carcinus maenas* and *Homarus americanus*, which has been linked to ROS clearance (Lorenzon *et al.,* 2004; Wang *et al.,* 2017). Also, Trombini *et al.* (2021) conducted a 21-day study on crayfish exposed to ibuprofen, ciprofloxacin, and flumequine mixtures at low and high concentrations (10 and 100g/L). Hexosaminidase A (HEXA) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels were found to be higher (GAPDH). The two enzymes work together to break down carbohydrates. HEXA is the enzyme that phosphorylates glucose to glucose 6-phosphate and initiates glucose metabolism via both glycolysis and the pentose phosphate pathway (PPP). GAPDH oxidises triose glyceraldehyde 3-phosphate (G-3P), a product of glycolysis, fructose catabolism, PPP, and glycerol metabolism, causing G-3P to follow its path to pyruvate. The increased HEXA activity observed in treated crayfish may activate the oxidative pentose phosphate pathway to generate NADPH and contribute to maintaining GSH levels, which are affected by excess ROS generated by drug mixed exposure (Tang *et al.,* 2015). Under oxidative stress conditions, the crustacean hyperglycemic hormone, (CHH), and an abundant neuropeptide stimulated lipid mobilization from storage tissues such as the hepatopancreas, including free fatty acids released into haemo-lymph to be used metabolically (Santos *et al.,* 1997; Lorenzon *et al.,* 2004). The levels of 3-hydroxyacyl-CoA dehydrogenase (HADH) resulted in an increase in the hepatopancreas of *P. clarkia* after mixed drug exposure. This enzyme is essential for catalyzing the penultimate reaction of β-oxidation.

In the current study, the three analgesic treatment groups (low dose, high dose, and mixer low dose) showed a significant increase in calcium content in the crayfish hepatopancreas. Calcium is a critical second messenger molecule in all cells and is required for synaptic transmission in neurons. Nonsteroidal anti-inflammatory drugs can cause mitochondrial Ca2+ release, interfering with Ca2+-dependent hormonal signaling processes (Salgueiro-Pagadigorria *et al.,* 2004). Naproxen significantly reduced mitochondrial Ca2+ retention and inhibited ATP-dependent Ca2+ uptake by microsomes. In addition to activating Ca-dependent signaling, calcium release in mitochondria can induce the transition of mitochondrial permeability, a crucial step leading to mitochondrial apoptosis (Jomova *et al.,* 2023).

In the present study, we don't use the mixed high concentration from the three analgesics because high concentrations damaged the microorganism's cell membranes, which accelerated interaction between analgesics and biologicals substances in the cells and the mixer of low concentration is the closest to the field water analysis. Ding *et al.* (2017) discovered that naproxen at relatively high concentrations caused an increase in LPO concentration in two microalgae, *Cymbella* sp. and *Scenedesmus quadricauda*, after 4 days of exposure. Both microalgae were completely inhibited (100% inhibition) by 100mg L-1 NPX after 24 hours.

*Procambarus clarkii* group co-treated with a mixture of low concentrations of the three analgesics plus the dried microalgae (*Chorella*) for 60-day, showed an improvement in all biochemical investigation. Microalgae have demonstrated the ability to detoxify a wide range of organic and inorganic compounds at various scales, from laboratory to full scale (Sutherland and Ralph, 2019) Such detoxification typically takes place via three main pathways: 1- **bio-adsorption**, in which the compound is adsorbed to cell wall components or onto organic extracellular excretions; 2- **bio-uptake**, in which the compound is actively transported into the cell; and 3- **biodegradation**, in which the compound is broken down into simpler molecules via catalytic metabolic degradation (Sutherland and Ralph, 2019). Data from laboratory studies also indicate that microalgae can metabolize those analgesics more efficiently than bacteria (Escapa *et al.,* 2016). The present study suggested that the bio-adsorption mechanism is the most one occurred. As a result, the biosorption mechanism progresses through several step processes: i) adsorbate mass transfer from bulk to adsorbent surface, ii) adsorption of adsorbate on adsorbent surface, and movement within the pores of sorbent material (Cheung *et al.,* 2007). Because adsorption on the surface is extremely fast, the biosorption method is controlled by the first and second steps (Aravindhan *et al.,* 2007). Previous studies have found that *Chlorella* sp. can degrade toxic organic aromatic compounds like 2,4-dimethyl phenol, 2-chlorophenol, and bisphenol A (Peng *et al.,* 2006); however, others have found that this genus can only tolerate organic pollutants (Rakaiby *et al.,* 2012). NSAIDs received little attention in the many studies on chemical remediation by microalgae. Xiong *et al.* (2018) demonstrated that microalgal inoculation in wastewaters improved pharmaceutical removal efficiencies by up to 80%.

Following 30 days of incubation, Ding *et al.* (2017) discovered varying rates of degradation of the NSAID naproxen between different microalgal species, with *Cymbella* sp. enhancing naproxen degradation by 27% above that in the control and *Scenedesmus quadricauda* inhibiting degradation by 23%. Photocatalysis has been shown to be the primary degradation pathway for those compounds in the environment. According to Baena-Nogueras *et al.* (2017), light during microalgae treatment may play a vital role in its removal. DCF can biodegrade or photodegrade into the following metabolites: 5,40-dihydroxy-diclofenac, 3-dihydroxy-diclofenac, 40-dihydroxy-methyl-diclofenac, 30-hydroxymethyl-diclofenac, 40-hydroxy-diclofenac, and 50-hydroxy-diclofenac (Deng *et al.,* 2003). The latter two are oxidized to benzoquinone imine intermediates, which are highly toxic to aquatic organisms (Oviedo-Gómez *et al.,* 2010). Under aerobic conditions, ibuprofen is rapidly degraded, with removal rates of up to 90%. (Ternes, 1998). Aryl-carboxyl IBP, hydroxy-IBP, and carboxy-IBP are the primary photo degradation metabolites (Carballa *et al.,* 2004; Méndez-Arriaga *et al.,* 2008). 4-isobutylacetophenone, 1-(6-methoxy-2-naphthyl) ethanol, and

2-acetyl-6-methoxy-naphthalene are among the photodegradation products of naproxen (Miranda *et al.,* 1991).

**3. Histological Studies:**

According to histological findings, *P. clarkii* hepatopancreas consists of numerous tubules with four types of cells: absorptive (A), secretory (S), fibrillar (F), and embryonic (E) cells with a central asterisk-like lumen. The absorptive cell has a small vacuole at the apex and a nucleus at the base. The secretory cell has a nucleus at the bottom of the cell with a large centra.

Other than the hepatopancreas, some authors investigated the effects of analgesics on various organs of different species of crayfish. Laurenz *et al.* (2020) investigated the effects of Diclofenac and Terbuthylazine on noble crayfish gonadal maturation. Zhang *et al.* (2021) reported on the effects of acute diclofenac exposure on intestinal histology in freshwater *P. clarkii*. Changes in tubular cells, particularly secretory ones, indicate an increase or decrease in the uptake of harmful substances (Laurenz *et al.,* 2020). Because it is the primary site of detoxification, Jaiswal and Sanojini (1990) proposed that histological changes in the hepatopancreas are direct toxic effects of toxicants on cells. We believe that the observed changes in secretory cells are the result of a greater need to lead off chemicals, which damage their membranes. The harmful effects on development, growth, and survival can occur during the extrusion of harmful substances that cause hepatopancreatic damage (Laurenz *et al.,* 2020). Lytic and highly vacuolated cytoplasm, as well as Pyknotic nuclei, could be the result of hyperactivity prior to cell necrosis (Roncero *et al.,* 1992). Furthermore, analgesics may interact with enzymes, causing oxidative stress and reactive oxygen formation, which may eventually lead to cell necrosis (Chio *et al.,* 2010). The slight improvement in the hepatopancreas after treatment with *Chlorella vulgaris* algae in the current study could be due to the bio-adsorption of these NSAIDS by the dried microalgae via the same mechanism discussed in the physiology section (Arami *et al.,* 2008). The findings of the hepatopancreas investigation show a wide range of sublethal effects on freshwater crayfish, leading to the assumption that other effects are possible and should be investigated in future studies. We hypothesized that combining analgesics with other introduced chemicals could reduce the effective doses of the pharmaceutical or create synergies with other unknown effects.

## CONCLUSION

The study of toxicological effects of ibuprofen (IBP), diclofenac (DCF), and naproxen (NPX) on the freshwater crayfish *Procambarus clarkii*. The results showed that lipid peroxidation, nitric oxide contents, glutathione-S-transferase, aspartate transaminase, alanine transaminase, and alkaline phosphatase activities were significantly increased in the hepatopancreas of the crayfish. On the other hand, total protein, glucose and calcium contents were significantly decreased after IBP, DCF, and NPX exposure versus the control. *Chlorella vulgaris* microalga improved the oxidative stress effect and alleviates the AST, ALT, and ALP enzymes, with increased sugar, protein, and calcium contents. This enhancement could be accomplished through the bio-adsorption of these NSAIDS by dried microalgae. Specific studies on this subject are thus required to gain a better understanding of the role of the

various processes accounting for micropollutant removal to optimize their efficiency and to assess further disposal and/or reuse of the biomass generated, as biological treatment technologies are generally more environmentally friendly and less expensive than physical and chemical treatment technologies. Based on these results, modified algal biomass could be used as a potential alternative to traditional, reusable sorbent for the removal of pharmaceuticals from wastewaters.

**Ethical approval:** This study was ethically approved by the Research Ethics Committee of Faculty of Women for Arts, Science, and Education, Ain Shams University, Cairo, Egypt (Code: Sci1332309002)

## REFERENCES

**Abdel-Satar, A.M.; Ali, M.H. and Goher, M.E.** (2017). Indices of water quality and metal pollution of Nile River, Egypt. Egypt. J. Aquat. Res., 43: 21-29.

**Abdel-Shafy, H.I. and Mohamed-Mansour, M.S.** (2013). Issue of Pharmaceutical Compounds in Water and Wastewater: Sources, Impact and Elimination. Egypt. J. Chem., 56: 449-471.

**Adam, S.A.; Marr, R.S. and Gerace, L.** (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J. Cell Biol., 111: 807-816.

**Aguirre-Martínez, G.V.; Del Valls, T.A. and Martín-Díaz, M.L.** (2013). Identification of biomarkers responsive to chronic exposure to pharmaceuticals in target tissues of *Carcinus maenas*. Mar. Environ. Res., 87-88: 1-11.

**Aguirre-Martínez, G.V.; Del Valls, T.A. and Martín-Díaz, M.L.** (2016). General stress, detoxification pathways, neurotoxicity and genotoxicity evaluated in *Ruditapes philippinarum* exposed to human pharmaceuticals. Ecotoxicol. Environ. Saf., 124: 18-31.

**Al Ketife, A.M.D.; Al Momani, F. and Judd, S.** (2020). A bioassimilation and bioaccumulation model for the removal of heavy metals from wastewater using algae: New strategy. Process Saf. Environ. Prot., 144: 52-64.

**Almeida, Â.; Solé, M.; Soares, A.M.V.M. and Freitas, R.** (2020). Anti-inflammatory drugs in the marine environment: Bioconcentration, metabolism and sub-lethal effects in marine bivalves. Environ. Pollut. Barking Essex 1987, 263: 114442.

**Angulo, E.; Bula, L.; Mercado, I.; Montaño, A. and Cubillán, N.** (2018). Bioremediation of Cephalexin with non-living *Chlorella* sp; biomass after lipid extraction. Bioresour. Technol., 257: 17-22.

**Arami, M.; Limaee, N.Y. and Mahmoodi, N.M.** (2008). Evaluation of the adsorption kinetics and equilibrium for the potential removal of acid dyes using a biosorbent. Chem. Eng. J., 139: 2-10.

**Aravindhan, R.; Rao, J.R. and Nair, B.U.** (2007). Removal of basic yellow dye from aqueous solution by sorption on green alga *Caulerpa scalpelliformis*. J. Hazard. Mater., 142: 68-76.

**Baena-Nogueras, R.M.; González-Mazo, E. and Lara-Martín, P.A.** (2017). Degradation kinetics of pharmaceuticals and personal care products in surface waters: photolysis vs biodegradation. The Science of the total environment, 590-591: 643-654.

**Bagnis, S.; Fitzsimons, M.; Snape, J.; Tappin, A. and Comber, S.** (2018). Processes of distribution of pharmaceuticals in surface freshwaters: implications for risk assessment. Environ. Chem. Lett., 16: 1193-1216.

**Bancroft, J.D.; Stevens, A. and Turner, D.R.** (1996). Theory and Practice of Histological Techniques. 4th Edition, Churchill Livingestone, New York.

**Bartoskova, M.; Dobsikova, R.; Stancova, V.; Zivna, D.; Blahova, J.; Marsalek, P.; Zelníckova, L.; Bartos, M.; di Tocco, F.C. and Faggio, C.** (2013). Evaluation of ibuprofen toxicity for zebrafish (*Danio rerio*) targeting on selected biomarkers of oxidative stress. Neuro Endocrinol. Lett., 34(2): 102-108.

**Belal, T.S.; Bedair, M.M.; Gazy, A.A. and Guirguis, K.M.** (2015). Validated selective HPLC-DAD method for the simultaneous determination of diclofenac sodium and lidocaine hydrochloride in presence of four of their related substances and potential impurities. Acta Chromatogr., 27: 477-493.

**Belfield, A. and Goldberg, D.M.** (1971). Revised assay for serum phenyl phosphatase activity using 4-amino-antipyrine. Enzyme, 12: 561-573.

**Bhattacharyya, S.N.; Habermacher, R.; Martine, U.; Closs, E.I. and Filipowicz, W.** (2006). Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell, 125: 1111-1124.

**Bogé, G.; Leydet, M. and Houvet, D.** (1992). The effects of hexavalent chromium on the activity of alkaline phosphatase in the intestine of rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol., 23: 247-260.

**Brozinski, J.-M.; Lahti, M.; Meierjohann, A.; Oikari, A. and Kronberg, L.** (2013). The anti-inflammatory drugs diclofenac, naproxen and ibuprofen are found in the bile of wild fish caught downstream of a wastewater treatment plant. Environ. Sci. Technol., 47: 342-348.

**Carballa, M.; Omil, F.; Lema, J.M.; Llompart, M.; García-Jares, C.; Rodríguez, I.; Gómez, M. and Ternes, T.** (2004). Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Res., 38: 2918-2926.

**Chafi, S.; Azzouz, A. and Ballesteros, E.** (2022). Occurrence and distribution of endocrine disrupting chemicals and pharmaceuticals in the river Bouregreg (Rabat, Morocco). Chemosphere, 287: 132202.

**Chen, B.; Gao, L.; Lavina, B.; Dera, P.; Alp, E.E.; Zhao, J. and Li, J.** (2012). Magneto-elastic coupling in compressed Fe7C3 supports carbon in Earth's inner core. Geophys. Res. Lett., 39(18): 1-4.

**Cheung, W.H.; Szeto, Y.S. and McKay, G.** (2007). Intraparticle diffusion processes during acid dye adsorption onto chitosan. Bioresour. Technol., 98: 2897-2904.

**Chio, C.P.; Chen, W.Y.; Chou, W.C.; Hsieh, N.H.; Ling, M.P. and Liao, C.M.** (2010). Assessing the potential risks to zebrafish posed by environmentally relevant copper and silver nanoparticles. Sci. Total. Environ., 420: 111-118.

**Deng, A.; Himmelsbach, M.; Zhu, Q.Z.; Frey, S.; Sengl, M.; Buchberger, W.; Niessner, R. and Knopp, D.** (2003). Residue analysis of the pharmaceutical diclofenac in different water types using ELISA and GC–MS. Environ. Sci. Tech., 37: 3422-3429.

**Ding, T.; Lin, K.; Yang, B.; Yang, M.; Li, J.; Li, W. and Gan, J.** (2017). Biodegradation of naproxen by freshwater algae *Cymbella* sp. and *Scenedesmus quadricauda* and the comparative toxicity. Bioresour. Technol., 238: 164-173.

**Doi, H.; Iwasaki, H.; Masubuchi, Y.; Nishigaki, R. and Horie, T.** (2002). Chemiluminescence associated with the oxidative metabolism of salicylic acid in rat liver microsomes. Chem. Biol. Interact., 140: 109-119.

**Doumas, B.T.** (1975). Standards for total serum protein assays--a collaborative study. Clin. Chem., 21: 1159-1166.

**Draper, H.H. and Hadley, M.** (1990). Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol., 186: 421-431.

**El-Sayed, A.B. and El Fouly, M.M.** (2005). Recovery of outdoor mass culture bleached *Scendesmus* sp. Pakistan journal of biological science, 8(3): 470-474.

**Ericson, H.; Thorsén, G. and Kumblad, L.** (2010). Physiological effects of diclofenac, ibuprofen and propranolol on Baltic Sea blue mussels. Aquat. Toxicol., 99: 223-231.

**Escapa, C.; Coimbra, R.N.; Paniagua, S.; García, A.I. and Otero, M.** (2016). Comparative assessment of diclofenac removal from water by different microalgae strains. Algal Res., 18: 127-134.

**Fareed, A.; Wahaab, R.; Mahmoud, T.; Souaya, E.R. and Hassan, A.** (2018). Occurrence, Sources, and Fate of Pharmaceuticals Products along Rosetta Nile River Branch. Middle East J. Appl. Sci., 8(3): 776-786.

**Fekadu, S.; Alemayehu, E.; Dewil, R. and Van der Bruggen, B.** (2019). Pharmaceuticals in freshwater aquatic environments: A comparison of the African and European challenge. Sci. Total Environ., 654: 324-337.

**Feyereisen, R.** (2006). Evolution of insect P450. Biochem. Soc. Trans., 34: 1252-1255.

**Gagné, F.; Blaise, C. and André, C.** (2006). Occurrence of pharmaceutical products in a municipal effluent and toxicity to rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Ecotoxicol. Environ. Saf., 64: 329-336.

**Gindler, E.M. and King, J.D.** (1972). Rapid colorimetric determination of calcium in biologic fluids with methylthymol blue. Am. J. Clin. Pathol., 58: 376-382.

**Gómez-Oliván, L.M.; Galar-Martínez, M.; García-Medina, S.; Valdés-Alanís, A.; Islas-Flores, H. and Neri-Cruz, N.** (2014). Genotoxic response and oxidative stress induced by diclofenac, ibuprofen, and naproxen in *Daphnia magna*. Drug and Chemical Toxicology, 37(4): 391-399.

**Gómez-Oliván, L.M.** (2020). Non-Steroidal Anti-Inflammatory Drugs in Water: Emerging Contaminants and Ecological Impact, The Handbook of Environmental Chemistry. Springer International Publishing, Cham.

**Gonzalez-Rey, M. and Bebianno, M.J.** (2011). Non-steroidal anti-inflammatory drug (NSAID) ibuprofen distresses antioxidant defense system in mussel *Mytilus galloprovincialis* gills. Aquat. Toxicol. Amst. Neth., 105: 264-269.

**Gonzalez-Rey, M. and Bebianno, M.J.** (2014). Effects of non-steroidal anti-inflammatory drug (NSAID) diclofenac exposure in mussel *Mytilus galloprovincialis*. Aquat. Toxicol. Amst. Neth., 148: 221-230.

**Guiloski, I.C.; Stein Piancini, L.D.; Dagostim, A.C.; de Morais Calado, S.L.; Fávaro, L.F.; Boschen, S.L.; Cestari, M.M.; da Cunha, C. and Silva de Assis, H.C.** (2017). Effects of environmentally relevant concentrations of the anti-inflammatory drug diclofenac in freshwater fish *Rhamdia quelen*. Ecotoxicol. Environ. Saf., 139: 291-300.

**Habig, W.H.; Pabst, M.J. and Jakoby, W.B.** (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem., 249: 7130-7139.

**Halliwell, B. and Gutteridge, J.M.C.** (2015). Antioxidants from the diet, in: Halliwell, B.; Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. Oxford University Press.

**Heckmann, L.-H.; Sibly, R.M.; Timmermans, M.J. and Callaghan, A.** (2008). Outlining eicosanoid biosynthesis in the crustacean *Daphnia*. Front. Zool., 5: 1-9.

**Henry, R.J.; Chiamori, N.; Golub, O.J. and Berkman, S.** (1960). Revised spectrophotometric methods for the determination of glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and lactic acid dehydrogenase. Am. J. Clin. Pathol., 34: 381-398.

**Huie, R.E. and Padmaja, S.** (1993). The reaction of no with superoxide. Free Radic. Res. Commun., 18: 195-199.

**Ibrahim, A.M. and Khalil, M.T.** (2009). Freshwater crayfish in Egypt. Center of Res. and Stud. Of Natur. Protec. Ain Shams University, Egypt.

**Jaeschke, H.; Knight, T.R. and Bajt, M.L.** (2003). The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. Toxicol. Lett., 144: 279-288.

**Jaiswal, K. and Sarojini, R.** (1990). Histopathological lesion in the hepatopancreas of freshwater prawn *Macrobrachium kistnensis* induced by naphthalene poisoning. Environ. Pollut. Hlth. Hazards, Environmental series, III: 151-157.

**Jomova, K.; Raptova, R.; Alomar, S.Y.; Alwasel, S.H.; Nepovimova, E.; Kuca, K. and Valko, M.** (2023). Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. Arch. Toxicol., 97: 2499-2574.

**Karatepe, M.** (2004). Simultaneous determination of ascorbic acid and free malondialdehyde in human serum by HPLC-UV. LC-GC N. Am., 22: 362-365.

**Kazakova, J.; Fernández-Torres, R.; Ramos-Payán, M. and Bello-López, M.Á.** (2018). Multiresidue determination of 21 pharmaceuticals in crayfish (*Procambarus clarkii*) using enzymatic microwave-assisted liquid extraction and ultrahigh-performance liquid chromatography-triple quadrupole mass spectrometry analysis. J. Pharm. Biomed. Anal., 160: 144-151.

**Koracevic, D.; Koracevic, G.; Djordjevic, V.; Andrejevic, S. and Cosic, V.** (2001). Method for the measurement of antioxidant activity in human fluids. J. Clin. Pathol., 54: 356-361.

**Kumirska, J.** (2020). Special Issue "Pharmaceutical residues in the environment." Molecules, 25: 2941.

**Láng, J. and Kőhidai, L.** (2012). Effects of the aquatic contaminant human pharmaceuticals and their mixtures on the proliferation and migratory responses of the bioindicator freshwater ciliate *Tetrahymena*. Chemosphere, 89: 592-601.

**Laurenz, J.; Brendelberger, H. and Lehmann, K.** (2020). Effects of diclofenac on the embryonic development of freshwater crayfish. Int. Aquat. Res., 12: 255-265.

**Li, Q.; Wang, P.; Chen, L.; Gao, H. and Wu, L.** (2016). Acute toxicity and histopathological effects of naproxen in zebrafish (*Danio rerio*) early life stages. Environ. Sci. Pollut. Res. Int., 23: 18832-18841.

**Li, Y.; Chen, Y.-F.; Chen, P.; Min, M.; Zhou, W.; Martinez, B.; Zhu, J. and Ruan, R.** (2011a). Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresour. Technol., 102: 5138-5144.

**Li, Y.; Zhou, W.; Hu, B.; Min, M.; Chen, P. and Ruan, R.R.** (2011b). Integration of algae cultivation as biodiesel production feedstock with municipal wastewater treatment: strains screening and significance evaluation of environmental factors. Bioresour. Technol., 102: 10861-10867.

**Lohner, T.; Reash, R. and Williams, M.** (2001). Assessment of Tolerant Sunfish Populations (*Lepomis* sp.) Inhabiting Selenium-Laden Coal Ash Effluents. Ecotoxicol. Environ. Saf., 50: 217-224.

**Lorenzon, S.; Edomi, P.; Giulianini, P.G.; Mettulio, R. and Ferrero, E.A.** (2004). Variation of crustacean hyperglycemic hormone (cHH) level in the eyestalk and haemolymph of the shrimp *Palaemon elegans* following stress. J. Exp. Biol., 207: 4205-4213.

**Masubuchi, Y.; Nakayama, S. and Horie, T.** (2002). Role of mitochondrial permeability transition in diclofenac-induced hepatocyte injury in rats. Hepatol. Baltim. Md, 35: 544-551.

**Méndez-Arriaga, F.; Torres-Palma, R.A.; Pétrier, C.; Esplugas, S.; Gimenez, J. and Pulgarin, C.** (2008). Ultrasonic treatment of water contaminated with ibuprofen. Water Res., 42: 4243-4248.

**Miranda, M.A.; Morera, I.; Vargas, F.; Gómez-Lechón, M.J. and Castell, J.V.** (1991). In vitro assessment of the phototoxicity of anti-inflammatory 2-arylpropionic acids. Toxicol. In Vitro, 5: 451-455.

**Miranda, K.M.; Espey, M.G. and Wink, D.A.** (2001). A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide Biol. Chem., 5: 62-71.

**Nautiyal, P.; Subramanian, K. and Dastidar, M.** (2017). Experimental investigation on adsorption properties of biochar derived from algae biomass residue of biodiesel production. Environ. Process., 4: 179-193.

**Nunes, B.; Daniel, D.; Canelas, G.G.; Barros, J. and Correia, A.T.** (2020). Toxic effects of environmentally realistic concentrations of diclofenac in organisms from two distinct trophic levels, *Hediste diversicolor* and *Solea senegalensis*. Comp. Biochem. Physiol., 231: 108722.

**Nunes, B.; Leal, C.; Rodrigues, S. and Antunes, S.C.** (2018). Assessment of ecotoxicological effects of ciprofloxacin in *Daphnia magna*: life-history traits, biochemical and genotoxic effects. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res., 2017: 835-844.

**Oviedo-Gómez, D.G.C.; Galar-Martínez, M.; García-Medina, S.; Razo-Estrada, C. and Gómez-Oliván, L.M.** (2010). Diclofenac-enriched artificial sediment induces oxidative stress in *Hyalella azteca*. Environ. Toxicol. Pharmacol., 29: 39-43.

**Parolini, M.** (2020). Toxicity of the non-steroidal anti-Inflammatory drugs (NSAIDs) acetylsalicylic acid, paracetamol, diclofenac, ibuprofen, and naproxen towards freshwater invertebrates: A review. Sci. Total Environ., 740: 140043.

**Paul-Clark, M.J.; van Cao, T.; Moradi-Bidhendi, N.; Cooper, D. and Gilroy, D.W.** (2004). 15-epi-lipoxin A4–mediated induction of nitric oxide explains how aspirin inhibits acute inflammation. J. Exp. Med., 200: 69-78.

**Peng, Z.; Wu, F. and Deng, N.** (2006). Photodegradation of bisphenol A in simulated lake water containing algae, humic acid and ferric ions. Environ. Pollut. Barking Essex 1987, 144: 840-846.

**Praskova, E.; Voslarova, E.; Siroka, Z.; Plhalova, L.; Macova, S.; Maršálek, P.; Pistekova, V. and Svobodova, Z.** (2011). Assessment of diclofenac LC50 reference values in juvenile and embryonic stages of the zebrafish (Danio rerio). Pol. J. Vet. Sci., 14: 545-549.

**Quinn, B.; Schmidt, W.; O'Rourke, K. and Hernan, R.** (2011). Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (*Dreissena polymorpha*) and their comparison with standardised toxicity tests. Chemosphere, 84: 657-663.

**Rakaiby, M.E.; Essam, T. and Hashem, A.** (2012). Isolation and characterization of relevant algal and bacterial strains from Egyptian environment for potential use in photosynthetically aerated wastewater treatment. J. Bioremediat. Biodegrad., S8: 1-5.

**Reitman, S. and Frankel, S.** (1957). A Colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. American Journal of Clinical Pathology, 28: 56-63.

**Rodriguez, I.; Rodriguez, C.; Alfonso, A.; Otero, P.; Meyer, T.; Breitenbach, U. and Botana, L.M.** (2014). Toxin profile in samples collected in fresh and brackish water in Germany. Toxicon Off. J. Int. Soc. Toxicology, 91: 35-44.

**Roncero, V.; Durán, E.; Soler, F.; Masot, J. and Gómez, L.** (1992). Morphometric, structural, and ultrastructural studies of tench (*Tinca tinca* L.) hepatocytes after copper sulfate administration. Environ. Res., 57: 45-58.

**Rowley, A.F.; Vogan, C.L.; Taylor, G.W. and Clare, A.S.** (2005). Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. J. Exp. Biol., 208: 3-14.

**Salgueiro-Pagadigorria, C.L.; Kelmer-Bracht, A.M.; Bracht, A. and Ishii-Iwamoto, E.L.** (2004). Naproxen affects Ca2+ fluxes in mitochondria, microsomes and plasma membrane vesicles. Chem. Biol. Interact., 147: 49-63.

**Santos, E.A.; Nery, L.E.; Keller, R. and Gonçalves, A.A.** (1997). Evidence for the involvement of the crustacean hyperglycemic hormone in the regulation of lipid metabolism. Physiol. Zool., 70: 415-420.

**Santos, J.L.; Aparicio, I. and Alonso, E.** (2007). Occurrence and risk assessment of pharmaceutically active compounds in wastewater treatment plants. A case study: Seville city (Spain). Environ. Int., 33: 596-601.

**Schmidt, W.; O'Rourke, K.; Hernan, R. and Quinn, B.** (2011). Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus* spp.) and their comparison with standardized toxicity tests. Mar. Pollut. Bull., 62: 1389-1395.

**Sheir, S.; Osman, G.; Galal, M. and Soliman, M.** (2015). Biohazards of the biofungicide, Trichoderma harzianum on the crayfish, *Procambarus clarkii*: Histological and biochemical implications. Egypt. J. Basic Appl. Sci., 2(2): 87-97.

**Sirisha, T.; Gurupadayya, B. and Sridhar, S.** (2014). Simultaneous determination of ciprofloxacin and tinidazole in tablet dosage form by reverse phase high performance liquid chromatography. Trop. J. Pharm. Res., 13: 981-987.

**Stainer, R.Y.; Kunisawa, R.; Mandel, M. and Cohin-Bazire, G.** (1971). Purification and properties of unicellular blue-green algae (order Chrococcales). Bacteriol. Rev., 35: 171-205.

**Sutherland, D.L. and Ralph, P.J.** (2019). Microalgal bioremediation of emerging contaminants - Opportunities and challenges. Water Res., 164: 114921.

**Tang, H.-Y.; Ho, H.-Y.; Wu, P.-R.; Chen, S.-H.; Kuypers, F.A.; Cheng, M.-L. and Chiu, D.T.-Y.** (2015). Inability to maintain GSH pool in G6PD-deficient red cells causes futile AMPK activation and irreversible metabolic disturbance. Antioxid. Redox Signal., 22: 744-759.

**Ternes, T.A.** (1998). Occurrence of drugs in German sewage treatment plants and rivers1Dedicated to Professor Dr. Klaus Haberer on the occasion of his 70th birthday.1. Water Res., 32: 3245-3260.

**Togola, A. and Budzinski, H.** (2008). Multi-residue analysis of pharmaceutical compounds in aqueous samples. J. Chromatogr. A, 1177: 150-158.

**Trinder, P.** (1969). Enzymatic determination of glucose in blood serum. Annals of Clinical Biochemistry, 6: 24.

**Trombini, C.; Kazacova, J.; Montilla-López, A.; Fernández-Cisnal, R.; Hampel, M.; Fernández-Torres, R.; Bello-López, M.; Abril, N. and Blasco, J.** (2021). Assessment of pharmaceutical mixture (ibuprofen, ciprofloxacin and flumequine) effects to the crayfish *Procambarus clarkii*: A multilevel analysis (biochemical, transcriptional, and proteomic approaches). Environ. Res., 200: 111396.

**Vulliet, E. and Cren-Olivé, C.** (2011). Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. Environ. Pollut. Barking Essex 1987, 159: 2929-2934.

**Wang, C.; Shi, H.; Adams, C.D.; Gamagedara, S.; Stayton, I.; Timmons, T. and Ma, Y.** (2011). Investigation of pharmaceuticals in Missouri natural and drinking water using high performance liquid chromatography-tandem mass spectrometry. Water Res., 45: 1818-1828.

**Wang, L.; Chen, H.; Xu, J.; Xu, O.; Wang, M.; Zhao, D.; Wang, L. and Song, L.** (2017). Crustacean hyperglycemic hormones directly modulate the immune response of hemocytes in shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol., 62: 164-174.

**Wang, L.; Ying, G.-G.; Zhao, J.-L.; Yang, X.-B.; Chen, F.; Tao, R.; Liu, S. and Zhou, L.-J.** (2010). Occurrence and risk assessment of acidic pharmaceuticals in the Yellow River, Hai River and Liao River of north China. Sci. Total Environ., 408: 3139-3147.

**Wang, Y.; Ho, S.-H.; Cheng, C.-L.; Guo, W.-Q.; Nagarajan, D.; Ren, N.-Q.; Lee, D.-J. and Chang, J.-S.** (2016). Perspectives on the feasibility of using microalgae for industrial wastewater treatment. Bioresour. Technol., 222: 485-497.

**Xiong, J.-Q.; Kurade, M.B. and Jeon, B.-H.** (2018). Can microalgae remove pharmaceutical contaminants from water?. Trends Biotechnol., 36: 30-44.

**Yu, C.-P. and Chu, K.-H.** (2009). Occurrence of pharmaceuticals and personal care products along the West Prong Little Pigeon River in east Tennessee, USA. Chemosphere, 75: 1281-1286.

**Yu, S.-H.; Zhu, K.-Y.; Zhang, F.; Wang, J.; Yuan, H.; Chen, Y.; Jin, Y.; Dong, M.; Wang, L.; Jia, X.-E.; Gao, L.; Dong, Z.-W.; Ren, C.-G.; Chen, L.-T.; Huang, Q.-H.; Deng, M.; Zon, L.I.; Zhou, Y.; Zhu, J.; Xu, P.-F. and Liu, T.-X.** (2018). The histone demethylase Jmjd3 regulates zebrafish myeloid development by promoting spi1 expression. Biochim. Biophys. Acta BBA - Gene Regul. Mech., 1861: 106-116.

**Zárate, A.M.; Florez, J.Z.; Angulo, E.; Varela-Prieto, L.; Infante, C.; Barrios, F.; Barraza, B.; Gallardo, D.I. and Valdés, J.** (2017). *Burkholderia tropica* as a potential microalgal growth-promoting bacterium in the biosorption of mercury from aqueous solutions. J. Microbiol. Biotechnol., 27: 1138-1149.

**Zhang, Y.; Sun, K.; Li, Z.; Chai, X.; Fu, X.; Kholodkevich, S.; Kuznetsova, T.; Chen, C. and Ren, N.** (2021). Effescts of acute diclofenac exposure on intestinal histology, antioxidant defense, and microbiota in freshwater crayfish (*Procambarus clarkii*). Chemosphere, 263: 128-130.

**Zhou, D.; Qiao, B.; Li, G.; Xue, S. and Yin, J.** (2017). Continuous production of biodiesel from microalgae by extraction coupling with transesterification under supercritical conditions. Bioresour. Technol., 238: 609-615.