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Expression of *Cyp1a* and *p53* Induced by Water-Soluble Fractions of Crude Oil in Juveniles of *Clarias gariepinus* (Burchell, 1822).

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Abstract

Oil spills, like most other sources of pollutants, have been shown to disrupt biochemical pathways and cause the death of organisms. In this study, the relative expression of *Cyp1a* and *p53* genes in juveniles of *Clarias gariepinus* was determined after exposure to sub-lethal concentrations of water-soluble fractions of crude oil. Fish (5.1 ± 0.3 g) were exposed to 4 sublethal concentrations (30, 45, 60, and 75% of the LC_{50} corresponding to 67, 101, 135, and 169 mg/L respectively) of the oil and a control. After 90 days of exposure, fish were sacrificed, tissue collected, and used for gene expression analysis. Total RNA was extracted using a modified CTAB extraction protocol. Gene quantification and amplification were carried out using standard methods. The *B-actin* gene was used as an internal control. The results revealed that the relative expression of *Cyp1a* mRNA increased in a dose-dependent manner, indicating the upregulation of *Cyp1a.* In contrast to *Cyp1a*, crude oil modulated the expression of p53, but the expression pattern did not vary in a dose-dependent manner. The expression of the *Cyp1a* gene can therefore be used as an important factor for assessing the toxicity of crude oil, especially at sub-lethal concentrations in fish species.

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Introduction

Oil pollution is the introduction of liquid petroleum hydrocarbons into the environment, primarily from human activities, and is a major environmental concern (Adesanmi *et al.*, 2021). In Nigeria, the majority of the oil pollution occurs as a result of shipping and terrestrial traffic accidents, improper disposal of drilling muds, depot leakage, tank washing and oil ballast discharges, failure or rupture in oil pipelines, and in the process of oil theft or artisanal refining (bunkering) of crude oil (Ayuba, 2012; Kadafa *et al.*, 2012). This can have cascading effects on the entire ecosystem and reduction of biodiversity (Eriegha and Sam, 2020). Studies have shown that oil pollution can lead to the death of fish, birds, and marine mammals due to direct toxicity or smothering effects. It can also disrupt important biochemical pathways, such as oxidative stress and detoxification mechanisms, leading to cellular damage and impaired physiological functions (Eriegha *et al.*, 2019; Eriegha, 2023; Eriegha *et al.*, 2023). Additionally, exposure to oil can cause DNA damage and chromosomal abnormalities, affecting the development and growth of organisms, including embryos and larvae (Santos *et al.*, 2016; Ardeshir *et al.*, 2018). Oil spills can also contaminate water sources, making them unsafe for human use and damaging the fishing and tourism industries, which are often important sources of income for local communities (Andrews *et al.*, 2021).

Recent advances in bioassay have expanded beyond traditional endpoints (such as enzymatic assays and protein quantification) to include gene expression studies. While enzymatic assays and protein quantification provide valuable information on the biochemical effects of toxic substances, gene expression studies offer insights into the molecular mechanisms underlying toxicity. Several studies have demonstrated the effectiveness of gene expression studies in toxicology using RT-qPCR (Piña *et al.*, 2007; Chivittz *et al.*, 2016; Santos *et al.*, 2018). By evaluating mRNA levels using techniques like RT-qPCR, researchers can identify specific genes that are upregulated or downregulated in response to exposure. This information can help identify potential biomarkers for toxicity and provide a deeper

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understanding of the biological processes affected by toxic substances. The RT-QPCR is an effective tool for gene expression studies due to the accuracy, sensitivity and speed of results (Derveaux *et al*., 2010).

The expression of genes such as Cytochromes P4501A (CYP1A) and p53, which plays a major role in the biotransformation of a variety of endogenous substances such as steroids, lipids, and vitamins, as well as environmental toxicants, is a veritable instrument for establishing the dynamics of the environment on cellular expression patterns (Kassahn *et al*., 2007; Ardeshir *et al*., 2018). The induction of *Cyp1a* by the various xenobiotics has led to its use as a biomarker in fish for assessing the level of contamination in an aquatic environment (Cousinou *et al*., 2000; Hassanin *et al*., 2009; Uno *et al*., 2012). The p53 gene, also known as tumour protein, encodes a protein that regulates the cell cycle and so works as a tumor suppressor. Tumor suppression is a critical process in multicellular organisms to prevent uncontrolled cell proliferation and the development of cancer (Vogelstein *et al*., 2000). As a transcription factor, p53 regulates the transcription of various genes involved in critical cellular processes. For example, it activates the transcription of gadd45, p21, and cyclinG1, which are genes responsible for cell cycle arrest. By upregulating these genes, p53 halts the progression of the cell cycle, allowing time for DNA repair or apoptosis if the damage is too severe (Speidel, 2015). Additionally, p53 also regulates the transcription of genes involved in DNA repair, such as DDB2 and XPC, ensuring the proper maintenance of the genome. Furthermore, p53 can induce apoptosis by activating the transcription of genes like bax and bcl-2, which play crucial roles in programmed cell death. Understanding the transcriptional regulatory functions of p53 provides insights into how it controls key cellular processes to maintain cellular integrity (Liebermann and Hoffman, 2008).

Fish are good indicators of water contamination in aquatic systems for several reasons. Firstly, they occupy different trophic levels, and therefore represent different positions in the food chain. This is important because contaminants can accumulate and biomagnify as they move up the food chain, making higher trophic level organisms more likely to show signs of contamination (Odiete, 2003). Additionally, fish are more sensitive to many toxicants compared to invertebrates, making them more likely to exhibit visible effects of contamination (Raimondo *et al*., 2007). Lastly, fish have the ability to accumulate contaminants in their tissues over time, providing a record of exposure to specific pollutants (Hermoso *et al*., 2010).

Clarias gariepinus is widely regarded as one of the most significant tropical catfish species for aquaculture, and is believed to have met all the attributes outlined for an ideal biosensor of environmental pollutants. These attributes may include the species' sensitivity to pollutants, its ability to accumulate contaminants in its tissues, and its responsiveness to changes in environmental conditions. This species has already been used in laboratory investigations and is a suitable organism for assessing the impact of xenobiotics (Olaifa, 2012; Obemeata *et al*., 2012). Several studies have shown that sublethal exposure to contaminants can lead to physiological and behavioral changes in fish. However, much is still unclear regarding the consequences of low-level exposure on organisms, particularly at the molecular level. Sublethal effects are essential for predicting habitat and fish population impacts accurately, since many more organisms are likely to be exposed to sublethal concentrations of contaminants than to lethal concentrations as a spill disperses, and many may experience chronic low levels of one or more contaminants for a long time. Hence, this study aimed to determine the relative expression of *Cyp1a* and p53 induction in juveniles of *C. gariepinus* after exposure to water soluble fractions of crude oil. The findings of this study will provide valuable insights into the potential impact of crude oil exposure on the genetic expression.

Materials and Methods

Water-Soluble Fractions of Crude oil

Crude oil was obtained from the Afiesere oil field, was transported to the Department of Chemistry of the University of Ibadan where water soluble fraction (WSF) of the oil was prepared using Anderson *et al*. (1974) with little modifications. Briefly, 1 ml oil was mixed with 9 ml of freshwater. The mixture was slowly stirred with a magnetic stirrer for a period of 20 h at room temperature. The bottle was capped with aluminum foil to minimize evaporation of the more volatile oil hydrocarbons. After mixing, the oil and water phases were allowed to separate for to 6 h before the water phase was siphoned off, and utilized in experiments. Different concentrations of the WSF were prepared by diluting the stock WSF with water.

Experimental Fish

Healthy juveniles of *Clarias gariepinus* (average weight = 5.1 ± 0.3 g) with a good physical appearance were purchased from Elivital fish farm, Isiokolo and transported to the laboratory of the Department of Fisheries and Aquaculture of the Nigeria Maritime University, Okerenkoko in oxygen bags. Health status of the fish was ascertained based on the absence of lesions and other morphological diagnostic symptoms as suggested by Wedemeyer (1997). The experimental fish were first kept in the laboratory for 2 weeks in natural photo-regime conditions in 30 L experimental tanks containing tap water. The juveniles of *C. gariepinus* were randomly allocated to triplicate groups with 20 fish per tank. To prevent the fish from escaping, the experimental tanks were fitted with a lid constructed of fine polyethylene gauze screen with a mesh size of 1mm. This type of lid also allowed for proper ventilation within the tanks and ensured that no unwanted materials entered the tanks, and thereby maintaining a clean and controlled environment for the experiments. They were fed with a commercial feed, Coppens® *ad libitum* two times a day during the acclimatization period.

Preliminary Bioassay

A preliminary toxicity test was conducted in a static exposure system to determine the lethal concentration 50 (LC_{50}). Specimens of *C. gariepinus* (n = 20 per tank) were randomly allocated in 15 litres of the test solution. The following concentrations of WSF of crude oil were used for the definitive (acute) test: 0, 100, 180, 320, and 576 mg/L. All treatments and control were conducted in triplicate. The set up was not aerated and fish mortality was documented every 24 h for 96 h. Fish were considered dead when they remained immobile on the bottom of the tank or suspended in the water column even after mechanical stimuli. Dead fish were removed immediately in other to avoid contamination of experimental setup. Mortalities recorded were expressed as percentages of the test populations and the median lethal concentration (LC_{50}) values was calculated by using regression equation method of probit analysis (Sakuma, 1998).

Exposure Assessment

The juveniles of *C. gariepinus* were treated with four different sub-lethal doses (30, 45, 60 and 75% of the LC_{50} (267 mg/L) corresponding to 67, 101, 135, and 169mg/l respectively) of WSF of crude oil and a control group. The test was carried out utilizing a semi-static renewal method in which the exposure medium was refreshed every three days to preserve the toxicant's strength while reducing the level of ammonia. The experimental fish were fed with a commercial feed, Coppens® *ad libitum* two times a day. After 90 days of treatment, fish were sacrificed, tissue collected and transported to the Biorepository and Clinical Virology Laboratory of the University College, Ibadan, Nigeria for gene expression analysis.

Analysis of *Cyp1a* and *p53* Gene Expression

RNA was isolated using an RNA extraction kit (YT9065, Yekta, Iran) following the manufacturer's instructions with little modification based on Santos *et al*. (2018). Briefly, fish tissue was thawed and approximately 100 mg of fish tissue was ground and mixed in nuclease-free tubes with 350 µL of RB buffer and 3.5 µL of beta-mercaptoethanol. The mixture was then passed through a filter column and centrifuged at 18,000g for 2 minutes. The supernatant was combined with 70% ethanol, passed through an RB mini-column, and centrifuged for 1 minute. To remove DNA contamination, 250 µL of washing buffer 1 was added to the column and centrifuge for 1 minute. The column membrane was then subjected to 60 µL of RNase-free DNase I solution (Roche) for 15 minutes. After cleaning and drying the column twice, ddH2O was added to it and centrifuged for one minute. To control the quality of extracted RNA, 2 µL of loading buffer and 5 µL of the RNA were loaded on 1% electrophoresis gel and electrophoresed for 30 min (85 V). A Nanodrop spectrophotometer (Thermo Scientific®) was used to measure the RNA concentration and purity. The absorbance of the RNA sample was measured at wavelengths of 230 nm, 260 nm, and 280 nm. cDNA synthesis was achieved using a commercially available kit (Thermo Scientific®). RiboLock RNase Inhibitor (1 µL) was applied after mixing and centrifuging random hexamer primer and RNA at 65 °C for 5 minutes (on ice). Then, RevertAid RT (1 µL), Reaction Buffer (4 µL), and dNTP (2 µL) were added. The reaction took place in a thermocycler (Biorad®) for 5 minutes at 25°C, 1 hour at 42°C, 5 minutes at 70°C, and an indefinite time at 20°C.

To design specific qPCR primers for the specific *Clarias gariepinus* genes, mRNA reference sequences of

individual genes were obtained from the genebank database on the NCBI website (National Center for Biotechnology Information) site was then accessed and sequence pasted in the sequence entry box and multiple intercalating dye PCR primers were generated. It is very necessary to ensure that the primers will have a perfect match, this will enhance primer annealing during PCR. To do this, primers must anneal to regions where the sequences are conserved. Each primer pair was then checked for specificity to be sensitive to only the genes of interest to which it was designed to detect and also the ability to cut across all aligned genes then the best primer was selected and synthesized at Inqaba Biotech Laboratory in South Africa. The forward and reverse sequences of designed primers are presented in Table 1.

For the treatment of RNA, 20 ng total RNA was treated with NEB DNase 1 (M0303) to eliminate extracted DNA. A mixture of 2µl of 10ng/ µl RNA, 10 µl DNase I Reaction Buffer (10X), 1 µl DNAse I (RNase-free) and up to 100 µl with Nuclease-free H_2O. After 10 minutes of incubation at 37°C, 1 µl of 0.5 M EDTA (to a final concentration of 5 mM) was added. The mixture was then heat-inactivated at 75°C for 10 minutes before being stored at -20°C until use. Gene quantification was done following the Luna® Universal qPCR Master Mix Protocol (M3003). This was also used to detect the presence of mRNA genes in the extracted RNA. Expression of the B-actin gene was used as an internal control. A mix of 10 µl Luna Universal qPCR Master Mix, 0.5 µl Forward primer (10 µM), 0.5 µl Reverse primer (10 µM) and 0.06 Reverse Transcriptase (Promega) made up to 18 µl with Nuclease-free Water to which 2 µl of the treated RNA Template was added. The mixture was run with the profile initial denaturation at 95°C for 60 seconds followed by 40-45 of Denaturation at 95°C 15 seconds extension and plate reading at 60°C for 30 seconds and thereafter a termination at 72°C for 10 minutes. Amplification was conducted using the cfx96tm real-time system from bio-rad following the manufacturer's manual. The relative expression of *Cyp1a* and *p53* genes was computed using the comparative CT ($2^{-\Delta\Delta CT}$) method of Pfaffl *et al*. (2002).

Results and Discussion

The result of the quality assessment from the Nanodrop spectrophotometer is presented in Table 2. The quantity assessment of RNA from the Nanodrop spectrophotometer showed that A260/280 ranged from 1.40 – 1.68 and A260/230 ranged from 1.62 – 1.92, implying suitable purity of the RNA.

The result of relative *Cyp1a* and *p53* mRNA expression in the tissue of *C. gariepinus* is presented in Figure 1. The result revealed that the relative expression of *Cyp1a* increased (upregulation) in a dose-dependent manner. The expression level increased as the concentration of the water-soluble fractions of crude oil increased. The control group had the least (3.39) while the highest relative expression (21.37) was observed in the group containing the highest concentrations of the toxicant. The upregulation of *Cyp1a* is crucial for xenobiotic metabolism and an indication of the activation of the aryl hydrocarbon receptor (AHR) pathway. This is because, polycyclic aromatic hydrocarbons (considered the most toxic component of crude oil) metabolism is mediated by the AHR, a ligand-binding transcription factor with a high affinity for PAH and polychlorinated biphenyl ligands (Billiard *et al*. 2002). Many genes in the AHR gene battery such as Cyp1a are involved in xenobiotic clearance (Dalton *et al*., 2002; Jones, 2019). Induction of Cyp1a transcription is such a ubiquitous action of AHR activation that its upregulation is considered a classic biomarker of contaminant exposure. *Cyp1a* is a well-known biomarker of exposure to PAH (Lee and Anderson, 2005). Hook *et al*. (2010) have reported the upregulation of *Cyp1a* by crude oil, heavy fuel oil, and gas oil, but not by kerosene. Because kerosene does not contain substantial concentrations of high molecular weight PAH. Sustained activation of the Cyp1a gene was predictive of persistent exposure to sublethal concentrations of crude oil components and negative population-level impacts in fish following the *Exxon Valdez* oil spill (Whitehead *et al*., 2012).

The findings of this study were in agreement with Sørhus *et al*. (2021) who reported a stable expression of *Cyp1a* in embryonic development in *Melanogrammus aeglefinus* after exposure to crude oil toxicity. They however reported a dynamic temporal expression of Cyp1b, Cyp1c, and Cyp1d indicating that these enzymes are actively involved in the metabolism of xenobiotics. Specifically, they are responsible for the biotransformation of these foreign compounds, converting them into more soluble forms that can be easily excreted. Upregulation in *Cyp1a* expression has also been reported in southern flounder (*Paralichthys lethostigma*) after exposure to crude oil. Elevated expression of *Cyp1a* is indicative of hydrocarbon

metabolism and is a commonly used biomarker for exposure to oil (Bilbao *et al*., 2010).

Crude oil exposure has been shown to trigger gene pathways involved in biotransformation such as *cyp1a* and *cyp1c1* mRNA expression, lipid metabolism, stress response, and ion regulation in early life stages of Atlantic haddock (Sørhus *et al.* 2017). Aranguren-Abadía *et al*. (2020) have also reported the expression of *Cyp1a* in the cardiac tissues, skin, and the developing liver of *Gadus morhua* at 8 and 10 days' post fertilization. *Cyp1a* also been shown to be protective and necessary for detoxification (Wincent *et al*., 2015) and may also result in the production of some reactive toxic metabolites. Nebert *et al*. (2004) have also noted other enzymes that can produce metabolites and increase the toxicity. Myocardial *Cyp1a* induction was observed in *Clupea pallasii* exposed to crude oil (Incardona *et al*., 2019).

The results of this study showed that water-soluble fractions of crude oil modulated the expression of p53 but the expression pattern did not vary in a dose-dependent manner (Figure 1). The lowest relative expression level (1.88) of p53 was observed in the group containing no crude oil while the highest relative expression (8.20) was observed in the group containing 45% of the LC_{50}. The influence of crude oil on the expression of the p53 gene can vary depending on the specific circumstances and exposure conditions. Studies have revealed that exposure to crude oil or its components can affect the expression of genes involved in various cellular processes, including those related to DNA repair and cell cycle regulation, such as the p53 gene. However, the exact mechanisms and outcomes of crude oil exposure on p53 gene expression can be complex and context-dependent. Conflicting results showing both upregulation and downregulation (decreased expression) of p53 in response to exposure to xenobiotics have been reported by many authors (Liu *et al*., 2011). The specific composition of the crude oil, the concentration and duration of exposure, and the cell or tissue type being studied can all influence the observed effects on p53 gene expression. Mitra *et al*. (2018) have reported the down-regulation of p53 in *Rita rita* from a polluted riverine environment. Similar down-regulation of p53 has also been reported in Japanese medaka after 2 days of exposure to nonylphenol and bisphenol (Min *et al*., 2003). Similar findings have also been reported in adult hermaphroditic fish; Kryptolebias marmoratus on exposure to endocrine-disrupting chemicals (Lee *et al*., 2008). On the other hand, Willams and Hubberstey (2014) demonstrated upregulation of p53 in brown bullheads from contaminated regions, compared to clean sites. Similarly, up-regulation of p53 has been reported in different tissues of *Mytilus edulis* in response to environmental genotoxicants (Di *et al*., 2011). Studies have suggested that p53 might be activated in fish due to genotoxic stress (Rau-Embry *et al*., 2006).

Conclusion

Crude oil is a compound mixture of hydrocarbons that can have a significant impact on gene expression in fish. Exposure to crude oil can lead to the upregulation of genes involved in detoxification, stress response, and cell death. The effects of crude oil exposure on gene expression can be complex and long-lasting. These effects can lead to a variety of negative consequences, including reduced growth, impaired reproduction, and increased susceptibility to disease. A comparative assessment of the target genes revealed that the relative expression of *Cyp1a* was upregulated in a dose-dependent manner. Despite the modulation of expression in p53, there was no definite pattern, as expression did not vary in a dose-dependent manner. The study therefore recommends the use of *Cyp1a* as a suitable gene in the assessment of crude oil toxicity. Further research is needed to better understand the mechanisms of the regulation of the p53 gene.

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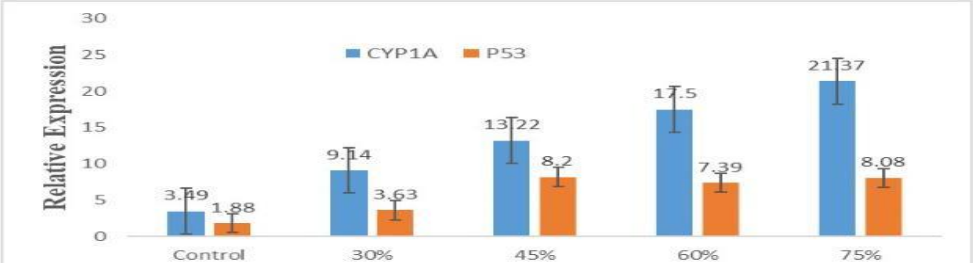

Figure 1: mRNA relative expression of *Cyp1a* and *p53* in the tissue of *C. gariepinus* after 90 days of exposure to WSFs of crude oil.

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Table 1. Gene-specific primers used for RT PCR analysis

Gene	Mechanisms	Forward primer sequence	Reverse primer sequence
CYP1A	Detoxifying	GAAACCCGGCTGACTTCATT	GCCTCTGCATGAACACGTTA
P53	Tumour suppressor	TATACTGGCCTGTACGCATTG	TCTTGATAGTGAATAGACGGTCTTC
b-actin	Endogenous control	TGATGAAATCGCCGCACT	CCACAATGGATGGGAAGACA

Table 2: Mean values of Nanodrop quantification

Sample group	Nucleic acid	A260 (Abs)	A280 (Abs)	260/280	260/230
Control	157.00 ng/µl	2.14	1.42	1.49	1.77
30% LC_{50}	255.55 ng/µl	5.11	3.63	1.68	1.92
45% LC_{50}	247.75 ng/µl	2.96	1.87	1.40	1.62
60% LC_{50}	182.00 ng/µl	3.64	2.31	1.58	1.77
75% LC_{50}	212.45 ng/µl	4.25	2.65	1.59	1.80