Biochemical and Histopathological Impacts of NSAID s on the Hepatopancreas of the Freshwater Crayfish Procambarus clarkii, and Possible Treatment with Chlorella vulgaris

Hanan Ahmed El-Rouby1, Elham Hassan Ahmed Ali2, Magdy Tawfik Khalil2, Asmaa Ali Baioumy Ali2*  
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: Asmaali86@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 alga. 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 (β-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 (Procamburus 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 toxicologic 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_{2}NO_{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°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°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, Elmansorua, 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.

![Sampling Sites along the River Nile](image)

**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 7.4 min, 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 aerated 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 26.8-34.5 g weight). At the beginning of the experiment, crayfish were divided into 9 groups in triplicate plastic tanks for every group in which one tank contains 8 crayfish, then acclimated in aerated, de-chlorinated freshwater 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°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 spectrophotometrically using 1-chloro-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≤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, Elmansoruiia, 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).

<table>
<thead>
<tr>
<th>Place</th>
<th>Alrahaway</th>
<th>Nakluh</th>
<th>Elmansoruia</th>
<th>Biba city</th>
<th>Bartas</th>
<th>Zat-Alcom</th>
<th>Kafar-Hakium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen</td>
<td>ND</td>
<td>ND</td>
<td>0.073</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.106</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>0.0022</td>
<td>ND</td>
<td>0.0194</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.0157</td>
</tr>
<tr>
<td>Naproxen</td>
<td>0.0719</td>
<td>1.9367</td>
<td>0.2031</td>
<td>0.1363</td>
<td>0.4948</td>
<td>0.6727</td>
<td>0.3541</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>ND</td>
<td>ND</td>
<td>0.1624</td>
<td>0.0152</td>
<td>0.2272</td>
<td>0.5065</td>
<td>0.2343</td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.0059</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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.

![Total antioxidant activity graph](image)

**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.

![Nitric oxide graph](image)

**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.

![Graph showing GST activity](image)

**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.

<table>
<thead>
<tr>
<th>Group</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.50±0.73a</td>
<td>0.17±0.00a</td>
<td>3.31±0.13a</td>
</tr>
<tr>
<td>Ibuprofen (low)</td>
<td>33.43±1.40b</td>
<td>0.24±0.04bcd</td>
<td>5.63±0.20d</td>
</tr>
<tr>
<td>Ibuprofen (high)</td>
<td>47.87±1.27c</td>
<td>0.39±0.03e</td>
<td>7.53±0.10f</td>
</tr>
<tr>
<td>Diclofenac (low)</td>
<td>35.64±1.52b</td>
<td>0.28±0.01ed</td>
<td>8.22±0.30g</td>
</tr>
<tr>
<td>Diclofenac (high)</td>
<td>26.67±3.70a</td>
<td>0.30±0.02d</td>
<td>10.13±0.19h</td>
</tr>
<tr>
<td>Naproxen (low)</td>
<td>26.71±1.08a</td>
<td>0.21±0.02abc</td>
<td>5.88±0.26de</td>
</tr>
<tr>
<td>Naproxen (high)</td>
<td>53.89±0.90d</td>
<td>0.27±0.02cd</td>
<td>6.47±0.31e</td>
</tr>
<tr>
<td>Mixed (low doses)</td>
<td>48.72±1.74c</td>
<td>0.25±0.02bcd</td>
<td>4.38±0.19c</td>
</tr>
<tr>
<td>Mixed (low+algae)</td>
<td>56.88±1.23d</td>
<td>0.18±0.03ab</td>
<td>2.63±0.10b</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (mg/g)</th>
<th>Total Proteins (mg/g)</th>
<th>Calcium (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.75±0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.46±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.14±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ibuprofen (low)</td>
<td>3.31±0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.35±0.02&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.03±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ibuprofen (high)</td>
<td>1.48±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.32±0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.06±0.01&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diclofenac (low)</td>
<td>2.57±0.12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.48±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07±0.01&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diclofenac (high)</td>
<td>1.39±0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.38±0.02&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>0.04±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Naproxen (low)</td>
<td>2.83±0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.42±0.02&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.03±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Naproxen (high)</td>
<td>2.55±0.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.33±0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.08±0.03&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mixed (low doses)</td>
<td>1.41±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.34±0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.10±0.01&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mixed (low+algae)</td>
<td>4.57±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.45±0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.06±0.01&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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, doxycycline 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 (Fe3) O2] - during NSAID biotransformation, resulting in the release of superoxide anion (O2) by uncoupling of this reaction (Doi et al., 2002). Because NSAIDs affect the mitochondrion and thus oxidative phosphorylation, increased ROS production, especially of O2, 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-Martinez 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-Martinez 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. clarkii* 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 (*Chlorella*) 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**


