Molecular Integrity and Antioxidants Status of Nile Tilapia (*O. niloticus*) Exposed to *Indigofera tinctoria*

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Abstract

Pollution in aquatic ecosystems by various pollutants has been a concern in recent decades because of the devastating effect posed on the stability between the receiving water bodies and the biodiversity therein. *Indigofera tinctoria* has been used in a variety of fields, including health and medicine, as well as industrial applications such as textiles. The impacts of *I. tinctoria* on freshwater fishes, as well as the mechanisms underlying the biological effects, are, however, sparse. The goal of the study was to see how *I. tinctoria* affected Nile Tilapia (*O. niloticus*) juveniles' oxidative stress response, biochemical indicators, DNA disintegration, and protein profile. For 21 days, 6 varying concentrations were applied to the fish, including a control. The biochemical parameters in the test fish revealed a considerable variation in a dose-response manner. In *I. tinctoria* treated fish, the enzyme activity, when compared with control treatment, lipid peroxidation, DNA fragmentation, and oxidative stress all increased relative to the toxicant doses used. The protein profiling revealed a modification in the protein credentials in *I. tinctoria*-treated fish when compared to the control. As per the study, *I. tinctoria* is toxic to *O. niloticus*, particularly at higher doses. The results of this research could be applied to biomonitoring studies of *I. tinctorial* toxicity, particularly in industrial applications.

Keywords: *Indigofera tinctoria*, Oxidative stress, *Oreochromis niloticus*, SDS-PAGE, DNA fragmentation

Introduction

Freshwater environments are often affected by human activity and xenobiotic effects from agricultural runoff, industry, mining development, and direct release from floating sediments [1]. Such problems in aquatic ecosystems that are severely affected by uncontrolled emissions can occur in developing countries such as Nigeria [2,3]. Therefore, the pollution in aquatic ecosystems by various pollutants has been a concern in recent decades. Water contaminants devastate the biological harmony between the receiving water bodies and their biodiversity [4].

Emissions of pollutants into rivers, swamps and lakes have led to mass mortality of aquatic animals, especially fishes [5]. Inland waters, unlike oceans that have open ocean volume, make them fragile and limit their ability to dilute pollutants [1]. It has been observed that large amounts of untreated waste, including toxic substances, are discharged into water bodies an act that is predominant in developing countries, disrupting aquatic ecosystems and having some impact on aquatic life there [6]. The utilization of plants for various purposes has been identified by several authors, as a source of raw materials for pharmaceuticals, food, and other industries. Alagbe *et al*. [7] reported that there are more than 250,000 medicinal plants worldwide, of which 21,000 are listed and *I. tinctoria* is one of the lesser-known medicinal plants. Aside from the medicinal use of *I. tinctoria* and other *Indigofera* species contain precursors of indigo dye compounds. After soaking in lime-added water, it is fermented and enzymatically hydrolyzed to produce a blue mixture. It is then dried, cut into cubes, and placed in water for coloring cloth [8]. The indigo residue of organic fertilizer has been reported to be useful for crops except for hygienic applications [9] and there is no information on its usefulness or its harmful effects on aquatic life and habitats.

Waste from textile dyeing activities associated with *I. tinctoria* is released untreated into water bodies, endangering life [10]. It accomplishes this by causing a toxic effect via a biochemical process that results in the generation of free radicals (ROS). Immune cells employ ROS to execute their regular duties, but too much ROS can assault and harm the cell's components. Lipid peroxidation (LPO) according to Javed *et al*. and Ahmad and Ahmad [11,12] is frequently used as a bioindicator in the research area of fish health because it is the most noticeable result of ROS. Cells may suffer from oxidative damage when reactive

oxygen species (ROS) are present, consequently, the antioxidant and detoxifying enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and non-enzymatic glutathione reductase (GSH) help to shield cells from this harm. Fish have an advanced antioxidant system that makes them susceptible to oxidative stress by producing ROS [13]. Acetylcholinesterase (AChE) activity is often employed in aquatic animal research as a diagnostic for pollutant exposure [14]. Toxins, at low doses, can block AChE and promote acetylcholine buildup at cholinergic central synapses and nerve terminals [15].

Inhibiting AChE has the potential to influence the movements and homeostasis of exposed organisms [16]. Malondialdehyde (MDA) being a lipid peroxidation (LPO) bioconjugate, the activation of antioxidant enzymes and enhanced lipid peroxidation in contaminants exposed to fish are biological factors used in measuring oxidative stress [17]. The use of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis to determine protein profiles at acute exposure levels of different pollutants in response to stress is affirmed by Muhammad *et al.* [18], to be a promising technique. It is assured by Frenzill *et al.* [19] that DNA fragmentation (oxidative DNA damage test) is an effective technique for evaluating the genotoxicity of environmental pollutants. Pollutants were noted to cause fragmentation of DNA in *Mytilus galloprovosystemis* [20,21], also in Semelidae bivalve mollusks, [22] reported the same observation, and in *Neocaridina davidi* by [23,24].

One of the most significant freshwater finfish in aquaculture is *O. niloticus*. Many of the tilapia-inhabited areas are threatened by various contaminants, particularly botanical pollutants. This fish species is frequently utilized in research due to its rusticity and adaptability to confinement circumstances [25]. Because of their flexibility, fertility, and quick growth, they are often employed in aquaculture. This study intends to confirm the potentially harmful effects that *I. tinctoria* might have on aquatic species by using *O. niloticus* as a model animal and oxidative stress indicators.

Materials and methods

Exposure of fish to the toxicant

Handling of experimental animals was done in conformity with OECD and EPA norms as required during experimental operations [26]. The Ethical Committee of the Federal University of Technology, Akure, gave the approval to conduct the experiment prior to the experiment. Nile Tilapia (*O. niloticus*) juvenile (wt. = 34.35 ± 0.067 g, L = 5.28 ± 0.12 cm) live was used for this experiment. The physicochemical characteristics of test media were determined on a daily basis for conductivity, pH, dissolved oxygen, temperature, and TDS, while ammonia, nitrite, and nitrate were weekly).

The lower *I. tinctoria* concentrations used in the chronic test arrived from the acute test in the previous study, and the fish samples were grouped into 6 (20 fish per each) including the control group ((0, 13, 27, 40, 53, 67 mg L^{-1}) reared for 21 days. Serum from pool blood obtained from 6 fishes from each group through the caudal vein was used for enzyme activities and lipid peroxidation assays. The same set of fishes were used for AChE and DNA fragmentation assays, and SDS-PAGE using muscle from the same set of fish.

Sampling and enzyme assay

Criteria of Del Maestro and McDonald [27] for evaluating SOD were used, and the absorbance was determined kinetically at 420 nm for 3 min at 25 °C. Each unit's SOD content was specified in IU/L. The method of Lück [28] was used to assess CAT activity in cell lysates from the indigo-exposed fishes and the absorbance change rate per minute was measured at 240 nm. Moron *et al.* [29] method was used to estimate reduced glutathione levels in cell lysates. The optical density was quantified at 405 nm and GSH levels were quantified in grams of GSH per milligrams of protein. In order to measure the absorbance at 340 nm and determine the GPx level in fishes exposed to toxicants, the procedure described by Paglia and Valentine [30] was used. The quantity of AChE was calculated by the Ellman *et al.* [31] method, and absorbances were measured at 412 nm for 2 min in IU/L. The method described by Bergmeyer *et al.* [32] was applied in ALP activity determination. The ALP activity was measured in IU/L. The MDA was measured using the Ohkawa *et al.* [33] method, and the values were given in nmol/mL of protein.

SDS-PAGE analysis and DNA fragmentation assay

Tilapia muscles (0.5 g) were suspended in lysing buffer (1.0 mL) and were steamed at 100 °C for 5 min, followed by centrifuging at 10,000 rev/min. The extracted protein treatment was used for protein analysis using SDS-PAGE and the low molecular weight standards (BioBasic, USA) were run simultaneously, and the protein molecular mass was determined using Gel-Pro Analyzer package V3.1 for Windows and the densitometric analysis of protein bands were estimated using Popoola and Udoh [34].

DNA fragmentation was determined according to Boraschi and Maurizi [35]. Spectrophotometric measurement was done at 575 nm to quantify the reaction. The ratio of DNA in the supernatant to total DNA was used to determine the percentage of fragmentation.

Data evaluation

Data were analysed in a descriptive manner using the appropriate statistics (mean, standard and scatter graph). The physicochemical features of the control and treatment groups were compared using 1-factor analysis (ANOVA) in SPSS version 21.

Results and discussion

Water quality parameters during 21 days of exposure of *O. niloticus* juvenile to *I. tinctoria* (Chronic concentration) revealed that temperature and pH were not statistically different ($P > 0.05$) across the varying concentrations including the control treatment. However, other parameters like DO, TDS, Conductivity, ammonia, nitrite, and nitrate, all show a significant variation ($P < 0.05$) within the treatment groups (**Table 1**).

The findings of this research show that the water quality parameters except pH and temperature were significantly altered by *I. tinctoria*. It has been reported that water quality largely regulates the distribution and productivity levels of aquatic organisms [36]. The observed significant variation in water quality may affect some biochemical processes and metabolic rates of the test organism [37].

Table 1 Water quality parameter during 21 days of exposure of *O. niloticus* juvenile to *I. tinctoria* (Chronic concentration).

Parameters	Concentration mgL^{-1}					
	Control	T1	T2	T3	T4	T5
T (°C)	25.27 ± 0.03^a	25.33 ± 0.09^a	25.37 ± 0.03^a	25.37 ± 0.03^a	25.30 ± 0.00^a	25.20 ± 0.06^a
pH	6.87 ± 0.02^a	6.66 ± 0.03^a	6.64 ± 0.03^a	6.60 ± 0.01^a	6.57 ± 0.02^a	6.54 ± 0.01^a
DO (mgL^{-1})	8.30 ± 0.44^d	8.10 ± 0.20^d	7.30 ± 0.12^c	7.50 ± 0.06^c	6.90 ± 0.00^b	5.80 ± 0.06^a
COND (μcm^{-1})	0.17 ± 0.00^a	0.20 ± 0.00^b	0.29 ± 0.00^b	0.31 ± 0.00^b	0.39 ± 0.00^c	0.41 ± 0.00^c
TDS (mgL^{-1})	116.67 ± 3.3^a	133.67 ± 3.33^b	133.33 ± 3.33^b	136.67 ± 3.33^c	136.33 ± 3.33^c	143.33 ± 3.33^d
NH$_3$+ (mgL^{-1})	0.00 ± 0.00^a	0.20 ± 0.00^b	0.50 ± 0.12^c	0.50 ± 0.01^c	1.00 ± 0.00^d	2.00 ± 0.00^e
NO$_2$- (mgL^{-1})	0.25 ± 0.02^a	0.25 ± 0.02^a	0.50 ± 0.02^b	1.00 ± 0.01^c	2.00 ± 0.00^d	4.00 ± 0.00^e
NO$_3$- (mgL^{-1})	2.50 ± 0.17^a	5.00 ± 0.17^b	10.00 ± 0.17^c	10.00 ± 0.09^c	20.00 ± 0.04^d	40.00 ± 0.02^e

Values are Mean ± SDEV, a row of letters with distinct letters in them indicates a difference. ($P < 0.05$).
(T; Temperature, DO; Dissolved oxygen, COND; Conductivity, TDS; Total Dissolved Solid, NH$_3^+$; Ammonia, NO$_2^-$ Nitrite, NO$_3^-$; Nitrate; a = control, T1 = 13 mgL^{-1}, T2 = 27 mgL^{-1}, T3 = 40 mgL^{-1}, T4 = 53 mgL^{-1} and T5 = 67 mgL^{-1}).

Measurement of antioxidants biomarkers

In comparison with control groups, the ALT level and other antioxidant enzymes (CAT, LDH, GPx, SOD, GSH and GST) were considerably elevated ($P < 0.05$) in the *I. tinctoria* exposed *O. niloticus* (**Figures 1(a)** - **1(c)**). Except for GSH (**Figure 1(c)**), the levels of enzyme activity assessed were statistically similar ($P > 0.05$) and concentration-dependent.
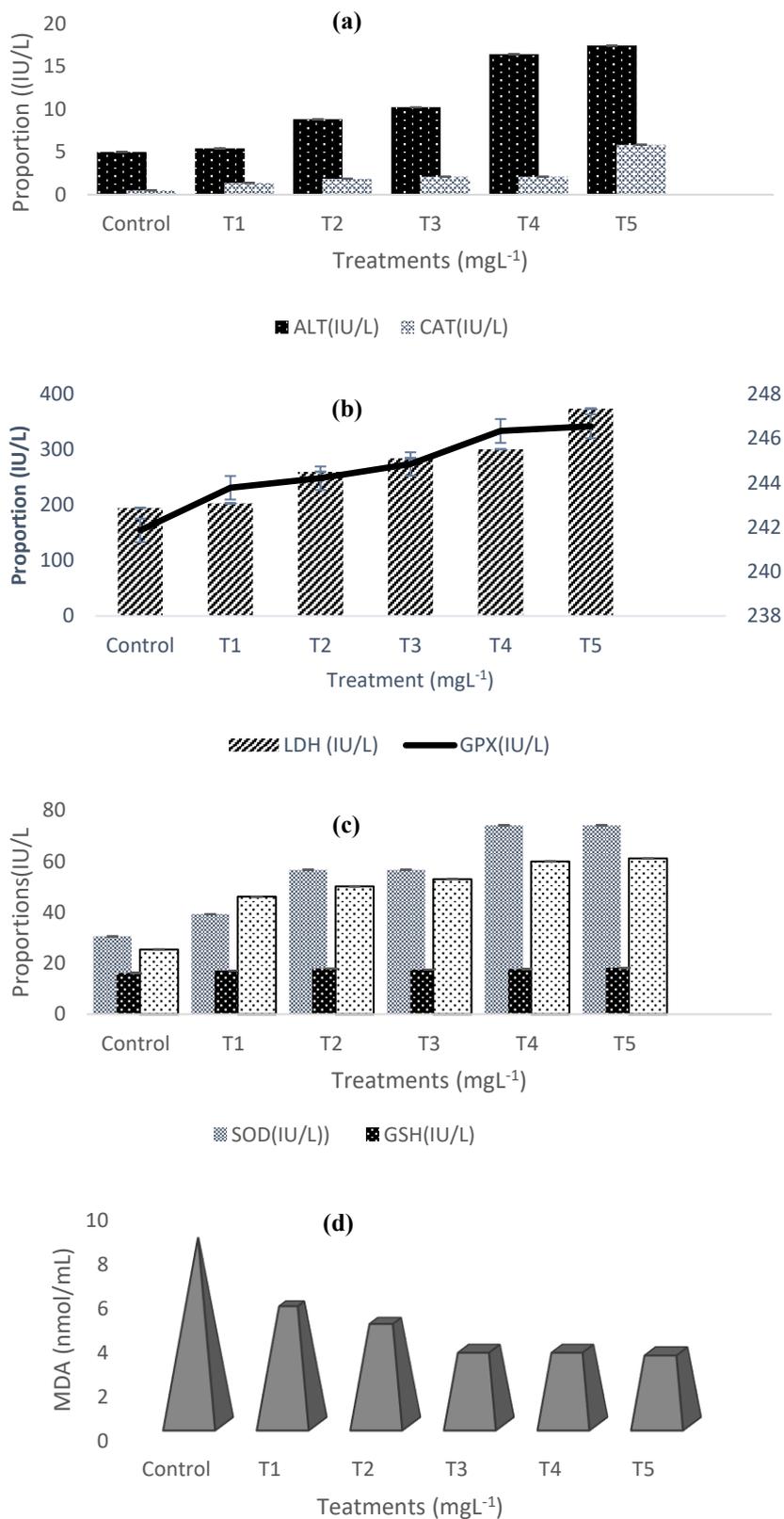
Figure 1 The effect of *I. tinctoria* on antioxidant indicators (a: ALT & CAT, b: LDH & GPx, c: SOD, GSH & AChE and d: MDA) in juvenile Nile tilapia *O. niloticus*) (a = control, T1 = 13 mgL^{-1}, T2 = 27 mgL^{-1}, T3 = 40 mgL^{-1}, T4 = 53 mgL^{-1} and T5 = 67 mgL^{1}).

A toxicant has been identified to significantly impair animal physiology [38], which is especially devastating in aquatic lives. Toxins have been related to oxidative-induced stress, which is characterized by disproportion favoring the ROS synthesis over the counter antioxidant mechanism. Antioxidant indicators have been employed to determine the impact of various adverse environmental conditions on several fins and shellfish [39]. Aquatic environment toxicology relies heavily on understanding oxidative stress in fishes and because numerous chemicals and/or xenobiotics cause oxidative damage, the action of pro-oxidant factors can be utilized in determining the level and nature of pollution. Antioxidant enzyme screening has been used to indicate an organism's level of antioxidant protection [40]. The current study revealed the activities of ALT, LDH, CAT, SOD, GPx, GSH and AChE and DNA fragmentation) to be considerable rise while the activity malondialdehyde (MDA) represents a substantial decrease, following the exposure to 13 to 67 mgL^{-1} of *I. tinctoria* for 21 days.

GSH is a molecule, functions as a cellular reducing and protecting agent against varieties of contaminants via the SH-group [41]. Its functions are linked with oxyradical scavenging and as a substrate of antioxidant enzymes in living cells [42]. GSH appears to be vital in protecting cells from the harmful effects of ROS this it does by interacting with them to synthesize glutathione disulfide. GSH levels in *O. niloticus* tissue were found to have significantly increased in the current investigation. In contrast to Ozoagudike, and Bawa-Allah [43] observation of a decrease in GSH activity in *Clarias gariepinus* subjected to a sub-acute concentration of *Carica papaya* seed and *Anacardium occidentale* bark for 28 days. Mowuogwu and George [44] reported increased GSH levels in *Clarias gariepinus* exposed to *Hibiscus sabdariffa* extract for 21 days and according to EL-Gazzar *et al.* [45], *O. niloticus* exposed to polluted discharge had greater GSH levels, which might be an antioxidant adaptation to long-term exposure. A shift in GSH levels could be a key sign of an organism's ability to detoxify the phytochemical components of the utilized aqueous extract, which have antioxidant properties and protect GSH, may also play a role in this association.

This study revealed that as the concentration of *I. tinctoria* increased, so did the relative level of CAT activity in the tissue. As a crucial antioxidant that guards against any potential lipid damage brought on by the toxicant, CAT is used in this study to protect against lipid disruption, however, the increase in CAT might well be connected to indigo-mediated oxy-radical generation [46]. The CAT and SOD systems are typically utilized as a biomarker to signal ROS generation and serve as the defensive line against oxygen poisoning [47]. According to Lushchak *et al.* [48], CAT produces water and molecular oxygen from hydrogen peroxide, whereas SOD converts superoxide anion radicals to oxygen and hydrogen peroxide. Hydrogen peroxide and lipid peroxide levels are reduced by GPx catalysis, which is regarded to be an efficient chemical defense against LPO [49].

I. tinctoria-treated *O. niloticus* tissue in this study showed a noticeably elevated level of GPx activity. Comparable studies have been done on HaCaT cultured cells exposed to herbicides, [50]. The reported increase may have stimulated the antioxidant system, resulting in increased peroxide generation, despite the fact that these enzymes are important in the elimination of organic peroxides and hydrogen peroxide metabolization [51].

Lipid peroxidase levels in the muscle were lowest in T5 (3.26 ± 1.27) and the highest concentration was recorded in control (8.40 ± 3.16). The lipid peroxidase concentration decreases with an increase in *I. tinctoria* concentration (**Figure 1(d)**).

Lipid peroxidation, one of the most detrimental results of oxidative stress, is caused when ROS that weren't neutralised react with membrane lipids [52]. In contrast to Nwani's [53] report, which claimed that *Clarias gariepinus* juveniles exposed to Primextra herbicide experienced a marked increase in LPO, this study found that lipid peroxidation in fishes decreased after 21 days of treatment. The contrasting report might be linked to species differences and toxicant used and possibly, the duration of exposure.

Because of a considerable rise in ALT, LDH, CAT, SOD, GPx and AChE activities prior to exposure, it may be extrapolated that the antioxidant defense in experimental fishes was adequate, resulting in decreased lipid peroxidation, which was sufficient to fight the ROS and therefore minimize the occurrence of oxidative damage.

SDS-PAGE and DNA fragmentation
According to the electrophoretic profile, the control treatment had a greater overall number of protein bands (7). With increasing *I. tinctoria* concentration, the number of protein bands in muscle reduced (**Figure 2**).

(a)

(b)

(c)

(d)

(e)

(f)
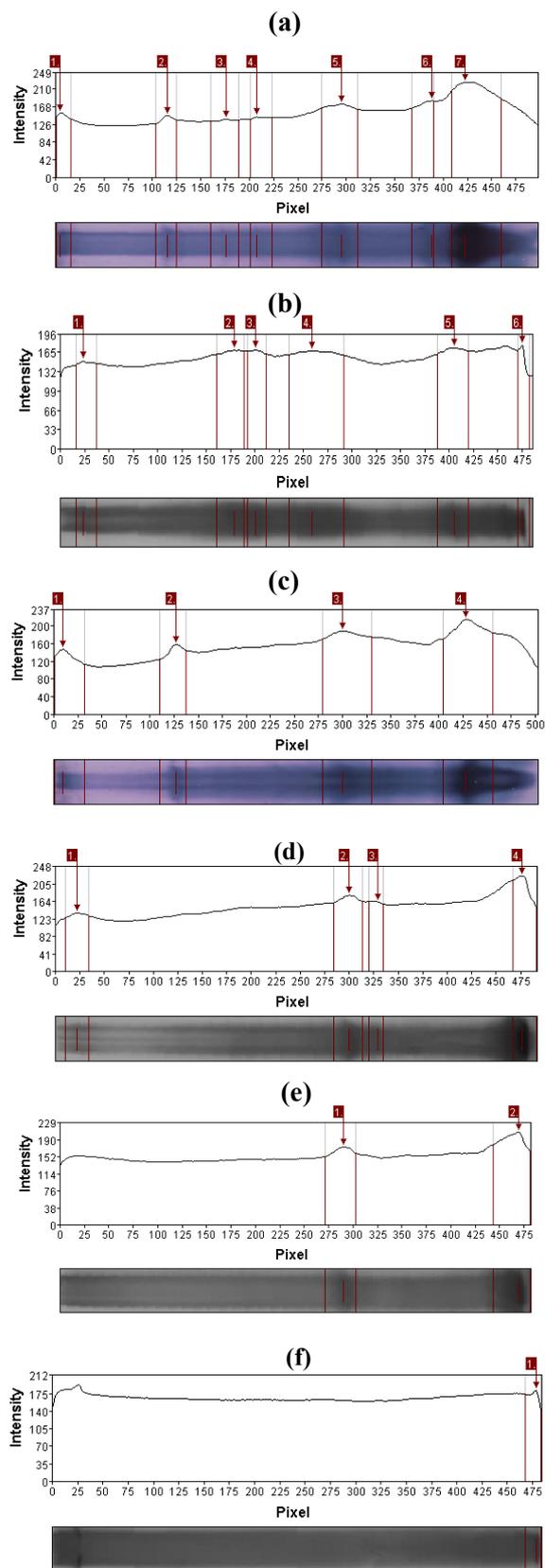
Figure 2 Electropherogram of muscle protein in *O. niloticus* exposed to varying concentration of *Indigoferra tinctoria* (a = control, T1 = 13 mgL^{-1}, T2 = 27 mgL^{-1}, T3 = 40 mgL^{-1}, T4 = 53 mgL^{-1} and T5 = 67 mgL^{-1}).

DNA fragmentation in *O. niloticus* demonstrated that *I. tinctoria*T5 (67 mgL^{-1}) produced 33.44 % fragmented DNA, meanwhile the control group produced a minimal 14.75 % disrupted DNA in *O. niloticus* (**Figure 3**). The increase in DNA fragmentation compared with the control is concentration-dependent.
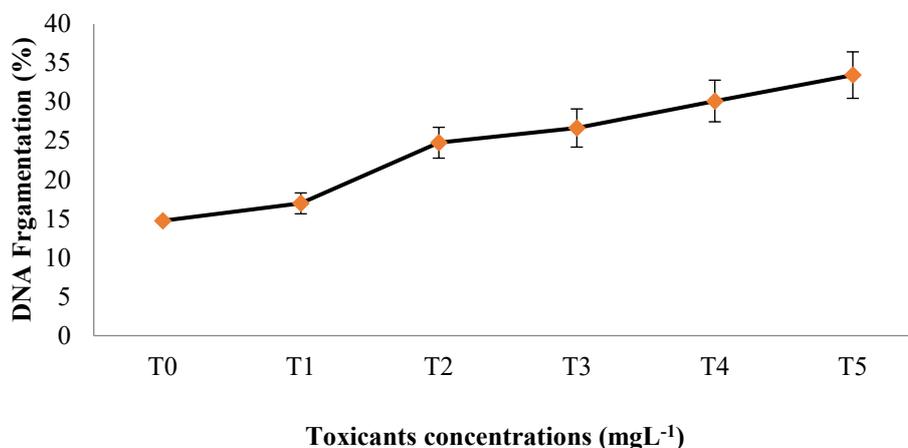
Figure 3 DNA fragmentation in *O. niloticus* exposed to sublethal concentration of *I. tinctoria*.

The electrophoretic profile in this investigation revealed that in the control treatment, the estimated number of protein bands was 7. After 21 days of exposure to 0.25 gL^{-1} *I. tinctoria*, the number of bands reduced to one. The shift in protein profiles could mean a change in metabolic activity (synthesis/degradation) as observed by Paruruckumani *et al*. [54]. Furthermore, it was suggested by Patterson [55] that contaminants impair protein production by interacting with nucleic acids, and our findings supported this idea. After copper exposure, the number of protein bands was reduced in *Lates calcarifer* [54] with reference to gills and muscles. According to Sharaf-Eldeen and Abdel-Hamid [56], some contaminants make certain blood protein fractions in Nile tilapia disappeared. Twenty-one days after exposure to endosulfan and malathion, *Clarias batrachus* intracellular protein pattern alterations were detected using electrophoresis [57]. In *Clarias gariepinus*, some serum protein fractions had lost their identity while others showed polymorphism, according to Badaway and El-Serafy [58], who collected the samples from various polluted water sources. The absence of several protein bands clearly demonstrates that exposure to the herbicide glyphosate over an extended period of time induced alterations in the pattern of serum protein expression in *Clarias gariepinus* [59].

Pollutants were found to break DNA in mussels (*Mytilus gallus*) [21,23]. Pollutant genotoxicity can result from 2 different situations: Direct DNA interactions, or too much formation of ROS [60]. DNA damage caused by *I. tinctoria* exposure in *O. niloticus* increased considerably ($P < 0.05$) with increasing concentration in the current investigation. The increase in DNA fragmentation may be attributed to oxidative stress mediated by oxygen radicals (ROS). This problem has been exacerbated by the antioxidant system's inability to eliminate ROS [61]. Long exposure of *O. niloticus* to *I. tinctoria* may cause cell damage and DNA breakage, according to Srikanth *et al*. and Popoola [62,63].

Conclusions

The information and findings from this study lead to the conclusion that *I. tinctoria* is toxic and, specifically at high doses stresses *O. niloticus*. Biomarkers for oxidative stress were found to increase in levels, some proteins are disappearing, and DNA fragmentation demonstrated clearly that long exposure of *O. niloticus* to *I. tinctorial* might change the pattern of muscle protein expression which may lead to genetic alteration. The result of this finding could be useful to *I. tinctorial* toxicity biomonitoring researches, mostly in the context of industrial applications.

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